IPCS

INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY

Environmental Health Criteria 244

Principles and methods to assess the risk of immunotoxicity associated with exposure to nanomaterials

World Health Organization

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PRINCIPLES AND METHODS TO ASSESS THE RISK OF IMMUNOTOXICITY ASSOCIATED WITH EXPOSURE TO NANOMATERIALS

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the United Nations Environment Programme, the International Labour Organization or the World Health Organization.

World Health Organization

Principles and methods to assess the risk of immunotoxicity associated with exposure to nanomaterials (Environmental health criteria 244)

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PREFACE

Objectives

In 1973, the WHO Environmental Health Criteria Programme was initiated with the following objectives:

(i)   to assess information on the relationship between exposure to environmental pollutants and human health, and to provide guidelines for setting exposure limits;
(ii)   to identify new or potential pollutants;
(iii)   to identify gaps in knowledge concerning the health effects of pollutants;
(iv)   to promote the harmonization of toxicological and epidemiological methods in order to have internationally comparable results.

The first Environmental Health Criteria (EHC) monograph, on mercury, was published in 1976, and since that time an ever-increasing number of assessments of chemicals and of physical effects have been produced. In addition, many EHC monographs have been devoted to evaluating toxicological methodology, such as for genetic, neurotoxic, teratogenic and nephrotoxic effects. Other publications have been concerned with epidemiological guidelines, evaluation of short-term tests for carcinogens, biomarkers, effects on the elderly and so forth.

The original impetus for the Programme came from World Health Assembly resolutions and the recommendations of the 1972 United Nations Conference on the Human Environment. Subsequently, the work became an integral part of the International Programme on Chemical Safety. The EHC monographs have become widely established, used and recognized throughout the world. The recommendations of the 1992 United Nations Conference on Environment and Development with the priorities for action in the six programme areas of Chapter 19, Agenda 21, the outcome document of the United Nations Conference on Sustainable Development "The future we want", and the WHO Chemicals Road Map approved by the World Health Assembly in decision WHA70(23) in 2017 all

lend further weight to the need for EHC assessments of the risks of chemicals.

Scope

Two different types of EHC documents are available: (a) on specific chemicals or groups of related chemicals; and (b) on risk assessment methodologies. The criteria monographs are intended to provide critical reviews on the effect on human health and the environment of chemicals and of combinations of chemicals and physical and biological agents and risk assessment methodologies.

The EHC monographs are intended to assist national and international authorities in making risk assessments and subsequent risk management decisions and to update national and international authorities on risk assessment methodology.

ACKNOWLEDGEMENTS

Scoping meeting

A scoping meeting, 8–9 April 2015, at RIVM, Bilthoven, the Netherlands, developed an outline of the document. The meeting was attended by the following:

Invited experts

James Bonner, North Carolina State University, United States of America

Yolanda Irasema Chirino Lopez, Universidad Nacional Autónoma de México, Mexico

Bengt Fadeel, Karolinska Institutet, Stockholm, Sweden

Sabina Halappanavar, Health Canada, Canada

Jun Kanno, National Institute of Health Sciences, Japan

Kee Woei Ng, Nanyang Technological University, Singapore

Kai Savolainen, Finnish Institute of Occupational Health, Finland

Henk van Loveren, RIVM, the Netherlands (Meeting Chair)

Il Je Yu, Hoseo University, Republic of Korea

Representatives (observers)

Laura Rossi, European Chemicals Agency (ECHA), Finland

Jihane El Gaouzi, Organisation for Economic Co-operation and Development, France

The WHO Secretariat was represented by Carolyn Vickers, WHO, Switzerland.

Discussion of first draft

The invited experts contributed to the development of a first draft, which was discussed at a meeting held from 30 March to 1 April 2016, at RIVM, Bilthoven, the Netherlands. The meeting was attended by the following:

Invited experts

Harri Alenius, Finnish Institute of Occupational Health, Finland

James Bonner, North Carolina State University, United States of America

Yolanda Irasema Chirino Lopez, Universidad Nacional Autónoma de México, Mexico

Wim H. De Jong, RIVM, the Netherlands

Janine Ezendam, RIVM, the Netherlands

Bengt Fadeel, Karolinska Institutet, Stockholm, Sweden

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Kee Woei Ng, Nanyang Technological University, Singapore

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Barbara Rothen-Rutishauser, Université de Fribourg, Switzerland

Kai Savolainen, Finnish Institute of Occupational Health, Finland

Henk van Loveren, RIVM, the Netherlands (Meeting Chair)

Susan Wijnhoven, RIVM, the Netherlands

Il Je Yu, Hoseo University, Republic of Korea

Representatives (observers)

Laura Rossi, European Chemicals Agency (ECHA), Finland

The WHO Secretariat was represented by Carolyn Vickers, WHO, Switzerland.

Revisions to the document were made by the experts following the March–April 2016 meeting, under the Chair of Henk van Loveren, in order to prepare the peer review version.

The peer review version was published on the WHO website for comment.

Final review group meeting

A final review group meeting was held during 2–4 October 2017, at RIVM, Bilthoven, the Netherlands, to consider the peer review comments received and to finalize the text subsequent to the meeting. Prior to the meeting, assistance to WHO was provided by Andrey Boyadzhiev, Canada, who catalogued the comments received.

The meeting was attended by the following:

Invited experts

Harri Alenius, Finnish Institute of Occupational Health, Finland

James Bonner, North Carolina State University, United States of America

Yolanda Irasema Chirino Lopez, Universidad Nacional Autónoma de México, Mexico

Wim H. De Jong, RIVM, the Netherlands

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Sabina Halappanavar, Health Canada, Canada

Jun Kanno, Japan Bioassay Research Centre, Japan

Kee Woei Ng, Nanyang Technological University, Singapore

Margriet Park, RIVM, the Netherlands

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Representatives (observers)

Laura Rossi, European Chemicals Agency (ECHA), Finland

The WHO Secretariat was represented by Carolyn Vickers, WHO, Switzerland.

Editing

Layout by

ABBREVIATIONS

3-OH FA	3-hydroxy fatty acid
4-HHE	4-hydroxy-2-hexenal
ABC	adenosine triphosphate-binding cassette
Ag-NP	silver nanoparticle
$Al(OH)_3$	aluminium hydroxide
AlOOH	aluminium oxyhydroxide
ALT	alanine aminotransferase
AOP	adverse outcome pathway
ARE	antioxidant/electrophile response element
AST	aspartate aminotransferase
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BMD	benchmark dose
BMDL	benchmark dose lower confidence limit
BrdU	5-bromo-2'-deoxyuridine
BX	bolus exposure
CARPA	complement activation-related pseudoallergy
CAS	Chemical Abstracts Service
CCL2	chemokine (C-C motif) ligand 2
CdSe	cadmium selenide
CED	critical effect dose
CeO_2	cerium oxide
CNT	carbon nanotube
COX	cyclooxygenase
CX	continuous exposure
CXCL1	chemokine (C-X-C motif) ligand 1 (etc.)
DAMP	danger-associated molecular pattern
DC	dendritic cell

Defra	Department for Environment, Food and Rural Affairs
DMSO	dimethyl sulphoxide
DNCB	2,4-dinitrochlorobenzene
DNEL	derived no effect level
DWCNT	double-walled carbon nanotube
EBC	exhaled breath condensate
EHC	Environmental Health Criteria
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ENM	engineered nanomaterial
EPA	Environmental Protection Agency
ERK	extracellular signal-regulated kinase
EUON	European Union Observatory for Nanomaterials
Fe_2O_3	iron oxide
Fe_3O_4	magnetite
G	generation
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
h-CLAT	human cell line activation test
HHRA	human health risk assessment
HMGB1	high-mobility group box 1
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
ICP-MS	inductively coupled plasma mass spectroscopy
ICRP	International Commission on Radiological Protection
IFN	interferon
IgA	immunoglobulin A (etc.)
Igf2r	insulin-like growth factor 2 receptor
IL	interleukin

InAs	indium arsenide
iNOS	inducible nitric oxide synthase
IPCS	International Programme on Chemical Safety
ISO	International Organization for Standardization
KLH	keyhole limpet haemocyanin
LAL	*Limulus* amoebocyte lysate
LD50	lethal dose 50%
LDH	lactate dehydrogenase
LLNA	local lymph node assay
LOAEL	lowest observed adverse effect level
LPS	lipopolysaccharide
M1	classically activated macrophages
M2	alternatively activated macrophages
MAP	mitogen-activated protein
MCP-1	monocyte chemoattractant protein-1
MDA	malondialdehyde
MDSC	myeloid-derived suppressor cell
MIP	macrophage inflammatory protein
MMAD	mass median aerodynamic diameter
MPO	myeloperoxidase
MPPD	multiple path particle dosimetry
MWCNT	multi-walled carbon nanotube
N_2	nitrogen
NaCl	sodium chloride
$NaCO_3$	sodium carbonate
NADPH	nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	sodium bicarbonate
ncRNA	non-coding RNA
NK	natural killer
NLR	NOD-like receptor

NLRP3	NLR family pyrin domain containing 3
NOAA	nano-objects and their agglomerates and aggregates
NOAEL	no observed adverse effect level
Nrf2	nuclear factor-erythroid 2-related factor 2
OECD	Organisation for Economic Co-operation and Development
OVA	ovalbumin
PAMAM	poly(amidoamine)
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PbSe	lead selenide
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PLGA	polylactic-co-glycolic acid
ppm	parts per million
PRR	pattern recognition receptor
PVP	polyvinylpyrrolidone
QSAR	quantitative structure–activity relationship
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RfC	reference concentration
RfD	reference dose
RIP	receptor-interacting protein
RIVM	National Institute for Public Health and the Environment, Netherlands
ROS	reactive oxygen species
RPT	rabbit pyrogen test
RT-PCR	reverse transcription polymerase chain reaction
SCENIHR	Scientific Committee on Existing and Newly Identified Health Risks

SCID	severe combined immunodeficient
SiO_2	silicon dioxide
STAT1	signal transducer and activator of transcription 1
SV	simian virus
SWCNH	single-walled carbon nanohorn
SWCNT	single-walled carbon nanotube
TDAR	T cell-dependent antibody response
TEM	transmission electron microscopy
TG	test guideline
TGF	transforming growth factor
Th	T helper
TiO_2	titanium dioxide
TLR	toll-like receptor
TNF	tumour necrosis factor
UV	ultraviolet
WHO	World Health Organization
wt%	weight per cent
$ZnFe_2O_4$	zinc ferrite
ZnO	zinc oxide

Measures and units

Length

m	metre
cm	centimetre
mm	millimetre
µm	micrometre
nm	nanometre

Mass

kg	kilogram
g	gram
mg	milligram
μg	microgram
ng	nanogram
pg	picogram
fg	femtogram

Capacity

L	litre
dL	decilitre
mL	millilitre
μL	microlitre

Other

eV	electronvolt
GPa	gigapascal
K	Kelvin
kDa	kilodalton
mM	millimolar
μM	micromolar
nM	nanomolar
μCi	microcurie
>	greater than
<	less than, fewer than

EXECUTIVE SUMMARY

Engineered nanomaterials (ENMs) may be associated with environmental and human health hazards, resulting in harmful effects on human health. There are different types of ENMs: carbon-based ENMs (examples include carbon nanotubes, graphene and fullerenes); metal-based ENMs (including nano forms of titanium dioxide, gold, silver and quantum dots); dendrimers (polymers that are mainly used in drug delivery); and composites (combinations of ENMs, including carbon nanotubes coated with nano metal oxides). These materials are being readily applied in various nanotechnology products, which can be categorized into four generations: (a) first-generation nanoproducts, including ENMs based on one material that are used in various consumer products such as cosmetics and food; (b) second-generation ENMs, including more complex nanostructures such as fertilizers; (c) third-generation ENMs, involving use of both first- and second-generation nanostructures to build nanosystems, as in the development of synthetic organs or engineered microbes, or self-assembling materials that assemble into new structures in the body upon their release; and (d) fourth-generation ENMs, which are still in the developmental phase, involving molecular nanosystems with a specific function, such as molecular devices used in genetic therapy. While the technology itself has progressed rapidly and thousands of consumer products containing ENMs are beginning to appear on the market, the exposure and toxicology of these materials are less understood. Lack of a clear understanding of how the general population is exposed and the extent of exposure, and the potential hazard of these ENMs, has been a major impediment to the implementation of nanospecific health and safety practices.

Extensive research conducted in the past 20 years has shown that not only the chemistry of nanomaterials but also their size, shape and surface characteristics influence the interaction of ENMs with biological systems. Consequently, toxicologists and risk assessors have found it virtually impossible to keep pace with the rapid development of technology, the sheer number of ENMs with diverse properties, and the constraints associated with conventional toxicity assessment methods. In addition to applicable conventional methodologies, addressing the challenges presented by ENMs

also requires novel approaches for exposure estimations, hazard identification, and risk assessment, integrating the physical, chemical and biological sciences.

The immune system consists of an innate component, which is able to directly respond to foreign agents immediately after exposure, regardless of the type of stimulus; and an adaptive component, which builds immunity over time. Interaction of exogenous agents, including ENMs, may result in immunosuppression, immunostimulation, hypersensitivity or autoimmunity. Recent reports have identified ENMs as potential stimulants of immune response that may culminate in eventual immunotoxicity. While there is no validated methodology available to assess the immunotoxicity of ENMs, this document outlines several assays conventionally used to assess chemical-induced immunotoxicity that may be compliant with nanomaterial testing. It is not realistic to expect that every ENM will be tested using all of the test methods available; however, simple rules may be followed, as outlined below.

Whereas exposure to ENMs can occur through all routes by which exposure to chemicals may occur, the lung has been predominantly investigated as the target organ, and lung inflammation has been the most reported outcome following exposure to ENMs. Other organ systems have been less studied so far. While lung inflammation in itself may not be considered as immunotoxicity, prolonged stimulation of the various components of the inflammatory system, including opsonization and complement activation, may incite pathological conditions such as asthma. Thus, ENMs that are immunostimulants should be investigated carefully for their potential to induce immunotoxicity. Several studies have shown that ENMs can translocate from the lungs to other immune-responsive organs, such as the spleen, liver and lymph nodes, depending on the ENM properties. Thus, in a scenario where lung exposure to ENMs results in lung inflammation, and if ENMs are found to be translocated to immune-responsive organs, those ENMs should be prioritized for a comprehensive investigation by other immunotoxicity-specific assays.

Similarly, absorption by and penetration through the layers of the skin are not anticipated for ENMs; however, if an ENM is shown to induce respiratory hypersensitivity, it should be investigated for

its potential to induce skin irritation as well. If the bulk counterpart of a nanomaterial is a known immunotoxicant, the nano form should be considered high-risk material and investigated by the appropriate methods. Although the choice of test methods should primarily be based on their potential application, in the case of skin application sprays, where bystander exposure to inhalation of spray mist is anticipated, lung toxicity should be investigated in addition to skin irritation and absorption tests. Thus, the choice of methods and the extent of investigation should carefully consider several factors, including the properties of ENMs, their potential application, and the route of exposure. Moreover, more than one test method targeting the same end-point should be included in the strategy to increase the confidence in the results derived.

There are currently no guidelines for assessing the immunotoxicological consequences of exposure to ENMs. For hazard identification a variety of methods are available that in principle have all been used for classical toxicity assessment of chemicals, including immunotoxicity. Given the multitude of ENMs, and the drive to minimize the use of laboratory animals for safety testing, emphasis has been on in vitro methodologies. However, many of these have not yet been standardized or validated for the use of testing for ENMs. In addition, there are limitations in representing the complexity of the immune system – in particular, the downstream response is difficult to mimic with cell culture experiments. In general terms, risk assessment of ENMs should follow the risk assessment paradigm for chemicals, namely hazard identification, hazard characterization, exposure assessment, and risk characterization. Currently, the design to perform risk analysis should be carried out flexibly on a case-by-case basis, including the components most appropriate for the material and its proposed use.

This Environmental Health Criteria document presents the current state of knowledge pertaining to principles and methods to assess the health risk of immunotoxicity associated with exposure to ENMs. Immunotoxicity assessment needs to be integrated in a broader context of ENM hazard and risk assessment. Therefore, even if the emphasis of the document is on immunotoxicity, common ENM-specific issues, such as characterization, sample preparation, and dosimetry, are also addressed.

1. INTRODUCTION

1.1 Scope of the document

The purpose of this document is to present an overview of the current knowledge and evidence on principles and basic mechanisms of immunotoxicity caused by engineered nanomaterials (ENMs). ENMs are encountered in their free or embedded forms occupationally during the manufacturing process and professional uses, as nanomaterials present in consumer products, or in the environment after release. ENMs have been shown to have the ability to influence the immune system. This document provides guidance on principles and methods for hazard and risk assessment of different ENMs and groups of ENMs on the immunological system in the body. Hence, the key cell types and elements and the functioning of the human immunological system will be described, and the effects of various ENMs on these cells and elements of the immune system will be addressed.

The potential users of the document include the scientific community, health care professionals, regulators and decision-makers at the national and international levels, industry and industrial associations, and relevant civil society organizations, such as social partners and other civil society interest groups.

1.2 Engineered nanomaterials and nanotechnologies

Nanotechnology – the manipulation of materials at or near the atomic scale to produce new structures, materials, and devices – holds possibilities for scientific advances in many areas, including consumer products, energy, and some areas of large-scale manufacturing. By arbitrary definition, nanotechnology deals with structures within the length range of approximately 1–100 nanometres (nm) *(1)*. However, in certain application areas a broader definition is used; for example, for nanomedicines a size of 1–1000 nm is used. The very small size results in a markedly increased surface area that conveys unique specific properties to the

nanoscale. Nanotechnology has the potential to improve existing technologies and to enable emerging technologies to blossom *(2, 3)*, for example in the construction, energy, automobile manufacturing, pharmaceutical, food and agricultural industries *(4, 5)*. Specific applications include energy production and storage, catalytic reactions, microelectronics, plastics and polymers, concrete, coatings and paints, cosmetics and medical products. It is also noteworthy that ENMs alone do not carry additional value in most cases – they enable added value when combined with existing and emerging technologies. Indeed, nanotechnology is considered as one of the "key enabling technologies" in the research and innovation programme of the European Commission *(6)*.

However, it has appeared over the years that some of the ENMs or groups of ENMs may be associated with harmful effects on health *(7, 8)*. Hence, it has become important to be able to distinguish those materials that may be harmful from those that are innocuous, and to develop methods for hazard identification, exposure and risk assessment for these materials, which in many ways differ from the soluble chemicals *(9)*. Because of the diversity of potential harmful effects of ENMs, it has also become necessary to identify the key target organs or organ systems for potential toxic effects and risk assessment. It is therefore also important to emphasize that describing ENMs as a uniform group of materials is misleading *(10)*. There may be hundreds of thousands of ENMs synthetized in the laboratory; carbon nanotubes (CNTs) alone exist in more than 50 000 different forms *(11)*. Hence, when discussing various ENMs, one has to be specific and indicate the special features of the nanomaterial in question, and properly characterize the physicochemical features in detail (for example, the size, surface area, surface–volume ratio, reactivity, chemical composition, structure, crystallinity, aspect ratio, tensile strength, electrical conductivity, persistency and dissolution rate, particle size distribution, and chirality), using appropriate methods *(12–14)*.

The ENMs in the widest use include carbon black, amorphous silica, nanoscale metal particles such as silver or gold, nanoscale metal oxides such as titanium dioxide (TiO_2), iron oxide (Fe_2O_3) or cerium oxide (CeO_2), as well as carbon-based materials such as fullerenes, single-walled carbon nanotubes (SWCNTs) and

multi-walled carbon nanotubes (MWCNTs), and carbon nanofibres. These ENMs possess specific properties that are determined by their shape, size and dissolution characteristics. In view of the multitude of ENM variations, a proper physicochemical characterization of the ENM under investigation is essential for both hazard and risk assessment *(15, 16)*. Such a characterization is also necessary for proper identification of the ENM (for example, ascertaining that the nanomaterial used in the final product is the same as the one for which the risk assessment is done).

It is not surprising that ENMs, consumer products incorporating ENMs, and nanotechnologies taking advantage of ENMs in industrial materials and processes have attracted a remarkable amount of attention during the past decade. The technological and beneficial characteristics of ENMs are attributable to their unique properties described accompanying the nanoscale *(12, 13)*. Several of the novel applications of ENMs have now been incorporated into a variety of popular consumer products, for example better protection against ultraviolet (UV) radiation by sunblock creams containing nanoscale TiO_2 or zinc oxide (ZnO) as effective UV filters *(17)*. ENMs are also now widely used in other consumer items such as electronics, cosmetics, clothes, cleaning materials (for example bleach), sportswear and other sport products (for example cross-country skis and tennis rackets). Nanotechnologies and materials are also used in a number of professional protective clothing items and several military applications *(18)*.

Industrial applications include, in addition to those mentioned above, computer hard drives with much higher memory capabilities, and incorporation of ENMs into semiconductors. The unique properties of ENMs may confer remarkable economic benefits by increasing the capacity of various types of information and communication technology devices. They may also enable a reduction in material needs – for example, increasing the strength of concrete in the construction industry through use of amorphous silica means that the amount of concrete and other building materials can be significantly cut back. The economically most important applications of ENMs are likely to be found in industrial applications such as coatings, optical and printed electronics, applications of nanocellulose, and energy production. A significant future benefit

will be for mitigation of climate change through clean technology applications, for example by reducing energy through more efficient batteries and power sources, or by use of lightweight composites for cars and aircraft, thereby reducing fuel emissions *(4)*. ENMs are also being used as additives to oil (nanodiamonds, to reduce friction) and gasoline (CeO_2, to decrease the release of carbon dioxide and improve burning efficiency) *(12)*.

As a consequence of this rapidly increasing range of technologies and applications, the number of consumer products and other products on the market incorporating ENMs has been predicted to grow dramatically by 2020 *(12, 18)*.

Notwithstanding these beneficial properties, in some cases ENMs can also be harmful to human health or the environment. In fact, many of the characteristic properties of ENMs that make them so valuable – small size (at least one dimension in the range 1–100 nm), large surface area per weight, and high surface reactivity – are unfortunately the very factors contributing to their potentially harmful effects *(7, 19–21)*. In fact, the small size of ENMs enables them to enter the body and penetrate biological barriers much more easily than their chemically identical but larger counterparts *(22)*. The small size may also have unexpected effects on the kinetics in terms of absorption, distribution, metabolism and excretion, especially with respect to distribution in the body.

Nevertheless, despite the increasing knowledge of the possible harmful health effects of some ENMs, the use and the number of applications of ENMs have dramatically increased during the last 10 years, and the growth continues at an increasing speed due to the technological and economic benefits of these materials.

One major challenge in assessing the risks of nanomaterials is the lack of systematic knowledge about exposure to these materials. There has been very limited systematic research into the hazards of ENMs for human health or the environment, with most of the studies concentrating on only a few nanomaterials *(23–25)*. The amount of data about exposure in workplaces and the general environment is even more restricted, and in fact only a few studies have been published *(13, 23, 25, 26)*. Without this basic information, it is not

possible to conduct an adequate assessment of risks of exposure to different nanomaterials.

Data on hazards and exposure are essential in performing a quantitative risk assessment, as indicated by the well known equation *(27)*:

hazard x exposure = risk.

Even though some ENMs have been identified as hazardous *(28–31)*, it is likely, as with other chemicals, that many if not most ENMs will prove to be harmless or only marginally dangerous *(32)*. However, the ability to identify the harmful materials from their harmless counterparts is currently very limited, complicating assessment of the safety and risks of ENMs. The challenges to differentiating between harmful and harmless ENMs, arising from the limitations of the current hazard, exposure and risk assessment methodologies, are a cause for concern among consumers, regulators and the industries using these materials. These concerns related to the uncertainties of the health and environmental effects of ENMs have been identified by the European Commission as a major obstacle preventing the efficient transfer of these materials into the activities of existing and emerging technologies *(32)*.

Concerns surrounding the potential health effects and safety of ENMs started to increase as the use of these materials became more widespread. The first publications on potential health effects of the materials were published in the 1990s *(33, 34)*. It was only at the beginning of this century that nanotoxicology became identified as a topic for ENM safety evaluation *(35–37)*. Similar to other particles, ENMs have the potential to induce lung inflammation after inhalation exposure, which resulted in an interest based on occupational safety. Oberdörster et al. *(38)* and Elder et al. *(39)* conducted work in experimental animals and reported that inhalation exposure to ENMs could lead to uptake of the particles by the brain. In this case, axons of the olfactory nerve in the olfactory epithelium could transport the particles into the olfactory bulb under the frontal cortex. This raised concern about the possibility of brain damage to humans, which was shown as largely unjustified by later studies. These materials can be distributed to other parts of the brain after their uptake through the nose

in experimental animals, but there is no evidence on effects in humans, though some effect on animals has been found. Several studies have associated TiO_2 nanoparticles with pulmonary inflammation *(34, 40)*, including transient effects *(41, 42)*. Exposure of SWCNTs resulted in progressive pulmonary fibrosis *(31)* and of MWCNTs in pulmonary granulomas and inflammation *(43)*, and even asbestos-like effects (pathology on the mesothelium) following exposure to certain types of MWCNT after intraperitoneal injection of experimental animals *(44–47)*. Ryman-Rasmussen et al. *(48)* have shown that CNTs can reach the subpleural tissue of the lungs and lead there to asbestos-like fibrosis and collagen accumulation. Also, more widespread tissue distribution was observed after respiratory exposure to CNTs *(49)*.

In spite of the expansion of the research into the potential health effects of ENMs, none of them has been systematically studied to allow the performance of a thorough evidence-based risk assessment of ENMs *(7, 25, 50, 51)*. The lack of information about exposure to these materials is even more noteworthy *(23, 26)*. In other words, the lack of both hazard and exposure data on these materials means that a realistic and reliable risk assessment is difficult, if not impossible, at present.

The predominating challenge associated with the use of these materials in various applications of nanotechnologies is the uncertainty associated with their potential health effects. A large research project funded by the European Union, ProSafe *(52)*, recently concluded that hazard, exposure and risk assessment of nanomaterials can be extremely complex, time consuming, costly, and hampered with uncertainties. The project further concluded that it was not realistic to investigate every nano form by applying all test methods. The main conclusions of the project, therefore, emphasized the importance of test method validation, and focused on the reliability and relevance of the methods used to assess the safety of nanomaterials. The project report also stressed the importance of developing novel testing strategies of nanomaterials to increase the reliability and predictive ability of nanomaterial safety testing *(52)*.

This uncertainty represents a major obstacle to the producers of these materials and is of concern to the enterprises that incorporate the materials into consumer and other products. Hence, one of the major

challenges facing the nanotechnology industry today is to alleviate the doubts about the safety of ENMs *(3, 53–55)*. Until the issue of safety assessment of ENMs has been resolved by research on both the potential hazards of and exposure to ENMs, uncertainty of the safety of these materials will remain an obstacle for the beneficial applications of ENMs in a number of technological applications worldwide.

1.3   Immunotoxicity testing

Immunotoxicity testing is usually done in rodent species. Whereas in general terms the immune systems in rodents and humans are similar, there are differences. In vitro testing for immunotoxicity is also carried out. Cell lines are often used for reasons of accessibility and reproducibility. They are surrogates for the specific cell types investigated. All of the above should be taken into account when devising tests or analysing test results.

The immune system evolved primarily to protect the host from infectious or neoplastic disease *(56)*. It consists of several organs and specialized cell types throughout the body *(57)*. As such, a normal functioning of the immune system presents a defence against invading microorganisms, foreign (xenobiotic) materials and tumours that would otherwise result in disease. However, deregulation of the immune system may result in allergic or autoimmune responses. In the modern world, humans are exposed to an ever-increasing variety of xenobiotics, including chemicals, metals, and drugs, and also in the form of ENMs that disrupt immune system homeostasis and normal functionality. Immunotoxicology is the study of the interaction of xenobiotic agents such as chemicals and drugs with the immune system. Immunotoxicity is defined as any adverse effect on the immune system following toxicant exposure that results in immune stimulation or immune suppression *(58)*. The immune system consists of an innate part that is able to directly respond to foreign agents and an adaptive part that needs some time to develop an immune response. Both of these may be affected by foreign agents. This interaction may result in immunosuppression, immunostimulation, or hypersensitivity and autoimmunity. Such conditions may result in depressed resistance to infections; play a role in the development of cancer; induce or facilitate allergic conditions such as asthma or

atopic contact dermatitis; or induce or facilitate autoimmune diseases. More recently, numerous reports have identified ENMs as a potential source for immunotoxicity *(59–61)*. This is not surprising, as ENMs in vivo end up in the mononuclear phagocytic system as part of the immune system. It should be realized that for ENMs, bacterial lipopolysaccharide (LPS) is a potential contaminant of many particle and nanoparticle suspensions. Possible LPS contamination should be assessed, as it has in itself profound effects on the immune system. On the other hand, LPS is ubiquitous in the environment and elucidating possible interactive effects of LPS on nanoparticle-induced signalling and inflammation is important.

Immunotoxicity caused by chemical exposure is a topic that has been addressed in previous Environmental Health Criteria documents. For example, Environmental Health Criteria monograph 180 of the International Programme on Chemical Safety *(58)* reviewed the causes, consequences, and detection of disorders mediated by immunotoxicity; Environmental Health Criteria monograph 212 *(62)* focused on mechanisms, clinical aspects, epidemiology, hazard identification, and risk assessment of allergy and hypersensitivity following exposure to certain chemicals; and Environmental Health Criteria monograph 236 *(63)* considered induction of autoimmunity associated with chemical exposure. A special issue of *Methods* published in 2007 was dedicated to the use of animal models for determining immunotoxicity *(64)*. It is not yet clear whether existing methods will be applicable to ENMs as well, or whether specific methods may be needed in view of the special characteristics of ENMs. In general, the testing guidelines of the Organisation for Economic Co-operation and Development (OECD) are applicable, but some adaptations may be needed in the way the actual testing is to be performed. This has already been accomplished in inhalation guidelines OECD TG 412 and OECD TG 413 *(65, 66)*.

Immunotoxicity testing of chemicals is done using a tiered approach. In the first tier, indications for effects on organs of the immune system are identified in regular toxicity tests, such as a 28- or 90-day repeated dose toxicity study. In the second tier, more specific immune function assays may be applied, such as antibody formation and resistance against infectious agents *(58)*. Although these tiered

studies are performed in animals, new and improved in vitro tests and in silico approaches are also being developed that will become available to study the immune system.

This criteria document is intended to focus on ENMs that would be encountered occupationally during the manufacturing process, nanomaterials present in consumer products, and nanomaterials that might be encountered in the environment after release from the manufacturing process or from consumer products. A special effort will be made to discuss only immunotoxicity studies that use well characterized ENMs. While it is acknowledged that nanomaterials used in emerging fields of nanomedicine (such as drug delivery and imaging) could result in immunotoxic side-effects, this topic will not be covered in this criteria document.

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2. TYPES OF NANOPARTICLES

The emergence of nanotechnology has brought an upsurge in the synthesis of a broad range of nanoparticles, which are not only difficult to define but also hard to classify, given their huge variety. However, some organizations, including the Royal Society and the Royal Academy of Engineering of the United Kingdom *(1)*, the Scientific Committee on Emerging and Newly Identified Health Risks *(2)*, the European Union *(3)*, and the International Organization for Standardization *(4)* have put forward definitions of nanomaterial. Even if they are not exactly the same, the definitions converge in the matter of structures sized between 1 and 100 nm in at least one of their dimensions (Table 2.1), independent of the shape, composition, solubility or other characteristics. On the other hand, according to the chemical composition, most nanomaterials can be categorized into carbon-based ENMs, metal-based ENMs, organic nanomaterials and composites. In this chapter, a description of the properties of these four types of nanoparticles is provided.

Table 2.1 Definitions of nanoparticles

Organization	Definition
International Organization for Standardization (ISO/TS 80004-2:2015) *(4)*	A nano-object, which is a discrete piece of material with one, two or three external dimensions in the nanoscale (length range approximately from 1 nm to 100 nm), where the lengths of the longest and the shortest axes of the nano-object do not differ significantly (typically by more than 3 times).
Royal Society and Royal Academy of Engineering (United Kingdom) *(1)*	Those which have structured components with at least one dimension less than 100 nm. Materials that have one dimension in the nanoscale (and are extended in the other two dimensions) are layers, such as graphene, thin films or surface coatings. Some of the features on computer chips come into this category. Materials that are nanoscale in two dimensions (and extended in one dimension) include nanowires and nanotubes. Materials that are nanoscale in three dimensions are particles, for example precipitates, colloids and quantum dots (tiny particles of semiconductor materials). Nanocrystalline materials, made up of nanometre-sized grains, also fall into this category.

Organization	Definition
Regulation (European Union) No 528/2012 of the European Parliament and of the Council *(5)*	Nanomaterial means a natural or manufactured active substance or non-active substance containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm. Fullerenes, graphene flakes and single-wall carbon nanotubes with one or more external dimensions below 1 nm shall be considered as nanomaterials.
Scientific Committee on Emerging and Newly Identified Health Risks (European Union) *(2)*	Any form of a material that is composed of discrete functional parts, many of which have one or more dimensions of the order of 100 nm or less.
European Union Recommendation 2011/696/EU *(3)*	Natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness, the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%.
NICNAS working definition of industrial nanomaterial (Australian Government, Department of Health) *(6)*	Industrial materials intentionally produced, manufactured or engineered to have unique properties or specific composition at the nanoscale, that is a size range typically between 1 nm and 100 nm, and is either a nano-object (i.e. that is confined in one, two, or three dimensions at the nanoscale) or is nanostructured (i.e. having an internal or surface structure at the nanoscale).
Environmental Protection Agency, United States of America *(7)*	The United States Environmental Protection Agency has no formal definition. The criteria considered are as follows: a substance that is solid at 25°C under atmospheric pressure, manufactured or processed so that its primary particles, aggregates, or agglomerates are 1–100 nm in size, giving them unique and novel characteristics or properties. A similar particle size distribution of more than 10% by weight in the range of 1–100 nm size is also recommended by the American Chemistry Council.
Health Canada *(8)*	Any manufactured substance or product and any component material, ingredient, device, or structure is considered to be nanomaterial if it is at or within the nanoscale in at least one external dimension, or has internal or surface structure at the nanoscale, or if it is smaller or larger than the nanoscale in all dimensions and exhibits one or more nanoscale properties/phenomena. "Nanoscale properties/phenomena" means properties that are attributable to size and their effects.

Source: Garduño-Balderas et al. *(9)*.

2.1 Categorization of nanoparticles

Based on the Project on Emerging Nanotechnologies *(10)*, the bulk of nanoenabled products that are commercially available today fall within the category of consumer products. Among these, the most commonly used ENMs are metal and carbon based (Figure 2.1). Here, ENMs are categorized into four main groups based on their chemical composition, namely carbon-based ENMs, metal-based ENMs, organic nanomaterials and composites. Some of their characteristics are described below.
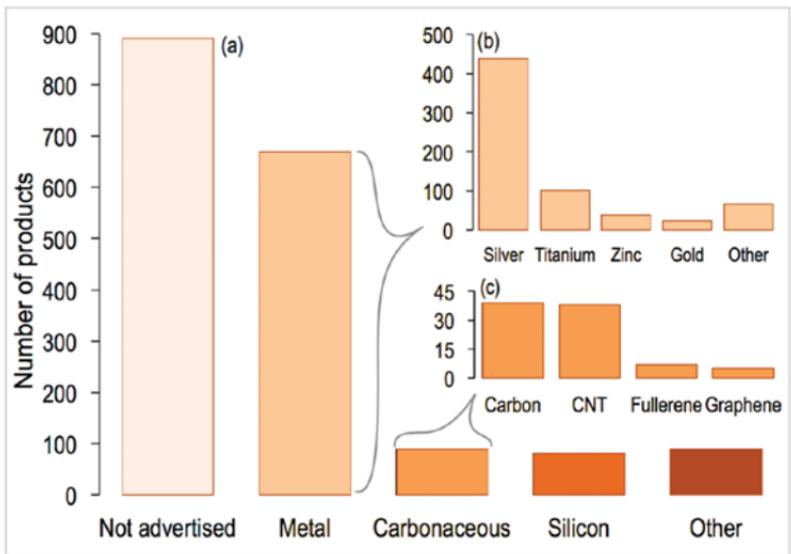
Figure 2.1 Composition of ENMs found in nanoenabled products
Source: Consumer product inventory maintained by the Project on Emerging Nanotechnologies; taken from Vance et al. *(11)*.

2.1.1 Carbon-based nanomaterials

(a) Carbon black

Carbon black nanoparticles contain more than 97% of carbon. They are manufactured by partial combustion or thermal decomposition of gaseous or liquid hydrocarbons. They are used

in rubber products and as pigment in plastics and paints. Recently, fluorescent black carbon nanoparticles have been synthetized by soot-based methods, producing different colours of nanoparticles that can be isolated by electrophoresis yielding less that 0.1% of fluorescent black carbon nanoparticles, and some other improved methods using a combination of solvents and centrifugation leading to yields close to 3% *(12)*.

(b) Single-walled carbon nanotubes (SWCNTs)

These structures can be viewed as cylinders of graphene rolled along a lattice vector in the graphene plane. This vector determines the chirality and diameter of the nanotube; the combination of these parameters, in turn, decides whether the nanotubes are metallic or semiconducting *(13)*. Chemical vapour deposition is one of the most common methods for SWCNT synthesis using temperatures greater than 800°C, which allows a highly dense and well aligned SWCNT that enhances electrical properties. Laser ablation and arc discharge are also used. The presence of metals within SWCNTs is a disadvantage of this method; this can be solved by selective electrical burning of metals *(14)*.

(c) Double-walled carbon nanotubes (DWCNTs)

DWCNTs are coaxial nanostructures composed of SWCNTs. The chemical vapour deposition method of methane is used for their synthesis, though the diameter cannot be entirely controlled by this method. However, iron silicide can be used for a higher nucleation efficiency to obtain a more restricted diameter of DWCNTs *(15)*. However, some other reagents used for catalysis, such as FeMo/MgO, in low-temperature (550°C) conditions yield high-purity DWCNTs *(16)*. The arc discharge and peapod methods are also used for their synthesis. SWCNTs are obtained as impurities during synthesis of DWCNTs, including amorphous carbon, but the synthesis can be purified by scavenging the compounds used as catalysts in low pH followed by high-temperature oxidation.

(d) Multi-walled carbon nanotubes (MWCNTs)

MWCNTs are multiple rolled structures. They can be synthetized by chemical vapour deposition of methane, laser ablation, or the

arc discharge and peapod methods. Metals (such as iron or nickel) used as catalysts are the main impurities during synthesis; they can be removed by graphitization. The inner and outer diameters of the MWCNTs can be modified by the catalyst particle size and the flow rate of hydrocarbon at the catalyst particle surface *(17)*.

(e) Graphenes

Graphene is defined as a single layer of carbon atoms with each atom bound to three neighbours in a honeycomb structure *(18)*. Derived from graphite, graphene is an allotropic, crystalline form of carbon that can be obtained in a two-dimensional or three-dimensional structure. Graphene-based nanoparticles can be classified based on the number of layers, the average lateral size and the carbon–oxygen ratio. Graphenes are produced as monolayer and multilayer nanoparticles. Graphene oxide and reduced graphene oxide can be synthesized and used in various applications *(19)*. Mechanical exfoliation, liquid phase exfoliation, assembly of tailored precursor molecules, epitaxy on silicon carbide and chemical vapour deposition are methods for graphene production *(20)*. Graphene is a zero band gap semiconductor with each carbon atom exposed on the surface, enhancing the surface area (approximately 2630 m^2/g) and leading to a high electrical conductivity nearly independent of temperature between 10 and 100 Kelvin *(21)*. Graphene-based nanoparticles are currently used in display devices, and are expected to have a higher number of applications in electronics and photonics. Physicochemical properties of graphenes include resistance to corrosive reactions, which makes them suitable for coating surfaces. They can also be used as a gas barrier material because graphene does not allow the diffusion of small gases through its plane.

(f) Diamonds

Nanodiamonds are synthetized by detonation using explosives in a chamber with nitrogen, carbon dioxide and water. Nanodiamonds are formed at high pressure – above 15 gigapascals (GPa) – and temperatures between 2000 Kelvin and 4000 Kelvin *(22)*. Pure diamond particles are optically transparent and completely non-fluorescent. Mechanical and optical properties, high surface areas and tunable surface structure are some of the useful properties for electronic and biomedical purposes. Functionalization of

nanodiamonds (hydrogen, hydroxyl, carboxyl, ethylenediamine, octadecylamine-functionalized nanodiamonds) renders fluorescent structures that are expected to replace (for instance) quantum dots, which are fluorescent and used in bioimaging but exhibit higher toxicity than nanodiamonds *(23, 24)*. Depending on the pH, nanodiamonds also exhibit different redox potential that can extend their usages *(24)*.

(g) Fullerenes

Harold Kroto, Robert Curl and Richard Smalley discovered in 1985 a new carbon allotrope in a truncated icosahedron structure, known as fullerene, which is a structure consisting of 60 atoms of carbon (C60). This discovery won the Nobel Prize for Chemistry in 1996. Fullerene is defined as a molecule composed solely of an even number of carbon atoms, which form a closed, cage-like, fused-ring polycyclic system with 12 five-membered rings, the rest being six-membered rings *(18, 25)*. The vaporization of graphite by resistive heating under carefully defined conditions, for example, produces fullerenes that can be isolated by chromatography *(26)*. C60 has one stable isomer but there are stable isomers for C76, C78, C84 and C100 *(26)*. The calcination of fullerenes leads to carbon dots, which are water soluble and photostable *(27)*.

2.1.2 Metal-based nanomaterials

(a) Titanium dioxide (TiO$_2$)

TiO$_2$ exists in nature mainly in three different crystalline forms: anatase, rutile and brookite, with rutile and anatase being more common. Anatase has a larger band gap – 3.2 electronvolts (eV) – which implies a higher conduction position compared to rutile (3.0 eV). For this reason, anatase is a better photocatalyst *(28)*.

TiO$_2$ can be engineered in various shapes, such as nanospheres or nanobelts. It is a highly stable semiconductor compound due to its wide band gap under UV light. It has electronic properties that allow its usage in the electronic field. TiO$_2$ is often used in consumer and personal care products (for example sunscreens and cosmetics) as a UV filter. Its ability to scatter light to give a consistent and stable whitening effect has also been exploited in its use as a pigment in

paints (PW6 or CI 77891) and as a food colouring additive (E171). Being photocatalytic, TiO_2 produces reactive oxygen species (ROS) under light and can act as an antimicrobial agent. TiO_2-based antimicrobial sprays have emerged as a result.

(b) Zinc oxide (ZnO)

ZnO is a semiconductor compound (band gap 3.37 eV) that exhibits a high surface area to volume ratio. ZnO nanoparticles are the third most commonly produced nanomaterial worldwide *(29)*. It can be synthetized by laser ablation, hydrothermal methods, electrochemical deposition, sol–gel methods, chemical vapour deposition and combustion methods *(30)*. Similar to TiO_2, ZnO is photocatalytic and is used in sunscreens as a UV filter.

(c) Iron

Magnetite (Fe_3O_4), haematite $(\alpha\text{-}Fe_2O_3)$ and maghemite $(\gamma\text{-}Fe_2O_3)$ are three of the most magnetic iron-based nanoparticles. Those nanoparticles have magnetic properties (specifically, superparamagnetic properties) that can be used in combination with external magnetic fields for different purposes.

(d) Silver

Silver is a metal that can be found pure in nature, and is used as an alloy with other metals. Silver nanoparticles (Ag-NPs) can be obtained by laser ablation and evaporation–condensation methods, among others *(31)*. Using reducing agents such as hydrazine, ethylene glycol, UV light and some others, and silver nitrate as a precursor, Ag-NPs can be obtained. Currently, silver is the most widely used ENM among known nanoenabled products (Figure 2.2). The most common reason to use Ag-NPs is their antimicrobial ability. Products that have exploited this property include textiles (clothing), electronics, food packaging, and wound dressings.

(e) Gold

Gold has been considered a precious metal throughout much of human history. It is an inert metal that can be found in nature but is

also alloyed with some other metals, such as copper. Synthesis of gold nanoparticles can be achieved by a two-phase transfer method (based on the Brust-Schiffrin method), which uses a thiol group that is bound to gold in an aqueous phase, which is then transferred to an organic phase. Because of their inertness, gold nanoparticles are often explored for applications in nanomedicine, for example as drug carriers.

(f) Quantum dots

Quantum dots are inorganic compounds with a core of elements from groups II–VI or III–V in a crystalline form made up of 100 to 100 000 atoms. They have high photostability, tunable fluorescence under single wavelength excitation, and a longer lifetime compared to conventional fluorophores *(32)*. Quantum dots have a metalloid core that is formed by cadmium selenide, lead selenide or indium arsenide (CdSe, PbSe or InAs) enclosed within a shell coating, comprising a different semiconductor material of higher band gap followed by a polymer coating *(33)*. The shell protects the core from oxidation and degradation, and the polymer coating can be an amphiphilic coating such as polyethylene glycol (PEG)-based bidentate ligands *(34)*.

2.1.3 Organic-based and other nanomaterials

(a) Polymer-based nanoparticles

Tomalia and colleagues were the first to describe the synthesis of dendrimers in 1984. They defined dendrimers as structures with an initiator core, interior layers called generations that are composed of repeating units radially attached to the initiator core, and an exterior layer attached to the outermost interior generation *(35)*. This first synthesis was developed using poly(amidoamine) (PAMAM) by a Michael addition reaction with ammonia and ethylenediamine as initiator cores.

PAMAM dendrimers from generation 1 (84 atoms) to generation 11 (nearly 300 000 atoms) can be synthetized by divergent methods, in which dendrimer grows outwards from a multifunctional core molecule, or by convergent methods, in which the dendrimer is starting from the end groups and progressing inwards *(36)*.

(b) Lipid-based nanoparticles

The synthesis of lipid-based nanoparticles can be achieved by emulsification methods. Stability can be modulated by the usage of cationic lipids *(37)*, but the symmetry of lipids, such as cholesterol, can influence the capability of interaction with other membranes *(38)*.

Solid lipid nanoparticles are synthetized by replacement of the lipid of an oil and water emulsion by a solid lipid. For instance, glyceryl monostearate could be used in a mixture of acetone and ethanol in a water bath to produce an organic solution that is mixed with an acidic aqueous phase to produce the solid lipid nanoparticles *(39)*. Organic solvent-free methods are also used for synthesis of solid lipid nanoparticles *(40)*. The usage of one or two surfactant reagents has strong influence on the size of this type of nanoparticle *(40)*.

Gelatin-based nanoparticles can be obtained by collagen alkaline hydrolysis. The most common methods include desolvation, emulsification and self-assembly technics.

(c) Nanoclays

Nanoclays are included in this category. Most of them have silicate-related compounds. The uses of nanoclays are expanding. It has been suggested that they can be used in pharmaceutical products and also for nanocomposite synthesis.

(d) Silicon dioxide (SiO$_2$)

SiO$_2$, or silica, exist both as an amorphous material and with different crystalline forms, including α-quartz, β-quartz, α-tridymite, α and β forms of cristobalite, keatite, coesite, and stishovite. Silicon–oxygen bond lengths vary between the different crystalline forms, but the Si–O–Si angle also varies between a low value in α-tridymite, up to high values in β-tridymite. Synthetic amorphous silica or SiO$_2$ nanoparticles can be produced by sol–gel or pyrogenic methods to obtain homogeneously sized nanoparticles. SiO$_2$ has very wide industrial uses, although they are not necessarily used in the nanoscale, as SiO$_2$ preparations mainly consist of aggregates or agglomerates of the primary nanosized SiO$_2$ particles. These applications range

from structural fillers in composite materials to abrasive agents in toothpastes and as anti-caking agents in food (E551).

2.1.4   Composite nanomaterials

It is also possible for nanomaterials to be made from combinations of the different multiphase materials described above, for example in the form of physical blends, copolymers, gels or core-shell structured nanoparticles. They can also be shaped as flowers, diamonds and other forms. There is no limit to the components that can be used to make a nanocomposite. Some are synthetized by few components, for instance, the supercapacitors made by reduced graphene oxide and TiO_2 nanorods; some others have a nanoparticle core and are coated with or functionalized by other nanoparticles, for instance, diamonds coated with nickel *(41)* or Fe_2O_3/gold placed on a substrate or matrix *(42)*. In addition, nanocomposites can be made using a multilayer system, such as those designed for solar cells, which can include layers of metal-based, carbon-based and organic nanoparticles (Figure 2.2). The combination of different types of nanoparticles leads to physicochemical properties that differ completely from those of the isolated components *(43)*.
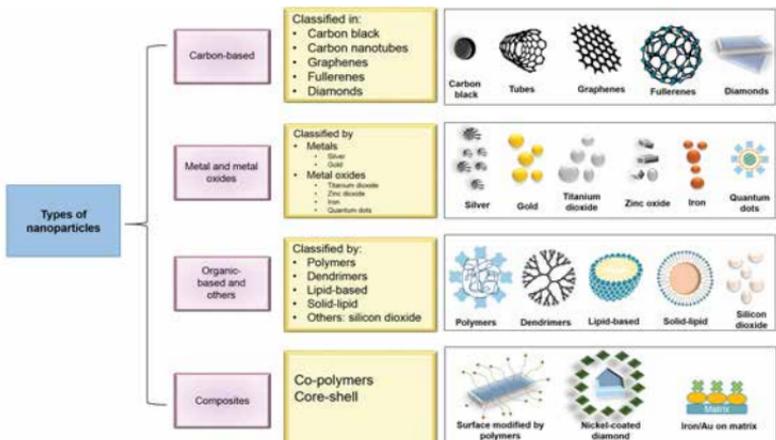
Figure 2.2 Types of nanoparticles according to chemical composition

2.2 Next ENM generations

ENMs are being readily applied in various nanotechnology products, and can be categorized into four generations.

- First-generation nanoproducts include simple passive nanostructures that are used in various consumer products such as cosmetics and food. The properties or functions of nanomaterials are not expected to change during their use.
- Second-generation products include more complex active nanostructures such as fertilizers, the properties or functions of which are expected to change during their use. These changes can be unintentional in response to their local environment or intentional, as in the case of drug delivery.
- Third-generation products involve uses of passive or active first- or second-generation nanostructures to build nanosystems, for example, the development of synthetic organs or engineered microbes, or self-assembling materials that assemble into new structures in the body upon their release.
- Fourth-generation nanostructures, which are still in the early stage of development, involve molecular nanosystems with specific functions, such as molecular devices used in genetic therapy.

The synthesis or the above-mentioned ENMs as primary structures gives rise to first-generation ENMs. The passive nanoparticles of the first generation, coated or functionalized with biological effects, belong to the second generation of ENMs, which have been produced since 2000. The third generation is related to the capability of assembling ENMs in more complex systems, for instance, polymer grafts used as assembly-regulating molecules able to bind to the surface of nanoparticles for use in spectroscopy. The fourth generation, which is expected to be developed in the coming decade, will be used in molecular devices with active functions.

2.3 Life cycle of nanomaterials

The focus of this document is on engineered nanomaterials (ENMs) – either intentionally produced to satisfy a particular function, or unintentionally produced because of anthropogenic

activities. Nanomaterials that occur due to natural processes, such as volcanic ash, are not within the scope of this discussion. Regardless of nanomaterial type, ENMs begin their life cycle at the point of synthesis, go through several stages of transformation, and end with final disposal *(44)*, usually in wastewater treatment plants or landfills. The life cycle of nanomaterials includes four stages.

Stage 1

The first stage is the manufacturing process, in which ENM exposure in the workplace represents a potential hazard. The manufacturing process can include synthesis of the nanoparticles or using nanoparticles acquired from manufacturers as ingredients to fabricate a nanoenabled product. Not only personnel in charge of production or direct handling of the nanomaterial could be exposed to inhalation, dermal contact or ingestion, but also maintenance and cleaning personnel and administrative staff. This first stage also includes storing nanomaterials or modifying the surfaces of primary nanoparticles for further processes. Textiles, paints, cosmetics, food and electronics industries involve personnel who can be exposed in occupational settings.

Stage 2

The second stage involves transporting nanoenabled products. In this stage nanomaterials could undergo some transformation as they can interact with packaging materials or may be subjected to varying conditions such as temperature fluctuations or UV exposure, with relevant risks to the personnel handing the materials. In addition, it has been estimated that between 0.1% and 2% of ENMs are released unintentionally to the environment, including water, the atmosphere and soil.

Stage 3

In the third stage, nanoenabled products are used by the consumer, resulting in ENMs being potentially released into the environment as a consequence of usage. As examples, clothing treated with antimicrobials can release Ag-NPs during the washing process, food can be poured into raw wastewater releasing food-grade TiO_2 and

SiO_2, or sunscreen containing ZnO or TiO_2 may be washed off in swimming pools. All of these ENMs may end up and accumulate in wastewater treatment plants and other disposal facilities.

Stage 4

The fourth stage occurs only when nanoenabled products are intentionally delivered as waste (end-of-life stage). In most cases, ENMs are disposed of in landfills, raw sewage or incinerators. These could result in uncontrolled release of ENMs into the environment through soil, water or air, and eventually result in their presence in the food chain.

Final disposal and recycling

In contrast to the recycling advances in other fields, there is no information about how to dispose of or recycle ENMs. Indeed, the technical data sheets for commercial nanomaterials for research have little or no information about final disposal of ENMs, and there are few regulations on the matter, even in developed countries. Most ENMs end up in landfills, while there are also significant accumulations in soil and water, followed by the atmosphere. Insoluble nanoparticles such as TiO_2 and SiO_2 remain as solid particles, including as agglomerates or aggregates, while some others release ions, for example iron and zinc nanoparticles. This represents a challenge in terms of recycling, as several of these materials cannot be recovered. In addition, the concept of recycling ENMs has not been fully addressed by researchers or industries, as recovery of ENMs from waste products or polluted water or soil is difficult and is rarely considered to be cost-effective.

2.4   Functionalization

Surface modification is performed to confer new properties to nanomaterials, which could include a decrease in toxicity (but also an inadvertent increase in toxicity), enhanced solubility, binding of specific molecules, and increased stability. Functionalization techniques might employ hydrophilic or hydrophobic molecules, which in turn can be organic or inorganic and can consist of polymeric or non-polymeric forms (coatings). Functionalization can confer new properties to the primary nanoparticle, for instance, graphene

oxide can be modified with poly(ethylene glycol)–poly(ethylene imine) copolymers tagged with folic acid for biological purposes. Such functionalization induces enhanced cellular uptake and photoluminescence *(45)*. For example, CNTs can be functionalized by covalently attaching carboxyl or amine groups to the surface to increase solubility or by atomic layer deposition coating with metal oxide for enhancing conductive properties. All of these functionalizations alter macrophage innate immune responses and either increase or decrease fibroproliferative responses in the lungs of mice *(46, 47)*. Another example is the attachment of fullerenes to SWCNT to change its functional properties *(48)*. Some types of functionalization have no influence on the intrinsic properties of nanoparticles, such as the functionalization of quantum dots with PEG, which does not interfere with their optical properties *(34, 49)*. However, the use of PEG as coating can change the toxicokinetic behaviour of the ENM as it prolongs the circulation time of ENMs in blood *(50, 51)*.

Iron-based nanoparticles are coated with PEGylated[1] citrate, polyacrylate and silica, while multicore iron nanoparticles can be coated with dextran or carboxydextran *(52)*.

Recently, molecules such as diazonium salts are being used for functionalization of gold nanoparticles, SWCNT, nanosized TiO_2 and nanodiamonds *(53)*.

2.5 Interaction of nanoparticles with physiological fluids

The specific primary routes by which engineered nanoparticles may interact with the human body include inhalation, ingestion and application to the skin. In addition, parenteral application by injection is a primary route for medical therapeutic purposes, but will not be discussed within this document. Independent of the entry route, the particles inevitably encounter a complex physiological fluid populated with (for example) proteins, vitamins, lipids, salts and ions. Different consequences of such encounters may include formation of a surface-bound protein layer, or particle dissolution or aggregation, which are expected to have a crucial impact on cellular interaction *(54–56)*.

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[1] PEGylation is the process of attachment or amalgamation of polyethylene glycol (PEG) polymer chains to molecules and macrostructures, which are then described as PEGylated.

Opsonization is the process by which a foreign organism or particle becomes covered with opsonin proteins, thereby making it more visible for phagocytosis, which typically occurs in the bloodstream. This phenomenon could be considered similar to biocorona formation, in which biomolecules surrounding nanoparticles are bound to their surface. Normally, water, proteins, lipids and ions form the biocorona and modify the physicochemical properties of nanoparticles, including charge and shape.

A tightly bound, immobile protein layer formed on the particle surface (the so-called hard corona) and possibly a weakly associated mobile layer (the soft corona) have been described *(57)*. In addition to protein binding, lipids such as the surfactant lipids in the lung or lipids in the blood should also be considered, as well as the possible exchange of the soft corona within time.

The determination of the protein or lipid corona is complex and technically challenging, and an overview of some relevant methods will be given in Chapter 6, on hazard assessment. The biocorona may be of particular importance for the interaction of ENMs with cells of the immune system, including phagocytic cells such as macrophages.

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3. HUMAN EXPOSURE

3.1   Exposure to nanomaterials

With the increasing number of nanomaterial applications, the production of nanomaterials is also increasing proportionally. Thus, there is an increased potential for human and environmental exposure to nanomaterials, including nanomaterials released from products containing nanomaterials and during the life cycle of nanomaterials, from production, use, and recycling to disposal *(1)*. Exposure can be defined as "contact with a chemical, physical, or biological agent by swallowing, breathing, or touching the skin or eyes". Exposure can be short term (acute exposure), intermediate duration, or long term (chronic) *(2)*. Exposure sources for humans include occupational settings for workers, through products for consumers, and from the environment for the general population (Figure 3.1).
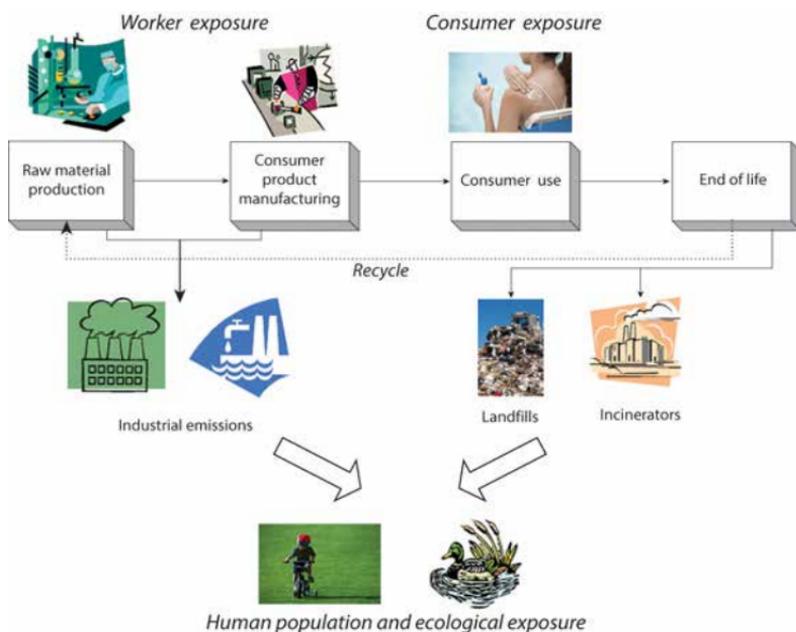
Figure 3.1 Life cycle exposure to nanomaterials
Source: Adapted from United States Environmental Protection Agency *(1)*.

3.1.1 Occupational exposure

Workers involved in the life cycle of nanomaterials, from the manufacturing, synthesis, formulation, handling, and packaging of nanomaterials, to the recycling of nanomaterials or products containing them, can be exposed to different amounts of nanomaterials depending on the work duration, frequency, occupational setting, and mitigation strategies. The various potential sources of occupational exposure depend on the manufacturing method, as shown in Table 3.1. Workplace air is known to contain manufactured nanomaterials, including nano-objects and their agglomerates and aggregates (NOAA). Along with the manufacturing process, handling, which includes coating, sonicating, dispersion, bagging, and cleaning the vacuum, can also generate NOAA in workplace ambient air.

Table 3.1 Potential sources of occupational exposure for various manufacturing methods

Manufacturing process	NOAA	Potential exposure source	Route of exposure
Gas phase	In air	Direct leakage from reactor	Inhalation
		Product recovery from bag filters in reactors	Inhalation or dermal
		Processing and packaging of dry powder	Inhalation or dermal
		Equipment cleaning or maintenance (including reactor and spent filters)	Dermal (and inhalation during reactor evacuation)
Vapour deposition	On substrate	Product recovery from reactor; dry contamination of workplace	Inhalation
		Processing and packaging of dry powder	Inhalation or dermal
		Equipment cleaning or maintenance (including reactor)	Dermal (and inhalation during reactor evacuation)
Colloidal	Liquid suspension	If liquid suspension is processed into powder, potential exposure during spray-drying to create powder, and processing and packaging of dry powder	Inhalation or dermal
		Equipment cleaning or maintenance	Dermal

Manufacturing process	NOAA	Potential exposure source	Route of exposure
Attrition	Liquid suspension	If liquid suspension is processed into powder, potential exposure during spray-drying to create powder, and processing and packaging of dry powder	Inhalation or dermal
		Equipment cleaning or maintenance	Dermal

Note: NOAA greater than 100 nm (ISO 12901-1).

Source: Adapted from Aitken, Creely and Tran *(3)*.

3.1.2   General population exposure through environment

Very little is known about general population exposure to nanomaterials, as to date there are very few quantitative and specific trace analytical methods for detecting nanomaterials in the environment. Notwithstanding, the general population can be exposed to nanoparticles in many possible scenarios, such as the release of nanomaterials from waste control systems, leakage, spillage, or other unknown or unexpected processes.

3.1.3   Consumer exposure

The potential use of ENMs in consumer products covers plastics, sporting equipment, electronic equipment and textiles – that is, applications where nanomaterials are embedded into a matrix. For textiles, ENM may be applied on the fibres or the surface. The main exposure from such consumer products would be from abrasion, grinding, cutting, washing and weathering of the products *(4–6)*. Dermal exposure via cosmetic products or sunscreens is also possible (for example, TiO_2 or zinc ENMs as present in sunscreens) along with minor inhalation or oral exposure. When compared with the data for occupational exposure, little is known about nanoparticles released from consumer products, which may differ from the original manufactured nanomaterials in terms of their physicochemical properties, such as size, surface area characteristics (chemical composition, corona), and shape. The physicochemical properties and hazard information related to released ENMs compared to pristine nanomaterials have not yet been studied extensively. Furthermore,

exposure to ENMs fabricated for nanobio or bionano products[2] may represent an important new safety concern, as exposure to these products and ENMs is usually via invasive administration. However, in this case, extensive data reviews and approval by regulatory authorities are normally required before use.

3.2   Route of exposure

Human exposure to nanomaterials can occur via various routes. Inhalation is an important route of exposure, and is the most studied. Depending on the use of nanomaterials, other exposure routes include oral ingestion, dermal exposure and parenteral exposure by injection.

3.2.1   *Inhalation exposure*

Inhalation is the main route for airborne NOAA to enter the bodies of workers. Once inhaled, nanomaterials are deposited in the various regions of the respiratory tract, according to particle size. As shown in Figure 3.2, the International Commission on Radiological Protection (ICRP) predictive mathematical model describes the fractional deposition of inhaled particles in the different regions of the human respiratory tract: head region (nasopharyngeal), tracheobronchial region, and alveolar region *(7)*. According to the ICRP model, for 1 nm particles, 80% are deposited in the head region, with 20% in the tracheobronchial region and less than 1% in the alveolar region. However, for 20 nm particles, 50% are deposited in the alveolar region, and 25% in the head and tracheobronchial regions. The main mechanism governing nanoparticle deposition is diffusion, whereas sedimentation and impaction govern the deposition mechanism for bulk particles.

For risk assessments, another means of predicting particle dosimetry in the human lung following exposure to airborne particulate matter is the multiple path particle dosimetry (MPPD) model *(8)*. In this model, the fractional depositions of particles in the human and rat lung were presented using three lung geometries: typical

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[2] Bionanotechnology generally refers to the study of how the goals of nanotechnology can be guided by studying how biological "machines" work, and adapting these biological motifs into improving existing nanotechnologies or creating new ones. Nanobiotechnology refers to the ways that nanotechnology is used to create devices to study biological systems.

Deposition probability
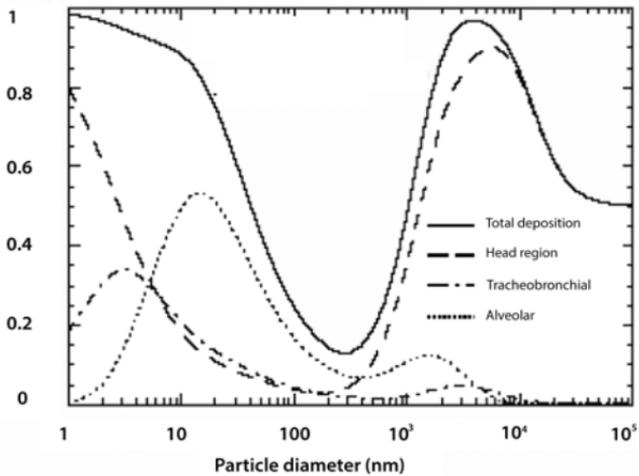
Figure 3.2 Predicted total and regional deposition of particles in human respiratory tract related to particle size using ICRP 66 model
Note: Deposition fraction includes probability of particles being inhaled (inhalability). Subject is a nose breather, performing standard work.

path symmetric, five-lobe symmetric, and five-lobe asymmetric (stochastic). The current version of the MPPD model (v3.04) is also applicable to prediction of deposition and clearance of nanoparticles *(9, 10)*. Little information is available on the magnitude of exposure, due to lack of personal sampling data. Most exposure studies have involved emission measurement from nanomaterial manufacturing or handling, or estimated emissions from nanocomposite product simulation studies. Although a harmonized approach to exposure to nanomaterials has been suggested *(11)*, standardized methods on nanomaterial exposure assessment have not been finalized.

3.2.2   *Oral exposure*

A number of nanomaterials are in use as food additives or in food contact materials. These applications include the nanoencapsulation of vitamins and flavours to protect them from deterioration during storage or allow controlled release, and the creation of specific nanosized micelles to deliver specific tastes. Synthetic amorphous silica (SiO_2 in

its aggregated form) is commonly used as an anti-caking agent in food products manufactured as powders. Many food and beverage products also contain nanoscale particles. Moreover, some of the nanomaterials used in food packaging can migrate into the food and be ingested, while some of the nanomaterials used in biocides can be ingested as food contaminants. Systemic exposure may be possible if insoluble nanoparticles are absorbed through the exposure routes. Once ingested, these nanoscale particles can be absorbed by the human gastrointestinal system and undergo various physical changes. Nanoscale particles may enter the systemic circulation intact. Some nanoparticles, such as silver nanoparticles (Ag-NPs), known and used as a biocide, can affect the gastrointestinal microbial milieu *(12)*. Standard methods for measuring nanomaterial characteristics and levels in complex matrices, such as food, are not yet available but are evolving. Furthermore, the absorption, distribution, metabolism and excretion of ingested nanomaterials are not well understood. Some attempts to characterize and estimate nanomaterials in food-relevant products internationally have been initiated by the International Life Sciences Institute NanoRelease project by organizing round-robin tests *(13)*.

3.2.3   Dermal exposure

In an occupational setting, in addition to inhalation exposure, dermal exposure to nanomaterials is also a concern, as certain skin areas can be exposed to nanomaterials. In addition, exposure through appendages (including hair follicles) may potentially occur *(14)*. However, dermal exposure may not necessarily result in the dermal penetration of nanomaterials into the epidermal layer and systemic uptake, especially in the intact skin. The keratin layer of the skin acts as the first barrier to dermal penetration. In general, uptake via the skin is considered negligible. However, a number of other factors also influence the dermal absorption of nanoparticles, including the location and skin conditions (for example, skin abrasions, atopic inflammation and allergic dermatitis) at the application site *(15, 16)*, physicochemical properties of the penetrating molecules, and physicochemical properties of the vehicle dispersing the penetrating molecules *(17)*. The lipophilic-hydrophilic gradient, pH gradient, and isoelectric point also influence the dermal absorption of nanoparticles *(18)*. Furthermore, the inclusion of solvents, surfactants, enhancers, and others molecules in a nanosuspension can alter or damage the

stratum corneum to induce a potential increase in the absorption of all or selected ingredients of the applied nanomaterial-containing formulation *(19–22)*.

3.2.4 Other routes

Although they are not normal routes of exposure to nanomaterials, intratracheal instillation, intraperitoneal injection and intravenous injection have been performed on experimental animals to identify potential hazards of nanomaterials for the respiratory tract, mesothelial layers and systemic circulation, respectively *(8, 9)*.

3.3 Epidemiology and health surveillance

Under the European Union-sponsored NANOSH project, Gulumian et al. *(23)* conducted a systematic review of health surveillance programmes used to protect workers from the potential risks of exposure to manufactured nanomaterials. A total of 30 citations related to health surveillance and epidemiology studies were identified and an additional 33 citations were identified from the references included in the initial 30 citations. After excluding five duplicate references, a total of 58 references were evaluated for inclusion in the systematic review, and five studies were finally selected, as shown in Table 3.2.

As the paper by Gause, Layman and Small *(24)* is more conceptual and introductory with no relevant health information, it is not discussed further here.

Lee et al. *(25)* conducted a health surveillance study of a silver nanoparticle (Ag-NP) manufacturing workplace, including an assessment of personal exposure levels to Ag-NPs, a walk-through evaluation of the manufacturing process, and the collection of blood and urine samples from the exposed workers. Two male workers, each with seven years in Ag-NP manufacturing, exhibited exposure at the work environment to silver concentrations of 0.35 and 1.35 micrograms per cubic metre ($\mu g/m^3$), respectively, where the blood silver levels were 0.034 and 0.135 micrograms per decilitre ($\mu g/dL$), respectively, and the urine silver levels were 0.043 $\mu g/dL$ and not detected, respectively. When reviewed by an occupational physician, their health status,

Table 3.2 Description of epidemiology and health surveillance studies included in systematic review

Study	Gause, Layman and Small (24)	Lee et al. (25)	Liou et al. (26)	Liao et al. (27)	Lee et al. (28)
Design	Descriptive programme case study	Descriptive programme case study	Cross-sectional; exposure case control study	Cross-sectional; exposure case control study	Cross-sectional; exposure case control study
Country	United States	Republic of Korea	Taiwan, China	Taiwan, China	Republic of Korea
Participants					
Exposure	Carbon-based nanomaterials	Silver nanomaterials	Metal, metal oxide and carbon-based manu-factured nanomaterials	Metal, metal-oxide and carbon-based manu-factured nanomaterials	MWCNTs
Exposure duration	NS	7 years	NS	NS	3.9 ± 3.9 years
Occupation or industry	Commercial research laboratory	Commercial, synthesis	Commercial, 14 plants handling nanomaterials	Commercial, 14 plants handling nanomaterials	Commercial, synthesis
Participant numbers	$N = 200$, of which ± 20% exposed	$N = 2$, both exposed	$N = 227$ exp $N = 137$ unexp	$N = 258$ exp $N = 200$ unexp	$N = 9$ exp $N = 4$ unexp
Participant age (years)	NS	37 and 42	Exp < 40: 173 Exp ≥ 40: 54 Unexp < 40: 89 Unexp ≥ 40: 48 ($P = 0.03$)	Exp < 40: 194 Exp ≥ 40: 64 Unexp < 40: 135 Unexp ≥ 40: 65 ($P = 0.12$)	Exp: 33.8 ± 4.9 y Unexp: 28.3 ± 4.4 y
Participant gender (% male)	NS	Male exp: 100%	Male exp: 78% Male unexp: 54%	Male exp: 76% Male unexp: 60%	Male exp: 10% Male unexp: 75%

Study	Gause, Layman and Small (24)	Lee et al. (25)	Liou et al. (26)	Liao et al. (27)	Lee et al. (28)
Medical surveillance					
Biomarkers or clinical	Clinical	Biomarkers, clinical	Biomarkers, clinical	Clinical	Biomarkers, clinical
Target	General health	"Routine" blood	Lung inflammation Oxidative damage Antioxidants Cardiovascular Neurologic Genotoxicity Lung function	Cardiovascular, respiratory, skin, neurological	Pulmonary stress biomarkers "Routine" blood
Indicators	"Periodic health status", lung function, chest X-ray	Silver (blood, urine)	Clara cell protein, heat shock protein 70, nuclear factor kB transcription factor activation, nitric oxide 8-ho-guanosine, mguanosine, isoprostane Superoxide dismutase, glutathione peroxidase, myeloperoxidase Fibrinogen, intracellular adhesion molecule-1, interleukin (IL)-6 Reaction time, memory test	Self-administered symptom question-naire, work-relatedness of symptoms	Malondialdehyde, H_2O_2, 4-HHE, nhexanal Cobalt, molybdenum (blood) Pulmonary function parameters

Table 3.2 (Contd)

Study	Gause, Layman and Small (24)	Lee et al. (25)	Liou et al. (26)	Liao et al. (27)	Lee et al. (28)
Programme outcomes					
Biomarkers	NA	Blood/urine silver not elevated	No difference in lung inflammation, oxidative damage or genotoxicity Antioxidants decreased Cardiovascular markers elevated	NA	Pulmonary stress increased, all three markers No difference in cobalt, molybdenum No changes in pulmonary function
Early signs	NA	1 No difference in lung function 2 Routine blood not deviant	1 out of 3 neuro-tests impaired No difference in lung function	NA	Routine blood not deviant
Clinical signs	NA	NA	NA	Increased prevalence of allergic dermatitis and "sneezing"	NA
Interpretation of outcomes	Nanospecific surveillance not deemed necessary	Surveillance measures did not detect any abnormalities	Although some biomarkers were elevated, these markers are not specific for nanomaterial exposure	Allergic dermatitis has multiple causes, not specific for nanomaterial exposure "Sneezing" non-specific symptom	Oxidative stress markers increased, but small sample and non-specific
Costs					
Monetary or resource use	NS	NS	NS	NS	NS
Coverage	NS	2 out of 5 workers participated	97% of workers participated	NS	NS

Note: exp = exposed, unexp = unexposed. NS = not stated, NA = not applicable.
Source: Gulumian et al. (23).

including blood chemistry and haematology, was determined to be within a normal range. In addition, the study reported that the silver nanomaterial manufacturing workers were exposed to much lower concentrations of silver dust and soluble silver threshold limit values, and no significant findings were recorded regarding their health status.

One additional case report of work-related argyria has been documented in the Republic of Korea. A 27-year-old male, employed as a technician plating mobile telephone subunits with aerosolized silver for four years, presented with asymptomatic blue-to-grey facial discoloration that had progressed over four months, consistent with general argyria. The patient also showed silver deposits in his sclera, conjunctiva and oral mucosa. No other adverse physical or organ effects were observed. A laboratory investigation confirmed that his complete blood count, chemistry panel, liver function test, and routine urinary analysis were all within a normal range. However, his serum silver level was elevated at 15.44 μg/dL (normal range, 1.1–2.5 μg/dL) and urinary silver was 243.2 μg/L (normal range, 0.4–1.4 μg/L). The lead, mercury and nickel levels were all within a normal range. A histopathological examination of a 3-millimetre punch biopsy specimen from the patient's face revealed silver granules in the epidermal basal layer. In addition, fine, minute, round, and brown-to-black silver granules were deposited in the basement membrane zone surrounding the eccrine sweat glands. The physician reported a case of generalized argyria resulting from the use of aerosolized silver in the mobile telephone subunit industry, which is apparently the first case of its kind based on an extensive literature search of English publications *(29)*. While unaware of the exact use of nanosilver in the workplace, the physician concluded that the patient's four-year work exposure to silver particles, whether nanoscale or non-nanoscale, seemed to induce the general argyria.

Lee et al. *(28)* studied an MWCNT manufacturing workplace with four office workers and nine manufacturing workers and used several biomarker tools, including levels of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), 4-hydroxy-2-hexenal (4-HHE) and n-hexanal in exhaled breath condensate (EBC), lung function parameters, blood metal (cobalt and molybdenum) concentration as a surrogate for MWCNT exposure, and routine haematology and biochemistry, along with the workplace air concentration of MWCNTs in terms of the total suspended particulate concentration

and elemental carbon concentration. The workers exhibited a normal range of haematology and blood biochemistry values and normal lung function parameters. When analysing the EBCs, the MDA, 4-HHE, and n-hexanal levels in the MWCNT manufacturing workers were significantly higher than those in the office workers. The MDA and n-hexanal levels were also significantly correlated with the blood molybdenum concentration, suggesting MDA, n-hexanal, and molybdenum as useful biomarkers of MWCNT exposure *(28)*.

Several epidemiological studies were recently conducted on workers in Taiwan, China (227 exposed and 137 unexposed workers) handling 14 different ENMs *(26, 27, 30)*. The 227 exposed workers showed a depression of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, and increased expression of cardiovascular markers, including fibrinogen, intracellular adhesion molecule, and IL-6 *(26)*. In a six-month follow-up study, 38 CNT handling and manufacturing workers showed a depression of glutathione peroxidase, significant changes in a comet assay (L/H ratio, tail-to-head ratio), and significant reduction of the lung function parameters, such as the peak expiratory flow rate and a forced expiratory flow of 25% *(27)*. However, since these studies lack proper measurement of the ambient nanomaterial concentrations the workers were exposed to, this limits any correlation between the biomarkers and the nanomaterial exposure.

Similar variables were also analysed in two studies that collected samples from 14 different nanomaterial handling plants *(26, 30)*. Due to the different settings from which the workers were recruited for these studies, the exposure to nanomaterials was heterogeneous and included exposure to metals, metal oxides and carbon-based nanomaterials *(26, 30)*. Both studies stratified the exposed participants into two risk groups. The risk stratification was conducted following the control banding tool developed by Paik, Zalk and Swuste based on the severity score of the nanomaterial toxicity and the score of the exposure probability *(31)*. Risk group 1 was considered the lower-risk group and comprised 139 participants (35 female, 104 male) in the study by Liao et al. *(30)* and 128 participants (31 female, 97 male) in the study by Liou et al. *(26)*. Risk group 2, the higher-risk group, had 119 participants (26 female, 93 male) in Liao's study and 99 participants (19 female, 80 male) in Liou's study. Liao's study also

used a self-reported symptom questionnaire to assess the prevalence of cardiovascular, respiratory, skin and neurological symptoms and diseases between the exposed and unexposed workers. The work-relatedness of the symptoms was also assessed through the questionnaire by asking whether or not the symptom was present before being exposed to nanomaterials. The reported symptoms and diseases were further checked by an occupational physician *(30)*. The only symptom identified as significantly work related was sneezing (5.88% in risk level 2 and 7.91% in risk level 1 versus 2.00% in controls, $P = 0.04$). The prevalence of a work-related dry cough ($P = 0.06$) and productive cough ($P = 0.09$) among the nanomaterial handling workers was also higher than that among the controls. The only disease significantly worsened by work was allergic dermatitis (4.20% in risk level 2, 0% in risk level 1 versus 0.50% in controls, $P = 0.01$). The incidence of angina was also higher among the nanoworkers than among the controls ($P = 0.06$).

More recently a cross-sectional health surveillance study on large-scale manufacturing of MWCNTs along with relatively high occupational exposure levels has been reported *(32)*. All air samples were collected at the workplaces from both specific areas and personal breathing zones using filter-based devices to quantitate elemental carbon and perform particle analysis by transmission electron microscopy (TEM). Biological fluids of nasal lavage, induced sputum and blood serum were obtained from MWCNT-exposed and non-exposed workers for assessment of inflammatory and fibrotic markers. The study found that exposure to MWCNTs caused significant increase in IL-1β, IL-6, tumour necrosis factor (TNF)-α, inflammatory cytokines and KL-6, a serological biomarker for interstitial lung disease in collected sputum samples. Additionally, the level of transforming growth factor (TGF)-β1 was increased in serum obtained from young exposed workers, indicating accumulation of inflammatory and fibrotic biomarkers in biofluids of workers manufacturing MWCNTs *(32)*.

Trop et al. *(33)* reported on a burn victim who had an argyria-like condition and elevated activities of liver-specific plasma enzymes when an Acticoat™ dressing (containing ionic Ag-NPs) was applied to his wound. No mention was made of silver being sequestered in the liver, although this was possible since levels of the metal were

elevated in plasma and urine. As reported by the authors, local treatment with Acticoat™ dressings for seven days caused the plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) to rise incrementally to 233 and 78 units per litre, respectively (upper limits of normal: 33 units per litre for ALT and 37 units per litre for AST). The concentration of silver in blood plasma was 107 micrograms per kilogram (μg/kg); this value subsequently dropped towards normal levels (13.3 μg/kg after 97 days) when Acticoat™ dressings were changed on day 8 to dressings containing betadine ointment. The C-reactive protein level was also elevated, in parallel with plasma silver levels, reaching a maximum concentration of 128 milligrams per litre (mg/L) after four days. The level of this liver-synthesized marker for acute inflammation was back to normal (5 mg/L) after eight silver treatment-free days. In a prospective study of 30 patients with graft-requiring burns, Vlachou et al. *(34)* found increased concentrations of silver in serum (median, 56.8 μg/L; range, 4.8–230 μg/L) when Acticoat™ dressings were applied to the wounds. They found no changes in haematological or clinical chemistry parameters indicative of toxicity associated with the silver absorption, and at the six-month follow-up, the median serum level had declined to 0.8 μg/L *(34)*.

3.4 Toxicokinetics

3.4.1 Introduction

In the safety assessment, toxicokinetics may be seen as bridging the gap between exposure and toxicology. Toxicokinetic testing gives information on the fate and behaviour of the substances under evaluation and provides insight into potential target organs and organ burdens that may ultimately result in toxicity. As in the case of other substances, it describes the study of absorption, distribution, metabolism and excretion as potentially sequential processes. However, especially for certain metal and metal oxide nanomaterials, it may be debatable whether metabolism does occur. It is very important to have good insight into the behaviour of nanomaterials in the body. The major risk associated with nanomaterials is related to the presence or release of free nano-objects, ions or components that comprise the individual nanomaterials, for example when nanoparticle

aggregates or agglomerates disintegrate and release the comprising primary nanoparticles. Biodistribution over the various organs will determine the possibility of inducing harmful effects.

The biodistribution of nanoparticles is influenced by a number of factors, including size, surface charge, and surface composition, such as protein binding and coating *(35–37)*. When nanomaterials are used in test systems, some of the properties that need to be determined can be affected and are largely dependent on the surrounding environment (for example, tissue culture media, blood or serum, or protein presence). Such interactions with the environment may result in a temporal evolution of the nanomaterials themselves, for example by obtaining or shedding a protein coating, the formation of nano-object agglomerates or aggregates, and other changes in the nanomaterials. Such changes may affect the nanomaterial characteristics, which can impact the toxicological profile of a nanomaterial. Factors such as route of administration, size of the nano-object or its aggregates or agglomerates, surface properties (chemistry and charge), animal species, dose and dosing methods have all been reported to influence the toxicokinetics in animal models *(35, 36, 38–40)*.

3.4.2   Methods to evaluate toxicokinetics of nanomaterials

OECD test guideline (TG) 417 describes the toxicokinetic studies performed for chemical substances and explicitly states that it is not intended for the toxicokinetic testing of nanomaterials. Analogously, OECD TGs 427 (in vivo) and 428 (in vitro) for dermal penetration were developed for chemicals and have not proven valid for nanoparticles. Therefore, the use of such methodologies should be evaluated on a case-by-case basis. However, a number of basic requirements, as indicated in OECD TG 417, can be applied also to nanomaterials or nanoparticles, for example the study design and number and sex of animals to be used. In view of the possible long retention time (as mentioned above), the time schedule indicated in OECD TG 417 of an evaluation typically at day 7 after administration is not applicable for nanomaterials, though prolonged evaluation up to 14 days is also mentioned. A more prolonged time schedule, for example of 90 days, as used by Geraets et al. *(41)*, might be more appropriate. For certain nanomaterials that shed ions or slowly dissolve or degrade (such as nanosilver, nano ZnO), shorter time periods may need to be

considered. Studies in spleen, liver, lung and fat, and even brain, need to be performed as these are likely to be depot sites for nanomaterials.

As for all (toxico)kinetic studies with nanomaterials, a critical point is the availability of a measurement system for detection of the nanomaterials. However, detection of nanoparticles in tissues and organs is complex. Electron microscopy is not generally applicable for quantitative measurements of nanomaterials or for all nanomaterials. To date, most studies on toxicokinetics of nanomaterials have used elemental analysis of the components of the nanomaterials, for example zinc for ZnO, titanium for TiO_2, or silver for Ag-NPs *(42)*. For metal and metal oxides, analysis could be performed by using inductively coupled plasma mass spectroscopy (ICP-MS) or atomic absorption mass spectroscopy. Although this provides a good indication of the possible tissue distribution, the limitation is that the nanoparticles themselves are not detected or measured. With ICP-MS generally the tissue including the nanomaterial is completely dissolved under acid conditions, so only the elements composing the nanomaterial are detected. Whether these are present as nanoparticles or as previously dissolved ions is unknown. In combination with separation techniques such as field flow fractionation it is possible to evaluate the presence of metallic particles in tissues using so-called single particle ICP-MS *(43)*.

Specific labelling of nanomaterials to follow their fate in vivo can be done by using radioactive isotopes as radiolabels or fluorescent dyes. This can be done by adding a label to the nanomaterial by specific binding, or by using a radioisotope of the nanomaterial itself. A disadvantage of specific labelling is that the label can detach from the nanomaterial *(44)*. In that case the label is measured while it is detached from the nanomaterial, so incorrect or false information is obtained on the distribution of the nanoparticles. Therefore, when using any specific labelling technique a careful evaluation of the integrity of the label–nanoparticle combination is necessary. Alternatively, radioactive isotopes may be used that are isotopes of a metal that is part of the nanomaterial (for example, gold or silver). With this approach, there is some certainty that the nanoparticles themselves are detected, although for Ag-NPs there is still uncertainty regarding the release of silver ions. In addition, naturally stable isotopes may be used to demonstrate uptake from the application site *(45)*. By using

ZnO nanoparticles enriched for [68]Zn it was demonstrated that zinc present as ZnO nanoparticles in sunscreen formulations was taken up from the skin application site *(45)*. The [68]Zn could be detected in the blood and urine of human volunteers. As the [68]Zn was evaluated using ICP-MS measurements it was not known whether [68]Zn had been absorbed as ZnO particles or soluble zinc or both. Also, in internal organs of mice, including the liver, the presence of [68]Zn was observed, but here also penetration of the ZnO nanoparticles themselves could not be established *(46)*. The zinc homeostasis was largely maintained and the presence of ZnO particles in the sunscreen did not elicit an adverse biological effect *(46)*.

So, depending on the methodology used for nanoparticle detection, there may be uncertainty whether the nanomaterial or the released ions are detected, especially for metallic nanomaterials that can release ions (such as silver or ZnO).

Another method that may be specifically suitable for metal nanoparticles is neutron activation of the metal nanoparticles. Using this method with [198]Au, very low levels of translocation from the lung were demonstrated for 1.4 nm and 18 nm gold nanoparticles and for ^{192}Ir aerosol of ultrafine particles varying in size between 15 and 20 nm *(47, 48)*. This neutron activation can also be done after the administration of the metal nanoparticles for detection of the presence in organs.

As uptake via the various routes of administration may generally be rather low (see below), the determination of such low uptake and translocation remains a challenge when studying the toxicokinetics of nanomaterials. For example, the limit of detection for the used ICP-MS method to evaluate tissue distribution of metal and metal oxide nanoparticles is about 50 nanograms per gram (ng/g) of tissue *(49)*. So, instead of using one of the major exposure routes (skin, gastrointestinal tract or lung), the intravenous route of administration is also commonly used for toxicokinetic studies in order to identify potential target organs. However, the route of administration may also affect the systemic distribution, as has been demonstrated for the intravenous versus the inhalation route *(40, 48, 50, 51)*. This can be explained by interactions of the nanoparticles with lung fluids or intestinal tract fluids before reaching the absorbing epithelium. The

nanoparticles can become coated with a range of different proteins when they come in contact with biological fluids; this is commonly referred to as the "protein corona" *(52, 53)*.

3.4.3 *Absorption, distribution, metabolism and excretion*

Absorption

Skin

Dermal penetration can be evaluated with various in vitro systems using the skin of many mammalian species, including humans. Nanoparticle quantitation remains a problem in these studies, especially in view of the fact that dermal penetration of nanoparticles is generally considered to be low or absent *(54–57)*. In general, nanoparticle penetration of the skin is limited to the first cell layers of the stratum corneum *(54)*. However, for some nanomaterials, there seems to be limited uptake. For example, when ZnO nanomaterial was applied on the skin in a sunscreen formulation, the presence of zinc in the blood originating from the ZnO in the sunscreen was observed *(45)*. However, it cannot be deduced from the study whether the zinc was taken up in particle or in an ionic form.

Ag-NPs are widely used as antimicrobial agents, for example in wound dressings *(58, 59)*. In an in vitro system using human skin exposed to Ag-NPs, a low translocation into the receptor fluid was found, which was increased fivefold in damaged skin *(60)*. The presence of elemental silver was determined with electrothermal atomic absorption spectroscopy, which cannot discriminate between silver ions and silver particles, so translocation of nanoparticles was not demonstrated. Some absorption of silver does occur; after treatment of burn patients with wound dressings containing nanocrystalline silver, increased blood silver serum levels were observed, similar to the situation for zinc absorbance from sunscreen formulations containing ZnO nanoparticles *(34, 45)*. The silver levels in blood were considered to be non-toxic to the patients *(34)*.

For skin penetration and absorption, the quality of the skin in terms of skin damage such as abrasions and UVB damage (sunburn), mechanical stressors (skin flexing), and the effects of solvents and vehicles used may affect the skin penetration *(57)*.

In a recent review a more balanced outcome was described, showing that for certain (rather) small nanoparticles skin penetration might be possible, especially for metal and metal oxide nanoparticles *(61)*. Both types of nanomaterials may consist of particles with a secondary size, which occur in physiological media after interaction with media components. A size-dependent skin penetration can be distinguished: nanoparticles ≤ 4 nm can penetrate and permeate intact skin; nanoparticles between 4 and 20 nm can potentially permeate intact and damaged skin; nanoparticles between 21 and 45 nm can penetrate and permeate only damaged skin; and nanoparticles > 45 nm cannot penetrate or permeate the skin. In addition, for metals, other aspects such as dissolution and tendency to aggregate may potentially play a role, resulting in local and systemic effects. Moreover, some metals, such as zinc and nickel, may bind to albumin, a known component of human sweat, which can migrate bidirectionally between distinct skin layers *(62–64)*. A decision tree is proposed to evaluate the potential risk for consumers and workers exposed to nanoparticles *(61)*.

Gastrointestinal tract

Another route for internal exposure is via the gastrointestinal tract. However, there is still a lack of knowledge as to what extent single particles, small aggregates or agglomerates, and larger aggregates or agglomerates can be translocated across the epithelium of the gastrointestinal tract. Uptake from the gastrointestinal tract was demonstrated for several nanomaterials *(65–70)*, but a lack of uptake of nanoparticles was also observed *(71)*. In general, smaller particles were found to have a higher uptake *(65, 69)*. However, large titanium particles with a size of 500 nm were also absorbed via the gastrointestinal tract *(66)*. In a human volunteer study, nanosilver (size around 60 nm) was orally ingested for up to 14 days *(72)*. Serum silver concentration was detected in 42% of subjects receiving 100 μg/day and in 92% of subjects receiving 480 μg/day, but was undetectable in the urine *(72)*. However, in the silver preparation the majority of the silver was present as silver ions, so uptake of nanoparticles could not be established.

As for skin penetration studies, in vitro models are available for the gastrointestinal tract to study migration over intestinal cells,

for which Transwell systems using Caco-2 cells are commonly used *(73–76)*. A translocation for polystyrene nanoparticles was noted, varying between 1.6% and 12.3% of the added dose *(77)*. The in vitro cellular models can indicate low, medium or high translocation, although low translocation can be especially difficult to determine in view of limitations in the sensitivity of the measurement technique. Although Caco-2 cells are commonly used as a model for the gastrointestinal tract epithelium, models with cells present in the epithelium of Peyer's patches (so-called M cells) have also been used *(76)*. These M cells were found to be more efficient for particles than normal enterocytes *(78)*. So, M cells may be an important addition as a model for gastrointestinal absorption studies *(79)*. Especially for the gastrointestinal tract, the potential interaction that may occur with all components and gastrointestinal fluids may warrant a more dedicated evaluation of these interactions *(76)*. For these studies, various in vitro non-cellular models are also available *(80–84)*. Some of these models are static, but dynamic models are also available, each with their own limitations *(76)*. Similar to skin penetration, the solubility of the nanomaterials might be an issue in the gastrointestinal tract as a factor facilitating absorption of shed ions or degradation products of nanomaterials.

Respiratory tract

With regard to the lung, the uptake of nanoparticles occurs primarily in the deep lung, and depends on the deposition of the particles in the alveoli (the respiratory part of the lung). This deposition depends on the size of the particles and can be calculated (modelled) by the ICRP or the MPPD model *(7, 10)*. The uptake from the lung is limited *(50)*. A small but significant fraction of the dose of nanoparticles may show systemic distribution, although the majority of the nanoparticles remained in the lung *(48, 85–87)*. The elimination half-time from the lung for both fine and ultrafine (nano)particles in rats was approximately 65 days *(88, 89)*. Part of the inhaled particles (nano and micro) will end up in the gastrointestinal tract due to the mucociliary cascade that removes inhaled particles from the lung, and will be excreted in the faeces *(87)*. The primary particle size of the nanoparticles is important, as smaller (7 nm versus 20 nm) nanoparticles have a higher uptake from the lung *(90)*. In this study,

macrophage-mediated mucociliary escalation, followed by faecal excretion, was the major pathway of clearing the inhaled nanoparticles from the lungs. For lung exposure, the potential of the nanoparticles to migrate along the olfactory nerve into the brain (olfactory bulb) should also be considered *(90, 91)*.

Distribution, metabolism and excretion

Following translocation across the portal of entry, distribution of certain ENMs to different organs and tissues may occur. Generally, metabolism of ENMs is considered to be minimal or absent, although metal and metal oxide ENMs especially may degrade and disappear slowly by shedding ions. After oral administration or exposure, excretion of non-absorbed ENMs occurs via the faeces from the gastrointestinal tract. When systemically available, a rapid sequestration of ENMs by cells of the mononuclear phagocytic system occurs, as indicated by high levels of intravenously administered nanoparticles into the liver and the spleen *(36, 41, 92)*. The nanoparticles are actively and quasi-irreversibly removed from the blood by the phagocytizing cells of the mononuclear phagocytic system, and the process is thus not concentration dependent. So, the concentration in blood or plasma generally has little value for internal dose estimation, while the concentration in organs does not depend on the blood concentration. Even with low blood concentrations, the uptake in organs of the mononuclear phagocytic system, notably the liver and spleen, can occur.

The ability to measure the internal dose of an ENM in tissues is essential in identifying potential target organs for toxicity testing and in the construction of an appropriate dose–response relationship. This is especially true for ENMs that can accumulate in tissues over time. For TiO_2, at 90 days after administration titanium could still be detected in considerable amounts, mainly in the liver and spleen *(41)*. This presents a new toxicological paradigm, particularly for organs that are normally protected against the entry of larger particulate materials. This also makes it essential that dose–response relationships for ENMs are determined for multiple target organs, including some that may not be a first-tier consideration in the risk assessment of conventional chemicals.

Oral and intravenous exposure to gold nanoparticles of different sizes resulted in an increased organ distribution in mice and rats with decreasing particle size *(36, 93)*. For TiO$_2$, after oral administration organ distribution was reported for 500 nm particles *(66)*. In contrast, minimal to non-existent translocation was observed for TiO$_2$ nanoparticles *(41)*. The size of the primary particles of the latter study was 20–25 nm, while the majority of particles occurred as agglomerates or aggregates measuring 80–150 nm. In another independent study, a low but significant amount of titanium could be detected in the liver or spleen at 24 hours after the last of five consecutively administered oral doses *(94)*.

For nanosilver, a dose-dependent increase of silver content was present in brain, kidneys, liver, lungs, stomach and testes after oral administration *(43, 68)*, though the translocation of silver from the gastrointestinal tract could be mainly attributed to migration of Ag$^+$ ions *(43)*. The oral administration of Ag-NP resulted in a widespread presence of silver in various organs, which was mainly cleared from the organs at eight weeks, with the exception of the brain and testes. The organ silver content was highly correlated with the presence of Ag$^+$ ions in the Ag-NP suspension, indicating that Ag$^+$ ions and to a lesser extent Ag-NP passed through the intestines. Remarkably, the presence of Ag-NPs could be demonstrated in animals treated with silver nitrate solutions, indicating the new formation of nanoparticles from Ag$^+$ in vivo. It was concluded that exposure to Ag-NPs appears to be very similar to exposure to silver salts *(43)*. Also, for two commercially available synthetic amorphous silica nanomaterials uptake from the gastrointestinal tract was demonstrated, with increase in silica tissue levels in the brain, kidney, liver and testes *(95)*.

In a more recent review, Hougaard et al. *(96)* evaluated the possibilities for migration of nanoparticles into the placenta and fetus. In addition to animal models (using intravenous administration), in vitro and ex vivo placental models are available of both animal and human origin. The migration of nanoparticles into the placenta and pups was considered possible. For various types of nanoparticles, migration into the placenta and pups was demonstrated, though of low extent *(96)*.

3.4.4 *Specific considerations for toxicokinetic studies on nanoparticles*

A major difference between the toxicokinetics of neat (soluble) chemicals and nanomaterials is that for neat dissolved chemicals the tissue distribution is concentration dependent, and an equilibrium is generally obtained between blood and organ concentration, whereas nanoparticles are rapidly removed from the circulation by cells of the mononuclear phagocytic system, as indicated by the distribution of a major fraction of an injected dose into the spleen and liver *(36, 41, 92)*. A toxicokinetic, physiologically based pharmacokinetic (PBPK) model indicated distribution to several compartments, in which the nanomaterials were quasi-irreversibly retained in the organs using two to five compartments for nanoparticle distribution *(92)*. There is no equilibrium concentration between tissue and blood. Uptake in organs can occur independent of the blood concentration; that is, even with a low blood concentration and high organ concentration, organ uptake can occur. This may result in the persistence of nanomaterials in organs for long periods: silver could still be detected in various organs at day 17 after intravenous administration of Ag-NPs in rats *(88, 92, 97)*. Titanium nanoparticles were still detectable up to 90 days after a single and repeated intravenous administration *(41)*. To identify tissue distribution and the potential for tissue accumulation and persistence of a nanomaterial, it is necessary to design single and repeated kinetic studies with a representative follow-up period of time. In OECD TG 417 on toxicokinetic testing, the follow-up period is typically up to seven days, which may be too short a period for nanomaterials in view of their potential persistence in organs.

The surface chemistry – possible coatings or adhesion of biomolecules to the surface of the nanoparticles – affects the tissue distribution and toxicokinetics. For polyethylene glycol (PEG)-coated nanomaterials, prolonged blood circulation was demonstrated *(35, 38)*. In this respect, the passage of the nanoparticles through the respiratory or gastrointestinal tract may also influence absorption and thus systemic organ exposure. For several metallic nanoparticles, the possibility of dissolution or degradation should be considered. Careful evaluation of the organ content is therefore needed when performing (toxico)kinetic studies for nanomaterials. A combination

of separation techniques, such as field flow fractionation, might be used together with single particle ICP-MS to identify the presence of particles in tissues.

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4. MECHANISMS OF IMMUNOTOXICITY

4.1 General principles of toxicity of nanomaterials

4.1.1 Role of physicochemical material properties

In order to systematically investigate the toxic effects of ENM, it would be desirable to correlate their toxic effects with their physicochemical properties. However, this approach is not straightforward, as many physicochemical properties are strongly entangled and are difficult to control independently *(1, 2)*. Nevertheless, this section discusses how material properties are linked to ENM toxicity, focusing on the importance of size, shape, charge, and solubility or dissolution.

Size matters, in particular for cellular uptake of nanoparticles. For some nanoparticles, size has been shown to drive toxicity through direct interactions with cellular receptors or intracellular structures. For instance, Tsoli et al. *(3)* found that Au55 clusters displayed cytotoxicity in a variety of different normal and cancer cell lines that seemed to be caused by the unusually strong interaction between the 1.4 nm particles and the major grooves of DNA. Only marginally smaller or larger particles of the same chemical composition showed drastically reduced toxicity. Sargent et al. *(4)* provided evidence of CNT interference with the mitotic spindle in human lung epithelial cells, leading to induction of aneuploidy, an early event in the progression of many cancers. The nanotube bundles are similar in size to the microtubules that form the mitotic spindle – such biomimicry may explain how these nanomaterials are incorporated into the mitotic spindle apparatus. Moreover, SWCNTs have approximately the same diameter (1–4 nm) as the DNA double helix and can physically intertwine with DNA *(5)*. It is generally believed that the greater the intracellular dose of nanoparticles, the more pronounced the toxic effects that they generate. For example, it has been demonstrated in mammalian cells, using gold nanoparticles, that there is an optimal size for cellular uptake *(6, 7)*. However, it is important to note that cellular uptake does not necessarily lead to cell death, as studies have shown

mesoporous silica nanoparticles or silica-coated iron oxide (Fe_2O_3) nanoparticles to be relatively inert at the doses tested, despite considerable cellular uptake in macrophages *(8, 9)*. Moreover, cellular uptake is not mandatory for cytotoxicity to occur: cobalt-chromium nanoparticles were shown to cause damage to fibroblasts across an intact cellular barrier without having to cross the barrier. The outcome, which included DNA damage without overt cell death, was different from that observed in cells subjected to direct exposure to nanoparticles *(10)*.

Nanoparticle shape is also important. In particular, phagocytosis of foreign materials such as ENMs can be influenced by their aspect ratios. Using HeLa cells as a model, particles of ellipsoid shape were found to be more readily engulfed than spherical particles *(11)*. In contrast, nanomaterials with dramatically high aspect ratios and rigidity (such as the rodlike CNTs) may resist uptake by macrophages, but could cause cellular damage through piercing of the plasma membrane (so-called frustrated phagocytosis), resulting in the leakage of cellular constituents, including cytokines and ROS *(12)*. Similarly, changing a relatively inert material such as TiO_2 into a fibrous structure results in a toxic nanomaterial that provokes inflammatory responses in macrophages *(13)*.

In addition to the overall shape of the nanoparticles, the smoothness or roughness of the particle's surface affects the opsonization of the particle and its subsequent uptake by phagocytes (discussed below). Furthermore, in a study using gold nanoparticles with different aspect ratios, Schaeublin et al. *(14)* found that the gold nanospheres were non-toxic, whereas the gold nanorods induced apoptosis. Notably, both nanoparticles formed agglomerates in cell culture medium, but the spherical particles had a large fractal dimension (tightly bound and densely packed) while the nanorod agglomerates had a small fractal dimension (loosely bound). Similarly, the in vivo pulmonary toxicity of SWCNTs in mice has been demonstrated to be highly dependent on their agglomeration; hence, fibrelike materials may sometimes behave as agglomerated particles and not as individual fibres *(15, 16)*. It has been suggested that the effective surface area of CNTs (based on geometrical analysis of the agglomerates) might be a more useful dose metric than specific surface area or particle number *(16)*.

Surface properties, such as the charge (zeta potential) and hydrophobicity, directly affect the interaction of nanomaterials with biological surfaces, cell membranes and proteins *(17)*. In general, owing to the overall net negative charge of cellular surfaces, positively charged nanoparticles are incorporated faster by cells than negatively charged ones, leading to high rates of non-specific internalization and a shorter half-life in the circulation. The higher toxicity of positively charged nanoparticles is generally correlated to their enhanced cellular uptake *(18)*. Schaeublin et al. *(19)* demonstrated the effects of surface charge on the modality of cell death induced by a particular nanomaterial. Thus, charged gold nanoparticles induced cell death through apoptosis whereas neutral gold nanoparticles triggered necrosis in a human keratinocyte cell line. To elucidate surface charge-dependent toxicity, nanoparticles with different surface charges for which the other physicochemical parameters remain constant are required, but this is often experimentally difficult to achieve. Overall, the physicochemical properties of nanoparticles are strongly linked and are difficult to control independently *(1)*. Moreover, size, shape and surface charge are altered when the nanoparticles interact with the biological environment. In addition, dissolution of metal and metal oxide nanoparticles in acidic cellular compartments (lysosomes) underlies the so-called Trojan horse-type mechanism of particle toxicity *(17)*. This mechanism has been attributed to oxides of zinc, iron, manganese, cobalt and copper nanoparticles, which drive toxicity through release of toxic ions into cells. Cho et al. *(20)* correlated the toxicity of 15 different metal and metal oxide nanoparticles with one of two physicochemical parameters: zeta potential under acid conditions for low-solubility nanoparticles, and solubility (degree of dissolution) for high-solubility nanoparticles. Hence, the authors found that the inflammogenic potential of high-solubility nanoparticles depends on the ions that are produced during their dissolution inside the acidic phagolysosomes of the cells *(20)*. For some (photocatalytic) particles, the crystal structure is an important determinant of toxicity. Sayes et al. *(21)* studied the effects of TiO_2 nanoparticles and found that the extent to which those particles affected cellular behaviour was not dependent on surface area; instead, the phase composition of the particles was important, insofar as anatase TiO_2 was 100 times more toxic than rutile TiO_2. The most cytotoxic nanoparticles were also the most effective at generating ROS *(21)*.

4.1.2 Direct cytotoxic effects of nanomaterials

With respect to immunotoxicity of ENMs, this could certainly result from a direct cytotoxic effect culminating in cell death. Understanding which cell death modality is engaged upon nanoparticle exposure could aid the development of strategies by which to mitigate these effects. There are several different modes of cell death, including apoptosis (a form of programmed cell death with numerous upstream and downstream regulators), and regulated necrosis or necroptosis (cell death dependent on receptor-interacting protein 1 (RIP1) and RIP3 kinase activation), as well as pyroptosis (cell death mediated by caspase-1 activation), ferroptosis (cell death mediated by lipid peroxidation), autophagy (controlled destruction of survival factors or organelles within a cell) and others *(22)*. For example, CeO_2, an ENM widely used as a fuel additive, induces apoptosis and autophagy in human peripheral blood monocytes *(23)*. Recent studies showed that ultrasmall (< 10 nm in diameter) poly(ethylene glycol)-coated silica nanoparticles triggered ferroptosis in starved cancer cells, which could potentially be exploited for therapeutic purposes *(24)*. Notably, acute and chronic exposure to the same nanomaterial could induce drastically different effects at the cellular level. Hence, exposure of cells to CNTs or CNT-based scaffolds induces apoptosis *(25, 26)*. However, Wang et al. *(27)* reported that chronic exposure to CNTs may yield an apoptosis-resistant and tumourigenic phenotype in lung epithelial cells. The same team also demonstrated the induction of cancer stemlike cells upon chronic exposure of human lung epithelial cells to CNTs *(28)*.

4.1.3 Altered cellular signalling by nanomaterials

In addition to direct killing of immune cells, ENMs also have the potential to disrupt extracellular or intracellular signalling and thereby impair cell function or enhance proinflammatory activity of the cells. For example, nickel nanoparticles have been shown to prolong platelet-derived growth factor (PDGF)-initiated phosphorylation of mitogen-activated protein (MAP) kinase in rat pleural mesothelial cells *(29)*. This resulted in enhanced production of chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 10 (CXCL10), which play important roles in the recruitment of macrophages, monocytes and lymphocytes during the pleural immune response

to ENMs, such as nickel or nickel-containing CNTs. Sustained phosphorylation of signalling intermediates such as MAP kinases or receptor tyrosine kinases is achieved by ROS generation by metal nanoparticles. ROS inactivate protein tyrosine phosphatases that are necessary to dephosphorylate activated kinases *(30)*. Tian et al. *(31)* reported that nanosized graphene oxide retarded cellular migration via disruption of the actin cytoskeleton. Kodali et al. *(32)* have shown that pretreatment of macrophages with Fe_2O_3 nanoparticles at non-cytotoxic doses caused extensive transcriptional reprogramming in response to subsequent challenge with bacterial LPS. Macrophages exposed to nanoparticles displayed diminished phagocytic activity towards certain bacteria. The authors concluded that the effects of nanoparticles may be indirectly manifested only after challenging normal cell function, such as phagocytosis of pathogens.

4.1.4   Role of oxidative stress for nanotoxicity

The generation of ROS in cells is a common feature of nanomaterial-induced toxicity *(33, 34)*. Two major mechanisms of ROS generation have been proposed. First, the direct interaction of nanomaterials with molecular oxygen generates free radicals such as superoxide ion and hydroxyl radical. The oxidative potential of metal oxide nanoparticles (for example zinc, copper, iron) is due to generation of ROS via Fenton-like reactions. Second, the activation of inflammatory cells (for example macrophages, neutrophils) by nanomaterials stimulates the release of ROS by these cells through activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Using NADPH oxidase-deficient mice, Shvedova et al. *(35)* showed that NADPH oxidase-derived ROS play a direct role in determining the course of the pulmonary response to CNTs. Nanomaterials can also disrupt mitochondrial homeostasis. For example, Ag-NPs inhibit the electron transport chain and adenosine triphosphate synthesis in cells *(36)*. Nanomaterials also have the potential to inhibit antioxidant pathways. ROS generated by nanomaterial exposure can, in turn, act as signalling intermediates to activate intracellular signalling targets, including receptor tyrosine kinases, MAP kinases, and transcription factors, leading to transcriptional activation and the expression of genes involved in pulmonary immune responses. However, while ROS generation or, more specifically, oxidative stress (that is, the imbalance between

ROS production and the cell's inherent ability to detoxify the reactive intermediates or repair the resulting oxidative damage), is often viewed as the final common pathway of nanoparticle-induced toxicity, it is prudent to ask whether oxidative stress is a specific event that plays a role in mediating cellular damage, for instance through the activation of apoptosis, or whether ROS generation represents a secondary event resulting inevitably from disruption of biochemical processes within the dying cell *(37)*. The answer to this question will have important ramifications for the development of strategies with which to reduce toxicity of ENMs.

4.2 Nanoparticle interactions with the immune system

The immune system represents the primary defence system against foreign intrusion, including both pathogens and particles. Immunity, in essence, is the balanced state of having adequate defences against infection or other biological intrusion, while retaining tolerance to avoid destructive actions towards the body's own cells. The immune system can be divided into the "primitive" or innate immune system, which is quick to react to foreign intrusion; and the more delayed, but highly specific, adaptive immune system *(38)*. Importantly, there is cross-talk between the innate and the adaptive arms of the immune system, and some cell types that were previously considered as being primitive innate immune cells, such as the neutrophil, are also involved in the orchestration of adaptive responses. In order to understand the potential toxicity of ENMs, it is necessary to understand the immune system, with its multiple, specialized cell types that work in concert, and its soluble mediators, including cytokines and complement factors *(39)*. Moreover, it is important to consider not only material-intrinsic properties of the pristine nanomaterial, but also the acquired, context-dependent "identity" of a nanomaterial in a living system resulting from the adsorption of biomolecules on its surface *(1)*. Furthermore, nanomaterials may adopt a "new" identity through the adsorption of biomolecules (proteins, lipids, and so on), a phenomenon that, in turn, is linked to nanomaterial intrinsic properties, such as size and hydrophobicity. This section discusses the structure and function of the immune system and reviews available literature on interactions of nanomaterials with the immune system following three main routes of exposure: dermal exposure, pulmonary exposure, and oral exposure.

4.2.1   Innate immune system

The immune system consists of an array of dedicated cell types that work in concert using complex detection, communication and execution systems to defend humans from any external or internal harm. The immune system is typically divided into the innate immune system, which is quick to react to foreign intrusion; and the more delayed but highly specific adaptive immune system, which is endowed with immunological memory after an initial response to a specific pathogen, leading to an enhanced response to subsequent encounters with that same pathogen. The latter mechanism is the basis for vaccination, one of the triumphs of modern medicine. It has been proposed *(39, 40)* that most if not all of the adverse effects of ENMs are exerted via direct effects on cells of the innate immune system, including macrophages, or via dendritic cells (DCs), phagocytosis-competent, antigen-presenting cells that act as a bridge between the two arms of the immune system, while the effects on the adaptive immune system – that is, B cells and T cells – are thought to be indirect. This is not surprising, as one of the main functions of the cells of the so-called mononuclear phagocytic system is to clear the body of unwanted foreign materials (pathogens, particles). In the following subsections, published data on in vitro and in vivo interactions of nanomaterials with the innate and adaptive immune system are highlighted, along with a discussion of the biocorona.

Physical and biological barriers

In addition to specialized immune cells belonging to the innate or adaptive immune system, which are found throughout the body, the organism is also protected by anatomical barriers, which may also be viewed as part of the innate immune system. These include physical, chemical and biological barriers. The skin is the main barrier that protects the body from the external environment and acts as the first line of defence against invading microorganisms. In the respiratory and gastrointestinal tract, movement due to cilia (the mucociliary escalator or peristalsis in the gastrointestinal tract) helps remove infectious agents. On the skin and in the airways, small peptides known as defensins are secreted and contribute to the "primitive" defence against infection. The microflora in the gut also protect the host organism by competing with pathogenic bacteria. Studies in

recent years have shown that the gut microbiome takes an active role in shaping and instructing the immune system *(41)*. This is an area of potentially great significance when it comes to exposure to ENMs *(42)*, and more research is therefore needed on the potential interplay between ENMs, the microbiome and the immune system.

Notably, the skin, gastrointestinal tract, and lungs are in direct contact with the environment, and direct exposure to nanomaterials may therefore occur. Consequently, understanding the anatomical, chemical and biological barrier function at these locations is of great importance. Furthermore, it is also important to note that nanoparticles may translocate across various biological barriers and reach organs distal to the portal of entry. Hence, nanoparticles may avoid macrophage clearance in the lungs and cross the air–blood barrier, thus entering the systemic circulation, leading to their distribution to other organs *(43)*. In the pregnant mother, the placenta protects the unborn child; studies in recent years have addressed whether nanoparticles could cross the placental barrier, causing harm to the fetus *(44)*. Recent evidence demonstrates that ZnO nanoparticles can cross the blood–placenta barrier after oral administration *(45)*. Moreover, ZnO nanoparticles can also penetrate the blood–testes barrier and the blood–brain barrier *(45–47)*. However, it cannot be deduced from the studies whether the zinc was taken up in particle or in an ionic form.

Phagocytosis and clearance of nanoparticles

Phagocytosis, the process whereby macrophages, DCs and other myeloid cells (such as neutrophils) internalize various targets, including microbes or cell debris, is a key mechanism of innate immunity. Numerous studies have demonstrated that phagocytosis of nanoparticles also occurs. However, when the target is physically too large for the macrophage to engulf it, this may trigger "frustrated phagocytosis", leading to the release of proinflammatory mediators; this is seen, for instance, when asbestos fibres or long, rigid MWCNTs are injected into mice *(40)*. If a foreign body is too large, a granuloma is formed. In some cases, macrophages fuse with each other to form "giant cells" that encapsulate the foreign body; this encapsulation of offending agents is an ancient defence strategy and may also be of relevance for immune responses to
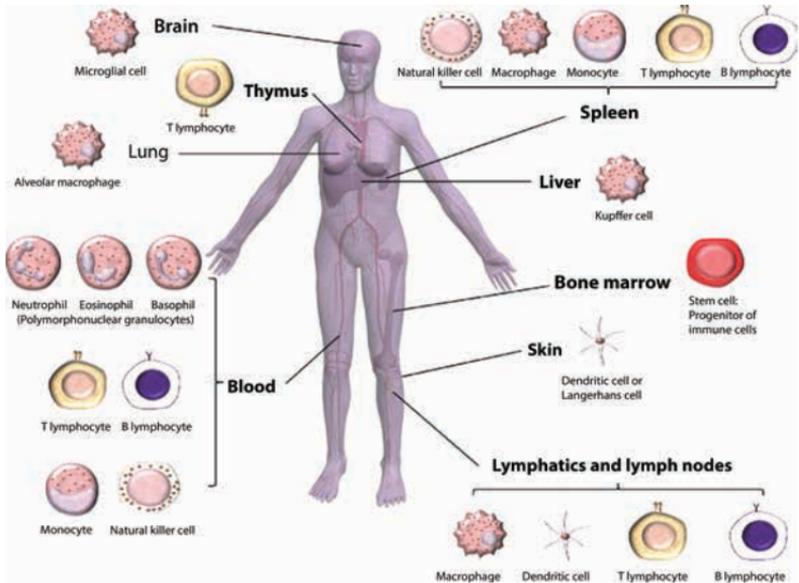
Figure 4.1 Organs of the immune system and distribution of innate and adaptive immune cells
Source: Elsabahy and Wooley *(54)*.

nanomaterials. Understanding how phagocytes (Figure 4.1) interact with nanoparticles is thus of key importance.

In addition, it may be important to consider differences between the various populations of macrophages, depending on tissue of origin and activation status. Activated macrophages are commonly divided into M1, or classically activated macrophages; and M2, or alternatively activated macrophages *(48)*. Hence, while M1 are thought to be more proinflammatory and oriented towards pathogen killing, M2 macrophages have been linked to anti-inflammatory, wound healing and tissue repair functions, and have been implicated in fibrosis and cancer. The presence of T helper 1 (Th1) cytokines has a tendency to polarize macrophages towards the M1 phenotype, while T helper 2 (Th2) immune responses can induce macrophage polarization towards the M2 phenotype *(48)*. It should be noted that the classical M1–M2 categorization has been viewed

as an oversimplification *(49)*. Nevertheless, it has been shown that macrophage phenotype determines uptake of silica particles *(50)* and gold particles *(51)*. Jones et al. *(52)* showed that mouse strains that are prone to Th1 immune responses clear nanoparticles at a slower rate than Th2-prone mice. Interestingly, the authors could show that granulocytes – mainly neutrophils – as well as macrophages participated in the clearance observed in Th2-prone mice. The implication of this preclinical study is that the immune status of an individual may impact nanoparticle clearance. In a subsequent study, the immune system was found to undergo changes in tumour-bearing mice leading to increased particle clearance caused by an increase in M2 macrophages *(53)*, further supporting the view that the immune status affects nanoparticle clearance.

An undesired effect of nanoparticles is the impairment of phagocytic activities. For instance, SWCNTs were described to impair macrophage engulfment of apoptotic target cells *(8)*. Similar observations have previously been made for ultrafine carbon particles, which impair ingestion of microorganisms by human alveolar macrophages *(55)*. Thus, inhalation of nanoparticles could lead to increased susceptibility to pulmonary pathogens, as their clearance might be impaired. Indeed, delayed bacterial clearance is seen in mice exposed to SWCNTs via pharyngeal aspiration *(35)*. Furthermore, as discussed above, Kodali et al. *(32)* showed that pretreatment of macrophages with Fe_2O_3 nanoparticles caused extensive transcriptional reprogramming in response to subsequent challenge with bacterial LPS. Macrophages exposed to nanoparticles displayed a phenotype suggesting an impaired ability to transition from an M1- to M2-like activation state, associated with a diminished phagocytic activity towards certain bacteria *(32)*.

Several studies in recent years have shown that CNTs are susceptible to degradation by naturally occurring plant and human enzymes (peroxidases) *(56)*. Myeloperoxidase (MPO) is a component of the microbicidal system of phagocytes, especially neutrophils, and thus an important part of the innate immune response. Notably, MPO expressed in primary human neutrophils is capable of degradation of oxidized SWCNTs *(57)*. Furthermore, neutrophil extracellular traps produced by activated neutrophils can "capture" and digest SWCNTs in an MPO-dependent manner *(58)*. Additionally, eosinophil

peroxidase, the major oxidant-producing enzyme in eosinophils, degrades SWCNTs *(59)*. CNTs can also undergo biodegradation in activated macrophages through a superoxide/peroxynitrate-driven oxidative pathway *(60)*. Thus, innate immune cells are apparently capable of enzymatic degradation of bacteria and fungi as well as carbon-based nanomaterials. However, it remains unclear how important these enzymatic biodegradation mechanisms are in the lungs of experimental animals and humans. For example, while MPO-dependent degradation of SWCNTs was demonstrated in the lungs of mice *(37)*, abundant intact MWCNTs remain biopersistent in the lungs and pleura of mice months after inhalation, suggesting that biodegradation may be limited *(61)*. However, it is noted that MWCNTs are not as readily degraded as SWCNTs *(62)*. This issue has important implications for the toxicity of ENMs, as biopersistence versus biodegradability may determine whether nanomaterials exert long-term adverse effects in the body.

4.2.2   Cellular and soluble mediators of inflammation

Inflammation is an adaptive response involving soluble factors and cells that is triggered by infection, trauma, ischaemia, or toxic or other injury *(63)*. In inflammation, foreign agents are detected by inflammatory cells upon entry into the body, resulting in the stimulation of these cells, with production of soluble factors such as cytokines and chemokines *(64)*. The most important inflammatory cells are the monocytes and macrophages, neutrophils, eosinophils and mast cells, all of which have been implicated in responses to nanomaterials. Importantly, both infectious and non-infectious inducers of inflammation trigger the activation of conserved signalling pathways, including the activation of the inflammasome in macrophages, with release of IL-1β, one of the key mediators of inflammation *(65)*. Regardless of the trigger, the purpose of the inflammatory response is to remove or sequester the offending agent, to allow the host to adapt and, ultimately, to restore functionality to the tissues *(64)*. However, if the process becomes chronic, other cell types such as lymphocytes and fibroblasts may become involved and the adaptive changes may damage the host. Thus, it is important to distinguish between transient, protective responses to an insult versus chronic, maladaptive ones.

Macrophages are a part of the mononuclear phagocytic system. These cells are professional phagocytic cells and are endowed with proteolytic and catabolic activities. Macrophages originate from monocytes that, when they become tissue bound, convert into cells with enhanced phagocytic capacity *(66)*, or may arise from self-renewal in the tissues. Macrophages are usually distinguished from DCs by their expression of F4/80, CD11b and Fc surface receptors, though some authors have argued that DCs are simply a heterogeneous subset of the mononuclear phagocytic system with no unique adaptation for antigen presentation *(67, 68)*. Macrophages detect foreign matter using receptors such as the toll-like receptors (TLRs) *(66)*. TLRs belong to the pattern recognition receptors (PRRs) expressed by cells at the front line of host defence, for example macrophages, DCs, and epithelial cells. PRRs enable these cells to detect and respond to the presence of danger- and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively).

Neutrophils or polymorphonuclear granulocytes are granular leukocytes possessing a nucleus with three or more lobes. They are the most short-lived cells in the bloodstream. The turnover is tremendous; it has been estimated that about 60% of haematopoietic activity in the bone marrow is directed towards neutrophil production. Neutrophils are specialized in the phagocytosis of bacteria and other pathogens and are capable of phagocytosis on their own (that is, without the participation of any other immune mechanism) or through antibody- or complement-mediated opsonization of pathogens *(69)*. Neutrophils are equipped with oxidative and proteolytic enzymes for the destruction of ingested pathogens. Neutrophils also release neutrophil extracellular traps, which contribute to the defence against extracellular bacterial and fungal pathogens; in addition, neutrophil extracellular traps play a role in non-infectious (sterile) inflammation *(70)*.

Eosinophils are the least common white blood cell. Their presence is associated with parasite infections, allergic diseases such as asthma, chronic lung inflammatory states, and hypereosinophilic syndrome *(71)*. Eosinophils are found in the circulation, but reside predominantly in mucosal surfaces, where they can remain for weeks. Eosinophils are particularly effective in ridding the organism of

multicellular pathogens known as parasites. Following activation, eosinophils degranulate to release cytotoxic granule proteins that are capable of causing tissue damage.

Mast cells play a role in innate immunity, host defence against parasites, tissue repair, and angiogenesis. Mast cells are similar to basophil granulocytes in the blood and are activated by a variety of stimuli through various receptors, including receptors for immunoglobulin E (IgE). Once activated, the mast cell produces histamine, leukotrienes, proteases, cytokines, chemokines and other substances that cause immediate airway inflammation, leading to asthma symptoms and in some cases anaphylactic shock *(72)*.

The key soluble mediators of the innate immune system are the cytokines, chemokines and complement factors. These proteins not only allow immune cells to communicate, but also facilitate the clearance of pathogens through opsonization – decoration of microbial surfaces to stimulate macrophage or neutrophil engulfment. Conceptually, this is similar to the biocorona formation on the surface of nanoparticles (discussed below). The major role of chemokines is to act as chemoattractants to guide the migration of immune cells *(73)*. The release of chemokines is often stimulated by proinflammatory cytokines. Monocytes and macrophages, neutrophils, eosinophils, and mast cells all express receptors for different chemokines. TNF-α is a key cytokine involved in inflammation; it is produced mainly by activated macrophages *(49)*. The IL-1 family is a group of cytokines that play a central role in the regulation of immune and inflammatory responses to infectious or non-infectious insults *(74)*. Among the family members, IL-1α and IL-1β are the most studied. Finally, the complement system is an ancient and integral part of the innate immune system that helps or "complements" antibodies. Historically, the complement system has been viewed as a supportive first line of defence against microbes, quickly tagging them for phagocytosis. However, it is now understood that complement factors not only complement but even orchestrate immunological and inflammatory processes, thus contributing substantially to tissue homeostasis *(75)*. The complement system is composed of 30 distinct plasma and cell-bound proteins that are activated through three different pathways – the classical, alternative and lectin pathways. Inadvertent complement activation could lead to anaphylaxis, a serious allergic

reaction that is rapid in onset and may cause death *(76)*. Thus, it is essential to evaluate nanomaterials with respect to their propensity for complement activation.

4.2.3   *Inflammasome activation by nanomaterials*

The inflammasomes are protein complexes in phagocytic cells that regulate the activation of IL-1β and IL-18, a proinflammatory cytokine involved in infectious and non-infectious inflammation *(77, 78)*. Inflammasomes are activated by DAMPs and PAMPs (see previous subsection). PAMPs include conserved molecular motifs found in bacterial cell wall components (for example LPS and peptidoglycan) or viral DNA/RNA, as well as fungal glucans. DAMPs, on the other hand, are endogenous stress signals (sometimes referred to as "alarmins"). Several such factors have been identified, including high-mobility group box 1 (HMGB1) protein, uric acid, nucleotides, and heat shock proteins *(79)*. DAMPs are released from activated or dying cells. Their recognition by DCs and macrophages via PRRs results in immune cell maturation and the production of proinflammatory cytokines *(77)*. Notably, several studies in recent years have shown that various classes of nanomaterials also trigger inflammasome activation, specifically the NLRP3 inflammasome[3] *(80)*.

Several members of the NOD-like receptor (NLR)[4] family, a subset of cytoplasmic PRRs, are able to sense DAMPs and PAMPs and subsequently induce the assembly of the inflammasome, which serves as an activation platform for caspase-1, a central mediator of innate immunity *(65)*. Active caspase-1, in turn, promotes the maturation and release of IL-1β and IL-18. It appears that DAMPs and PAMPs synergize to permit secretion of IL-1β: PAMPs (such as LPS) stimulate synthesis of pro-IL-1β, but not its secretion; while DAMPs can stimulate assembly of an inflammasome and activation of caspase-1, which cleaves proIL-1β into IL-1β. Dostert et al. *(81)* initially demonstrated that asbestos fibres and crystalline silica trigger activation of the NLRP3 inflammasome. Several other studies have subsequently shown that the NLRP3 inflammasome responds to a

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[3] NLRP3 = NLR family pyrin domain containing 3.
[4] NOD-like receptor (NLR) = nucleotide-binding oligomerization domain-like receptors.

range of different ENMs, including metal and metal oxides and carbon-based nanomaterials *(82–88)*. It is worth noting that there are several inflammasomes *(89)*. However, while all inflammasomes recognize certain DAMPs or PAMPs, it is a distinctive feature of the NLRP3 inflammasome that it is activated by many diverse stimuli. Overall, it seems that innate immune cells may respond in a similar manner to pathogens as well as to ENMs and other exogenous substances, for example alum *(90)*.

The mechanism of activation of the NLRP3 inflammasome has been the subject of much research, and ROS production (either through the activation of the NADPH oxidase or through perturbation of mitochondrial function), lysosomal damage, and a drop in intracellular potassium concentrations have all been implicated in this process. Specifically, the release of the cysteine protease cathepsin B from lysosomes has been implicated in inflammasome activation, not least by nanomaterials *(80)*. Li et al. *(91)* reported that rare earth oxide nanoparticles triggered inflammasome activation through a biotransformation process within lysosomes resulting in lipid membrane dephosphorylation and organelle damage, with release of cathepsin B. In a subsequent study, rare earth oxide nanoparticles were found to interfere with autophagosome fusion with lysosomes, thereby disrupting the homeostatic regulation of activated NLRP3 complexes, leading to enhanced IL-1β production *(92)*. Sun et al. *(93)* provided evidence for NADPH oxidase-generated ROS in lysosomal damage and subsequent IL-1β production in macrophages exposed to MWCNTs. In a recent study, caspase-1 activation and production of IL-1β in bone marrow-derived macrophages was shown to occur upon simultaneous exposure to SiO_2 and TiO_2 nanoparticles at concentrations at which these nanoparticles individually did not cause macrophage activation, and marked lung inflammation was observed in mice treated with both SiO_2 and TiO_2 *(94)*. Notably, SiO_2 nanoparticles, but not TiO_2 nanoparticles, localized in lysosomes, while only TiO_2 nanoparticles triggered ROS production, suggesting distinct forms of cell damage leading to inflammasome activation.

Overall, the NLRP3 inflammasome appears to function as a sensor and integrator for proinflammatory stimuli. It should be noted that the in vitro assessment of inflammasome activation is usually

performed using LPS-primed cells, implying that nanoparticle exposure alone might not be sufficient to induce inflammasome activation and that other concomitant signals would be required in a living organism. MWCNT exposure was recently shown to increase secretion of HMGB1 (an endogenous stress signal or DAMP) in alveolar macrophages, and neutralization of extracellular HMGB1 reduced MWCNT-induced IL-1β secretion in vivo *(95)*. These findings provide a basis for the sterile (non-infectious) inflammation triggered by MWCNTs and perhaps other nanomaterials.

4.3　Nanomaterials and the adaptive immune system

The innate and adaptive immune systems work together in a coordinated manner. The innate immune system is the first line of defence against foreign intruders and its activation leads to signals (for example, cytokine secretion) for adaptive immune response activation. The innate immune response is immediate and occurs, for instance, through engulfment of the offending pathogen, while the adaptive immune response takes days or weeks; however, once an immune response has been established, the immune system can respond rapidly to a new encounter with the same pathogen. Exposure to nanomaterials and other xenobiotics could in principle lead to immune suppression, in which the immune system would fail to expand in response to a pathogen, or immune stimulation, in which case the immune system would overrespond, leading potentially to autoimmune or allergic disease *(38, 96)*.

The adaptive immune system is composed of B cells and T cells. B cells are responsible for humoral (antibody-mediated) immunity, while T cells are involved in cell-mediated responses. T helper (Th) cells (CD4+) are needed to support the production of antibodies by B cells; Th cells, in turn, are subdivided into Th1, Th2, and Th17, depending on their specific role and cytokine profiles. Cytotoxic T cells (CD8+) are required for killing virus-infected and malignant cells, while regulatory T cells are required for maintenance of immune tolerance, which is important for the discrimination between "self" and "non-self" (that is, pathogens). Dendritic cells (DCs), in turn, constitute the bridge between the innate and the adaptive arms of the immune system. These cells are effective phagocytic cells that also exhibit a capacity for processing and presentation of antigens

(substances that provoke an adaptive immune response) *(97)*. Importantly, DCs migrate from peripheral tissues to lymph nodes, where they can stimulate T cells and B cells. Targeting DCs may be advantageous, for instance, in vaccination strategies *(98)*. However, undesired targeting of DCs may be linked to nanomaterial toxicity. In fact, several studies have reported that ENMs may disturb the functions of DCs, which may affect B cells or T cells. For instance, MWCNTs were found to alter the capacity of human monocytes to differentiate into DCs *(99)*, and pulmonary exposure to SWCNTs has been shown to induce diminished proliferation of splenic T cells in mice through direct effects on DCs *(100)*. Nanoparticulate carbon black present in cigarette smoke was found to accumulate in human myeloid DCs from emphysematous lung, and administration of carbon black induced inflammation and Th17-dependent emphysema in mice *(101)*.

Importantly, surface modification may serve to mitigate or otherwise modulate ENM effects on immune cells. Zhi et al. *(102)* studied the effects of graphene oxide with and without polyvinylpyrrolidone (PVP) coating on human DCs, T cells and macrophages, and found that PVP-coated graphene oxide exhibited lower immunogenicity compared with uncoated graphene oxide in terms of inducing DC differentiation and maturation. It is noteworthy that hydrophobicity has been hypothesized as a conserved DAMP that plays a role in activating the immune system *(103)*. The suggestion is that hydrophobic portions of proteins are normally hidden from the immune system, but act as danger or damage signals upon exposure or release. Recent studies using nanoparticles appear to support this view. Moyano et al. *(104)* generated a panel of gold nanoparticles with varying degrees of surface hydrophobicity and measured cytokine responses in splenocytes after exposure to the nanoparticles. Splenocytes mainly comprise B cells, but also include T cells and monocytes, and thus represent both the innate and adaptive arms of the immune system. The authors could show a direct correlation between the hydrophobicity of nanoparticles and cytokine expression, and a similar result was also noted in mice following a single intravenous injection of the nanoparticles *(104)*. In line with these findings, Shahbazi et al. *(105)* reported that surface chemistry of porous silica particles dictated the immunostimulatory effects on human DCs.

4.4 Immunosuppression versus immune activation

Nanomaterials can elicit either immunostimulatory or immunosuppressive effects *(106)*. Mitchell et al. *(107)* found that inhalation of MWCNTs produced a systemic immunosuppression in mice, with decreased T cell proliferation in the spleen due to a signal from the lung, most probably TGF-β secreted by alveolar macrophages. As already mentioned, nanoparticles have been shown to influence T cell proliferation, typically through an effect on antigen-presenting cells, leading to an enhanced T cell stimulatory capacity *(39)*. Using a fully human autologous immunological construct as a non-animal alternative to monitor nanoparticle responses, Schanen et al. *(108)* showed that exposure to TiO_2 nanoparticles led to elevated levels of proinflammatory cytokines and increased maturation of DCs. Additionally, the nanoparticles effectively primed activation and proliferation of naïve CD4+ T cells in comparison to micrometre (μm)-sized TiO_2.

Myeloid-derived suppressor cells (MDSCs), a heterogeneous population of myeloid progenitors, are increasingly being recognized as important players in the immunosuppression induced by tumours. This is accomplished either through direct or indirect mechanisms (that is, by directly influencing effector T cells), or through the generation or expansion of other regulatory cell populations, such as regulatory T cells *(109)*. Shvedova et al. *(110)* reported that metastatic establishment and growth of Lewis lung carcinoma, a commonly used model of pulmonary metastatic disease, is promoted by SWCNTs, and that this effect is probably mediated by increased accumulation of MDSCs, as their depletion prevented the pro-tumour activity of the nanomaterials. Additionally, acute exposure to SWCNTs induced recruitment of MDSCs in the lungs of exposed mice and MDSC-derived production of TGF-β, resulting in an increased tumour burden in the lungs *(111)*.

Mast cells are well known to act in response to danger signals through a variety of receptors and pathways, including IL-33 and the IL-1-like receptor ST2, and as such are critical in innate and adaptive immune responses, while playing a role in allergic conditions. Katwa et al. *(112)* demonstrated the crucial involvement of mast cells and the IL-33/ST2 axis in pulmonary and cardiovascular responses to

MWCNTs. Thus, toxicological effects of MWCNTs were observed only in mice with a sufficient population of mast cells, and were not observed when mast cells were absent or incapable of responding to IL-33. Furthermore, in a more recent study, Rydman et al. *(113)* compared two types of MWCNTs – rigid rodlike and flexible or tangled MWCNTs – in terms of their effects following inhalation exposure in mice. Mast cell-deficient mice were used to evaluate the role of these cells in the inflammatory process. The authors found that even short-term inhalation of the rodlike MWCNTs induced allergic airway inflammation. Mast cells were found to partially regulate the inflammation caused by rodlike MWCNTs, but alveolar macrophages were also suggested to play an important role in the disease. Unlike rodlike MWCNTs, flexible or tangled MWCNTs do not appear to directly cause allergic airway inflammation when administered to the lungs of mice, but tangled MWCNTs do exacerbate allergen-induced lung inflammation by enhancing levels of growth factors (PDGF and TGF-β1) or mucins (MUC5AC and MUC5B), as has been demonstrated in ovalbumin (OVA) allergen and house dust mite allergen mouse models *(61, 114)*. Li et al. *(115)* reported that SWCNTs exacerbated OVA-induced allergic asthma in rats and that this exacerbation was counteracted by concurrent administration of vitamin E. This work thus points to an important role of ROS in this model. Shurin et al. *(116)* reported that graphene oxide attenuates Th2 immune response in a model of OVA-induced asthma, but leads to potentiation of airway remodelling and hyperresponsiveness. Moreover, exposure to graphene oxide increased the macrophage production of the mammalian chitinases, CHI3L1 and AMCase, whose expression is associated with asthma *(116)*. Subsection 4.10.2 on the respiratory system further discusses the pulmonary effects of ENMs. In general, there is considerable evidence that ENMs either cause or exacerbate allergic lung inflammation in rodents, suggesting that certain ENMs might pose a health concern for individuals with pre-existing asthma or as an inciting agent for asthma.

4.5 Immunogenicity of engineered nanomaterials

An important issue that needs to be addressed is nanomaterial immunogenicity, that is, the ability to elicit a specific adaptive immune response *(117)*. To date, immunogenicity of nanomaterials has been observed only rarely. It has been shown that PEG-coated nanoparticles

(for example, liposomes) are able to act as T cell-independent immunogens and elicit a direct B cell response with the production of anti-PEG immunoglobulin M (IgM) antibodies, responsible for accelerated clearance of the nanoparticles upon subsequent administrations *(118, 119)*. Recognition of PEG represents a problem in the context of nanomedicine, as PEG is widely used for particle coating. However, no response against the particles themselves was detected in the aforementioned studies. Nanoparticles of metals such as nickel and cobalt are immunogenic by acting through TLR-4 *(120)*.

It is well known that certain small molecules, referred to as haptens, can elicit an immune response when coupled to a protein carrier *(121)*. Indeed, immunization of mice with a C60 fullerene derivative conjugated to a protein (bovine thyroglobulin) yielded a population of fullerene-specific immunoglobulin G (IgG) antibodies *(122)*. Similarly, induction of specific antiparticle antibody responses was reported upon in vivo inoculation of PAMAM dendrimers conjugated with a protein (for example, the cytokine IL-3), constructed to increase the bioavailability and half-life of the protein *(123)*. Indeed, while the dendrimer was not immunogenic by itself, it became so when conjugated with IL-3, as well as with other proteins, such as bovine serum albumin. Furthermore, it is possible that the acquired biocorona on the surface of nanoparticles could induce specific immune responses if proteins bound to the nanoparticles were to undergo conformational changes, thereby revealing cryptic epitopes. Further studies are required to address this possibility.

4.6   Nanoparticles as adjuvants

Nanosized particles have for a long time been used as adjuvants in vaccine preparations *(124, 125)*. Particulates and crystals induce inflammatory responses and thereby stimulate the immune system. One of the most commonly used adjuvants in vaccines is aluminium hydroxide ($Al(OH)_3$) *(125)*. The vaccine adjuvant preparation can be prepared by adding aluminium salts to a solution of antigen (aluminium-precipitated method) or by adding antigen to previously prepared $Al(OH)_3$ (aluminium-adsorbed method). The $Al(OH)_3$ particles may have a rather heterogeneous size distribution. It was reported that the smaller particles induced a better immune response. Antibody

titers were higher in the vaccine adsorbed with $Al(OH)_3$ with mean diameter of 200 nm compared to that containing $Al(OH)_3$ particles of 600 nm diameter. In addition, the adjuvant with smaller particle size had better physical characteristics and absorption efficiency *(126)*. He et al. *(125)* concluded that nanoscale $Al(OH)_3$ induces higher antibody responses in various antigen–adjuvant combinations and would be a good candidate for a vaccine adjuvant. Sun et al. *(127)* synthesized a library of aluminium oxyhydroxide (AlOOH) nanorods and found that the crystallinity and surface hydroxyl group display of AlOOH nanoparticles impacted the activation of the NLRP3 inflammasome in human THP-1 myeloid cells or murine bone marrow-derived DCs; thus, shape, crystallinity, and hydroxyl content play an important role in NLRP3 inflammasome activation and are potentially useful for boosting antigen-specific immune responses.

Polylactic-co-glycolic acid (PLGA) and liposomal nanoparticulates are now under investigation as adjuvants in cancer vaccines *(128)*. PLGA nanoparticles induced an increase in mucosal and systemic immune responses when the particles were used as a nanocarrier for hepatitis B surface antigen *(129)*. For thiol-organosilica particles (size range 100 to 925 nm) the smallest particles induced an immune response in the Peyer's patches, as indicated by an increased number of DCs and immunoglobulin A (IgA) levels in intestinal secretions *(130)*. Enhancing pulmonary immunity may be accomplished by modification of the surface of polyanhydride nanoparticles. The surface modification with carbohydrates to target C-type lectin receptors on antigen-presenting cells resulted in an increased uptake by alveolar macrophages and the cells displayed an enhanced activation phenotype *(131)*.

In addition to the use as adjuvant itself, nanomaterials are also studied for their capacity as antigen carriers. Different PLGA polymers showed a size-dependent uptake in antigen-presenting cells, while the uptake was independent of the polymer composition *(132)*. Both in vitro and in vivo OVA-directed immune responses were enhanced compared to OVA alone. Thus, important lessons may be learned from the extensive work on adjuvants in terms of how the immune system responds to nanoparticles. This has implications for innate and adaptive immune responses to coexposure to nanoparticles and allergens, for instance in the lungs or on the skin.

4.7   Immunologically susceptible populations

Life expectancy is increasing. Consequently, the health of the elderly is rapidly becoming a major public health concern. Specific immune responses in the elderly are less effective than in adults, with decreased T and B cell activation, while innate immune reactions may be increased due to a constitutively inflammatory microenvironment *(133)*. However, several reports show that macrophages and DCs, being constitutively activated in the elderly, are less responsive to activation by external stimuli. Moreover, immunological frailty due to chronic diseases, infection and malnutrition is often associated with a state of enhanced inflammation that induces constitutive macrophage and DC activation. Therefore, it may be surmised that nanomaterials, which interact mainly with phagocytic cells, as discussed in preceding sections, may trigger fewer unwanted reactions in the elderly population. However, information is lacking and research on this topic is certainly needed. The situation is different in infants and very young children, in which the immune system is immature and therefore less able to cope with external challenges, but not constitutively inflamed as in the elderly. It is also important to note that barrier functions in infants may differ from those in adults *(134)*. This could certainly impact the biodistribution of nanomaterials and, therefore, the outcome of nanomaterial exposure in infants versus adults. In any case, focused studies using relevant models are required to assess the detrimental effects of nanomaterials in immunologically susceptible or frail populations (elderly, chronically ill) and individuals with pre-existing conditions, including infections, allergy or asthma, with particular consideration of the effects of chronic exposure, in order to understand the real-life impact of ENMs.

4.8   The role of the biomolecule corona

The interaction of ENMs with the surface of cells or with biomolecules has been termed the "nano–bio interface" *(135)*. These interactions are of critical importance for understanding the biological behaviour of nanomaterials, not least the effects of nanomaterials on the immune system. Indeed, the immune system has evolved to recognize and respond to foreign intruders with or without a coating of endogenous opsonins such as complement factors or immunoglobulins, and it stands to reason, therefore, that immune

cells would also be able to respond to nanoparticles with or without a surface corona of biomolecules *(39)*.

It is important to consider that nanomaterials do not present pristine surfaces in a biological system. Instead, upon entry into a biological system, nanomaterials rapidly adsorb biomolecules to form a biocorona on the surface *(135)*. It is generally believed that the biocorona has two components – a "hard" corona that is stable, and a "soft" corona. The hard corona is thought to bestow a "biological identity" on the nanomaterial. In other words, the hard corona is what the cells "see". Whether or not the hard corona covers the surface of the nanomaterial completely remains unresolved.

Notwithstanding, the binding of proteins to nanoparticle surfaces may not only afford a new identity to the nanoparticle, but this interaction may also affect the proteins that are adsorbed. In an illustrative example, Deng et al. *(136)* showed that poly(acrylic acid)-coated gold nanoparticles bind fibrinogen, a protein involved in blood clot formation, in a charge-dependent manner, inducing unfolding of the protein, and that binding to integrin receptors on the surface of a monocytic cell line led to activation of the NF-κB pathway and secretion of the proinflammatory cytokine, TNF-α. Moreover, Yan et al. *(137)* noted that albumin undergoes conformational changes upon adsorption onto nanoporous polymer particles, leading to a significant decrease in internalization in undifferentiated THP-1 cells in comparison to the bare particles, while scavenger receptor-mediated uptake in differentiated, macrophage-like THP-1 cells was enhanced by the presence of the unfolded albumin. The latter study emphasizes that adsorbed proteins, such as albumin, may act as opsonins to promote uptake, or, on the contrary, as dysopsonins, depending on the cellular context. In a comprehensive study using silica and polystyrene nanoparticles of various sizes and different surface functionalizations, Tenzer et al. *(138)* could show that plasma protein adsorption occurs very rapidly and that this affects haemolysis (lysis of red blood cells), thrombocyte activation, cellular uptake and endothelial cell death.

However, it remains to be firmly demonstrated if and how the specific identity of the corona proteins is linked to toxicity or other cellular responses. Dobrovolskaia et al. *(139)* reported that the

composition of the protein corona did not correlate with compatibility of colloidal gold nanoparticles with cells of the blood. Ge et al. *(140)* reported that the toxicity of SWCNTs was mitigated after the binding of serum proteins, but this does not necessarily prove that specific proteins are involved; the serum proteins may have saturated the binding sites of the CNTs, thereby preventing further interactions with cellular proteins. Furthermore, in vitro studies using various cell lines have demonstrated that the presence of a protein corona affects the toxicity of silica nanoparticles, possibly through passivation of the particle surface *(141–143)*. The relative proportion of protein versus the amount of nanoparticles also influences the extent of corona formation. An increase in serum concentration approaching in vivo serum levels suppressed in vitro cytotoxic activity of nanoparticles *(144)*.

Thus, while there is increasing evidence for the importance of the protein corona in regulating the cellular interactions of nanoparticles, not least cellular uptake of nanoparticles, it appears difficult at this stage to identify a role for individual proteins in the corona. However, some recent studies have provided evidence for a role of specific proteins in the regulation of cellular uptake. Hence, Fedeli et al. *(145)* identified histidine-rich glycoprotein as the major component of the plasma-derived hard corona on silica nanoparticles and showed that this protein functioned as a dysopsonin when particles were incubated with cells. Saha et al. *(146)* reported that surface functionality can be used to tune the protein corona formed on the surface of cationic gold nanoparticles, dictating the interaction of the nanoparticles with macrophages, and evidence was provided for recognition of specific complement proteins in the biocorona.

Furthermore, while the majority of biocorona studies to date have been performed using human plasma or fetal bovine serum as a source of proteins, it is important to note that the biocorona may consist not only of proteins, but also of lipids, sugars, nucleotides and other biomolecules. Moreover, the biocorona composition may of course vary depending on the portal of entry. Hence, nanoparticles that are inhaled into the airways are more likely to be covered with a corona of lung surfactant consisting of both lipids and proteins, while nanoparticles that are taken up through the oral route would encounter an entirely different mix of biomolecules in the gastrointestinal tract.

Kapralov et al. *(147)* found that pharyngeal aspiration of SWCNTs in mice resulted in adsorption of lung surfactant proteins and surfactant lipids and, furthermore, that this protein–lipid biocorona facilitated uptake of SWCNTs by murine macrophage-like cells. Gasser et al. *(148)* found that precoating MWCNTs with porcine pulmonary surfactant (Curosurf) affected their oxidative and proinflammatory potential in vitro. Raesch et al. *(149)* performed proteomic and lipidomic analysis of nanoparticle corona upon contact with lung surfactant and found differences in protein but not lipid binding for nanoparticles with different surface functionalizations. For the gastrointestinal tract, food content as a source for biomolecules in the corona might also need to be considered. However, there are very few studies to date on the gastrointestinal biocorona on nanoparticles *(150)*. It will also be important to understand whether the biocorona would change as nanoparticles cross biological barriers in the body, or whether a biological "fingerprint" of different anatomical compartments is retained on the surface.

The adsorption of complement factors may be viewed as a special case of biocorona formation and could have pronounced effects if complement is activated *(151)*. Polymer coating of nanomaterials may influence complement activation. Complement activation can be prevented by modifying the density of the polymers on polystyrene nanoparticles *(152)*. Moreover, Hamad et al. *(153)* found that alterations of copolymer architecture on nanoparticles from "mushroom" to "brush" configuration not only switched complement activation from the C1q-dependent classical pathway to the lectin pathway, but also reduced the level of generated complement activation products. These findings provide a rational basis for the intelligent design of immunologically safer nanosystems for clinical applications. In a more recent study, Chen et al. *(154)* reported that dextran-coated superparamagnetic Fe_2O_3 core-shell nanoparticles incubated in human serum and plasma are rapidly opsonized with the third complement component (C3) via the alternative pathway. Thus, complement factors may bind to the nanoparticle surface, or to the biocorona.

The conjugation of common allergens, such as birch or grass pollen or house dust mites, to gold nanoparticles was explored in a recent study, and enhanced allergic responses were found to such

conjugates *(155)*. Specifically, in this in vitro study, gold nanoparticles were conjugated with the major allergens of birch pollen (Bet v 1), timothy grass pollen (Phl p 5) and house dust mites (Der p 1), and differences in the activation of human basophil cells derived from birch or grass pollen-allergic and house dust mite-allergic patients in response to free allergen and nanoparticle–allergen conjugates were determined using the basophil activation assay. The formation of a stable corona was found for all three allergens used. The data suggested that, depending on the allergen, different effects could be observed after binding to nanoparticles, including enhanced allergic responses against Der p 1 and also, for some patients, against Bet v 1 *(155)*. In summary, the data showed that conjugation of allergens to ENMs may modulate human allergic responses. Thus, the formation of an allergen corona could perhaps lead to the exacerbation of symptoms in sensitized individuals. Moreover, adsorption of endogenous "self" proteins to nanoparticles may result in denaturation, where the denatured protein is recognized by immune cells as "non-self" *(156)*.

4.9   The microbiome and the immune system

A wide variety of microorganisms inhabit the body surfaces of essentially all vertebrates. The microbiota is the ecological community of microorganisms that share our body space. In the lower intestine these organisms have evolved to degrade a variety of dietary substances that enhance host digestive efficiency and ensure a steady nutrient supply for the microbes. The microorganisms that are present on human skin, our largest body part, were traditionally considered as a cause of infections, but it has become clear that the specific microbes are necessary for defence and thus for the health of the skin. Invasion of host tissue by resident bacteria has serious health consequences, including inflammation and even sepsis. It is evident that this host–microbiota relationship plays an important role in the development and functioning of the immune system, and that the immune system is crucial to preserving the symbiotic relationship between host and microbiota *(157)*.

Much of our current understanding of the microbiota–immune system interactions has been acquired from studies of germ-free animals. Such gnotobiotic animals are housed in sterile isolators to control their exposure to microorganisms, including viruses,

bacteria, and parasites. More recently, next-generation sequencing technologies have opened new avenues to investigate microbiota complexity. Although microbial cell cultures are still needed, the complex composition of the microbiota can be efficiently studied by 16S ribosomal RNA sequencing and by unbiased deep sequencing of microbial DNA (shotgun sequencing). These high-throughput analyses have made possible the construction of defined microbiotas in different anatomical locations of the host as well as comparison of microbiotas between different animal species and between individuals. Recent technological advances have also provided tools (such as transcriptomics, epigenomics and metabolomics) to explore how microbiota shape many aspects of host physiology and immunity. The application of these new approaches in the context of older technologies has revolutionized the study of interactions between the microbiota and the immune system *(157)*.

Unravelling the complex interactions between the microbial environment and host tissues in the context of exposure to various types of ENMs will provide important insights into how ENMs influence mechanisms of maintenance or disruption of tissue homeostasis. Studies in recent years have underscored that the human microbiome is critically implicated in many diseases, such as obesity, diabetes, atherosclerosis, cardiovascular disease, rheumatoid arthritis, chronic obstructive pulmonary disease, asthma and skin diseases. Analysis of the ENM–microbiota interaction will generate information on possible deleterious effects of ENMs on microbiota and associated pathophysiologies, providing the basis for development of effective post-exposure treatments that might attenuate or eliminate the impact of ENMs on pathological processes mediated by the microbiome *(42)*.

Skin provides a unique opportunity for host–microbiome interaction studies, due to its continuous interactions with the environment and the optimal accessibility (for sampling) of both microbiota and underlying tissues *(158)*. As an ENM exposure route, skin is a relevant target because of the extensive use of cosmetics and sunscreens, and contact with ENM-coated surfaces. Intact skin is an efficient penetration barrier to topical ENMs but there is a lack of knowledge with regard to the effects of ENM exposure in the case of weakened or disrupted skin, for example in conditions such as contact, atopic and irritant dermatitis. More importantly, virtually nothing

is known about the possible effects of cutaneous ENM exposure on the composition of the skin microbiome and host–microbiome interactions in ENM-related tissue inflammation.

The airways are probably the most relevant route for ENM exposure in occupational settings. Until recently, the airways were thought to be sterile unless infected. However, 16S and shotgun sequencing approaches have revealed that the airways harbour a unique steady-state microbiota *(159)*. Virtually nothing is known about the effects of ENM exposure on the airway microbiome and its role in the maintenance or disruption of airway tissue homeostasis. The gastrointestinal tract is an important exposure route for consumers, but it is considered less relevant for workers. It should be noted, however, that a substantial percentage of inhaled nanoparticles are cleared by the mucociliary escalator cells into the oral cavity and thereafter into the gastrointestinal tract, and that ENMs deposited in the skin may reach the gut lumen through hand–mouth contact. Therefore, the gut microbiome is probably involved even in the case of pulmonary or skin exposure *(42)*.

The consequences of ENM interactions with human and environmental microbiota and the outcome of this interaction are probably among the most challenging tasks facing research in nanotoxicology in the coming years. However, only a limited amount of information about the effects of ENMs on microbiota exists in the literature. Most of the previous articles on ENMs focus on in vitro settings and single microbes, or use microbial culture methods for intestinal microbial samples. Available data suggest that ENMs may cause adverse health effects through the direct killing of microorganisms, or through alterations of their function. It should be noted, however, that microbiota research in nanotoxicology is still in its infancy. To date, only a few large-scale microorganism analyses using modern 16S sequencing or similar approaches have been conducted to investigate the effects of ENMs *(160)*. More recent studies have found that while Ag-NPs did not elicit any overall toxicity in mice following oral exposure to human-relevant doses, the particles could induce microbial alterations in the gut *(161)* and associated alterations in the gut immune responses *(162)*. Ag-NPs are expected to have antibacterial effects, but data are lacking for other ENMs.

4.10   Immunotoxicity depending on route of exposure

Inadvertent exposure to nanomaterials may occur primarily via three different routes: on the skin, via inhalation into the lungs, or through oral exposure with transmission via the gastrointestinal tract. Therefore, the following subsections focus the discussion on these three routes of exposure, with a main emphasis on pulmonary exposure, as this is by far the most commonly studied route. Translocation of nanoparticles to the systemic circulation may occur, but direct intravenous administration of nanomaterials will not be discussed, as this is relevant only in the context of nanomedicines, which are, in principle, covered by existing regulations for medicines or medicinal products and subjected to the same scrutiny as any other medicinal products. Nevertheless, investigating the distribution and fate of nanomaterials in the body is of utmost importance for our understanding of nanomaterial toxicity. In particular, particles that enter the bloodstream are rapidly cleared by phagocytic cells belonging to the mononuclear phagocytic system, and subsequently end up primarily in the liver or spleen. This, again, points to the importance of the innate immune system in the surveillance and clearance of foreign objects. However, nanoparticles may also be excreted from the body. For an overview of the adsorption, distribution, metabolism, and excretion of a nanomaterial see section 3.4 on toxicokinetics. It is important to keep in mind that the immune system executes its surveillance role in the whole body, not least in the lungs and in the gastrointestinal tract, as these organs are in direct contact with the external environment.

4.10.1   Dermal exposure to engineered nanomaterials

Overview of skin immunology

Skin is the largest human organ by size and also by surface area in contact with the external environment (average surface area of 2 square metres in adults). This necessitates an impeccable defence system to keep foreign insults at bay. The skin does this with an intricate combination of physical and immunological barriers. The physical barrier is made up of tightly packed corneocytes and an impermeable envelope of highly cross-linked proteins and lipids. The immunological barrier provided by the human skin is expectedly complex and results from intricately orchestrated interaction cascades between multiple

cell types (epithelial, stromal and immune cells) and the foreign stimulus *(163–165)*. In the epidermis, keratinocytes and non-epithelial immune cells, including Langerhans cells and epidermal DCs, form the first line of immunological defence. In the dermis, stroma cells such as fibroblasts and endothelial cells, and a variety of immune cells including macrophages, mast cells, dermal DCs, dermal T cells and innate lymphoid cells, fortify the skin against deeper threats.

Keratinocytes play an integral role in the innate defence system of skin. Upon contact with a wide variety of exogenous stimuli, potentially including nanomaterials, keratinocytes serve as the first line of immunological defence by acting as cellular signal transducers. They do this by secreting a myriad of cytokines and chemotactic agents, as well as by expressing cell surface adhesion molecules (Figure 4.2). These molecules facilitate the recruitment and retention of circulating immune cells into the epidermis. Keratinocytes then amplify this
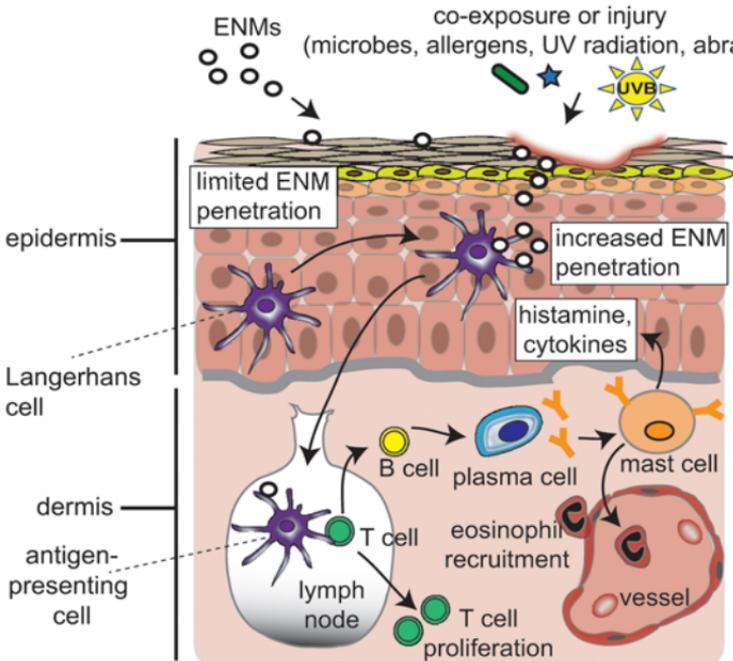
Figure 4.2 Schematic figure showing immune responses to ENMs in the skin

initial immune response by producing additional immunostimulatory molecules once the circulating immune cells arrive in the skin. In addition, keratinocytes themselves can act as antigen-presenting cells. Collectively, keratinocytes therefore constitute a major component linking the innate and adaptive immune systems in the skin, in order to support rapid and distinct responses of human skin to exogenous stimuli *(166)*.

Langerhans cells are the resident antigen-presenting cells in the epidermis. Upon activation they migrate to the lymph nodes, where they interact with T cells and B cells to initiate and shape the adaptive immune response *(167)*. In skin contact hypersensitivity, Langerhans cells activate regulatory T cells while inhibiting cytotoxic T cells *(168)*. Normal human skin is home to approximately 20 billion T cells, of which 80% are $\alpha\beta$ T cells *(169)*. T cells in the epidermis are dominated by CD8+ cells, while CD4+ T cells are the majority in the dermis *(170)*. Dermal DCs are critical in cross-presenting keratinocyte-derived antigens for the activation of cytotoxic T cells *(171, 172)*. Aside from multiple immune cells, an arsenal of antimicrobial peptides are present in the skin, which are significant contributors in preventing microbial growth in healthy skin *(173)*. However, it has not been established if antimicrobial peptides play any role in helping skin cope with exposure to ENMs.

In a study of dermal sensitization, an enhancement of reactivity to the model sensitizer 2,4-dinitrochlorobenzene (DNCB) was reported when nano TiO_2 was administered subcutaneously at the base of the ear in a local lymph node assay (LLNA) *(174)*. Augmentation of the Th2 response was indicated by the increased production of IL-4 and IL-10 by the lymphocyte population isolated from the draining lymph node. The effective concentration for inducing a threefold increase in cell proliferation compared to controls (EC3), used as a threshold value in the LLNA, was decreased for DNCB when nano TiO_2 was used, but not with pigment TiO_2. When amorphous silica nanoparticles were intradermally injected together with mite antigen of *Dermatophagoides pteronyssinus*, an aggravation was observed in the *D. pteronyssinus*-induced atopic dermatitis in a manner dependent on nanoparticle size (range investigated from 30 to 1000 nm), with the smaller nanoparticles inducing the most severe effects *(175)*. IgE and Th2 responses were also enhanced. The dose of silica nanoparticles

was fixed at 250 µg, resulting in both a higher number of nanoparticles and a higher surface area for the smaller nanoparticles. So, it is not clear whether the effect was a matter of size only (the smaller, the more active) or depended on the dose itself, as higher dosages were used when the dose was expressed as a different metric (for example, number of particles or surface area).

Both diesel exhaust particles and carbon black had adjuvant activity in a murine OVA sensitization model when OVA and diesel exhaust particles or carbon black were coadministered. Both local lymph node responses in terms of cell proliferation and systemic IgE production were enhanced in the diesel exhaust particles and carbon black treated animals compared to controls *(176)*.

So far, the mechanism of enhanced immune responses by (nano) particles has not yet been elucidated, even in the use of particles as vaccine adjuvants that have been applied for decades. However, it is evident that coexposure to particulates and antigen poses an increased risk for sensitization.

Dermal toxicity of nanomaterials

To answer the question of whether skin exposure to nanomaterials is a threat to the human immune system, the possibility of the nanomaterial in question penetrating the skin will first need to be established. It was widely accepted that penetration of engineered nanoparticles across the skin was highly unlikely *(177–179)*. Any penetration, if at all, was considered to be trivial, and happened through hair follicle openings rather than across the stratum corneum *(180– 182)*. However, the bulk of these early studies on the topic focused on penetration across animal or healthy adult human skin models. More recently, reports have emerged that nanomaterial penetration through skin will increase significantly when the barrier function of skin is compromised, such as in UV-damaged or tape-stripped skin *(183, 184)*. Quantitatively, Miquel-Jeanjean et al. *(185)* calculated that 0.19 $\pm$ 0.15 weight per cent (wt%) of TiO_2 nanoparticles can reach viable cells in skin after 24 hours exposure on intact skin, which doubled to 0.39 $\pm$ 0.39 wt% when the skin was damaged by UV irradiation. There is also a current lack of knowledge in relation to nanomaterial penetration across the skin in situations of long-term use and across

skin of varying states of health *(179, 186)*. From the perspective of chronic skin exposure and potential prolonged low-level penetration of nanomaterials across the skin, there is therefore a significant need to establish immunotoxicity influences of nanomaterials in the skin. Representative reports of such studies are discussed below.

Results from in vivo studies

Skin irritation and sensitization tests make up the bulk of available reports about skin immunotoxicity of ENMs. Most of these evaluate the influence of metal-based (pure metal and metal oxide) nanoparticles, due to the frequent use of these in cosmetics and personal care products, primarily as UV filters and antimicrobial agents. In a recent study, TiO_2 nanoparticles (anatase; < 25 nm diameter) were evaluated for local immune effects after dermal exposure on the ears of female BALB/c mice *(187)*. Auricular lymph node cell proliferation was not affected by exposure to the TiO_2 nanoparticles, based on the LLNA. However, skin irritation, based on measuring the percentage of ear thickness change, was observed with TiO_2 exposure. Following these results, the group sensitized mouse ear skin with TiO_2 nanoparticles and subsequently challenged the skin with further exposure to TiO_2, with no significant change in ear thickness found. Using a similar mouse model, Smulders et al. *(188)* exposed mouse ear skin with TiO_2, silver and SiO_2 nanoparticles for a day before applying DNCB as a known dermal sensitizer for three days. It was found that dermal exposure to silver or SiO_2 nanoparticles prior to DNCB sensitization did not influence the stimulation index, which was calculated after six days of exposure. However, application of TiO_2 nanoparticles prior to DNCB sensitization resulted in a significant increase in the stimulation index, along with increased titanium concentration in the draining lymph node cells of this group.

In an attempt to understand skin sensitization of nanosized UV absorbers, ZnO, TiO_2 and SiO_2 nanoparticles embedded in textiles were applied onto rabbits for acute dermal irritation evaluation (OECD TG 404) and guinea-pigs for sensitization tests (OECD TG 406) *(189)*. Results suggest that none of the analysed materials or modifiers induced major skin reactions. Only a TiO_2 (TK44) and ZnO (Z11) based modifier were classified as mild sensitizers based on the calculated primary irritation index.

Guinea-pigs were also used for testing skin sensitization from repeated exposure to high aspect ratio hydroxyapatite nanoparticles of minor axis greater than 50 nm *(190)*. Through visual inspection and analysing blood parameters, oxidative stress in the liver and brain, and DNA damage in the liver, it was concluded that the hydroxyapatite nanoparticles did not induce skin sensitization or oxidative damage up to a delivered dose of 100 micrograms per millilitre (μg/mL).

Carbon-based nanoparticles have been explored as potential antioxidants in products for topical application but few in vivo data are available, probably due to the policy of not using animal testing in the cosmetics industry and the availability of an in vitro alternative for skin irritation testing, as described in guideline OECD TG 439. In one such study, single-walled carbon nanohorns (SWCNHs) were exposed onto the back skin of rabbits by applying a cotton lint laden with 0.015 g of the as-grown SWCNHs *(191)*. The primary irritation index was found to be 0, suggesting low acute toxicities caused by dermal exposure to SWCNHs.

Because of the uncertainty regarding nanomaterial influence on skin of various conditions, there is a need to adopt in vivo models of skin diseases to study nanomaterial immunotoxicity. In this respect, atopic dermatitis models are interesting because of the hypothesis that skin with defective physical barriers would facilitate higher levels of nanomaterial penetration, which could elicit an immune response. However, there are limited reports of such studies, and most are focused on studying metal-based nanoparticles. Yanagisawa et al. *(192)* found that repeated exposure of 20 μg of TiO_2 nanoparticles (15, 50, or 100 nm) in 10 microlitres (μL) of saline to the ear skin of NC/Nga mice over a 17-day period resulted in significant increase of histamine levels in blood serum and IL-13 expression in the ear, independent of nanoparticle size. Moreover, the TiO_2 nanoparticles aggravated epidermis thickening when exposed together with mite allergen extracts, along with elevated levels of IL-4 in the skin, total IgE and histamine in blood serum. The authors concluded that dermal exposure to TiO_2 nanoparticles when skin barrier function is compromised will exacerbate atopic dermatitis symptoms through Th2-biased immune responses. The same group repeated a similar study to evaluate size effects of polystyrene nanoparticles on atopic dermatitis *(193)*. Similar to TiO_2 nanoparticles, polystyrene

nanoparticles also aggravated epidermis thickening when exposed together with mite allergen extracts. In contrast to TiO_2, this effect was more significant with smaller polystyrene particles (25, 50 nm) compared to larger ones (100 nm). However, it should be noted that the nanoparticles were injected intradermally rather than applied topically in these studies.

In comparison, Hirai et al. *(194)* carried out an immunotoxicity study via repeated topical application of silica nanoparticles (30 nm) on the ears and upper backs of NC/Nga mice. When silica nanoparticles were applied together with mite allergen extracts, atopic dermatitis-like lesions were aggravated, based on ear thickness and histology assessments, compared to application of either the silica nanoparticles or the mite allergen extracts alone. In addition, concurrent application of silica nanoparticles and mite allergen extracts resulted in the production of allergen-specific Th1-related IgG2a and Th2-related IgG1, which were absent when the mice were exposed to nanoparticles applied separately from the allergen or to well dispersed nanoparticles. It was concluded that the presence of allergen-adsorbed agglomerates of silica nanoparticles led to a low IgG–IgE ratio, which is a key risk factor in human atopic allergies.

Besides a transgenic model, it is also possible to evoke local inflammation and allergy in normal skin by sensitizing with an allergen or superantigen to model atopic dermatitis. Using the back skin on BALB/c mice and OVA/staphylococcal enterotoxin B as the allergen/superantigen cocktail, Ilves et al. *(195)* showed that nanosized ZnO (< 50 nm), as opposed to bulk ZnO (> 100 nm), is able to reach the dermis of the allergic skin. Both particle types were able to alleviate local allergic effects on the skin, although this ability was more pronounced with the nanosized ZnO. However, ZnO nanoparticles induced systemic production of IgE more significantly than larger ZnO particles, providing evidence of the allergy-promoting potential of ZnO nanoparticles.

Collectively, there are a few in vivo studies that have investigated immunotoxic effects of nanoparticles. These studies suggest a lack of effect on intact skin but a worsening of symptoms in atopic skin exposed to nanoparticles and allergens.

Results from in vitro studies

Nanoparticles of various configurations are being produced for a wide range of applications. These may be doped or surface treated with other materials to achieve a particular function. It is prudent that immunotoxicity of such hybrid nanoparticles be evaluated in relation to their stoichiometric composition or spatial arrangement. In this respect there is significant literature on using in vitro models, primarily monolayer skin cell cultures, for nanoimmunotoxicity studies. Similar to the in vivo studies, most in vitro studies have focused on metal-based (pure metal and metal oxide) nanoparticles due to the relevance of their application in commercial products.

Schaeublin et al. *(14)* found that nanoparticles of differing aspect ratios could elicit different cellular response in human keratinocytes. Gold nanospheres (20 nm diameter) were compared with gold nanorods (16.7 nm diameter, 43.8 nm long) for their toxic effects on immortalized human keratinocytes (HaCaT). The nanorods were found to be cytotoxic from 25 μg/mL onwards and caused more significant ROS production compared with the nanospheres. Notably, the nanorods induced higher levels of IL-1α, IL-1β, IL-18 and TNFSF10,[5] suggesting that gold nanoparticles of a higher aspect ratio induce greater immune response in keratinocytes. However, the nanospheres were coated with mercaptopropane sulphonate, while the nanorods were coated with polyethylene glycol (PEG). Although both coating materials are known to be biocompatible, some differential effects could be present.

Ryman-Rasmussen, Riviere and Monteiro-Riviere *(196)* determined that surface coatings can affect the irritation potential of quantum dots in epidermal keratinocytes. Quantum dots of a spherical core-shell structure with a diameter of 4.6 nm and quantum dots of an ellipsoid core-shell structure with diameters of 6 nm by 12 nm were coated with PEG, PEG-amine or carboxylic acid to create neutral, cationic and anionic surfaces, respectively. Primary human keratinocytes were exposed to these quantum dots and were found to internalize them after 24 hours. Neutral quantum dots did not result in significant cytotoxicity in the keratinocytes. Conversely,

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[5] Tumour necrosis factor (ligand) superfamily, member 10.

only quantum dots with anionic surfaces significantly increased the release of proinflammatory cytokines IL-1β, IL-6 and IL-8. These data indicate that surface coating on nanoparticles can be an important determinant of immunotoxicity in human keratinocytes.

A valid concern of in vitro nanotoxicology studies is the relevance of the dosage used in relation to real-life exposure levels. In this aspect, a recent report demonstrated that TiO_2 nanoparticles can induce autophagy in primary human keratinocytes at serial diluted concentrations as low as 100 femtograms per millilitre (fg/mL) *(197)*, which would be a realistic level of cellular exposure even with the anticipated low levels of nanoparticle penetration across the skin. Although autophagy is inherently a survival mechanism, its induction suggests some level of sinister influence by the nanoparticles. Autophagy was recently implicated in keratinocyte inflammation response by negatively regulating p62 expression *(198, 199)*, which is involved in increased inflammation and tumourigenesis *(200)*.

Carbon-based nanoparticles are the next most common group of nanoparticles used in immunotoxicity studies. For example, fullerene-based amino acids were tested with human epidermal keratinocytes over a concentration range *(201)*. The fullerene-based amino acids induced high levels of IL-8, IL-6 and IL-1β. Conversely, TNF-α and IL-10 levels were insignificant. Various configurations of CNTs have received significant attention due to reports of cellular responses that are mechanistically similar to those seen in asbestosis *(202, 203)*. In terms of immunotoxicity, exposing HaCaT cells to SWCNTs resulted in elevated oxidative stress levels shown by formation of free radicals, accumulation of peroxidative products and antioxidant depletion *(204)*, suggesting the initiation of an immune response by keratinocytes.

4.10.2 *Impact of nanomaterials on the respiratory system*

Nanomaterials pose potential risks for immune-mediated lung diseases through occupational, consumer, or environmental exposure *(205, 206)*. Immunotoxicity is defined as any adverse effect on the immune system following toxicant exposure that results in immune stimulation or immune suppression *(207, 208)*. In the lung, immunostimulation increases the incidence of allergic reactions,

inflammatory responses or autoimmunity, while immunosuppression suppresses the maturation and proliferation of immune cells, resulting in increased susceptibility to infectious diseases or tumour growth. ENMs have been reported to exhibit either immunostimulatory or immunosuppressive effects in the lung, and this largely depends on the specific type of nanomaterial in question. However, the effects of nanomaterials on the immune system can also depend on the context of exposure; for example, repeated exposures versus nanomaterial exposure after the establishment of allergic inflammation.

Methods of administration to the lungs

Inhalation is a major route of exposure for ENMs, and therefore the lungs are a major target of ENM-initiated immunotoxicity. Because of their small size inhaled nanoparticles easily reach the lower respiratory region, principally through diffusion, where they deposit on the alveolar epithelium. Larger aggregates of nanoparticles may also be subjected to the forces of sedimentation and deposit in the lower airways of the lung and at alveolar duct bifurcations. Depositions of (nano)particles in the various regions of the lung can be modelled by using the ICRP or MPPD models as described above *(209–211)*. Once deposited, ENMs trigger a complex sequelae of events that activate the immune system in the lungs, beginning with stimulation of the respiratory epithelium to release soluble chemotactic factors that attract resident alveolar macrophages as well as neutrophils, eosinophils and lymphocytes to the lungs (Figure 4.3). Epithelium-derived chemokines also attract DCs, which sample and transport inhaled foreign substances, including nanoparticles, to draining lymph nodes to programme naïve Th cells to differentiated Th phenotypes (Th1, Th2, Th17, T-reg). These diverse cell types participate in the complex immunotoxic response to ENMs.

The deposition of inhaled ENMs is determined by a number of factors, including particle size, shape, electrostatic charge and aggregation state. For example, inhalation exposure to well dispersed CNTs in mice results in deposition in the distal regions – the alveolar duct bifurcations and alveolar epithelial surfaces – of the lungs of mice or rats *(213, 214)*. Agglomeration of ENMs, alternatively referred to as state of dispersion, refers to nanoparticles that loosely adhere to one another through non-covalent interactions, such as electrostatic charge,
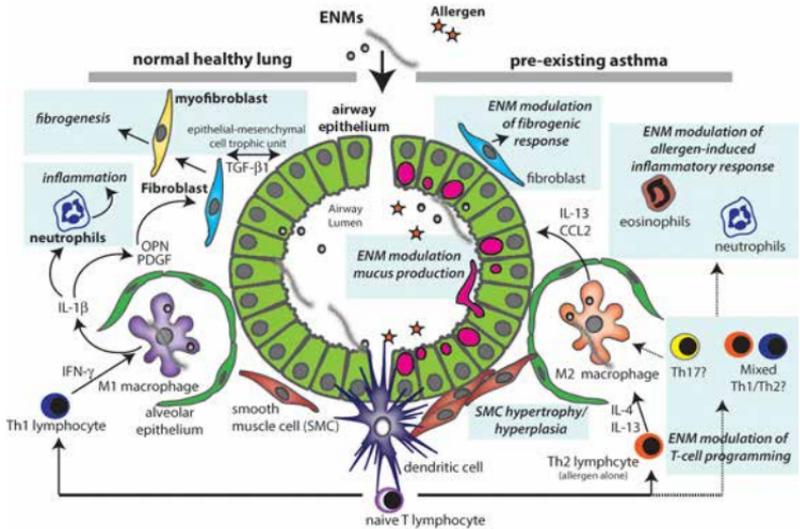
Figure 4.3 Interaction of ENMs with immune cells in the lung under normal conditions and pre-existing asthma
Source: Adapted from Thompson et al. *(212)*.

whereas aggregation refers to strongly bound particles by covalent interaction between particles. Dispersion of agglomerated ENMs can be achieved through surface functionalization to reduce electrostatic charge, or by suspension of ENMs in surfactant-containing media. The relative state of dispersion often influences the type of immune or pathological response to ENMs. For example, dispersed CNTs cause diffuse interstitial fibrosis throughout the lower lung, whereas agglomerated CNTs tend to cause focal granuloma formation.

Aggregation of ENMs also depends to some extent on the method of delivery to the lungs. Intratracheal instillation or oropharyngeal aspiration techniques for delivery to the lungs of rats or mice can result in more aggregation of ENMs and generally do not faithfully reproduce deposition patterns that are achieved with inhalation exposures to dry aerosolized or nebulized suspensions of ENMs.

There may also be a difference in the local dose, as instillation results in the delivery of the total dose as a bolus whereas inhalation presents a more evenly distributed exposure dose. However, major advances have been made in methods for dispersing ENMs in aqueous suspension using surfactant-containing media prior to instillation or aspiration in rats or mice. Inhaled or well dispersed instilled ENMs also reach the subpleural region of the lungs via either macrophage-dependent or -independent processes. Persistent ENMs such as CNTs may remain embedded within the subpleural tissue of mice for months *(61)*. Some CNT-bearing macrophages exit the lung via the pleural lymphatic system and enter the pleural space or can be found in lung-associated lymph nodes. This type of translocation of ENMs is discussed further below.

Translocation of nanomaterials

Most of the surface area of the alveolar region of the lung is lined with thin, pancake-shaped type I alveolar epithelial cells, and a lesser surface area of the alveolus is occupied by type II epithelial cells, which produce surfactant that lines the alveolus and serve as progenitor cells for the type I epithelial cells. ENMs that enter the alveolus must pass through the surfactant coating covering the type I epithelium, then pass either through or around the epithelial cells and the underlying interstitial space, finally passing through or around the capillary endothelial cells to enter the bloodstream. Following inhalation exposure, it is estimated that nanoparticles with a diameter less than ~34 nm have the ability to cross the alveolar epithelium of the lung and endothelium of the pulmonary capillaries to enter the circulation *(215)*. The translocation of gold nanoparticles across the alveolar barrier (air–blood barrier) has been demonstrated to be inversely related to nanoparticle size; smaller particles with greater specific surface area crossed this barrier most effectively *(216)*, though translocation was low when expressed as a percentage of the administered dose. Moreover, translocation was greater for negatively charged nanoparticles than positively charged nanoparticles. Once in the circulation, nanoparticles can again cross the endothelial barrier of the vascular system to reach organs such as the heart, liver, spleen, kidney and brain. There is also evidence that ENMs can cross the placental barrier in pregnant mothers to gain access to the developing fetus *(217)*.

ENMs may also translocate from the lungs via the lymphatic system. The pulmonary lymphatic system is a vascular network that serves to remove excess fluid from the connective tissue spaces of the lung parenchyma. The lymphatic system is also known to play an important role in clearing particulate material from the lung to the lymph nodes *(218)*. The lymphatic system in the lung is divided into superficial and deep portions, but these two portions are connected. The superficial portion is located in the connective tissue of the pleura lining the lung. The deep portion is in the connective tissue surrounding the bronchovascular tree. The two portions connect in the interlobular septa. The lymphatic vessels are structurally similar to thin-walled veins. The presence of valves in the lymphatic vessels and the movement of the lung during respiration promote the flow of lymph from the periphery and pleura towards the hilus. Afferent lymphatics from the lung drain into the tracheobronchial lymph nodes. Lymph from the tracheobronchial and hilar nodes drains into the thoracic, right, and left lymphatic ducts, and from these ducts the lymph then drains into the systemic venous system. Therefore, ENMs can reach the systemic circulation via lymphatic clearance. For fibrelike ENMs such as CNTs, lymphatic clearance could be an important conduit for translocation to other tissues. It has been reported that MWCNTs delivered to the lungs by oropharyngeal aspiration are found in extrapulmonary organs such as the liver and kidney *(219)*.

ENMs, either by direct translocation or through the release of soluble mediators from the lungs, are capable of activating immune responses in tissues and organ systems beyond the lungs, including the spleen and heart. For example, inhaled MWCNTs cause systemic immunosuppression in mice through a mechanism that involves the release of TGF-β1 from the lungs, which enters the bloodstream to signal cyclooxygenase(COX)-2-mediated increases in prostaglandin E2 and IL-10 in the spleen, both suppressing T cell proliferation *(107, 220)*. Also, SWCNTs or MWCNTs delivered to the lungs of mice have been reported to exacerbate cardiovascular dysfunction and disease *(221)*. These studies showed no evidence of CNT translocation from the lung. Rather, the systemic effects were probably due to the release of soluble cytokines or growth factors from the lung. However, it is possible that at least some ENMs will translocate from the lung to distant organs to modulate immune responses.

Nanomaterial interaction with lung cells

Lung epithelium

The lung epithelium is the primary site of deposition for ENMs. The lower respiratory tract comprises the conducting airways (trachea, bronchi and bronchioles) and the lung parenchyma, which consists primarily of gas exchange units (alveoli) (see Figure 4.3). The trachea, bronchi and bronchioles conduct air to the pulmonary parenchyma. The bronchi bifurcate to form bronchioles and continue to progressively bifurcate in a treelike fashion to form bronchioles of decreasing diameter. The most distal conducting segment of the tracheobronchial tree is called the terminal bronchiole, which bifurcates to form respiratory bronchioles that contain some alveolar ducts and terminate in clusters of alveolar sacs. Well dispersed ENMs reach the alveolar region, where they interact with two primary alveolar epithelial cell types: type I and type II cells. Type I cells cover the majority of the alveolar surface and constitute part of the air–blood barrier, which also includes the capillary endothelium and interstitial compartment sandwiched between the type I epithelium and endothelium.

Type I cells comprise 8–11% of the structural cells found in the alveolar region, yet cover 90–95% of the alveolar surface *(218)*. Their major function is to allow gases to equilibrate across the air–blood barrier and to prevent leakage of fluids across the alveolar wall into the lumen. The epithelial type I cells are particularly sensitive to damage from a variety of inhaled toxicants, including ENMs, due to their large surface area. Moreover, their repair capacity is limited because they have few organelles associated with energy production and macromolecular synthesis. Type II cells comprise 12–16% of the structural cells in the alveolar region, but cover only about 7% of the alveolar surface. They are cuboidal cells with a microvillus surface and unique organelles called lamellar bodies that store surfactant. The major function of type II cells is to secrete surfactant to lower the surface tension in the alveoli, thereby reducing the filling of the alveolar compartment with fluid and alveolar collapse. Type II cells also serve as a progenitor cell for type I cells, which cannot replicate. Therefore, type II cells are critical to alveolar epithelial repair after injury. The wall of the alveolus is composed of the alveolar epithelium, a thin layer of collagenous and elastic connective tissue interspersed with fibroblasts (termed the pulmonary interstitium),

and a network of capillaries lined by endothelial cells. This distance between the alveolar space and the capillary lumen is known as the air–blood barrier. The air–blood barrier is a multilayered structure approximately 0.4 μm in thickness that consists of an alveolar type I cell, alveolar basement membrane, interstitial space, endothelial basement membrane, and capillary endothelial cell.

ENMs that deposit on the type I epithelium either translocate across the air–blood barrier or remain on the surface, or are taken up by the epithelium; this depends largely on the physicochemical features of the ENMs, such as size, shape and charge. ENMs are further modified by biocorona formation once they interact with the surfactant layer that covers the alveolar epithelium. ENMs are also taken up by alveolar macrophages (discussed below). As an example, inhaled MWCNTs that deposit on the alveolar surface in mice are taken up to some extent by macrophages, but some singlet MWCNTs and small agglomerates penetrate the type I epithelium. This is probably an important event in mediating alveolar cell injury and stimulating the type I cells to produce chemokines that attract circulating inflammatory cells (for example, neutrophils) from the blood to migrate across the air–blood barrier to the alveolus. Neutrophilic influx is regulated by CXC chemokines, such as CXCL1, CXCL2 and CXCL5.

While well dispersed ENMs act in the alveolar region, agglomerated ENMs deposit on the airway epithelium or in particular at regions where airways and alveolar ducts bifurcate (alveolar duct bifurcations). The airway epithelium forms a continuous lining for the conducting airways. The varied composition of the epithelium allows it to perform a variety of functions. First, the epithelium, along with its apical mucus layer and its basal lamina, comprise an important barrier against inhaled ENMs. The apical surfaces of the airway epithelial cells are connected by tight junctions and effectively provide a barrier that isolates the airway lumen. Second, the various airway epithelial cells produce a mixture of secretions composed of (a) an aqueous "sol" phase containing proteins, lipids and ions; and (b) a gel phase containing mucus. Third, ciliated cells comprise the largest proportion of exposed cells in the normal airway and, as discussed above, they propel the mucus within the airway lumen proximally, thereby mediating clearance of inhaled particles and debris. Fourth, the airway epithelium exhibits repair following injury, thereby establishing

normal airway architecture. Fifth, the airway epithelium can produce a variety of soluble mediators (cytokines, growth factors, protease and lipid mediators) that modulate the responses of other lung cells, including airway smooth muscle cells, fibroblasts, immune cells and phagocytes. During an immune response to inhaled ENMs, the airway epithelium is stimulated to secrete chemokines that attract DCs to the basal side of the epithelium, where they insert their dendritic processes between the epithelial cells to contact ENMs on the cell surface and after migration present ENMs to naïve T cells in draining lymph nodes to promote T cell differentiation (discussed further below).

Alveolar macrophages

Alveolar macrophages serve a central immune defence role against inhaled exogenous agents, including microorganisms, particles and fibres. Most inhaled ENMs deposited in the distal regions of the lung are avidly taken up by alveolar macrophages. However, the majority of engulfed ENMs are in an agglomerated or aggregated form (clusters of nanoparticles). While aggregated ENMs are engulfed by macrophages, individual nanoparticles can escape immune surveillance and phagocytosis. For example, individual CNTs evade phagocytosis or uptake by macrophages and can be detected by TEM within epithelial or mesenchymal cells *(222)*. Agglomerated or aggregated ENMs taken up by macrophages via phagocytosis are cleared from the lungs through two primary mechanisms: the mucociliary escalator, and the lymphatic drainage system. The mucociliary escalator comprises a coating of mucus on the surface of the airways that is constantly moving up the airways by the coordinated movement of cilia on the airway epithelium *(218)*. Macrophages with engulfed particles or fibres migrate to the distal portion of small airways, where they are transported by the escalator to larger airways and ultimately out of the trachea to the larynx, where they are swallowed or expelled through coughing. A secondary macrophage-mediated clearance passage for ENMs out of the lung is the lymphatic drainage system, which includes lymphatic vessels that drain into the pleural cavity. Rigid, high aspect ratio ENMs (fibre- or tube-shaped) can present a problem for macrophage-mediated clearance if the nanofibre or nanotube exceeds the width of the engulfing phagocyte. For example, migration of macrophages containing CNTs across the pleura could cause DNA damage to mesothelial cells similar to asbestos fibres *(223)*.

However, whether CNTs possess pleural carcinogenicity, as in the case of asbestos fibres, remains unknown. It seems likely that when the CNTs share similar characteristics to asbestos fibres in terms of fibre length, rigidity and biopersistence, they can induce tumours *(224, 225)*. Whereas these non-degradable persistent CNTs with a rigid structure and a certain length did induce tumours, more tangled CNTs did not *(226)*. Inhaled MWCNTs also reach lung-associated lymph nodes in rats, and macrophages probably play a role in the trafficking of ENMs to lymph nodes.

Certain high aspect ratio ENMs (rigid CNTs, nanowires, nanofibres) are capable of disrupting macrophage function by causing frustrated phagocytosis, which results in the release of inflammatory mediators (ROS and cytokines) and cell death *(12, 223)*. The innate immune function of macrophages could also be compromised by formation of bridges composed of parallel bundles of CNTs that link two or more macrophages *(227)*. Macrophage phagocytosis and chemotaxis in rat alveolar macrophages in vivo is impaired by exposure to TiO_2 nanoparticles *(228)*. ENMs have also been reported to impair phagocytosis of microbes. For example, mice exposed to SWCNTs have impaired clearance of the bacterium *Listeria monocytogenes (229)*. ENMs can also cause cell death of macrophages by inducing mitochondrial stress. For example, CeO_2 nanoparticle toxicity to human peripheral blood monocytes was found to be caused by mitochondrial damage and overexpression of apoptosis-inducing factor, but was independent of ROS production, and resulted in autophagy *(23)*. Furthermore, autophagy induced by CeO_2 nanoparticles was further increased after pharmacological inhibition of tumour suppressor protein p53. Inhibition of autophagy partially reversed cell death by CeO_2 nanoparticles.

Macrophage activation by ENMs involves a complex network of intracellular signalling pathways, some of which are designed as protective responses to oxidative stress and cell injury. Specific types of ENMs have been reported to either enhance or suppress ROS-mediated events in macrophages. For example, Ag-NPs increase the expression of NF-κB and COX-2 in RAW 264.7 macrophages, whereas no such proinflammatory effect was found for gold nanoparticles *(230)*. MWCNTs also increase levels of the COX-2 enzyme and do so through a MAP kinase-dependent signalling pathway in mouse RAW

264.7 cells in vitro *(231)*. Also, MWCNTs activate the antioxidant mediator Nrf2 in cultured human THP-1 cells *(232)*. Both COX-2 and Nrf2 are protective factors against lung disease that are increased to counteract ROS-induced cellular stress initiated by CNTs. In contrast to MWCNTs, platinum nanoparticles suppress inflammatory responses of RAW 264.7 cells by reducing bacterial LPS induction of ROS, extracellular signal-regulated kinase (ERK) phosphorylation, and levels of COX-2 and inducible nitric oxide synthase (iNOS) *(233)*. The studies collectively suggest that the effects of ENMs on either inducing or suppressing proinflammatory signalling pathways in macrophages could be determined by multiple factors, including shape, elemental composition, and ROS-generating potential of the ENM.

The uptake of ENMs could have a variety of consequences related to macrophage biology and function. The high aspect ratio ENMs (for example, CNTs) cause inflammasome activation in macrophages, which results in the processing and secretion of the proinflammatory cytokines IL-1β and IL-18 *(80, 82, 234)*. Inflammasome activation by MWCNTs and other high aspect ratio materials (for example, nanofibres, TiO_2 nanobelts) is mediated by lysosomal disruption and ROS production *(80)*. Inflammasome activation leading to the release of mature IL-1β has been proposed as a pro-fibrogenic event, and downstream targets of IL-1R activation include upregulation for growth factor receptors such as PDGF-Rα *(235, 236)*.

While inflammasome activation and subsequent IL-1β release has been proposed as contributing to ENM-induced immunotoxicity and lung disease pathogenesis, macrophage IL-1β release is also an important innate immune response for recruiting neutrophils to the lung to participate in microbial killing and the resolution of inflammation *(237)*. Exposure to certain ENMs (TiO_2, ZnO, CNTs) results in a Th1 immune cell microenvironment that promotes polarization of classically activated macrophages (M1). M1 macrophages are capable of inflammasome activation and are thought to play essential roles in microbial killing and innate immune responses. In allergic asthma or fibrosis, macrophages are polarized to alternatively activated macrophages (M2) in the presence of Th2 cytokines IL-4 or IL-13. M2 macrophages are thought to play an important role in fibrosis and cancer *(238)*. While little is known about inflammasome activation and IL-1β release by M2 macrophages

compared with M1 macrophages, a decrease in caspase-1 expression and pro-IL-1β processing has been described for human monocytes treated with IL-13 *(239)*. While the M1/M2 nomenclature is widely used, recent evidence suggests that there is a greater complexity of macrophage subtypes *(49)*. IL-13 or IL-4 suppresses inflammasome activation in monocytes in vitro, and mice with Th2-type inflammation following house dust mite allergen sensitization display reduced inflammasome activation and IL-1β production following exposure to MWCNTs, yet have increased airway fibrosis *(114)*. Also, ENMs alone could modify macrophage phenotype, as discussed above. For example, alveolar macrophages from rats exposed to CeO_2 nanoparticles had increased levels of arginase-1 mRNA, which is a marker of M2 macrophages *(240)*.

Neutrophils, eosinophils and mast cells

Neutrophils are cells that rapidly infiltrate the lungs following initiation of a Th1 immune response. They are an important component of the innate immune response, and play a role in microbial killing via extrusion of cytoplasmic "nets" composed of nucleic acids decorated with proteinases. Neutrophilic inflammation can be rapidly resolved via recognition and phagocytosis by macrophages. A variety of ENMs stimulate neutrophilic inflammation in the lungs of mice or rats, including SWCNTs, MWCNTs, TiO_2, silver, SiO_2 and nickel *(27, 241–245)*. While neutrophils participate in the inflammatory response to many types of ENMs, there is little evidence that ENMs are engulfed by neutrophils. However, nickel nanoparticles are avidly engulfed by neutrophils, as well as macrophages, lavaged from the lungs of mice one day after oropharyngeal aspiration exposure *(245)*. In addition, inhalation exposure to CeO_2 nanoparticles results in phagocytosis by both neutrophils and macrophages *(246)*. These demonstrate that some types of ENMs directly interact with neutrophils and thus could impair the normal immune functions of these inflammatory cells.

Eosinophils are cells that rapidly infiltrate the lungs following initiation of a Th2 immune response. They are the hallmark inflammatory cell of allergic lung diseases, particularly asthma. Allergen challenge is commonly used in rodents to elicit an allergic eosinophilic lung inflammatory response. Some ENMs have also been reported to cause eosinophilic inflammation in the lungs of mice, including certain types of MWCNT (rodlike), and certain forms of

TiO$_2$ (nanobelts). Like neutrophils, there is little information showing that ENMs are taken up by eosinophils. However, mice challenged with fungal spores and then exposed to gold nanoparticles showed eosinophilic lung inflammation, and approximately 14% of eosinophils contained gold nanoparticles within intracellular vesicles *(247)*.

Mast cells are also important in the allergic immune response *(248)*. Upon activation mast cells release histamine, which alters vascular permeability. Mast cells have been shown to participate in ENM-induced immune and inflammatory responses after pulmonary exposure, influencing physiological responses in other organ systems such as the heart. For example, mast cells play a central role in the activation of the IL-33/ST2 axis to mediate adverse pulmonary and cardiovascular responses to MWCNTs *(112)*. Mast cells also contribute to altered vascular reactivity and ischaemia–reperfusion injury following CeO$_2$ nanoparticle instillation *(249)*. These findings demonstrated that CeO$_2$ nanoparticles activate mast cells, contributing to pulmonary inflammation, impairment of vascular relaxation and exacerbation of myocardial ischaemia–reperfusion injury. In addition, anatase and rutile TiO$_2$ nanoparticles induces histamine secretion in mast cells *(250)*.

Table 4.1 summarizes the types of lung inflammatory cell infiltration observed in rodents following exposure to ENMs.

Table 4.1 Types of lung inflammatory cell infiltration observed in rodents following exposure to selected ENMs

ENM	Shape or characteristic	Species or strain	Inflammatory cell type	Reference
MWCNT	Flexible tube	Mouse, rat	Neutrophilic	Rydman et al. *(113)* Bonner et al. *(241)*
MWCNT	Rodlike tube	Mouse	Eosinophilic	Rydman et al. *(113)*
SWCNT	Flexible tube	Mouse, rat	Neutrophilic	Shvedova et al. *(251)* Mangum et al. *(227)*
TiO$_2$	Sphere	Mouse, rat	Neutrophilic	Gustafsson et al. *(242)* Bonner et al. *(241)*
TiO$_2$	Rigid nanobelt	Mouse, rat	Neutrophilic, eosinophilic	Bonner et al. *(241)*
Silver	Sphere	Mouse	Neutrophilic	Silva et al. *(243)*
SiO$_2$	Sphere	Mouse	Neutrophilic	Brown et al. *(244)*

Dendritic cells

Dendritic cells (DCs) are important immune initiators that serve to capture and present allergens to naïve T cells, thereby driving T cell polarization *(252)*. DCs mediate the first step in the allergic immune response through uptake and presentation of allergen to naïve T cells. Macrophages and B lymphocytes may also serve as antigen-presenting cells. DCs acquire and "sample" allergens deposited on the airway epithelium. Chemokines released by the airway epithelium upon allergen stimulation attract DCs. Following recognition and uptake, DCs migrate to the T cell-rich area of draining lymph nodes, display an array of antigen-derived peptides on the surface of major histocompatibility complex molecules, and acquire the cellular specialization to select and activate naïve antigen-specific T cells. Allergen targeting to the DCs occurs via membrane-bound IgE. DCs interact with many cell types, including mast cells, epithelial cells and fibroblasts. Mediators released by these cells can activate the DCs so that they are induced to mature and attract memory Th2 cells through release of Th2-selective chemokines. Mature effector Th2 cells play a central role in asthma pathogenesis by releasing cytokines (for example, IL-13) that stimulate eosinophil recruitment, smooth muscle cell cytokine and chemokine production, and goblet cell hyperplasia.

Understanding the effects of ENMs on the function and phenotype of DCs is an important area of study. The exacerbation of allergen-induced airway disease discussed above could be partly through the inappropriate activation of antigen-presenting DCs. Both ENMs and diesel pollutant nanoparticles have been shown to activate DCs *(253)*. Other work has shown that ZnO nanoparticles cause DC death at relatively low concentrations, whereas these ENMs had no effect on peripheral blood mononuclear cells (PBMCs) *(254)*. Interestingly, CNTs have been reported to inhibit the differentiation of peripheral blood monocytes into DCs *(99)*. The consequence of this would most probably be depletion of antigen-presenting DCs in the lung, which would presumably reduce immune recognition and subsequent T cell differentiation in draining lymph nodes.

Lymphocytes

The type of immune and inflammatory response elicited by inhaled ENMs depends largely on T lymphocyte programming. As discussed above, this occurs via presentation of the ENM to naïve T cells in the draining lymph nodes and is regulated by the cytokine microenvironment. Four different T lymphocyte populations can result as a consequence of programming: Th1, Th2, Th17, and T-reg. Th1 lymphocyte differentiation is driven by IL-12. Th1 cells produce interferon (IFN)-γ and TNF-α, which regulate cell-mediated immunity and IgG2 production. IFN-γ also stimulates macrophages towards an M1 phenotype, which in turn secrete IL-1β through the inflammasome mechanism. IL-1β then orchestrates downstream CXC chemokines (in mice) or IL-8 (in humans) to promote neutrophilic inflammation. Many types of ENMs have been shown to cause a Th1-type immune response in the lungs of rodents, with subsequent neutrophilic inflammation. For example, MWCNTs or TiO_2 nanoparticles primarily elicit neutrophilic inflammation in the lungs of mice or rats *(241)*. However, in this study the type of MWCNT used was tangled and flexible. As discussed below, more rigid MWCNTs promote a Th2 immune response.

Immunological research into the mechanisms of allergy has identified cytokine production by Th2 effector lymphocytes as being critical for orchestrating allergic inflammation rich in eosinophils. Upon recognizing their cognate antigen, Th2 lymphocytes produce cytokines that regulate IgE synthesis, growth and activation of eosinophils and mast cells, and expression of endothelial cell adhesion molecules. The first step in the allergic immune response is the uptake and presentation of allergen by DCs, as described in the previous subsection. Certain types of ENMs stimulate a Th2 immune response in the lungs. For example, while tangled MWCNTs elicit a Th1 immune response with neutrophilic inflammation, rigid rodlike MWCNTs stimulate a Th2 immune response characterized by eosinophilic inflammation *(113)*. Mice exposed to these MWCNTs exhibited elevated levels of Th2 cytokines (for example, IL-13) and mucous cell metaplasia of the airway epithelium. Interestingly, proteomic analysis of the secretome of monocyte-derived macrophages after exposure to tangled MWCNTs, rodlike MWCNTs,

or crocidolite asbestos revealed different protein profiles for tangled and rodlike MWCNTs, but similar profiles for rodlike MWCNTs and asbestos *(255)*.

Animal models of immune-mediated lung disease

Pulmonary fibrosis

Pulmonary fibrosis is an occupational hazard following exposure to many particles and fibres. Innate immune responses could play an important role in the progression or resolution of fibrosis. ENMs, because of their increased surface area–mass ratio and ROS-producing potential, could pose a significant risk for the development of fibrosis. In addition, persistence is an important factor. Certain assumptions have been made with respect to ENM toxicity and expected disease outcome. For example, CNTs share some features with asbestos fibres, mainly with regard to their fibrelike shape and aspect (length to width) ratio. Asbestos fibres are a known cause of fibrosis and mesothelioma in humans. However, CNTs also have some uniquely different properties from asbestos, including nanoscale width and highly conformal structure. Therefore, some caution should be taken in making comparisons of fibrelike or tube-shaped ENMs with asbestos fibres with respect to fibrogenic potential.

Nevertheless, pulmonary fibrosis is also a common pathological feature observed in numerous rodent studies after exposure to CNTs *(251, 256, 257)*. As mentioned above, aggregated CNTs tend to produce granulomatous lesions *(16, 258)*. In contrast, diffuse interstitial pulmonary fibrosis is associated with well dispersed CNTs that are readily taken up by macrophages and cause greater growth factor (PDGF-AA, TGF-β1) and IL-1β production than non-dispersed CNTs *(27)*. Other factors contribute to the fibrogenic potential of ENMs, including shape, composition, electrostatic charge, and ROS-generating capacity. High aspect ratio ENMs impede clearance, and structures longer than 10 to 15 μm (the approximate width of an alveolar macrophage) are difficult to clear from lung tissues via macrophage-mediated mechanisms. For fibre or tube-shaped ENMs, diameter is also a determinant of toxicity. For example, thinner (~10 nm diameter) MWCNTs are more toxic in the lungs of mice than thicker (~70 nm diameter) MWCNTs *(259)*. Rigidity of ENMs is also a factor. Therefore, for long CNTs, durability is an important factor in promoting

chronic fibrogenesis. For example, long SWCNTs are flexible and, when folded, are taken up by macrophages without causing frustrated phagocytosis or impeding macrophage clearance. The composition of ENMs must also be carefully considered. For example, metals used as catalysts in the manufacture of CNTs (such as nickel, cobalt, iron) are known to mediate pulmonary fibrosis in humans *(260)*. For example, nickel is known to cause occupational asthma and contact dermatitis, whereas iron and cobalt cause interstitial pulmonary fibrosis in occupations related to mining and metallurgy. Studies in mice show that metal nanoparticles (for example, nickel or iron) cause pulmonary fibrosis in the lungs of mice after oropharyngeal aspiration *(245, 261)*. Since some of these same metals are present as residual catalysts in CNTs, the combinations of CNTs and metal nanoparticles should be considered in the hazard assessment of immunotoxicity.

The emergence of the myofibroblast, a collagen-synthesizing mesenchymal cell, is a key step in the progression of lung fibrosis. A variety of growth factors, cytokines and chemokines that stimulate myofibroblast differentiation, growth, migration, and extracellular matrix production are induced in the lungs of rats or mice after exposure to ENMs *(262)*. For example, SWCNTs or MWCNTs delivered to the lung by intratracheal instillation in rats or inhalation in mice increase mRNA and protein levels of PDGF *(222, 263)*. PDGF stimulates the replication, chemotaxis and survival of lung mesenchymal cells (fibroblasts, myofibroblasts and smooth muscle cells) to promote lung fibrogenesis *(264)*. CNTs delivered to the lungs of mice increase levels of TGF-β1, a central mediator of collagen production by fibroblasts and myofibroblasts *(251, 265)*. In addition to TGF-β1, osteopontin levels stimulate collagen deposition and fibroblast migration, and levels of osteopontin are increased in the lungs of rats exposed to SWCNTs or MWCNTs *(266, 267)*. Alveolar macrophages, as well as airway epithelial cells and fibroblasts, produce PDGF, TGF-β1 and osteopontin. Several chemokines are also induced by CNT exposure and drive the inflammatory response in the lung. CXCL8 (IL-8), a potent neutrophil chemoattractant, is produced by a human bronchial epithelial cell line in vitro after exposure to MWCNTs *(268)*. CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), is produced by macrophages and airway epithelial cells and is increased in the bronchoalveolar lavage fluid (BALF) of mice after CNT inhalation exposure. In general, a

complex interaction of cytokines, chemokines and growth factors contributes to the progression of pulmonary fibrosis.

Asthma

Asthma features a chronic airway remodelling response that is characterized by (a) eosinophilic inflammation; (b) airway smooth muscle thickening; (c) mucous cell hyperplasia and mucus hypersecretion; and (d) subepithelial fibrosis. Allergic diseases, including asthma, are thought to result from a dysregulated immune response to commonly encountered antigens in genetically predisposed individuals. Immunological research into the mechanisms of allergy has identified cytokine production by Th2 effector lymphocytes as being critical for orchestrating allergic inflammation rich in eosinophils. Upon recognition of their cognate antigen, Th2 lymphocytes produce cytokines that regulate IgE synthesis, growth and activation of eosinophils and mast cells, and expression of endothelial cell adhesion molecules. Common allergens that cause asthma in the human population include house dust mite and cockroach antigens. In addition to allergens that directly cause Th2 cell programming and allergic disease, a variety of environmental agents (mould, ozone, pesticides) exacerbate pre-existing asthma. There is evidence that some ENMs are capable of either directly acting as allergens or exacerbating pre-existing allergic lung disease.

Malignant disease

In addition to airway and interstitial lung diseases, the pleural mesothelial lining surrounding the lungs is a potentially important site of toxicity for certain ENMs. Of particular concern are high aspect ratio ENMs such as CNTs, nanofibres and nanowires that have asbestos-like shape and therefore could be persistent in lung tissue. ENMs contained within macrophages cross the pleural lining via the lymphatic drainage and thereby interact with the mesothelial lining of the pleura. Here, the durable nature of CNTs, nanofibres or nanowires, coupled with fibrelike shape and reactivity (that is, ROS-generating capacity), could result in immune reactions, pleural inflammation or DNA damage to mesothelial cells. While unknown at the present time, it has been speculated that such high aspect ratio ENMs could have asbestos-like behaviour and long-term immune or inflammatory effects that could

lead to tumour formation, lung cancer and mesothelioma. Exposure of mice to MWCNTs via inhalation following tumour initiation by methylcholanthrene led to promotion of lung adenocarcinoma *(269)*. Furthermore, inhalation exposure of rats to the same type of MWCNTs used in the study by Sargent et al. *(269)* resulted in bronchoalveolar carcinomas but no pleural mesothelioma *(270)*. Single intraperitoneal injection of long MWCNTs in mice induced inflammation and granuloma formation on the mesothelial surface of the peritoneum *(202)*, and a similar strategy with CNTs using mice deficient in the tumour suppressor p53 showed mesothelioma formation in the abdominal cavity after injection of CNTs *(224)*. A more recent study showed that several different MWCNTs caused mesothelioma after intraperitoneal injection in rats *(271)*. Moreover, this study found that nanotube curvature, in addition to aspect ratio, was an important parameter influencing the carcinogenicity of MWCNTs.

In addition to in vivo studies, in vitro studies show that CNTs activate the tumour suppressor p53 in mouse embryonic stem cells *(272)*. MWCNTs delivered to the lungs by inhalation or aspiration accumulate in subpleural tissue, and some tubes penetrate the pleural lining *(61, 257, 273)*. The inhalation of MWCNTs in mice has been shown to produce proinflammatory lesions on the pleural surface that have been referred to as mononuclear cell aggregates *(61)*. These same mice had elevated levels of PDGF and CCL2 in lavage fluid. Interestingly, PDGF and nickel nanoparticles synergistically increased CCL2 production by cultured rat mesothelial cells *(29)*. The mechanism involves nickel enhancement of PDGF-induced CCL2 expression by prolonged MAP kinase activation in mesothelial cells. The accumulation of mononuclear cell aggregates at the pleural surface after exposure to CNTs, or presumably nickel nanoparticles, could be mediated by PDGF secreted by activated macrophages. PDGF, in turn, stimulates the production of CCL2, which serves to recruit mononuclear cells to the pleural surface. Moreover, CCL2 is produced by pleural mesothelial cells and is a candidate chemokine that could participate in the formation of mononuclear cell aggregates observed at the pleura of mice after inhalation of MWCNTs *(274)*. The issue of whether ENMs are capable of causing immune, inflammatory or carcinogenic effects at the pleura in humans remains a key topic of research and will have important implications for the future use and development of ENMs for a variety of applications.

Animal models of disease susceptibility

It is likely that ENMs will have the most profound adverse health effects on individuals with pre-existing respiratory diseases such as asthma, bronchitis or chronic obstructive pulmonary disease *(275, 276)*. For example, MWCNTs exacerbated allergic airway inflammation in mice caused by OVA sensitization as determined by amplified lung levels of Th2 cytokines and chemokines, as well as serum IgE levels, compared with allergen alone *(277)*. SWCNTs have also been reported to exacerbate allergic airway inflammation in mice via enhanced activation of Th immunity and increased oxidative stress *(278, 279)*. MWCNTs have been shown to exacerbate airway fibrosis in mice that were first prechallenged with OVA, and the increase in fibrosis was associated with elevated levels of pro-fibrogenic cytokines PDGF-AA and TGF-β1 in lung lining fluid that were induced by MWCNTs and OVA, respectively *(222)*. Repeated exposure to MWCNTs has also been shown to induce Th2 allergic responses in the absence of any allergen pre-exposure *(280)*. Table 4.2 summarizes the effect of selected ENMs on allergen-induced lung inflammation.

Table 4.2 Effect of selected ENMs on allergen-induced lung inflammation

ENM	Allergen	Species/ strain	Effect	Reference
MWCNT	Ovalbumin	Mouse	Exacerbation of airway fibrosis; enhanced Th2 cytokines and IgE	Ryman-Rasmussen et al. *(222)*; Inoue et al. *(277)*
MWCNT	House dust mite	Mouse	Exacerbation of airway fibrosis	Shipkowski et al. *(114)*
SWCNT	Ovalbumin	Mouse	Enhanced allergic response	Nygaard et al. *(279)*
TiO$_2$	Diisocyanate	Mouse	Enhanced airway hyperresponsiveness	Hussain et al. *(281)*
TiO$_2$	Ovalbumin	Mouse	Suppression of allergen-induced airway inflammation	Rossi et al. *(282)*
Gold	Diisocyanate	Mouse	Enhanced airway hyperresponsiveness	Hussain et al. *(281)*
SiO$_2$	Ovalbumin	Mouse	Adjuvant effect; exacerbation of airway inflammation	Brandenberger et al. *(283)*

Other ENMs also exacerbate allergic inflammation in mice. TiO$_2$ or gold nanoparticles enhanced airway hyperresponsiveness in a mouse model of diisocyanate-induced airway inflammation and increased numbers of inflammatory cells *(281)*. However, the majority of inflammatory cells in that study were neutrophils, which suggests a shift from the classic Th2 response to one that primarily features eosinophils. While these studies suggest that individuals with allergic asthma are susceptible to lung and airway disease caused by exposure to ENMs, it remains unknown whether ENMs will cause or exacerbate asthma in humans. While there is a significant body of evidence in rodents suggesting that ENMs (such as CNTs) would be a hazard to individuals with asthma through exacerbation of Th2 inflammation, some studies with ENMs show suppression of allergen-induced airway disease in mice. For example, it has been shown that TiO$_2$ nanoparticles cause neutrophilic inflammation in healthy mice, but suppress allergic airway inflammation in mice sensitized with OVA allergen *(282)*. Therefore, there is evidence that ENMs can cause immunostimulation or immunosuppression of local immune responses in the lung.

When nanoparticles (nano TiO$_2$ or carbon black) were coadministered with an antigen (such as OVA) in mice, an enhancement of pulmonary inflammation and allergic airway sensitization was observed *(284)*. Also, particulate pollutants, such as diesel exhaust particles, have been shown to increase airway response after intratracheal instillation with an antigen, and are considered to contribute to allergic diseases *(285)*. SWCNTs, MWCNTs and ultrafine (nano) carbon black were demonstrated to promote allergic responses in the murine OVA allergic airway model. Following a subcutaneous or intranasal coadministration of OVA antigen with the various carbon nanomaterials, after three weeks an intranasal challenge was given to the animals. Serum levels of OVA-specific IgE and cell numbers in BALF were strongly increased by the carbon nanomaterials *(279)*. An IgE adjuvant effect was demonstrated for polystyrene particles of size 100 nm in a mouse OVA IgE model *(286)*. Irrespective of the route of administration (intranasal instillation, intratracheal instillation or intraperitoneal injection) or immunization protocol, polystyrene particles in combination with OVA elicited increased levels of both allergen-specific and total IgE in mice. Promotion of allergic airway inflammation in the OVA

model in mice was demonstrated for TiO_2, diesel exhaust particles, carbon black, MWCNTs, SWCNTs, SiO_2, and polystyrene particle nanomaterials *(277, 279, 283–287)*. For silica (SiO_2) nanoparticles, the enhanced reactions in the OVA airway allergic model could be attenuated by amino and phosphate surface modifications of the SiO_2 nanoparticles *(287)*. In an airway asthmatic hyperreactivity model with toluene diisocyanate immunization, pulmonary pretreatment with TiO_2 (15 nm) and gold (40 nm) nanoparticles one day before oropharyngeal challenge with toluene diisocyanate resulted in a threefold to fivefold increase in lung inflammatory cells (neutrophils and macrophages), and histologically in increased oedema, epithelial damage and inflammation *(281)*. So, for allergic lung diseases, both particulate airborne pollutants (such as diesel exhaust particles) and manufactured nanomaterials can exacerbate existing allergic disease in the lung and act as an adjuvant for the induction of such allergies. Both workers and consumers with lung problems are at risk for adverse effects after airway exposure to (nano)particles.

The effects of ENMs in the lung could also be exacerbated by pre-existing bacterial or viral infection. Bacterial LPS is a potent proinflammatory agent and has been implicated in a number of occupational and environmental lung diseases in humans, including bronchitis, chronic obstructive pulmonary disease and asthma. CNTs (either SWCNTs or MWCNTs) have been reported to increase the severity of LPS-induced lung inflammation, pulmonary vascular permeability and production of proinflammatory cytokines in the lungs of mice *(288)*. Moreover, pulmonary fibrosis induced by MWCNTs is increased by LPS pre-exposure in rats, and CNT-induced production of PDGF by rat alveolar macrophages and lung epithelial cells is enhanced by LPS pre-exposure *(263)*. These studies provide evidence that LPS-induced lung inflammation is a susceptibility factor that increases the severity of fibroproliferative lung disease caused by CNT exposure.

In addition to environmental susceptibility factors, a variety of genes play important roles in determining susceptibility to CNTs and other ENMs. Transgenic mouse models are valuable for elucidating the role of specific immune mediators (such as transcription factors or enzymes). For example, signal transducer and activator of transcription 1 (STAT1) transcription factor plays a central role in

Th1 immunity by mediating many of the biological effects of IFNs. STAT1 knockout mice are susceptible to exacerbation of allergen-induced airway fibrosis by MWCNTs *(289)*. The transcription factor T-bet maintains Th1 immunity, and knockout of T-bet in mice results in a spontaneous shift towards a Th2 phenotype. T-bet knockout mice display enhanced airway remodelling (for example, mucous cell metaplasia) and exaggerated levels of CCL2 after exposure to nickel nanoparticles *(245)*. In addition to transgenic models of transcription factor deficiency, knockout mice lacking specific enzymes reveal clues about their role in immune reactions to ENMs. Mice deficient in the COX-2 enzyme are susceptible to exacerbation of allergen-induced airway remodelling by MWCNTs *(290)*. While COX-2 mediates proinflammatory effects, it has also been implicated in the pathogenesis of asthma and pulmonary fibrosis *(262)*. Mice deficient in MPO have fewer MWCNTs in their lungs compared to wild type counterparts, suggesting that MPO mediates the degradation of some CNTs in the lungs *(37)*.

4.10.3   Exposure to nanomaterials via the gastrointestinal tract

Overview of gastrointestinal tract immunology

The gastrointestinal tract derives from the endoderm during embryonic development. It is responsible for the breakdown and absorption of food and liquids, which starts with mechanical digestion and continues with chemical digestion through the action of enzymes in the mouth. The oesophagus, stomach, small intestine (including the duodenum, jejunum and ileum), large intestine, rectum and anus are the main components of the gastrointestinal tract. Accessory organs, such as the salivary glands, tongue, liver, gall bladder and pancreas, are also closely involved in the functions of the gastrointestinal tract.

The main structures of the stomach are the cardia, fundus, body and pylorus (including the pyloric antrum, pyloric canal and pyloric sphincter). The tissue of the gastrointestinal tract comprises four layers: the mucosa, submucosa, muscularis externa and serosa. In the mucosa, the epithelium is composed of parietal cells, which secrete hydrochloric acid; the chief cells, which release pepsinogen; and the enteroendocrine cells, which mainly produce gastrin. The small and large intestines absorb nutrients and water through the crypts. Stem cells in the crypt produce various other cell types, including microfold

cells and goblet cells. The gastrointestinal tract also contains non-lymphoid and secondary lymphoid tissues. The gut-associated lymphoid tissue comprises immune cells, located in the epithelium and lamina propria, while mesenteric lymph nodes and Peyer's patches are constituents of the secondary lymphoid tissues. The villi, located in the small intestine, have thin walls and are specialized for absorption of nutrients in the bloodstream.

A number of cell types are found in the intestine. The stem cells of the intestine include the pluripotent cells, located at the base of the crypts, which are capable of proliferation and differentiation into the various specialized cells of the body. Some of the differentiated epithelial cells have endocrine functions through the secretion of different molecules. For instance, Paneth and goblet cells produce antimicrobial proteins, which are released into the intestinal lumen. Specifically, Paneth cells – which are present in the small intestine but absent from the large intestine – produce alpha-defensin, lysozyme and cathelicidins, while goblet cells are specialized for mucin 2 production as a protection barrier. Alpha-defensins are expressed prenatally and increase after birth. Their functions, as first described, were related to their antimicrobial properties in combating pathogenic organisms, but they have subsequently been found to play a role in the release of inflammatory cytokines *(291–293)*. Intestinal epithelial cells, also known as enterocytes, produce antimicrobial proteins, including C-type lectin regenerating islet-derived protein 3γ. They are fundamental for the transcytosis of immunoglobulins from the lamina propria to the intestinal lumen. The microfold cells, also called M cells, are responsible for antigen uptake. They facilitate the antigen-specific immune response, as they have basolateral invaginations that harbour immune cells. Peyer's patches have three zones: the follicular area, the interfollicular area and the follicle-associated epithelium. Here, the proliferative B lymphocytes, follicular DCs and macrophages are located. B cells, T cells and DCs surround the Peyer's patches, which are connected to lymphatic and endothelial vessels (Figure 4.4).

In addition, the gastrointestinal tract is colonized by a community of bacteria, fungi, protozoa and viruses, including the Firmicutes and Bacteroidetes, two major phyla of the domain Bacteria. Collectively, this community is termed the microbiota of the gut.
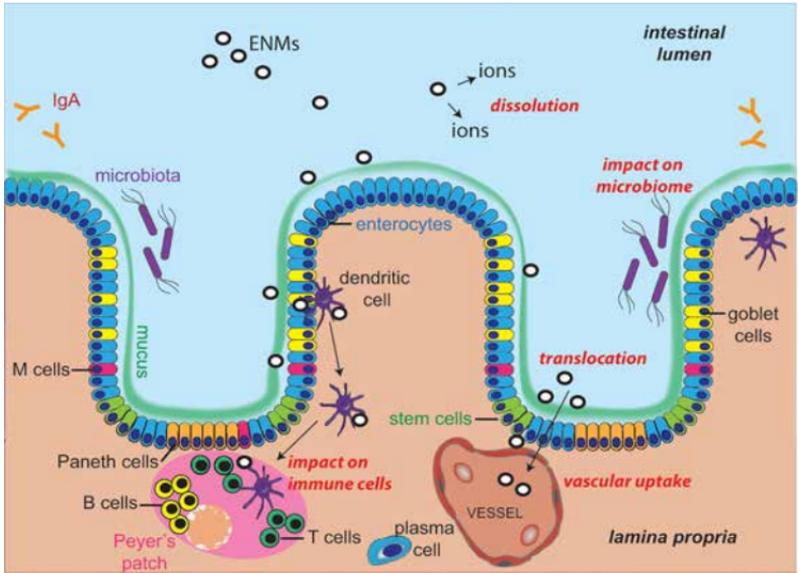
Figure 4.4 Schematic representation of immune cells in the small intestine

In general, the gastrointestinal tract protects itself against pathogens through the innate immune system, which releases various molecules that prevent the entrance of the invasive organism into the deeper layers of the gut. However, if pathogens overcome this strategy, the adaptive immune system responds through the deployment of B and T cells. Thus, if nanoparticles are consumed in the diet, they could end up being internalized by the immune system. So far there have been limited studies on the specific immunotoxicity of the oral cavity, oesophagus and stomach, compared to the more extensive research undertaken on the small and large intestines. However, the evidence of accumulation in those tissues raises the question of possible immunotoxicity. For instance, TiO_2 nanoparticles can accumulate in the oral cavity, while silver and ZnO nanoparticles can accumulate in the stomach of rats *(294)*. However, evidence indicates that lesser amounts of nanoparticles can be absorbed in the oral cavity, oesophagus and stomach than in the small and large intestines *(295)*. For TiO_2 a higher absorption rate has been estimated at 4% for the large intestine, and lower for the rest of the gastrointestinal tract

(296). However, more recently much lower percentages (< 1%) of absorption of the administered TiO$_2$ nanoparticle doses were reported *(297, 298)*. Indeed, there is evidence of accumulation of particles in Peyer's patches, which could affect the function of the T and B cells.

Once nanoparticles have reached the intestine there are several routes of translocation, for example paracellular transport through tight junctions, transcytosis and endocytosis (including processes such as pinocytosis and phagocytosis, mediated by caveolae formation and clathrin protein). As the normal function of M cells is the transport of molecules and microorganisms by transcytosis, more attention should be addressed to this cell type as a possible route for transport of nanoparticles, and the possible loss of antigen-presenting functions.

The more insoluble nanoparticles can still be observed as nanoparticles, including as agglomerates or aggregates, while some others, such as ZnO, cannot. Some soluble, organic or functionalized nanoparticles (such as amylases, lipases and proteases) could undergo enzymatic degradation. They can be affected by the environment in which they are located, for instance low pH in the stomach versus alkaline pH in the duodenum, or high mucus content in the small and large intestines. In contrast, internalization of coated nanoparticles could occur (for example, chitosan promotes the transcytosis of nanoparticles in the intestine) *(299)*.

Importance of oral exposure to nanomaterials

The increasing use of nanomaterials in foods, food packaging and pharmaceuticals has brought concern about their effects after oral exposure. The fact that some of the inhaled nanoparticles can be cleared by mucus and reach the gastrointestinal tract has heightened that concern, especially because inhalatory exposure to nanomaterials has been shown to have toxic effects. Consequently, nanomaterials used in the food and pharmaceutical industries *(300, 301)* have attracted the attention of toxicologists. For instance, nanoparticles have been shown to migrate from packaging into food, raising concerns about the safety of nanoparticles and also about modifications in the organoleptic properties of the food. Some nanocomposites are being used as monitors of food stability and quality, and also as antimicrobial

agents *(302)*. Nanocomposites have also been suggested as useful tools for drug or calcium delivery. Taking into account all the possible oral applications, it is probable that oral exposure will be increased.

Nowadays a number of nanomaterials are intentionally added to food as additives, either to act as preservatives or to improve the organoleptic properties of the food. The letter "E" identifies such additives in the European Union. Some of the currently used food additives, such as TiO_2 *(303)*, designated as E171, have been used since 1966. At that time E171 was produced in the form of microparticles, but it has been demonstrated that it also contains a nanofraction with a size below 100 nm at reported percentages of 10–36% *(304–306)*. SiO_2, designated as E551, and silver particles, designated as E174, are other food additives that are being used at the nanometric scale *(307, 308)*. Several other additives, including calcium carbonate (E170) and calcium silicate (E552), are being used in food in nanoparticle form but have been little investigated thus far. In addition, some other nanoparticles, such as ZnO, are not considered as food additives but are present in foodstuff-containing plastics for long-term storage *(309)*.

There is the potential for adverse effects on human health of the use of nanoparticles as food additives or in food packaging. One of the first studies related to the effects of nanoparticles used in food additives was performed by the American Cyanamid Company in 1963. In this study, rats were exposed for 30 days to a diet with very high TiO_2 concentrations of 100 000 milligrams per kilogram (mg/kg) of diet. Subsequently, only a low accumulation of titanium in muscle was reported *(310)*. During the following decades, in vitro and in vivo experimental models have been used to determine if oral exposure to nanoparticles might have an impact on the alimentary tract. More recently, studies have been undertaken of oral exposure to nanoparticles used in medical applications, and inhalation of nanoparticles that could reach the gastrointestinal tract as a consequence of clearance from the respiratory system *(311)*. The need to evaluate the effects of nanoparticles in the alimentary tract has been strengthened by evidence that inhaled nanoparticles – as well as those ingested orally – can reach the gastrointestinal tract and translocate to the circulatory system, reaching such organs as the liver, kidney, brain and cardiovascular system *(312)*.

Despite the growing concern regarding the use of nanoparticles as food additives and in food packaging, as well as their potential application for the oral administration of drugs, only a few types of nanoparticles have been investigated so far with respect to oral exposure. Here, the effects of exposure to carbon-based, metal-based and organic nanoparticles on the alimentary tract are considered, drawing on in vivo and in vitro data, and human studies.

Certain limitations need to be taken into account when assessing the toxicity of nanomaterials upon oral exposure. First, the lack of a standardized methodology for the characterization of nanomaterials makes it difficult to compare their effects, independent of the route of exposure. Second, preparation of nanomaterial stock and the dispersion media of nanomaterials exert a strong influence on the biological effects, and there are still no unified criteria to prepare a stock solution and to select a proper dispersion medium for each study. Third, the dose and concentration employed in research are often higher than occur under realistic human exposure; however, the lack of quantification of orally ingested nanomaterials adds to the difficulty of designing experimental studies. Fourth, the genetic variations associated with the strains of experimental animals, and variations in the age of animals used, have been poorly recorded, and the passage numbers of cell culture experiments are rarely reported. Fifth, few studies take into consideration the interaction of nanomaterials with saliva, gastric fluids and intestinal fluids, which might modify the nanomaterials, and few studies address the effect of food or pharmaceutical processes on the properties of nanomaterials and their impact on pharmacokinetics.

The current and possible future use of different types of nanomaterials resulting in oral intake has resulted in increased attention in studies to the possible adverse effects of nanomaterials on the gastrointestinal tract, and the possible immunotoxicological outcomes in humans *(295, 313)*. The lack of consensus on the experimental design of in vivo and in vitro studies hinders accurate interpretation of the available information, due for example to the overloads used for in vivo studies and the high concentrations used for in vitro studies. However, samples taken from human biopsies demonstrate that nanoparticles can reach important tissues involved in the immune response, which could have a detrimental impact on

human health, specifically under prolonged exposure or when pre-existing diseases are present in adults or children *(313)*. Diseases related to the gastrointestinal tract could be exacerbated by deposition of TiO_2, SiO_2 and other particles in the tissues of the gastrointestinal tract. The adverse effects of nanoparticles on the various components of the immune system and the gastrointestinal tract have not been deeply evaluated. For instance, oral intake of nanoparticles could impair macrophage phagocytosis, or nanomaterial internalization could inhibit the release of cytokines needed for a full immune response. The effect of further intake of nanomaterials could also be investigated, since antigen presentation could have deteriorated following previous exposure, and damaged macrophages are unable to accomplish their physiological function. Figure 4.4 depicts immune cells in the gastrointestinal tract.

Information on the oral intake (that is, consumption) of nanomaterials would contribute to a better understanding of the current situation regarding the exposure of the human alimentary tract to nanomaterials, and would provide a basis for improved research design. A model for such work is provided by the estimation of ingested nanomaterials by Weir and colleagues *(304)*, which estimated TiO_2 consumption at 1 to 2 mg/kg in children and less than 1 mg/kg in adults.

Results from in vivo studies

Carbon-based nanomaterials

Evaluation of the effect of some carbon-based nanoparticles on the gastrointestinal tract is still limited. There are some data available using carbon black particles dosed using intragastric administration in Zucker rats with metabolic syndrome. In that work carbon black administration induced lipid load in the liver, associated with hepatic steatosis *(314)*. SWCNTs or C60 fullerenes intragastrically administered in rats showed DNA damage in liver and lung tissues, with no alterations in the colon. Although there was increased mRNA expression of 8-oxoguanine DNA glycosylase in the liver of C60 fullerene-treated rats, exposure to C60 fullerenes was not associated with an increase in DNA repair activity *(315)*. SWCNTs administered orally in mice showed no behavioural changes, and brain, kidney, spleen, liver and blood analysis showed no alterations except for

elevated creatinine concentration in the blood *(316)*. SWCNTs can be eliminated through the kidney and bile ducts and detected in urine and faeces after oral administration *(316)*. However, intraperitoneal administration of SWCNTs, tested at different doses and lengths of nanomaterial, coalesced inside the body, and agglomerates higher than 10 µm induced granuloma formation. Accumulation of SWCNTs in the peritoneal cavity was observed after intraperitoneal exposure, with no apparent alterations *(316)*. Functionalized 125I-labelled graphene oxide delivered orally to BALB/c mice resulted in high levels of radioactivity in the stomach and intestine but not in the liver, spleen, kidney, heart, lung, skin, muscle, bone, brain or thyroid. In addition, retained radioactivity was detected only between 2% and 3% after one day of oral administration and was undetectable after seven days *(317)*.

Metal-based nanomaterials

Jani et al. *(296)* reported that TiO_2 (12.5 mg/kg) particles of 475 nm administered to rats by oral gavage for 10 days were located in the granular areas of Peyer's patches, in mesenteric nodes and in the connective tissues of the mesenteric network, but also in sinusoidal liver cells. Tissue in the colon showed the highest uptake, followed by Peyer's patches, liver, lungs, peritoneum and small intestine *(296)*. In the following decades, several studies were performed to investigate the effects of oral exposure to microparticles. With the development of nanotechnology, TiO_2 nanoparticles of non-food grade have been extensively investigated, though fewer studies have been undertaken of food-grade E171 nanoparticles. In this regard, E171 particles administered intragastrically in BALB/c mice enhanced tumour formation in the colon in a chemically induced colorectal cancer model using azoxymethane and dextran sodium sulphate *(318)*. In this model, E171 particles were not able to induce tumour formation after 10 weeks of administration, but enhanced tumour formation in the distal and medium colon. The goblet cells in the colon decreased dramatically with the sole E171 administration. Tissue isolated from E171-exposed mice was cultured (ex vivo), and the cells showed that some particles remained internalized in the cells after one week in culture *(318)*. The decrease in goblet cells in the colon resulting from administration of food-grade TiO_2 in mice might reduce the ability of the mucus barrier to protect against pathogens and may also affect the

enzymes of the mucus layer, which are required for proper functioning of the absorption process in the large intestine.

The biodistribution of three different sizes of TiO_2 nanoparticles (25, 80 and 155 nm) was investigated by oral administration in mice dosed at 5 mg/kg, which is a high dose for an in vivo model using CD-1 mice. The study showed that nanoparticles of 25, 80 and 155 nm dispersed in 0.5% hydroxypropyl methylcellulose were deposited in the liver at rates of 106, 3970 and 107 ng/g of tissue, respectively, and in the kidney at 375, 440 and 170 ng/g of tissue, and were undetectable in red cells *(319)*. Some histological alterations were found in the liver, such as hydropic degeneration, as well as spotty necrosis of hepatocytes. Increased ALT serum levels, swelling in the renal glomerulus, and inflammation of the stomach were found in mice that had received TiO_2 sized at 80 nm after two weeks. No abnormal findings in the heart, lung, testicles, ovary, and spleen tissues were reported, but fatty degeneration of brain tissue in the hippocampus was found. TiO_2 sized at 25 nm and 155 nm showed less alterations than at 80 nm *(319)*. In another study, TiO_2 nanoparticles sized at 25 nm were dispersed in distilled water and dosed at 1 and 2 mg/kg in rats. The effects on the spleen were analysed and compared between males and females. Accumulation of TiO_2 nanoparticles was found in the white pulp of the spleen and ovaries of female rats. Histological alterations at both doses were found in the thyroid gland and adrenal medulla, with no changes in thyroid function. An increase in testosterone levels was found in male rats *(320)*. In a further study, the impact of accumulation in the spleen on the immune response was not evaluated. TiO_2 sized at 40, 40–50, 120 and 5000 nm was administered to Sprague-Dawley male rats at 4.6 mg/kg dispersed in deionized water or 5% OVA solution. Interestingly, a background of titanium was detected in the blood (4–21 ng/mL) and urine (12–46 ng/mL) of control rats fed with a normal diet. It was found that cardboard toys provided for environmental enrichers contained 4.5 μg/g of titanium *(321)*. The dispersion media had no effect on tissue uptake, and samples taken from the gastrointestinal tract of rats exposed to 120 and 5000 nm had 86.7 ng/g of titanium *(321)*. Oral administration of SiO_2 nanoparticles dosed at 30 or 100 mg/kg during six days to mice with colitis showed that labelled SiO_2 nanoparticles with a hydrodynamic size of 101 nm were able to accumulate in inflamed tissue, while healthy tissue exhibited limited particle accumulation

(322). The study showed the potential usage of SiO_2 nanoparticles for the treatment of inflammatory bowel diseases when nanoparticles are bound to a therapeutic agent *(323, 324)*, but also raised the question of whether accumulation in inflamed tissue could worsen the inflammatory process under chronic conditions.

A more recent in vivo study found that amorphous SiO_2 with a diameter of 70 nm, 300 nm or 1000 nm orally administered at 2.5 mg/mouse/daily for 28 days induced no significant differences in body weight or liver and kidney function markers, and no abnormalities in the liver, kidney, large intestine, small intestine, brain, lung, spleen, heart or stomach *(325)*. Haematological analysis found that white blood cells, lymphocytes, monocytes, granulocytes, platelets and red blood cells also remained without alteration *(325)*. Intake of oral Ag-NPs sized between 53 and 71 nm, administered at 30, 300 and 1000 mg/kg/day during 28 days in rats, showed a slight hepatotoxicity effect with no alterations in food consumption, in body weight or in organ weight *(326)*.

Fe_2O_3 nanoparticles have also been investigated, though little information is available on the alimentary tract, as there are few oral consumption applications for Fe_2O_3, and most oral exposure in humans can be attributed to accidental ingestion or ingestion through pharmaceutical products. However, rats dosed at 500, 1000 and 2000 mg/kg of 30 nm Fe_2O_3 of < 5 μm (bulky) showed no genotoxicity in leucocytes. Micronucleus tests were also negative in peripheral blood cells. The biodistribution of iron was analysed at 6, 24, 48 and 72 hours after treatment in the liver, spleen, kidney, heart, brain, bone marrow, urine and faeces. Internalization of Fe_2O_3 nanoparticles was size and dose dependent in all tissues. The spleen, kidney and brain were able to take up nanosized Fe_2O_3, while bulk Fe_2O_3 was not detected. Bulk Fe_2O_3 was undetectable in blood and urine samples but dose-dependent distribution was detected in bone marrow and urine for nanosized Fe_2O_3. Large excretions in the faeces were found in both types of Fe_2O_3 *(327)*. A chronic study of oral exposure to γ-Fe_2O_3 coated with amino-dextran was carried out in growing chickens *(328)*. The nanoparticles (12 nm) were administered in the diet at concentrations ranging from 6 to 24 mg/kg. Consumption of γ-Fe_2O_3 in food increased during the study period yet no nanoparticle accumulation was observed in liver, spleen and duodenum. Changes

in liver iron levels reflected the bioavailability of the iron released from the partial transformation of γ-Fe$_2$O$_3$ nanoparticles in the acid gastric environment *(328)*. Studies to compare different types of metal-based nanoparticles showed that 14-day administration of SiO$_2$ (12 nm), silver (11 nm) and Fe$_2$O$_3$ (60 nm) nanoparticles dosed at 1959, 2061 and 2000 mg/kg, respectively, resulted in no alterations in clinical observations, body weight, haematological analysis, serum parameters or histological examinations in male and female Sprague-Dawley rats. A chronic 13-week study with these nanoparticles showed no toxicity of SiO$_2$ and Fe$_2$O$_3$, though Ag-NPs showed systemic toxicity *(329)*.

Organic nanomaterials

Despite the extensive literature on dendrimers, there is still limited information available about their effects on the gastrointestinal tract after oral administration. One of the earliest studies showed that uptake of poly(amidoamine) (PAMAM) dendrimers with a size of 2.5 nm (6300 kDa) dosed at 14 mg/kg accumulate in the blood, liver, spleen, kidney, small intestine and large intestine at 3%, 1.5%, 0.1%, 0.5%, 15% and 5%, respectively, after 6 hours of oral administration in rats *(330)*. A comparison of lymphoid and non-lymphoid tissue uptake showed that dendrimers are internalized at 1% after 3 hours in the lymphoid tissue of the small intestine, and in the non-lymphoid tissue of the small intestine at 3.8% after 3 hours. Lymphoid tissue from the large intestine showed no uptake, while non-lymphoid tissue increased from 1% after 3 hours to 3.8% after 12 hours of oral exposure *(330)*. This study revealed an opposite effect on the uptake of dendrimers in non-lymphoid tissue in the small and large intestines. With regard to dendrimer branches, the number of repeated branching cycles – classified by generation (G) – has been shown to have absorption and cellular effects. For instance, G3 dendrimers dosed at 3.4 mg/kg had higher accumulation in the kidney after 48 hours of administration, while G5 and G7 dendrimers were internalized in the pancreas after 24 hours of administration. G3 dendrimers also accumulated in the liver, kidney and spleen. In this study, G7 dendrimers showed high urinary excretion (74%) during the first 4 hours after injection *(331)*. Subsequently, several studies have been undertaken on the toxicity of dendrimers according to the number of generations and modifications

in the surfaces, showing that both exert distinct biological effects. G3.5, G4, G6.5 and G7 dendrimers with different surfaces, orally administered at doses between 30 and 500 mg/kg, were tested. Only G7-NH2 dendrimers dosed at 50 mg/kg showed haemobilia. G7-OH dendrimers dosed at 300 mg/kg showed splenomegaly, and G4-OH dendrimers dosed at 300 mg/kg showed an increase in blood urea nitrogen, a marker of renal function *(332)*. The study showed that the maximum tolerated dose was obtained by smaller dendrimers (G3.5 and G4). Signs of toxicity were observed from administration of G7-NH2 and G7-OH dendrimers. In addition, a study of oral bioavailability in mice found that radiolabelled G6.5-COOH (125I) dendrimers, orally administered at 1 mg/kg, did not show signs of toxicity after 10 days of exposure. These dendrimers remained intact under simulated gastrointestinal tract conditions, and 70% of the dose was recovered, mainly from the urine. The dose remaining in the gastrointestinal tract was located in the stomach, small intestine, caecum, postcaecum, stool and carcass *(333)*.

As dendrimers are being developed for drug delivery systems and imaging, studies of variations in the central core of dendrimers (for example, whether ammonia or ethylenediamine), and variations in the peripheral groups, have been designed to test anticancer drugs and imaging molecules. Biodistribution and pharmacokinetics of these types of dendrimers, which have drugs or molecules attached, have been tested, though studies on some of these promising dendrimers (for drug delivery systems or imaging) generally do not include consideration of biodistribution or toxicity of dendrimers without bonded drugs. Some of the studies claim that dendrimers have undetectable toxicity, and good transepithelial absorption occurs in the intestine after oral administration and other routes of exposure *(333–338)*.

Results from in vitro studies

Carbon-based nanomaterials

Carbon black, SWCNT and C60 fullerene nanoparticles were associated with lipid accumulation in human HepG2 hepatocytes after 3 hours of exposure to nanoparticles (0, 0.1, 10, 50 and 100 μg/mL) followed by 18 hours of incubation with oleic/palmitic acid, which was used to mimic some features of metabolic syndrome *(314)*.

This study highlighted that exposure to carbon-based nanoparticles followed by these fatty acids could worsen the accumulation of lipids in hepatocytes. Functionalization of some carbon-based nanoparticles, including SWCNTs and fullerenes (from 15.6 to 1000 µg/mL), showed a very low cell viability decrease of Caco-2 cells but a slight increase in the cytotoxicity of functionalized SWCNTs after 72 hours of exposure, and also caused disruption of tight junction integrity after 24 hours of cell culture exposure *(339)*. In addition, SWCNTs functionalized with carboxylic acid induced a decrease in transepithelial electrical resistance, reflecting enhanced paracellular transport following weakening of the tight junctions, while SWCNTs functionalized with poly(ethylene glycol) had no effect on this parameter *(339)*. Both types of functionalized SWCNTs inhibited the P-glycoprotein efflux pump, which is responsible for preventing xenobiotic cell accumulation. Collectively, these results suggest that functionalized carbon-based nanoparticles could be useful as modulators for oral drug delivery *(339)*, though they could also be associated with unspecific xenobiotic accumulation.

Human colon carcinoma cell line HT29 exposure to SWCNTs (0.1 and 0.2 µg/mL) during 48 hours and 72 hours induced a decrease in cell growth and mitochondrial activity and an increase in ROS generation. Induction of DNA strand breaks could be detected after 3 hours of incubation with SWCNTs at concentrations $\geq$ 0.0001 µg/mL. DNA damage started at 0.00005 µg/mL under alkaline conditions. Induction of p53 phosphorylation was also observed, which is triggered by DNA damage *(340)*. Exposure to SWCNTs at 100 µg/mL induced reorganization of clathrin in mast cells, which has an impact on the inflammatory response *(341)*. In addition, lower exposure (10 µg/mL) to SWCNTs inhibited calcium response after 30 minutes and disrupted kinase signalling pathways without alteration in proliferation, even after five days of exposure *(341)*. On the other hand, SWCNTs attenuated the decrease in cell viability induced by *Escherichia coli* and *Staphylococcus aureus* infection in Caco-2 cell cultures in concentrations ranging from 1 to 10 µg/mL. In addition, SWCNTs reduced the NLRP3 inflammasome-mediated IL-1β secretion of Caco-2 cells induced by bacterial infection *(342)*. This study highlights that SWCNTs could interact with gut microbiota, and that SWCNTs are capable of reducing components of the inflammatory response.

MWCNTs with a length of 5–10 nm and a diameter of 20–30 nm induced a slight decrease in the viability of co-cultures of Caco-2 and HT29 cells exposed to 100 µg/mL but upregulated cell proliferation, antiapoptotis and DNA repair pathways associated with cell survival *(343)*. A comparison with TiO_2 nanoparticles shaped as belts showed that there were general early cellular responses to MWCNTs and TiO_2 after 1 hour of exposure, but unique gene and protein expression patterns were associated with each type of nanomaterial after 24 hours of exposure to 10 µg/mL and 100 µg/mL *(343)*. MWCNT exposure to Caco-2 cell cultures at 12.5, 25 and 50 µg/mL showed interaction with the cell membrane without affecting cell morphology and cell membrane integrity but increased the concentration of MDA, a lipid peroxidation marker, in cell cultures exposed to 25 and 50 µg/mL. A downregulation of adenosine triphosphate-binding cassette (ABC) transporters was found in cells exposed to 12.5, 25 and 50 µg/mL, which could be useful to enhance cellular drug uptake *(344)*.

In vitro studies of graphene oxide flakes are very limited, but no cytotoxicity was reported on Caco-2 cell culture exposed to 166 µg/mL of graphene oxide after 24 hours of exposure, and no cytotoxicity on *E. coli* exposed to 400 µg/mL, though a slight decrease was found in the viability of these bacteria after 10 hours of exposure to graphene oxide flakes at 300 µg/mL *(345)*. However, graphene oxide sheets can act as a biocompatible surface for *E. coli* and promote proliferation *(346)*, highlighting the importance of shape and focusing attention on the effects induced in the microbiota of the human gastrointestinal tract.

Metal-based nanomaterials

Studies have been conducted to evaluate the uptake of nanoparticles in gastrointestinal tract-related cell cultures. TiO_2 nanoparticles can be internalized by Caco-2 cells *(347–349)*, though rates of absorption were lower than for lung epithelial cell cultures *(347)*. TiO_2 nanoparticles sized 12 nm (anatase) and 22 nm (rutile) were accumulated in Caco-2 cell cultures exposed to 50 µg/mL, with no alterations in cell viability or DNA damage despite increased ROS generation, which was sustained for 48 hours. In addition, induction of multidrug resistance protein 1, 2, and 4 and breast cancer resistance protein gene expression was observed in cell cultures exposed to both

types of nanoparticles, but the expression of their proteins was higher in cells exposed to rutile *(350)*. These proteins are associated with solute-liquid carriers and efflux pumps from the ABC transporter family. Their increase has been associated with xenobiotic resistance under pathological conditions.

In a study of the effect of TiO_2 nanoparticles on the intestinal barrier and absorption, the cytotoxicity and translocation of both native TiO_2 nanoparticles and TiO_2 nanoparticles pretreated with digestion simulation fluid (50 and 200 µg/mL) were investigated in Caco-2 cells. The influence of digestion simulation fluids, including saliva TiO_2 nanoparticles exposed to gastric fluids, induced a slight decrease in cell viability and membrane integrity and an increase in ROS generation only in undifferentiated cell cultures, and had no effect on differentiated ones. Undifferentiated Caco-2 cells internalized native nanoparticles easily, but not pretreated nanoparticles, compared to differentiated cells *(349)*. Food-grade TiO_2 (E171) induced disruption of the brush border of Caco-2 cell culture exposed to 350 ng/mL, an effect that was not associated with nanoparticle sedimentation *(348)*.

TiO_2 shaped as spheres have previously been evaluated, but more recently studies have been undertaken of nanoparticles shaped as belts, including nanobelts with a 7 µm length, 0.2 µm width and 0.01 µm thickness in Caco-2/HT29 co-cultures, showing induction of inflammation pathways, cell cycle arrest, DNA replication stress, genomic instability and apoptosis *(343)*.

A study of the effects of particles such as SiO_2 was published in 1980 by O'Neil et al., with the hypothesis that SiO_2 particles found in Mediterranean grass *Phalaris minor* could be the etiology of oesophageal cancer, which had a high incidence in Iran, since this plant was present as a wheat contaminant in the Middle East and proliferation of fibroblast cell cultures was observed after incubation with fibres isolated from *P. minor (351)*. In the following decades, some in vitro evidence pointed to limited cytotoxicity of SiO_2 particles in gastrointestinal tract-related cell culture, including colorectal cell lines showing that SiO_2 nanoparticles sized 25 nm and 100 nm had low cytotoxicity in a concentration between 10 µg/mL and 150 µg/mL *(352)*. Caco-2 cell lines were able to internalize 20, 60 and 90 nm nanoparticles in a time-, concentration- and size-dependent manner.

Mesoporous silica nanoparticles offer the potential to achieve enhanced bioavailability of poorly soluble hydrophilic drugs *(353)* or oral insulin delivery *(354)*. Further study is needed of the potential of SiO_2 nanoparticles to act as enhancers for the delivery of other types of molecules, and their possible adverse effects on cells.

Undifferentiated Caco-2 cells were found to have greater sensitivity to nanoparticle toxicity. However, no effects of gastric fluid simulation on ROS generation were observed *(355)*. In this study, a solution of 34 millimolar (mM) NaCl/HCl at pH 2.7 followed by an incubation in an intestinal solution (carbonate/bicarbonate buffer 50 mM at pH 9.5 including 1.68 g of $NaCO_3$, 7.16 g $NaHCO_3$ and 4 g NaCl in H_2O) was used as gastric fluid simulation.[6] After incubation the samples were diluted in cell culture medium. Further evaluation of the effects of digestive fluids on nanoparticles is needed in order to mimic more realistically the conditions of exposure to nanomaterials. However, the methodology to simulate gastric conditions has not yet been standardized.

SiO_2 nanoparticles obtained from different companies were tested on human gastric epithelial cells. A decrease in cell viability of between 40% and 60% was observed after 72 hours of exposure to 200 μg/mL, regardless of the company. The same nanoparticles exposed in Caco-2 cell cultures showed less toxicity, though the Caco-2 cell cultures showed a higher percentage of cells accumulated in the G2/M cell cycle phase than gastric epithelial cells, for which this cell cycle phase exhibited no changes *(356)*. This highlights the cell type response associated with nanoparticle exposure irrespective of nanoparticle uptake, since both types of cell culture showed comparable endocytic vesicles in the cytoplasm. Other studies have shown limited toxicity associated with the size of SiO_2 nanoparticles of 25 and 100 nm on colorectal HT29 cell cultures *(352)*. However, SiO_2 nanoparticles of 70, 100, 300 and 1000 nm had an inverse correlation with the amount of albumin, transferrin, and IgG human proteins bound to the nanoparticle surface *(357)*.

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[6] NaCl, sodium chloride; HCl, hydrogen chloride; $NaCO_3$, sodium carbonate; $NaHCO_3$, sodium bicarbonate.

Fe_2O_3 nanoparticles of 26, 53, 76 and 98 nm exposed to 0.1, 0.2 and 0.3 mg/mL in Caco-2 cell cultures showed higher adsorption for 76 and 98 nm nanoparticles when rates were expressed as $mg/m^2/min$. However, when adsorption rates were expressed as m^2/min, the rates were higher for smaller 26 and 53 nm nanoparticles. After 3 hours of exposure, transepithelial electrical resistance was affected followed by disturbances of microvilli *(358)*. The degree of substitution of carboxylic groups in Fe_2O_3 nanoparticles had a positive effect on uptake in Caco-2 cell cultures, which demonstrated the effect of nanoparticle charge on internalization *(359)*.

Organic nanomaterials

Cytotoxicity of dendrimers has been associated with destabilization of cell membranes and haemolysis, especially cationic dendrimers compared to their anionic counterparts *(360)*. Increased generation of cationic dendrimers can induce a greater degree of cell damage in colon epithelial cell cultures *(361)*. Internalization of cationic G3.5 dendrimers occurs through clathrin endocytic pathways, and can promote tight junctional opening in intestinal epithelia *(362, 363)*. G3 and G5 dendrimers induced a decrease in cell viability in lung fibroblast cell cultures at 1 mM (high) and 10 micromolar (μM) (intermediate), respectively. However, G7 dendrimers showed toxicity in 1 mM, 10 μM and 100 nanomolar (nM) (low) concentration *(331)*.

Further studies have evaluated the effect of different generations of dendrimers (G0 to G4), using Caco-2 cells. G0, G1 and G2 dendrimers had similar apical to basolateral permeability that was higher than for G3 dendrimers. In addition, basolateral to apical permeability was higher than apical to basolateral permeability, except in the case of G4 dendrimers, which showed no permeability in both cases. Cell viability decreased only with G2 dendrimers at 10 mM after 210 minutes, and with G3 dendrimers at 0.1, 1 and 10 mM after 210 minutes. G4 was toxic after a short exposure time (90 minutes) in all tested concentrations *(362)*. Further studies have shown that PAMAM G4, G5 and G6 dendrimers with a diameter of 4.5, 5.4 and 6.7 nm, respectively, had an increasing generation effect on toxicity of colon SW480 epithelial cells compared to HaCaT keratinocytes *(364)*. The "generation" refers to the number of repeated branching cycles resulting from the synthesis of these particles; each successive

generation results in a dendrimer roughly twice the molecular weight of the previous generation. In the latter study, higher generations were associated with an increase in ROS generation and internalization of dendrimers in the mitochondria. DNA breakage was generation-increasing dependent in HaCaT exposed cell cultures *(364)*. In addition, co-localization of PAMAM dendrimers of 45 nm with the mitochondria of human lung cells was associated with release of cytochrome C. A decrease in cell viability of 39% induced by a dendrimer concentration of 2 mg/mL was associated with DNA fragmentation, activity of caspases 3 and 9, increased Bax expression and decreased Bcl-2 expression, which are apoptosis markers *(365)*.

A three-dimensional organoid kidney proximal tubule epithelial cell culture was proposed to compare in vitro and in vivo studies on the effects of dendrimers. In a study using G5-OH PAMAM dendrimers, the effects of kidney clearance by intravenous administration were compared to the effects found in a three-dimensional cell culture model *(366)*. The results showed that dendrimers were accumulated in the organoids, with upregulation of kidney injury molecule-1 and N-acetyl-β-D-glucosaminidase, which are used as kidney acute renal failure markers. However, G5-OH PAMAM dendrimers induced low cell damage and limited cytokine release *(367)*. This three-dimensional organoid model could be useful for kidney toxicity assessment, since the results correspond to in vivo findings.

Dextran nanoparticles with a mean size distribution of 5 nm can be endocyted by kidney proximal tubules of male C57BL/6 mice (8 months of age) dosed at 40 mg/kg through the tail vein. Dextran nanoparticles were detected after 24 hours following injection, with no detection after seven days post injection. In addition, dextran nanoparticles increased the albumin uptake in proximal tubules, which was associated with an increase in megalin expression, a receptor involved in albumin uptake. In spite of changes in endocytosis, no alterations in renal function were reported *(368)*.

Nanocomposites

Some nanocomposites have been tested for therapeutic purposes, which are not addressed in this document. However, some of those studies show interactions with the gastrointestinal tract, even if they

are not based on oral administration. For instance, the pores formed on mesoporous silica nanoparticles, modified by undecylenic acid with chitosan as a surface modification, were loaded with glucagon-like peptide-1, with hydroxypropyl methylcellulose acetate succinate MF used as an enteric coating. This nanocomposite was given by gavage to diabetic animals, in which a 33% decrease in glucose concentration in blood was observed. Due to the chitosan component, which is able to open the tight junctions and has mucoadhesive properties, these nanocomposites have good epithelial adhesion in the intestine *(369)*. Fullerene functionalized with hydroxyl groups, known as fullerenol, has been suggested as possible therapy against immunological alterations derived from bone marrow transplantation. However, fullerenol nanoparticles have been found to have some effects in the small intestine, since fullerenol administered by intraperitoneal injection prevented the disruption of the epithelium and translocation of bacteria *(370)*. Some other nanocomposites not related to the gastrointestinal tract, such as nanocomposites of polycaprolactone coated with polyvinylpyrrolidone, have shown induction of cell differentiation on goblets cells in the conjunctiva with undetectable immune cell response *(371)*. Even if this nanocomposite is used in artificial conjunctiva, the effects on goblets cells could be relevant in terms of the gastrointestinal tract, in which goblet cells play a protective role through mucin synthesis. Early goblet cell differentiation and evasion of immune cell detection must be evaluated by similar nanocomposites with potential usage in the gastrointestinal tract. The coatings, such as those able to increase the adhesion in the epithelium, must also be evaluated, specifically if those nanocomposites are designed for longer treatments. The disruption of tight junctions caused by some coatings could have detrimental effects, and might cause translocation of microbiota. In addition, if the core or matrix of the nanocomposite is a metal- or a carbon-based component, those nanoparticles could end up in in the surrounding cells, with possible effects on the epithelium or cells from the immune system.

4.11   Human studies of nanomaterials

There are few human studies of exposure to nanomaterials, given ethical considerations, though some human studies have been undertaken for research on metal-based and organic nanomaterials used as food additives or in food packaging.

One early study in humans found the presence of dark pigment deposits in macrophages of Peyer's patches. The samples were obtained from postmortem and intestinal resections. Further analysis revealed the presence of aluminium, silicon and titanium *(372)*. Those elements were also found in mesenteric lymph nodes, and in some transmural inflammatory aggregates of patients with Crohn's disease *(372)*. Later, TiO_2 sized in 100 to 200 nm, aluminosilicates < 100–400 nm in length, and silicates ranging from 100 to 700 nm in length were found to be present in the basal areas of ileal Peyer's patches and colonic lymphoid aggregates from 20 adult patients diagnosed with Crohn's disease, ulcerative colitis, and colonic carcinoma *(373)*. Also, Gatti *(374)* demonstrated the presence of particles of various sizes in diseased colon mucosa. At that time, it was suspected that oral exposure to certain foods could include those particles. For example, TiO_2 could be contained in food as the additive E171, as it had already been approved for human consumption *(373)*.

Confirmation of human absorption of TiO_2 was reported later. Particles of 160 nm diameter showed higher absorption than particles of 360 nm in six male volunteers aged between 24 and 66 years *(375)*. The hypothesis that macrophages from the lung can be deposited in the bowel or in the lymphatic vessels through the bloodstream led to investigation of the presence of pigments in the colon, terminal ileum, duodenum, stomach and oesophagus in biopsies from children suspected of having inflammatory bowel disease. Aggregates of particles in the terminal ileum and Peyer's patches were found in 63 children (age ranging from 3.7 to 18.1 years). The presence of pigment was higher in patients with ulcerative colitis than in children with Crohn's disease *(376)*. Unfortunately, this study did not properly analyse the aggregates, but again it was suspected that TiO_2 and aluminosilicates could be the pigments identified, and that they could be orally obtained through the diet and intake of pharmaceuticals. In a more detailed absorption study, nine human volunteers aged 30 to 56 years received a single 5 mg/kg dose of TiO_2 dispersed in water. Volunteers received particles sized in 15 nm (study 1), 100 nm (study 2) or 5000 nm (study 3). The majority of them participated in the three studies. No differences in gastrointestinal absorption were found between the three types of particles, with absorption below 0.1% *(377)*.

While SiO_2 nanoparticles are being used as a food additive, the effects of oral consumption are still poorly investigated in human studies. The effects of SiO_2 particles were first reported in 1982, when O'Neill and colleagues demonstrated that these particles were located in the mucosa of oesophageal tumours from Chinese patients, probably because SiO_2 was present as an impurity in some foods such as millet bran *(378)*. The size of those particles was not identified, but the study highlighted the possibility of harmful effects on human health if they are orally ingested.

A 60-patient volunteer study was performed to evaluate the effect of 14-day oral exposure to two types of commercial Ag-NPs. Average daily ingestion of silver colloid formulation was estimated at 100 μg/day for 10 parts per million (ppm), and 480 μg/day for 32 ppm. Silver was detected in serum but no alterations were found in metabolic, haematological, urine, or physical parameters, and sputum morphology and imaging remained without changes *(379)*. Serum silver concentration was detected in 42% of subjects receiving 100 μg/day and in 92% of subjects receiving 480 μg/day, but was undetectable in the urine *(379)*.

4.12 Other exposure routes relevant for occupational settings

Incidental ophthalmic exposure can result from manufacturing processes in which workers without eye protection have eye contact with nanoparticles. Evidence of ophthalmic toxicity in environmental settings is limited, but ocular exposure to TiO_2 nanoparticles in rabbits reduced goblet cell density. Those cells are responsible for gel-forming mucin secreted in the conjunctival epithelium *(380)*. Long-term ocular exposure (90 days) to ZnO nanoparticles in rats showed not only nanoparticle deposition in the eyeball, muscle and surrounding tissues, but also retinal atrophy between the inner nuclear layer and the outer nuclear layer of exposed eyes *(381)*.

The translocation of inhaled particles through the olfactory bulb has raised concerns about toxicity in this structure, and there is direct evidence of an olfactory bulb–brain translocation pathway in rats *(382)*. In olfactory deposition, the size of nanoparticles is critical. In one study, smaller nanoparticles, sized at 3 nm, were deposited in

the anterior nose, while larger particles were uniformly distributed throughout the nasal structures *(383)*. In terms of toxicity, some metal oxide particles, such as ZnO, activated the Nrf2-mediated oxidative stress response after a short period of exposure (2 hours) in human olfactory neurosphere-derived cells, while longer exposure (6 hours) induced an activation of DNA damage repair pathways *(384)*. After 24 hours of exposure cell death markers were positive, and in all cases the coating of ZnO nanoparticles decreased the toxicity *(384)*. In in vivo systems, the intranasal administration of ZnO induced disruption of the olfactory epithelium, accompanied by infiltration of macrophages and neutrophils in the lamina propria *(385)*. The ZnO damage was associated with mitochondrial destruction *(385)*. There is evidence of alteration in neurotransmitter secretion in the olfactory bulb after nanoparticle exposure, as has been demonstrated after intranasal exposure to copper nanoparticles *(386)*. One main concern is that olfactory bulb–brain translocation of nanoparticles is strongly associated with apparent low olfactory bulb toxicity, though there is some alteration in the brain related to lactate dehydrogenase release and an increase in glutamate brain concentrations. However, no evidence of proinflammatory cytokines was detected in the brain *(387)*. Another concern of olfactory bulb–brain translocation is the possibility of deposition in the deeper brain regions, including the cerebral cortex, hippocampus and cerebellum. Intranasal instillation of TiO$_2$ nanoparticles caused pathological alterations in the olfactory bulb and hippocampus and induced higher TNF-α and IL-1β release *(388)*. As has been demonstrated in inhalatory and oral exposure, the crystalline phase has an impact on the release of these cytokines, since anatase TiO$_2$ nanoparticles have a higher proinflammatory potential that rutile *(388)*. Similar effects were reported in olfactory bulb–brain translocation of iron nanoparticles, in which pathological alterations in the olfactory bulb, hippocampus and striatum were found. Microglial proliferation and activation in the olfactory bulb were also reported *(389)*. It is important to highlight that microglia represent the immune cell response of the brain, and their activation and proliferation seem to be a direct consequence of the stress caused by nanoparticle deposition.

4.13 Placental exposure

Exposure during pregnancy in occupational settings has also been addressed, given concern about inhalation or some other

route of exposure. Some types of nanoparticles were not found in the placenta or fetal tissues of pregnant C57BL/6 mice, such as gold nanoparticles sized in 2 nm and 40 nm injected intravenously or intraperitoneally and traced after 24 hours *(390)*. Another study, however, found that gold nanoparticles modified by ferritin or PEG showed a higher internalization rate in the placenta and fetus than gold nanoparticles coated with citrate, which suggests that nanoparticles with a negative surface charge have less capability to be internalized *(391)*. However, Fe_2O_3 nanoparticles with either positive or negative surface modification had the same ability to cross the placenta, though positive surface modification induced higher toxicity in CD-1 mice *(392)*.

In addition, a study of exposure in the late phase of pregnancy found that gold nanoparticles coated with PEG and citrate can be taken up by the placenta without reaching the fetus *(391)*. These findings highlight the importance of gestational age exposure and the effect of coating. It was found that nanoparticles can be delivered from the lactating dam via the milk to the gastrointestinal tract of the pup, following administration of fullerenes via the tail vein in rats *(393)*. Oxidized SWCNTs administered in five-day pregnant mice induced fetal malformations and miscarriages *(394)*, which contrasted with the undetectable or low toxicity induced by gold nanoparticles. However, sole accumulation in the absence of damage can cause intrauterine inflammation, specifically following administration of gold nanoparticles sized at 3 nm and 13 nm *(395)*. Gold nanoparticles can also be translocated to the fetus through transtrophoblastic channels or via transcellular processes, rather than through amniotic fluids *(396)*. In addition, syncytiotrophoblast could be involved in the route of internalization, at least for polystyrene nanoparticles *(397)*. Functionalization of SWCNTs with PEG at low dose (10 μg/mouse) in pregnant CD-1 mice exhibits less toxicity compared to a higher dose (30 μg/mouse). The results showed teratogenic effects and reduced vascularization in the placenta *(398)*.

Inhaled cadmium nanoparticles have an impact on embryo implantation, as was demonstrated in a study using CD-1 pregnant mice. Cadmium was detected only in the placenta of pregnant mice that inhaled nanoparticles every other day. However, if inhalation was conducted daily, placental weight decreased and neonatal growth

was delayed *(399)*. Inhaled copper nanoparticles induced a decrease in the maternal weight of pregnant mice, with similar effect on the weight of newborns. While no copper was found in the placenta or fetus, alterations in gene expression of the spleen of the newborn were detected, mainly related to Th1 and Th2 responses *(400)*.

Despite the high doses (10, 100, and 1000 mg/kg/day) used for evaluation of oral effects of Ag-NPs sized at 6.45 nm in pregnant Sprague-Dawley rats, no alterations in fetus development were found. Hepatotoxicity in dams was the main toxic effect *(401)*. Intravenous administration of Ag-NPs induced an upregulation in meiosis, which was associated with a reduction in the DNA methylation levels of Zac1 transcription factor, while those of the insulin-like growth factor 2 receptor (Igf2r) gene showed an increase in placentas derived from Ag-NP-exposed mice *(402)*. ZnO nanoparticles were dispersed in 5% glucose solution and administered by the tail vein in 9-week-old Sprague-Dawley rats from day 6 to day 20 of gestation. High levels of zinc were found in the liver, lung and kidney, and in the liver of the fetus, but no evidence of toxicity related to nanoparticle administration was found *(403)*.

Placental barrier models have compared the possible toxicity among different nanoparticles. According to some findings, Fe_2O_3 nanoparticles could have a higher toxicity potential than SiO_2 nanoparticles *(404)*. This may be of relevance in developing systems to predict the most toxic characteristics of nanoparticles in terms of placental damage. Some other models, such as amnion epithelial cells or the BeWo b30 cell line, both derived from human placenta, can be useful in understanding the cellular mechanism of damage after internalization of nanoparticles. For instance, zinc ferrite ($ZnFe_2O_4$) nanoparticles can induce ROS generation, mitochondrial membrane disruption, DNA damage and inflammation *(405)*. Fe_2O_3 nanoparticles can also cause disruption of tight junctions *(406)*.

There are several limitations in the current studies of placental exposure to nanoparticles. Some of the most important for in vivo studies are related to the selected dose and the route of exposure for pregnant animals. Those studies showing placental translocation through inhalation of nanoparticles have high relevance in terms of occupational exposure, as do those in which animals receive the

nanoparticles by oral consumption, since both are realistic routes of exposure. Tail vain injection of nanoparticles is still helpful to understand if nanoparticles can reach the placenta and fetus once they enter the bloodstream, independent of the exposure route. While the immunotoxicity of nanoparticles in pregnant experimental models has been less explored, the toxicological studies that have been performed reveal that inflammation in the placenta and possible spleen immune responses in the fetus could have additional impacts. Indeed, most studies have been focused on the evaluation of the fetus or newborn, and there have been few studies on further effects, for instance on the growth of newborns until they reach adulthood, or on subsequent pregnancies of dams if they are exposed again, or on the impact of exposure of the fetus to nanoparticles on the second generation. Higher susceptibility for complications or alterations during the following pregnancies could also be observed. In addition, there is still limited information about the translocation of internalized nanoparticles in the fetus to other tissues, for instance during the clearance attempt. For example, in the dams, if macrophages have internalized nanoparticles, translocation to lymph nodes can occur.

Another important issue is the timing of exposure during the pregnancy. Animal models have shown that the effect during the early stages of pregnancy could have greater impact on the development of the fetus than exposure in the late stages. This is significant, given that women are not generally aware of pregnancy during the first weeks.

Even if there are still unknown mechanisms for translocation of placental nanoparticles, and the evidence of real toxicity in the dam, the fetus and the newborn is still incomplete, the risk for placental translocation in pregnant women exposed to nanoparticles poses a warning that merits further study.

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5. CONTROLLED EXPOSURE METHODS AND DOSIMETRY

For a correct interpretation of the results, experimental in vitro and in vivo studies with ENMs require adequate control of the exposure, in terms of both the methods and the exposure levels used. This chapter will outline some important considerations regarding exposure methods and levels to take into account when performing experimental studies with nanomaterials.

5.1 Dispersion methods

The appropriate dispersion method for an experimental study with ENMs will largely depend on the route of exposure of interest. For inhalation exposure, aerosols have to be (freshly) generated, whereas other routes of exposure may require dispersion in fluids. Dispersion, in general, is defined as an act to distribute particulate matter (dispersion phase) uniformly into another matter (continuous phase) or in materials such as air, liquid (culture medium) or solid via consolidation of the liquefied base. In the case of ENMs, dispersion may mean another step, that is, uniform distribution of primary particles by breaking down secondary particles. In any case, the dispersion is flexibly defined according to the aim of dispersion. For nanomaterial safety studies, the aim of dispersion is to reproduce or simulate the status of exposure at the target sites of organisms in experiments conducted for the assessment of its toxicity. For ENMs, this may require different techniques compared to larger particulate. The reason is because the smaller particles have a larger specific surface area and tend to agglomerate faster and stronger. There are three major forces: electrostatic, steric hindrance and van der Waals forces. Dispersion methods, especially in solution, are more or less designed to counteract those forces.

5.1.1 Aerosol generation

Methods for aerosol generation, and their advantages and limitations, have been described in the ISO/TR 19601 document

Nanotechnologies: aerosol generation for air exposure studies of nano-objects and their aggregates and agglomerates (NOAA) (1). In this document, summarized by Ahn et al. *(2)*, aerosol generation methods of ENMs are largely divided into two categories; (a) direct dispersion of dry (powder) sample in air (dry dispersion); and (b) suspension in liquid from which droplets are introduced in air (wet dispersion).

Dry dispersion is represented by the use of a dust feeder, which has normally been used for non-nano powder inhalation studies in the past and present. For better dispersion, the dispersed powder is introduced to a cyclonic airflow or acoustic vibration chamber for rigorous agitation, sometimes combined with a final filtration to remove large aggregates or agglomerates *(3)*.

Wet dispersion consists of two steps. The first step is to disperse an ENM in suspension. The most popular method is sonication of various energy levels and duration *(4)*. The purpose of dispersion is to homogeneously suspend the ENMs. Sonication is often used not only for dispersing primary particles but also for obtaining either primary particles from aggregates or the smaller agglomerates from larger ones. High energy and long sonication often results in changing the size or shape of the primary particle; fibrous particles may become shorter in length. The choice of dispersant in the suspension is made according to the nature of the ENMs and required characteristics of the aerosol. Pure water, aqueous solutions with detergents or proteins, high molecular weight adsorptive polymer, and oily low surface tension solvents are often used. As described by Deloid et al. *(4)*, adding molecules to water to improve the stability of the suspension may at the same time substantially affect the effective density and size of the ENM agglomerates. The second step is to generate dry aerosols from the suspension by removing the fluid. In order to avoid reaggregation by surface tension of the solvent, there are a few methods that use critical point drying.

A well known method is to introduce aqueous suspensions under controlled pressure and temperature conditions into a well designed nozzle to continuously create critical point drying at the tip of the nozzle for the continuous generation of dry aerosol (which inevitably includes some moisture).

Another method is to use tertiary butyl alcohol as a medium for dispersion and filtering in the liquid phase, followed by solidifying by freezing and sublimation under vacuum conditions to obtain the dry dispersed particles *(5)*. This method was inspired by the sample preparation method for scanning electron microscopy.

Removal of the aquatic component from the suspension by simple (diffusion) drying is possible, as is the case in "spray-and-dry" type systems at near room temperature and atmospheric pressure. In these systems, agglomeration of the sample within a droplet of the spray can be commonly seen; the fineness of the spray droplets often determines the level of dispersion. Non-volatile components of the dispersant, if any, will coexist with the final aerosol sample, thereby affecting the surface chemistry and potentially the outcome of the experimental study.

The first method, using pressure and heat, can bring strong sheer force to the suspension at the nozzle and results in shortening of fibrous ENMs and fracture of aggregates and fragile ENMs. The latter two methods can control the sheer force during dispersion so that the original size and shape of primary particles can be preserved. For all methods, filtration of the suspension by fine sieves can be applied to remove large aggregates or agglomerates above the pore size of the sieve used.

Table 5.1 presents a summary of modes of aerosol exposure.

Table 5.1 Modes of aerosol exposure

Mode/generation techniques	Advantages	Disadvantages
Dry dispersion		
Wright dust feeder	Small, simple and compact structure Small amount of material required for generation ENMs can be dispersed	Unstable concentration Feeder cannot be used for certain kinds of dust, such as highly cohesive samples Mechanical pressure to the dust may increase level and amount of agglomeration
Brush type aerosol generator	Small, simple and compact structure Possible to use test material as it is manufactured Less test material is required	Possible triboelectric charging may occur from friction while brushing off materials from pellet

Mode/generation techniques	Advantages	Disadvantages
Small-scale powder disperser	Possible to use test material as it is manufactured Small and compact structure	Unstable concentration, which is affected by shape or cohesiveness of particle when vacuuming the particle-loaded groove Intertwined and tangled CNTs may not be vacuumed evenly, or particles may stick together Applies relatively weak forces for dispersing an agglomerate
Fluidized bed aerosol generator	Small, simple and compact structure Possible to use test material itself	Variable aerosol concentration and alteration of test substance Ambient humidity, shape or cohesiveness of particles may cause unstable concentration Relatively weak mechanism for dispersing an agglomerate Fibrous test substances may exhibit breaking of individual fibres
Acoustic dry aerosol generator/ elutriator	Generates a stable aerosol Suitable for less cohesive powder such as silica (SiO_2) Possible to use test material itself	Affected by ambient humidity
Vilnius aerosol generator	Possible to use test material as it is manufactured Suitable to generate an aerosol for small volumes of powder Simple structure Possible to generate large amount ($1 \sim 2500$ mg/m^3) of test aerosol for a long time ($0.5 \sim 6$ hours)	Unstable concentration Weak mechanisms for dispersing an agglomerate Test particles may adhere to vanes, which will hinder aerosol generation process Unsuitable for generating aerosols from fibrous material
Rotating drum generator	Possible to use test material itself Small, compact and easy to use	Concentration of generated aerosol is unstable and affected by shape or cohesiveness of particles Relatively weak mechanisms for dispersing an agglomerate Unsuitable for generating aerosols from fibrous material Differences in concentration of aerosol generated over time

Table 5.1 (Contd)

Mode/generation techniques	Advantages	Disadvantages
Wet dispersion		
Atomizer/ nebulizer	Particles suspended or dispersed in liquid can be generated as aerosols	Particles may form from impurities in a solvent such as deionized water
	Small, compact and easy to use	Possible to change properties of ENMs such as CNTs by contact with a liquid
		Concentration of aerosol can also increase over time as liquid evaporates
		Difficult to generate particles when the particles are not well or uniformly dispersed
Electrostatic assist axial atomizer	Effective dispersing of CNTs by using ultrasonic energy	Possibility of damage of test substance by ultrasonic energy and introduction of impurities such as biological agents from deionized water
Freeze and sublimate	Simple procedure	Trace amount of dispersant may remain in dispersed particles
	Applicable to various ENMs	Needs more validation
Phase change		
Evaporation/ condensation generator	Simple and stable method of generating metal nanoparticles	Difficult to generate materials with high melting temperature and low evaporation rate
	Produced nanoparticles can be completely contamination free	
	Can obtain highly concentrated and non-agglomerated nanoparticles	
Spark generator	Can generate nanoparticle aerosols in the entire range (1–100 nm)	Few commercially available electrodes for aerosol generation, and differences in properties from actual ENMs exposed to workers in workplace air
	Produced nanoparticles can be completely contamination free and composed of one or more materials, depending on requirements and system used	

Mode/generation techniques	Advantages	Disadvantages
Condensation nanoaerosols	Might be only way to make controlled source of aerosol of appropriate particle sizes and concentration	Limited to materials with appropriate vapour pressure and temperature characteristics and stable under applied temperatures
		Coagulation with resulting particle size growth over time may limit ability to generate high concentrations
Chemical reaction		
Chemical reaction	Simple to use, effective method for generating ENMs	By-products are generated
		Use of inert gas may affect inhalation tests, dilution and other gas conditioning may be required

Source: Adapted from Ahn et al. *(2)*.

5.1.2   *Liquid suspension*

Special attention is needed for preparation of liquid suspensions of ENMs for toxicity studies, such as vehicles used in dermal or oral toxicity studies or cell culture medium for in vitro studies *(4, 6)*. The behaviour of insoluble ENMs in liquids is rather different from that of water-soluble materials. The extent to which particles agglomerate in liquids depends on electrostatic and van der Waals forces and steric hindrance. Other specific forces could be added, such as magnetic force for magnetized particles. An indication of the electrostatic properties of the surface of a particle can be obtained by measuring its zeta potential, taking into account that the zeta potential is a combined function of the properties of the material surface and the solution used to disperse the particle. Adhesive molecules in the dispersant, especially proteins, are known to form a corona around the particles and mask the original surface properties. The adhesiveness is again a function of original surface properties and conditions of the medium, including pH, salt concentration or polarity, and status of hydrated water. Using high molecular weight molecules such as PVP may improve the stability of the ENM suspension because of the steric hindrance that is caused by their adherence to the surface of ENMs. However, such molecules may have their own effect in safety studies and removing them from the dispersion is difficult, as

is defining proper controls. If the concentration of the high molecular weight molecules is lowered by dilution, the particles generally reagglomerate.

To improve the stability of the particle dispersion, suspensions are often sonicated using either an ultrasonic bath or probe. Rigorous ultrasonication using an ultrasonic probe can introduce contamination of the particle suspension by at least two possible mechanisms. First, the friction by the suspended particles may cause mechanical abrasion of the probe. It is known that MWCNT is hard enough to abrade the nickel metal of an ultrasonic probe. Second, contaminants may be introduced from chemical reactions that are reported to take place during sonication. When water is sonicated at the strength where cavitation bubble formation is seen around the probe, nitrous and nitric acid can be formed from H_2O and atmospheric nitrogen (N_2) dissolved in the water. Where the cavitation bubbles collapse, water can reach 5000 Kelvin (K) of high temperature and 1000 atmosphere of high pressure, breaking down H_2O molecules to reactive species to react with N_2. Where other chemical components are present, more complicated reaction may take place.

The complex and dynamic behaviour of ENMs in dispersions is thought to affect the outcome of toxicity studies. For oral and dermal in vivo studies, it is therefore recommended to use a matrix that resembles the exposure conditions in real life as closely as possible.

In order to facilitate comparison of results among in vitro studies, it has been suggested by various research groups that standardized dispersion protocols be developed and validated. A number of techniques and dispersion protocols for in vitro studies with nanomaterials are already available in the public literature, varying in ultrasound energies applied, dispersion stabilizers and their concentrations used, and different sequences of preparation steps *(4, 6–8)*, though none of these protocols has been formally standardized and validated.

Within the European Union-funded project NANoREG *(9)*, protocols were developed for probe sonication and preparation of dispersions for in vitro studies, both of which are available in an open access deliverable of the project. Although these protocols are not yet

available in the peer-reviewed literature, they have been performance tested and validated using an interlaboratory comparison approach.

5.1.3   *Characterization of nanosuspension*

For correct interpretation of study results, it is widely recognized that characterization of ENMs is essential. For example, in the case of studies using spray apparatus, light microscopy of the sprayed sample on a slide glass immediately before exposure to the animal and immediately after the end of exposure is recommended, as well as electron microscopy of the samples in suspension of the working concentration. In the case of in vitro cell culture studies, light microscopy views of the final medium smeared on a slide glass, or electron microscopy (EM) of the cell surface (scanning EM) and cytoplasm (transmission EM) or an equivalent image, are recommended. Various research groups have reported minimum lists of physicochemical properties to be measured in the context of toxicity studies with ENM *(10–14)*. These lists are largely overlapping. It is important to keep in mind that the properties to be measured are dependent on the purpose of the study – that is, they should be fit for purpose *(15)*. In addition, validated methods are not always available for every property of every type of ENM. ISO/TR 13014 and ISO/TR 16196 *(16, 17)* can be consulted for further consideration. An example of such a list of properties, along with the challenges in determining them, is shown in Table 5.2.

Table 5.2 Minimal characteristics and metrics recommended for every field of research investigating the health impact of nanomaterials

Characteristic	Metric	Challenges and comments
Essential metrics		
Size distribution (of primary particles)	Diameter: not appropriate for high aspect ratio nanoparticles	Different measurement methods investigate different submetrics, e.g. mobility diameter versus visual diameter
		Distribution of sizes needs to be reported
		Nanomaterials can agglomerate or aggregate
		Nanomaterials coated (corona) with biomolecules, depending on matrix – which diameter to assess?
		Depends on medium

Table 5.2 (Contd)

Characteristic	Metric	Challenges and comments
Essential metrics		
Chemical composition	Chemistry Purity/impurities	Chemical composition can be determined, but structural information is difficult to obtain due to complex measurements. Ideally this would be provided by manufacturers
		Impurities may be as important for health impact as the basic material
Nanomaterial surface	Surface area Chemistry Surface charge	Different measurement methods investigate different submetrics, e.g. BET surface, Fuchs surface, visual surface, mobility diameter surface
		There are no simple methods to assess the chemistry of the surface of nanomaterials; thus, provide at least information on the synthesis method used, and if/what surface treatment or stabilization method had been used
		Zeta potential and pH measurements should be reported for all particles in appropriate test media
Structure	Agglomerate size (distribution)	Agglomeration status is in equilibrium with the matrix. No commonly agreed-on metric exists to define the agglomeration status. Also, information about the stability of agglomerates in different media would often be very useful. Aggregation is a more fixed status, and should not be mixed up with agglomeration
Often important metrics		
Shape	Aspect ratio	Aspect ratio determines if an object falls within the WHO definition of a fibre, and is very important for health impact assessment purposes
Persistence	Solubility UV stability Thermal stability	These different types of metrics give information about the persistence of materials in biological media, and environmental compartments. These factors (UV, heat) may also affect ENM surface properties and agglomeration

Source: Adapted from Bouwmeester et al. *(10)*.

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5.2 Exposure methods

Ideally, when designing an in vivo toxicity study for ENM exposure, the actual workplace exposure or consumer exposure scenario should be simulated. However, in practice, there are a myriad of potential exposure scenarios for one material that may vary widely; therefore, exposure methods in regulatory in vivo studies generally follow standard requirements outlined in OECD test guidelines. Concentration in terms of mass or number, shape, size and size distribution of ENM, and frequency of exposure, need to be specified, so that these exposure concentrations can be translated to real-life situations. For the biopersistent ENMs, the accumulated amount of ENM in a target organ, such as the lung (lung burden), might be used as an indicator of exposure. For immunotoxicological assessment, the lymph node burden might become a subject of interest.

5.2.1 *In vivo exposure methods for inhalation studies*

A number of exposure methods can be used in in vivo studies investigating the toxicity of ENMs to the lung. Most inhalation toxicity studies use rats or mice as an experimental animal species. It should be noted that the structure of human lungs is significantly different from that of rodents, which has a profound effect on the fraction of particles deposited in different parts of the lung. Models such as the multiple path particle dosimetry model can aid in calculating these different deposition fractions.

Whole-body exposure systems use chambers in which the animals can move around in cages. These systems allow exposure of a large number of animals and are relatively non-stressful and less labour intensive, and convenient for chronic exposure studies where daily exposure durations are 6–24 hours. However, these systems expose all parts of the animal surface, entail provision of food and water, and require large volumes of test articles. They occupy a large area of the building and are expensive to build, install and maintain. There is a new system reported for small-scale whole-body inhalation studies with less expensive investment and lower maintenance costs *(5)* but wider evaluation is still needed on its performance and stability.

Nose/head-only exposure offers advantages over whole-body exposure, including less space needed to install and manage, minimum skin contamination, smaller quantity of test article required and better dose control compared with the whole-body system. However, higher stress to the animals and labour intensiveness to expose a large number of animals prohibit using this exposure method for chronic studies.

Other non-physiological methods in delivering a test article to the respiratory system are intratracheal instillation and pharyngeal aspiration methods. These methods are good for screening, and only require a small amount of the test article, but they require anaesthesia during administration and may induce tissue damage and occasional death due to handling of animals. These direct dosing methods are currently not used for risk assessment of pulmonary toxicity. Kinaret and colleagues *(18)* demonstrated that inhalation and oropharyngeal aspiration of CNTs resulted in similar airway inflammation and biological response in mouse lungs, although the dose levels at which these effects start to occur cannot easily be derived.

Table 5.3 presents the advantages and disadvantages of pulmonary exposure methods.

Table 5.3 Advantages and disadvantages of pulmonary exposure methods

Method	Advantages	Disadvantages
Whole-body exposure	Physiological way of exposure Long-term studies, repeated exposure possible Allows for a large number of animals No anaesthesia or discomfort for animals Labour efficient	Large quantity of material needed Occurrence of dermal, eye, oral exposure Dose not well defined Large space occupied Expensive
Nose/head-only exposure	Relatively physiological way of exposure Repeated exposures possible No anaesthesia Minimum skin contamination Better control of dose Relatively small space occupied	Stressful Labour intensive Animal training may be needed for placement in tube

Method	Advantages	Disadvantages
Intratracheal instillation	Delivers dose directly to lungs Good for screening of respiratory toxicity Can be used for toxicokinetic study	Non-physiological exposure currently not used for risk assessment Anaesthesia needed No repeated dosing Tissue injury Labour intensive Bolus administration (high dose rate) Bypasses the upper respiratory tract
Oropharyngeal instillation	Delivers dose directly to lungs Good for screening of respiratory toxicity Can be used for toxicokinetic study	Non-physiological exposure currently not used for risk assessment No repeated dosing Labour intensive Bolus administration (high dose rate) Bypasses the upper respiratory tract
Oropharyngeal aspiration	Delivers dose directly to lungs Good for screening of respiratory toxicity Can be used for toxicokinetic study response	Non-physiological exposure currently not used for risk assessment Potential aspiration of oral content into lungs No repeated dosing Labour intensive Bolus administration (high dose rate) Bypasses the upper respiratory tract

The uses and limitations of the intratracheal instillation method are well described in the paper by Driscoll et al. *(19)*. Use of intratracheal instillation has been recommended to address certain questions important to pulmonary toxicology: (a) screening panels of test materials for their relative potential to produce toxicity in the lower respiratory tract or to screen for effects over a range of doses; (b) comparing the effects of a new material to similar materials for which

inhalation data are available; and (c) comparing the toxicity of fractional components of a mixture with toxicity of the whole mixture to obtain information on which constituents may be most toxicologically relevant *(19)*. The limitations of intratracheal instillation are (a) difficulties in extrapolating a lung dose administered by intratracheal instillation to an inhalation exposure concentration; and (b) clumping (aggregates or agglomerates) of particles and local inflammatory responses that can affect local lung clearance process and biopersistence of particles. Therefore, intratracheal doses below 100 μg/rat should be used. Intratracheal instillation should not be used (a) when determining particle deposition patterns in the lungs that would occur following inhalation; (b) when information on the upper respiratory tract toxicity of a material needs to be obtained; (c) when evaluating short-term clearance; and (d) when materials are reacting with the vehicle or when a change in the vehicle may alter the material's toxicity.

Table 5.4 presents a comparison of lung exposure methods.

5.2.2 Dermal exposure methods

Dermal exposures are of particular concern in certain applications, often where nanoparticles are dispersed in liquid matrices. Methods should ensure that the nanoparticles are dispersed in a way that is uniform and that can be correlated to pertinent realistic exposures. In a study on ZnO, Song et al. used human skin and a commercial sunscreen as the source of nanoparticles *(21)*. This approach clearly attempted to develop a realistic exposure scenario that closely modelled human exposure and ENMs in commercial use *(17)*.

5.2.3 Oral exposure methods

Nanosuspensions can be administered through oral gavage or drinking water or mixed with food. The nanosuspension can be mixed with appropriate vehicles such as corn oil or carboxymethylcellulose or other vehicular material. In any case, the dose should be monitored regularly to identify whether the proper dose is being delivered to experimental animals.

Table 5.4 Comparison of lung exposure methods

Method	Anaesthesia	Deposition throughout respiratory tract	Evenness of deposition	Realistic dose rate	Toxicity ranking	Use for quantitative risk assessment	Nanoparticle treatment artefact	Mechanistic data caveats	Long-term exposure	Costs
Intranasal instillation	Yes	(No)	–	No	Yes	No	Yes	(Yes)	–	Low
Intratracheal instillation[a]	Yes	No	–	No	Yes	No	Yes	(Yes)	–	Low
Oropharyngeal aspiration[b]	Yes	No	(–)	No	Yes	No	Yes	(Yes)	–	Low
Laryngeal aspiration	Yes	No	(–)	No	Yes	No	Yes	(Yes)	–	Low
Intratracheal microscopy[a]	Yes	No	(+)	No	Yes	No	Yes	(Yes)	–	Low
Intratracheal insufflation[a]	Yes	No	(+)	No	Yes	No	Yes	(Yes)	–	Low
Inhalation										
Whole body[c]	No	Yes	+	Yes	Yes	Yes	(No)	No	+	High
Nose only	No	Yes	+	Yes	Yes	Yes	(No)	No	+	High
Intratracheal[d]	Yes	No	+	Yes	Yes	Yes	(No)	No	–	High

Key: a, synchronize with inspiration; b, may cause significant inflammation in rats; c, inhalation requires larger amounts of material; d, does not work well in mice.
– or + indicate negative or positive attribute of method (for example, for evenness of deposition, – indicates not even, while + indicates that evenness is good). Parentheses indicate uncertainty, depending on other factors.
Source: Adapted from Oberdörster et al. *(20)*.

5.2.4   Other exposure methods

Nanosuspensions can be administered intraperitoneally or intravenously to study the effect of nanomaterials on mesothelial layers and systemic circulation, respectively. As with other liquid media, consideration should be given as to how nanoparticles may transform from dry particles to particles in a liquid medium. If dispersing practices are used, how the dispersing method is relevant

to the realistic and potential biological effects from any dispersing agents should be considered and addressed. Exposure scenarios need to be described so it is clear whether a scenario represents potential exposures in the use of a nanomaterial or if the exposure is an overexposure intended to elicit a biological response *(17)*.

5.2.5 *In vitro exposure methods*

For the immune system, only parts of a complete immune response can be studied in vitro, such as a specific pathway or induction of immune response mediators. A number of additional challenges occur specifically when studying ENMs in vitro.

Static, submerged exposure to ENMs

In in vitro studies, administering materials under static conditions to cells cultured at the bottom of a culture plate will in most cases lead to a different interaction rate of the materials with cells compared to in vivo situations, leading to different cellular concentrations. In addition, similar nominal exposure concentrations may lead to different cellular concentrations for different ENMs due to differences in (effective) density of the material and resulting gravitational and diffusional forces *(22)*.

Moreover, when inhaled, ENMs interact with lung cells at an air–liquid interface, whereas for most in vitro studies materials are suspended in a cell culture medium with all the components they interact with, again resulting in a different identity and behaviour compared to materials dispersed in air.

For the reasons just mentioned, effect concentrations found in vitro cannot be readily extrapolated to in vivo. Often, effects in vitro are found at concentrations that are much higher than could ever be achieved in vivo and therefore may not be relevant. Determination of cellular concentrations rather than nominal concentrations may improve the extrapolation, or at least allow for a better comparison of results between different in vitro studies. A computational in vitro sedimentation, diffusion and dosimetry model was introduced by Hinderliter et al. *(23)* to calculate the cellular concentrations of ENMs depending on particle size, density and their aggregation state. More recently, a more advanced model (at present not freely available)

has been developed *(24)*. This so-called distorted grid model allows rapid modelling that accommodates polydispersity, dissolution, and adsorption, and takes into account the actual effective density of the ENMs in the suspension used for cell exposures.

Novel in vitro exposure technologies

In recent years, new technologies have made it possible to perform more realistic or dynamic exposures for cells of the respiratory system. One example of such a system is an air–liquid exposure chamber that allows for exposure of cells in culture plates to an aerosol containing ENMs, thereby avoiding potential artefacts caused by the interaction of the ENM with cell culture medium components *(25, 26)*. Another example of a relatively new technology is the use of flow-through systems in which cells can be exposed to a continuous flow of nanomaterials, such as a perfusion platform for skin exposure *(27)*. The advantage of such systems is not only that there is a continuous (rather than a single) bolus exposure, but also that exposure conditions are more realistic and better resemble what will occur in vivo, although studies comparing data from these systems and in vivo experiments are scarce. A disadvantage of air–liquid and flow-through systems is that the exposure concentrations may be more difficult to characterize and control, and these systems are often more laborious and less adequate for high-throughput screening purposes compared to simple submerged in vitro systems.

5.3   Metrics of dose

Traditionally, administered mass is used to describe doses of conventional chemical substances in experimental studies, both in vitro and in vivo. For deriving dose–effect relationships of ENMs having the same chemical composition but different physical characteristics, mass alone may not adequately describe the dose. ENMs with the same chemical composition can have completely different internal doses and distributions within as well as among organs *(28, 29)*. Other dose metrics such as particle number, volume or surface area have been suggested, but consensus is still lacking. An approach to determine the most adequate description of dose has been suggested by Delmaar et al. *(30)*, which could in theory also include particle properties such as particle size, zeta potential, surface reactivity

and dispersity index, or a combination thereof. However, it should be realized that these properties are dependent on the experimental condition, and therefore the most adequate dose metrics for ENMs are also likely to be different for different experimental and real-life situations, or even for different toxicity end-points.

For ENMs that readily dissolve in the body, mass may be a useful metric, similar to conventional substances *(31)*. Surface area has been shown to be a useful metric to extrapolate across ENMs of a range of sizes *(28)*, as well for classification of ENM powders *(32)*.

For ENMs that follow the WHO definition of a fibre (fibre length (L) > 5 μm, fibre diameter (D) < 3 μm and aspect ratio (L/D) > 3), particle number has been suggested to be the appropriate metric, as the fibres may follow the same mechanism of action as asbestos fibres. At present it is not clear if this WHO definition is sufficient to cover this aspect.

Combinations of two or more metrics have been suggested for toxicity assessment of TiO_2 as it was concluded that nanoparticle size distribution, along with mass or total surface area concentrations, contributes to a more mechanistic discrimination of pulmonary responses to nanoparticle exposure *(33)*.

5.4   Dosimetry

Special care should be taken that exposure concentrations administered in an in vivo study are not too high. In inhalation studies, high exposure concentrations could lead to overload conditions, while in oral studies, high doses that lead to aggregation may lead to lower uptake of materials than lower doses *(34, 35)*.

The rest of this section will compare continuous and bolus exposure. The major and toxicologically significant differences between continuous exposure (CX) to smaller doses and bolus exposure (BX) are the differences in the time course of organ burden and the dose rate. The organ burden is a function of input and output. For oral and dermal routes of exposure, differences in organ burden as a result of different dose rates of ENMs have rarely been investigated. For the inhalation route, the organ burden is the result of the speed

of lung deposition (dose rate) and clearance. In CX studies, the lung burden either reaches equilibrium or will gradually increase with time of exposure, depending on the nature and the concentration of the ENMs. For BX studies the same principles will apply; however, the dose is applied all at once (very high dose rate), whereas the lung burden will decrease gradually in time.

The decrease function seems to be a combination of two curves: (a) a decay curve reflecting clearance mechanisms such as the mucociliary escalator and particle-laden macrophage migration towards the oesophagus and local lymph nodes; and (b) a fixation curve reflecting particles embedded in granulomas or fibrotic scars of the lung tissue. As a whole, the shape of the curve is similar to a decay curve, except that the asymptote is not zero. In the case of well dispersed nanoparticles without aggregates or agglomerates the decay is rapid and the level of the asymptote is relatively low, whereas in the case of a sample with large aggregates or agglomerates the decay is minimal, with a very high level of asymptote due to massive formation of granulomas against the aggregates or agglomerates.

Although the total dose can be very similar for BX and CX studies, large differences in dose rate and distribution within an organ (in particular the lung) at the onset of the exposure may result in substantial differences in the responses, for example due to exhaustion of the host defence systems, such as antioxidant and phagocytizing capacity of the target organs (with the exception of the skin).

Figure 5.1 shows the time course of organ burden after a single bolus exposure. The blue dotted line indicates the organ burden. The line follows the shape of a decay curve except that the asymptote (red line) is not zero, indicating that some of the substance remains in the organ. The distance of the asymptotic line from the x-axis indicates the amount of substance trapped permanently in the organ (vertical red line with arrow heads).

In CX studies with soluble chemicals given by oral route or gaseous chemicals given by inhalation, chemicals are metabolized and cleared daily and the body burden or the target organ burden reach a constant value (asymptotic value) within the first few days or weeks of the study. Thus, the body burden of the test chemicals can
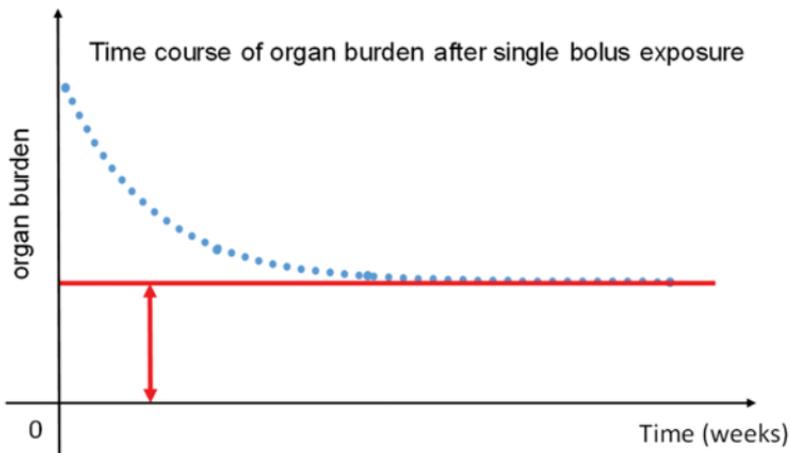
Figure 5.1 Time course of organ burden after a single bolus exposure

be considered virtually constant during the whole study period. When the body burden of such chemicals is plotted on a graph with y-axis as body burden and x-axis as day of the inhalation, the area under the curve is rectangular, with its height depicting the asymptotic value and its width as the time span of the study (Figure 5.2A).

On the other hand, in the case of CX inhalation studies with biologically persistent particulate matter including biopersistent ENMs, when constant concentration of the aerosol is given daily to the test animal, the material constantly accumulates in the lungs. The graph of the organ burden will be a linear line from zero at day 0 to a certain value at 2 years, making a triangle-shaped area under the curve. The particulate matter inhalation under this exposure protocol will give half of the area under the curve compared to gaseous chemicals (Figure 5.2B).

In the case of carcinogenesis studies, the area under the curve may affect the overall sensitivity. It is generally accepted that the accumulation of damage to the DNA via various mechanisms is closely related to the induction of tumours. On the other hand, immunotoxicological end-points, if any, are not well linked mechanistically to the organ burden of ENMs. Therefore, it is not

Difference in area under the curve (AUC) of organ burden given by low
molecular weight chemicals and ENMs by current exposure scenario
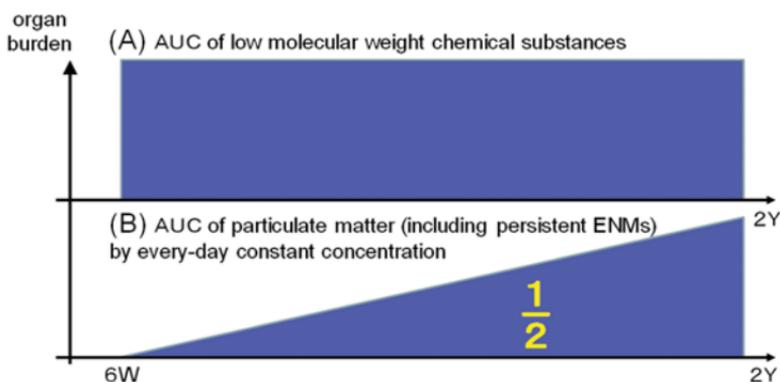
Figure 5.2 Comparison of continuous exposure study of (A) low molecular
chemicals that reach a constant organ burden and (B) biopersistent particulate
matter

clear at this moment whether the differences in area under the curve
and time course of the organ burden affect the sensitivity of a study to
investigate any immunotoxicity.

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6. HAZARD ASSESSMENT

6.1 Introduction

As discussed in the previous sections, several studies have demonstrated that nanomaterials interact with immune cells and tissues of the immune system. Such interaction is shown to trigger cytotoxicity, oxidative stress, proinflammatory response, complement activation-related pseudoallergy (CARPA), genotoxicity, alteration of metabolism, and proliferation of immune and inflammatory cells *(1)*.

As for chemicals, immune toxicity of nanomaterials can be associated with several adverse outcomes *(2)*: (a) immunosuppression – decreased host resistance to infectious agents; (b) immune activation – increased risk of developing autoimmune diseases; (c) increased risk of developing allergic diseases – atopy, food allergies and asthma; (d) hypersensitivity to the chemical or substance – repeated exposure; (e) abnormal inflammatory responses or unresolved inflammation – tissue or organ damage and dysfunction; and (f) abnormal inflammatory responses, altered adaptive immune response – disease.

Nanomaterial-induced immune modulation and dysregulation can result from inhalation, dermal exposure or oral ingestion of nanomaterials in the workplace and environment or via direct injection of drugs that contain nanomaterials. While most of the effects may be anticipated to occur locally at the site of exposure, there is evidence showing low levels of translocation of nanomaterials to blood from primary organs such as the lung *(3, 4)* or gastrointestinal tract, depending on the size and properties of the nanomaterials. For example, translocation of polystyrene microspheres across the gastrointestinal tract or translocation of ultrafine insoluble iridium particles from the lung epithelium to the extrapulmonary tissues was shown to depend on their size, with smaller size particles exhibiting higher translocation *(5, 6)*. In addition, there is evidence that orally administered silver can be distributed to other organs such as the liver *(7)*, and translocation of TiO_2 nanoparticles from the lungs of mice exposed via intratracheal instillation to the liver and heart was reported by Husain et al. *(3)*. The translocation of TiO_2 nanoparticles to the heart

coincided with the activation of complement proteins in the heart *(3)*. Once in the biological fluid, many types of nanomaterials are shown to form protein coronas, resulting in changes to their primary physical and chemical properties and hence their interactions with cells of the immune system (see section 4.8) and their ability to translocate. Several classes of nanomaterials with diverse properties are shown to persist in the lungs, spleen, liver and other immune responsive tissues, which will result in tissue injury and toxicity *(3, 8–10)*. Although the extent of such translocation is generally negligible, the resulting systemic consequences may depend on the primary properties of nanomaterials, such as solubility or size. In some cases, indirect systemic responses can be anticipated based on the magnitude of the target organ toxicity. Robust lung inflammation involving multiple immune cell types, as observed following exposure to carbon-based nanoparticles including carbon black or MWCNTs, tends to culminate in systemic responses in the liver *(11, 12)*. However, such responses are not observed after lung inhalation exposure to low toxicity nanomaterials, including TiO_2 nanoparticles *(13)*.

The OECD, ISO, and International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) have developed international guidelines for the assessment of immunotoxicity induced by chemicals and drugs. These test guidelines (OECD TGs 407, 412, 413, ISO 10093-4 and ICH S8) describe various parameters to consider in determining the appropriate test methods and provide specific details of the method, animal handling and dosing considerations, as well as the reporting standards. However, no single guideline on its own is encompassing enough to cover all aspects of immunotoxicity testing. Hence, based on the amount of nanomaterial used and the anticipated exposure route, a flexible combination of methods described in these documents may be necessary to accurately assess the nanomaterial-induced toxicity.

The research conducted so far does not reveal immunotoxicity specific to nanomaterials, even if the spectrum of immunological effects may be different. This suggests that the existing chemical-specific immunotoxicity testing guidelines and tools may in principle be applicable to nanomaterials, but will in some cases need further modifications. The tiered approach suggested in Environmental Health Criteria 180 (Principles and methods for assessing direct

immunotoxicity associated with exposure to chemicals) *(14)* may be applicable to some nanomaterials that are directly acting on components of the immune system. However, interaction of nanomaterials with cells and tissues of the immune system is suggested to be distinct from that of chemicals (described in the previous sections). Moreover, due to their distinct properties, nanomaterials have been shown to interfere with some of the components of the conventional immunotoxicity assays, leading to misinterpretation of their toxic potential. In addition, the sensitivity of the conventional test methods to assess or predict nanomaterial-induced immunotoxicity is not completely known. For other immunotoxic effects, such as autoimmunity and induction of CARPA, globally harmonized technical test guidelines are lacking altogether *(15)*. Overall, a major challenge for nanomaterials lies in precisely understanding their interaction with the immune system, determining the appropriate assays and their suitability, and knowing the necessary modifications required to make those assays applicable for testing nanomaterials.

Given the complexity associated with nanomaterial testing, the purpose of this chapter is to recommend an immune toxicity testing strategy as it relates to specific routes of exposure to nanomaterials. Figure 6.1 shows several questions that have to be addressed before initiating the specific experiments to assess nanomaterial-induced immunotoxicity. The obvious starting point would be to determine whether there is a likelihood of exposure to the nanomaterial that is being investigated in the environment. This can be derived from information on their potential applications. If the answer to this first question is yes, then the route of exposure and duration of exposure need to be determined. Next, if the nanomaterial of interest is suspected to interact with tissues and cells of the immune system, then tissue accumulation and biopersistence of nanomaterials has to be investigated. Intravenously injected nanomedicines are anticipated to impact the immune system due to their direct interaction with blood, depending on the time in blood (how long do nanomaterials remain in blood) and their dose. For nanomedicines, a tiered testing approach has been developed that includes screening for immunotoxicity in the first tier using immunopathology, humoral and cell-mediated immune testing *(16)*. Subsequently, for those materials screened positive in the first tier, confirmation and characterization of immunotoxicity using additional assays for assessing humoral, innate and cell-mediated
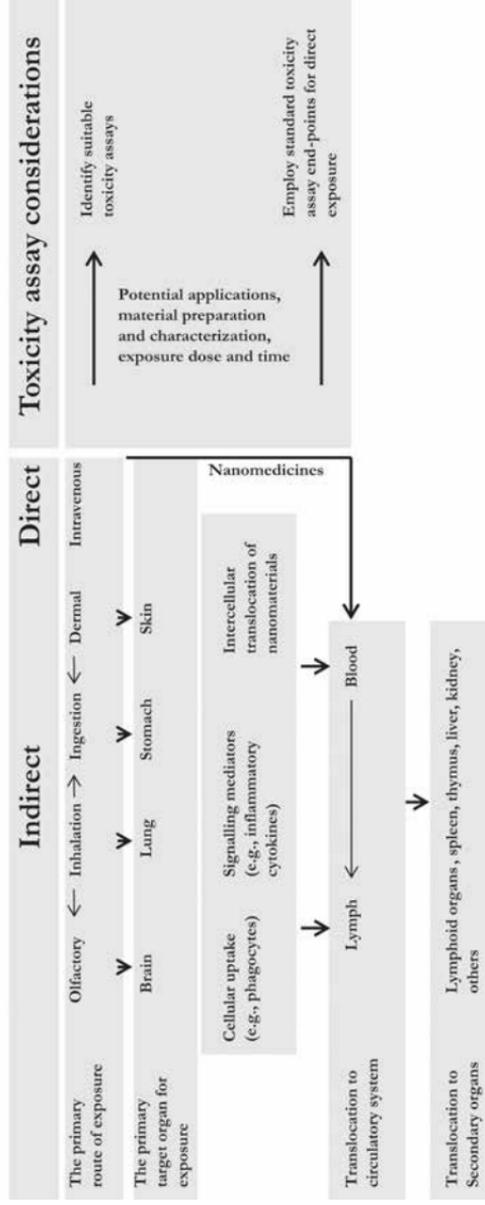
Figure 6.1 Primary questions to be addressed, considerations of exposure routes, and cells and tissues with which nanomaterials interact

effects on immunity, along with effects on host resistance, are conducted. Although nanomedicines are excluded from the scope of this document, the testing strategy may still be relevant for nanomaterials that become systemically available after exposure through other routes. In addition, structural similarities to known immunotoxicants, such as immunotoxicity of parent bulk materials, should be taken into consideration. Depending on the answers to the questions above, a selection of assays can be considered for testing immunotoxicity of the nanomaterials.

The following sections are intended to provide some guidance on current practices of immunotoxicity testing of nanomaterials with a special emphasis on such important factors as handling and preparation of nanomaterials, physical-chemical characterization, and appropriate dose regime.

6.2   Test material considerations

In general, nanomaterials are synthesized by a variety of techniques that often result in contamination with different types of impurities, including detergents, metal impurities and endotoxins. The following subsection will provide details on the assessment of endotoxin, as it is one of the major impurities and can impact the immune system directly.

6.2.1   Endotoxin contamination

Endotoxins, also known as lipopolysaccharide (LPS), are large thermostable molecules that are found on the outer cell membrane walls of gram-negative bacteria such as *Escherichia coli* and *Salmonella*, *Shigella*, and *Haemophilus* spp. Endotoxins are made up of a bioactive lipid component (lipid A) covalently bound to hydrophilic heteropolysaccharides of variable length *(17)*. Endotoxins are ubiquitous in our environment and exposure to a small amount of endotoxins can result in systemic reactions, including respiratory distress, inflammation, pyrogenic reaction, shock and coagulation-related events *(18)*. In an experimental condition, 6 picograms (pg) is the accepted safe level of endotoxin that is shown not to induce increases in cytokine levels when administered to a 20 g mouse via intravenous injection over a 1-hour period.

Many commercially available nanomaterials are not produced in a sterile environment, as they may not need to be sterile for the purposes of their applications. As a result, these nanomaterials are often found contaminated by endotoxin, levels of which vary with different batches even if synthesized in the same facility or laboratory, due to variations in the synthesis and handling procedures.

Endotoxin can bind to the surface of nanomaterials; hydrophobic nanomaterials and nanomaterials with a positive surface charge are known to adsorb biomolecules and hence may influence binding of endotoxin to surfaces. Since contamination with a very minute amount of endotoxin can trigger a large immune response, it is imperative to assess endotoxin contamination for each nanomaterial before their use in experiments *(2)*. For example, gold nanoparticles contaminated with endotoxin during the sample preparation process were shown to interfere with in vitro biocompatibility tests *(19)*. In a study *(20)* investigating the influence of endotoxin contamination induced by nanomaterials, mice were exposed to LPS only or LPS with carbon black nanoparticles (14 nm or 56 nm) and lung inflammation response was assessed. The results showed that in the presence of LPS, 14 nm carbon black aggravated the LPS-induced lung inflammation and oedema with enhanced lung expression of IL-1β, macrophage inflammatory protein-alpha (MIP-α), MCP-1, MIP-2 and keratinocyte chemoattractant, whereas 56 nm carbon black did not show apparent effects. These results indicate that nanomaterials can aggravate lung inflammation in the presence of bacterial endotoxin, which is more prominent with smaller particles *(20)*. A simple and straightforward method to check whether nanomaterial samples are contaminated with bacteria is to inoculate a small amount of it onto agar plates and check for growth of bacterial colonies over a few days. However, the absence of bacterial growth does not guarantee the absence of endotoxins, since these molecules may still be present in the sample but in the absence of live bacteria.

There are different types of endotoxin methods that can be used to test nanomaterials. The choice of method depends on the physical-chemical properties of the nanomaterials and their behaviour in aqueous media. In addition, special consideration should be given to their optical behaviour, as some of them are shown to interfere with the optical reading of endotoxin assay results *(15, 16)*. Some others,

owing to their hydrophobic or positively charged surfaces, more readily adsorb endotoxins. These nanomaterials should immediately be flagged for possible endotoxin contamination testing.

6.2.2 Storage and handling of nanomaterials in laboratories

Nanomaterials can be contaminated with endotoxin in the environment. They should be stored in commercially available endotoxin-free glassware or polystyrene containers. Equipment and glassware used for the preparation of samples for endotoxin tests can be treated by heating to a temperature of greater than 250ºC for at least 30 minutes prior to their use. A separate laboratory with clean water and air supply is recommended for working with nanomaterials. In addition, everything involved with the preparation of nanomaterials, including pipettes, dispersion equipment, dispersion liquid, pipette tips, containers and water, should be endotoxin free. ISO 29701:2010 *(21)* suggests that 0.05% polysorbate 20 for extraction of airborne endotoxin from glass fibre filters and 0.1% vitamin E surfactant for extraction of endotoxin from carbon-based nanomaterials were found to improve the extraction of endotoxins. More details of equipment and laboratory vessels used in the preparation of test samples are provided in ISO 29701:2010 *(21)*.

6.2.3 Endotoxin detection methods

The rabbit pyrogen test (RPT) and the *Limulus* amoebocyte lysate (LAL) test are the most commonly used, internationally harmonized and validated tests for assessing the endotoxin contamination of chemical substances, biological products, drugs and medical devices *(18)*.

The RPT involves measuring the rise in temperature of rabbits following the intravenous administration of a test solution at a certain concentration over a set period of time. The RPT is cost and time intensive and requires animal testing. For these reasons, it is no longer preferred. However, it is recommended when the test results of more than one type of LAL method are inconsistent.

The LAL test uses the cell lysate obtained from the circulating amoebocytes of horseshoe crab (*Limulus polyphemus*) as a test reagent, which, when reacted with endotoxins present in the test

substance, initiates a cascade of enzymatic reactions leading to a clot formation *(22)*. The LAL test is administered in three different ways: chromogenic, kinetic turbidity, and gel clot, all of which assess different aspects of this enzymatic reaction leading to the end result of clot formation.

First, the chromogenic method is based on the activation of a serine protease (coagulase) by the endotoxin, critical for the development of a clot. In the test, the natural coagulate that serves as a substrate in the reaction is replaced by a chromogenic substrate. In the presence of endotoxin, the chromogenic substrate is cleaved, releasing a chromophore, which is then measured by spectrophotometry. Second, the kinetic turbimetric method is based on the fact that in the presence of endotoxins LAL turns turbid. The time taken to reach a level of turbidity (onset time at which turbidity appears) is directly proportional to the amount of endotoxin present in the test substance; the higher the amount of endotoxin, the shorter the turbidity onset time. Third, the gel clot method is based on the presence or absence of a gel clot in the test sample, the end result of the reaction between the LAL and the endotoxin. The gel forms when proteins are coagulated due to the presence of endotoxins. All three methods have been used to test endotoxin contamination in nanomaterial formulations. These studies demonstrate that nanomaterials interfere with various steps of these assays and that appropriate controls and assay modifications are required *(15, 23)*. The colorimetric methods in general are not suitable for assessing nanomaterial-induced effects. The optical features of the nanomaterials should be taken into consideration in selecting the appropriate method.

In an end-point chromogenic LAL test, spiking a known amount of endotoxin into citrate-stabilized colloidal gold nanoparticles showed 50% lower endotoxin recovery compared to the actual endotoxin spiked in, whereas higher recovery rates than the original endotoxin amounts was observed for poly(β-L-malic acid) nanoparticles, suggesting both inhibitory and enhancement activity of nanoparticles in the LAL test *(23, 24)*. Similar interference was also observed in gel clot LAL assay. The same nanoparticle formulations tested by the kinetic turbidity LAL assay showed no such interference. Thus, LAL assays to detect endotoxin contamination should include appropriate controls to exclude enzyme inhibition or enzyme activity enhancement by the nanomaterials. Inclusion of

spike-in control (known amount of endotoxin standard into LAL-grade water and into the nanomaterial suspension) should be routine. If interference is observed, different dilutions of nanomaterial suspensions should be tried *(23)*. In a chromogenic end-point assay, chromogenic colour intensity is measured at 405 nm, which overlaps with extinction peaks of some nanomaterials, such as Ag-NPs. This will mask the colorimetric signal arising from the assay. Therefore, for Ag-NPs or for those nanomaterials that have overlapping extinction peaks with the LAL assay, the chromogenic LAL test is not applicable *(25)*. For such nanomaterials, the kinetic turbidity LAL assay is recommended. However, it is important to note that in the kinetic turbidity assay optical density is read at 660 nm, which can again overlap with extinction peaks of larger aggregates of nanomaterials such as Ag-NPs. Also, depending on the concentrations, the nanomaterial suspension may cause turbidity. In this case, samples should be diluted to minimize the number of aggregates or suspensions should be centrifuged to remove particles, and only the supernatants without any particles should be used. Additionally, minimizing the time duration used to collect baseline optical density, resulting in fewer opportunities for nanomaterials to aggregate, will reduce some of the interference *(24)*.

In any case, more than one LAL format should be used; not all nanomaterials inhibit or enhance LAL activity in a similar manner. Where possible, samples should be centrifuged to remove the nanoparticles and only the supernatant should be used in the testing. In the case of suspected false positive or negative results, samples can be tested for the presence of endotoxin-specific LPS using gas chromatography. It should be noted though that this method only identifies the presence of the LPS in the sample and gives no information on the activity of the endotoxin. If inhibition and enhancement controls yield inconsistent results, an in vivo RPT should be performed. Thus, the choice of the test method will depend on the type of nanomaterial and its various properties. Details of the endotoxin assay for nanomaterials have been reviewed by Dobrovolskaia and McNeil *(26)*.

Recently, a TLR-4 transfected reporter cell line has been used to verify the LAL results *(27)*. The macrophage activation test is another test method recognized as an alternative to the LAL test by the current

pyrogen and endotoxin testing guidance *(28)*. A TNF-α expression test using primary human monocyte-derived macrophages and inhibitor of the endotoxin polymyxin B sulphate is another method for endotoxin assessment that was developed for nanomaterials such as graphene that interfere with the LAL assay. However, these methods are limited to nanomaterial suspensions that do not cause cytotoxicity or nanoformulations that do not contain cytotoxic drug components. Other assays, such as the human PBMC activation assay and the human monocyte activation assay recommended by the European Centre for the Validation of Alternative Methods, may also be applicable to nanomaterials. However, pyrogen-induced inflammatory mediators may not be specific to endotoxins, as nanomaterials themselves may also act as pyrogens. Similarly, The human PBMC/IL-6 in vitro pyrogen test method recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods can be used.

It is important to note that often endotoxin is found adherent to the nanomaterial surface. With the tests mentioned above, it is not known if endotoxin that is adsorbed onto the nanomaterial surface can be detected. It is also not possible to distinguish between the free endotoxin present in the suspension and endotoxin that is adherent to the surface. The lack of sensitivity to detect endotoxin adhered to nanomaterial was addressed by a method that is based on the detection of 3-hydroxy fatty acids (3-OH FAs) using liquid chromatography in combination with mass spectrometry *(29)*. Endotoxins, as mentioned earlier, are LPS consisting of a terminal lipid region (lipid A) and a polysaccharide chain with the O-specific chain (O-antigen) and the core oligosaccharide. The toxic component of LPS is lipid A, which contains ester- or amide-bonded 3-OH FAs. Lipid A on its own is capable of inducing similar toxic effects to LPS in the test systems. Moreover, the chain length of carbon atoms in 3-OH FAs can determine the bacterial species or source of LPS contamination *(30)*. Thus, the method of detection of 3-OH FAs by liquid chromatography in combination with mass spectrometry not only alleviates the issues related to nanomaterial interference with the routine LAL assays, it also enables detection of LPS integrated or adsorbed onto the structure of nanomaterials with greater sensitivity.

While some methods are routinely used and applied, selection of a specific endotoxin testing method should consider the properties

of nanomaterials, as some nanomaterials may interfere with these methods *(15, 23, 25)*.

6.2.4 *Recommendations or suggested modifications for testing endotoxin contamination*

The following recommendations are offered for testing endotoxin contamination:

- inoculate small amount of nanomaterial on agar plate to check for bacterial growth;
- assess for inhibition or enhancement interference by spiking a known amount of endotoxin into LAL-grade water and nanomaterial suspension;
- for highly aggregating nanomaterials and where aggregation is known to interfere with the optical density reading, reduce the time required to collect baseline optical density data and read the sample immediately after the preparation;
- prepare as uniform a suspension as is feasible, as large aggregates of some nanomaterials interfere with the extinction peaks;
- use a series of dilutions to investigate the optical interference; if this does not resolve the issues then the colorimetric assay should not be used;
- use more than one type of LAL assay; if feasible, assess the contamination using all three LAL assay types;
- in the case of suspected false positive results, check for the presence of endotoxin-specific polysaccharides using gas chromatography;
- if the results obtained from two LAL assay types are inconsistent, RPT should be used to confirm the results.

6.3 Preparation of nanomaterials for exposure and characterization

For detailed guidance on how to prepare nanomaterials for exposure and characterization methods, see OECD ENV/JM/ MONO(2012)40 and ENV/JM/MONO(2014)15 *(31, 32)*. The biological behaviour of nanomaterials is linked to their physical and chemical properties, which include their size, shape, solubility, surface chemistry (charge, functionalized groups), and agglomeration

status. These primary material properties change in the exposure medium in which they are dispersed depending on the methods used. These properties determine how nanomaterials interact with cells and tissues, how deep they travel in a tissue, their cellular or tissue localization, toxicokinetics and ultimate fate. The property dynamics make it difficult to assess the dose dependency of toxicological effects of nanomaterials *(33, 34)*.

Nanomaterials might not disperse homogeneously in the suspension medium due to their aggregating behaviour, and their dispersion is influenced by the addition of factors that aid in uniform dispersion. The aggregation and agglomeration of nanomaterials, which is strongly dependent on their colloidal stability, influence the sedimentation rate of the nanomaterials and hence affect the deposited dose *(35)*. Additionally, the biomolecules, proteins and other constituents of the biological medium that get adsorbed to the particle surface affect their uptake by specific cells in the tissue (see section 4.8). This becomes more complicated when cells or animals are exposed to aerosolized nanomaterials, where the deposition of nanomaterials is dependent on the aerosolizing method and the exposure system used *(36–38)*.

Dispersability of nanomaterials will depend on the surface properties, the amount of particles to be dispersed and the mechanical treatment (such as sonication or milling) *(39)*. The choice of vehicle for dispersion of the nanomaterial should be based on the nanomaterial properties and route and method of exposure *(40)*. Ideally, more than one type of dispersant should be tested before settling on one type. It is important to note the influence of the dispersant on the behaviour of particles in the exposure medium as well as on the resulting toxicity *(41)*. Saline is used as a common vehicle to prepare nanomaterial suspension. Water or phosphate-buffered saline should be the vehicles of choice as they may aid in eventual deagglomeration of particles. However, BALF, bovine serum albumin, diluted serum from the test animals, and phosphate-buffered saline supplemented with surfactants have been routinely used as vehicles for dispersing nanomaterials *(42)*. For in vitro studies with lung cells, the coating of pulmonary surfactant has also been highly recommended, since this might influence the interaction with the cells *(43)*. However, it should be noted that the presence of bovine serum albumin in suspension containing carbon-

based nanomaterials may interfere with characterization of the suspension *(40, 44)*. A BALF mimic consisting of phosphate-buffered saline free of calcium and magnesium ions (Ca^{2+} and Mg^{2+}) with serum albumin and the lung surfactant (1,2-dipalmitoyl-sn-glycero-3-phosphocholine, or DPPC) is recommended to circumvent the issues related to retrieval of BALF from animals *(31)*. BALF mimic contains low levels of protein and surfactant found in the lung alveolar lining fluid. Pluronic F68, PEG, Triton, dimethyl sulphoxide (DMSO) and Tween are the other chemical surfactants used to aid dispersion of nanomaterials in suspension. The volume of the vehicle consisting of these various dispersants should be tested for their inherent toxicity and the exposure volume should be adjusted accordingly. Freshly prepared suspensions are the choice. Since nanomaterials can agglomerate very quickly in suspension, periodic monitoring of the dispersion rate is necessary, especially if suspended nanomaterials are used for aerosolization. The pH of the solution and temperature should be routinely checked *(32)*. The exposure concentration should be maintained at a constant level, especially for the inhalation chamber, by checking the stability of the suspension over time. Dynamic depolarized light scattering, dynamic light scattering, ultraviolet-visible spectroscopy, differential centrifugal sedimentation, and modified light microscopy can be used for this purpose *(45, 46)*.

Since the nanomaterial dose will impact the agglomeration and aggregation, independent measurements should be reported for each dose. As some dispersing methods such as sonication or milling may alter the primary particle properties, care must be taken to characterize the particles before and after the dispersion. The report should include information on number of particles, particle concentration, and for aerosol, aerosol characteristics *(32)*.

Thus, a detailed characterization of nanomaterials as produced and in use is essential, and should include characterization in dry as well as in suspended status. A number of techniques applicable to characterizing properties of nanomaterials, both in dry and in liquid suspension, are discussed in the previous chapters and are provided in the OECD guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials *(31, 32)*. The guidance documents also provide a list of techniques that can be used for generation of exposure aerosols.

6.4 Animal species, routes of exposure, and exposure duration and levels

6.4.1 *In vivo: animal species*

Special attention should be given to choosing the species and strain of animals, age of animals, duration and level of exposure, and route of exposure. For nanomaterials, although strain- or species-specific effects are not extensively characterized *(47)*, it may be better to start with the species and strains included or recommended in the routine testing of chemical-induced immune toxicity (OECD TG 407). For inhalation studies, see OECD guidance document number 39 *(48)*. Among the rodents, the murine immune system is well characterized and assays for immune toxicity testing in mice and rats are well established. Thus, mice and rats as the first choice for testing may be supported. On the other hand, for specific immunotoxicity tests such as CARPA, the miniature pig has been reported to be the preferred model *(49)*. For immunological diseases such as allergic asthma, the guinea-pig is considered a reliable animal model *(50)*. Since chemical-induced immune effects are pronounced during the developmental phase, if a nanomaterial is expected to induce immune effects (thymocyte proliferation and differentiation), in utero or neonatal exposure should be considered. For developmental studies, weaning animals are considered a better choice compared to adult animals. In pharmacological studies, guinea-pigs may be a better model as they are suggested to closely mimic human physiology.

6.4.2 *Routes of exposure*

The specific routes by which nanomaterials may enter the human body and potentially elicit adverse immune effects are the lung via inhalation, the gastrointestinal tract via digestion, the skin via application of nanoenabled skin products (sunscreens, moisturizers), and blood vessels via intravenous injection *(33)*. As nanomaterials gain contact with the respiratory tract, the gastrointestinal tract and the skin, these biological compartments are innately designed to act as barriers to the passage of nanosized materials into the organism. The following subsections discuss toxicity testing methods in the context of the specific routes of entry mentioned above.

Inhalation route

Inhalation is the physiological way by which organisms are exposed to respirable substances, and inhalation is thus the "gold standard" with respect to the choice of method for lung exposure to nanomaterials. While lung deposition via pharyngeal aspiration and intratracheal instillation are also considered relevant, the doses deposited via inhalation, instillation and aspiration vary considerably, with significant implications for dosimetry and consideration of the data for risk assessment. As a consequence, quantitative dose–response data derived from inhalation exposure form the basis for human health risk assessment, and data derived from other methods are usually used as weight of evidence in support of the conclusions reached.

The deposition of nanomaterials in the various regions of the respiratory tract after inhalation depends on (a) the physicochemical properties of the inhaled nanomaterials (including size); (b) the breathing frequency and tidal volume; and (c) anatomical features of the airway *(4, 51)*. Once deposited, further interactions of nanomaterials with surrounding milieu (including mucus, proteins, surfactants, and biomolecules) determine the biokinetics of particles over time and their effects. Inhalation of well dispersed particles results in even distribution of particles among the different lobes of the lungs. In the case of instillation, the particles are already suspended before their direct delivery into the lungs, which is anticipated to alter the dosimetry and lung distribution patterns. For example, inhalation exposure to well dispersed CNTs results in deposition of CNTs in the alveolar duct bifurcations and alveolar epithelial surfaces in rodents, whereas bolus delivery of nanomaterials results in central lung deposition *(36)*. Moreover, bolus administration of very high doses (possibly crossing the maximum inhalable dose) can alter the way in which nanomaterials interact with the surrounding milieux, and in some studies the effects of particles introduced via instillation have been found to be much greater compared to those of particles introduced via inhalation *(52, 53)*. However, several studies have reported no such differences in either the distribution patterns or lung responses following exposure via the two methods *(54)*. For respirable nanomaterials, aerosol characterization is important to ensure the exposure dose.

Oral route

Regarding the oral route, exposure via oral gavage or through feed is preferred over exposure via drinking water. For guidance on exposure via the oral route, see OECD TG 407 and TG 408. Some nanomaterials tend to precipitate in water, and thus it is difficult to control the delivered dose. A thorough characterization of nanomaterials at various stages before the exposure, including in their dry form and in the exposure vehicle, is essential. If nanomaterials are fed through the diet, characterization of nanomaterial transformation in the food matrix is necessary for accurate interpretation of the results. In addition, nanomaterials are likely to go through a number of transformations as they travel from the acidic environment of the stomach to the alkaline environment in the intestines, although the information available on the presumed transformation is limited *(55, 56)*.

Dermal route

Exposure via the dermal route is generally conducted under occlusion to prevent oral exposure via grooming. Nanomaterials need to be dispersed in an appropriate vehicle that does not induce a dermal response itself, and at the same time allows for maximum uptake of the nanomaterials through the skin. The majority of nanoparticles will remain in the stratum corneum, and so will not become systemically available. If any uptake through the skin occurs, it may take significantly longer than it would for soluble chemicals. Thus, before executing an in vivo dermal study, it may be important to conduct in vitro skin uptake studies with reconstituted skin, using sufficiently long exposure and evaluation times to determine whether systemic uptake can be expected and identify the post-exposure time at which this might occur *(57)*.

Compared to raw nanomaterials, characterization of nanomaterials contained in the skin care products or cosmetics used in dermal absorption studies is challenging. Thus, a separate characterization approach may be needed that includes methods to isolate, purify and concentrate nanomaterials before their characterization in cosmetic products. Some of the properties that are known to influence dermal penetration of nanomaterials include

particle size, chemical composition and surface chemistry. Adequate characterization of nanomaterials for these properties before exposure in dry state and in formulation should be conducted *(57)*.

It is suggested that nanomaterials in their dry state cannot penetrate the skin. Therefore, skin penetration studies for nanomaterials have to be conducted in their suspended forms. Adequate characterization of the solvent or formulation in which the nanomaterials are suspended is critical to the interpretation of the results. Use of standard compounds (positive and negative controls) is essential for validation of the results and should be routinely incorporated in the study design.

6.4.3   Duration and levels of exposure

For chemicals, a 28-day repeated exposure regimen is recommended prior to the assessment of immune parameters (OECD TG 407). However, the effects leading to immune dysfunction following exposure to nanomaterials may take a longer time than is known for chemicals. An understanding of the toxicokinetics of the nanomaterials being investigated prior to the immune toxicity assessment may thus be important.

In general, higher toxicity is observed at higher doses; however, nanomaterials do not show a dose-dependent transition in the toxicity observed *(58)*. This means that the toxicity induced at a lower dose may be different from the toxicity observed at a higher dose. This is mainly due to the fact that nanomaterial aggregate (a state of dispersion where nanomaterials are loosely clumped together through non-covalent interactions) in suspension acts in a dose-dependent manner. Aggregation of nanomaterials interferes with their uptake and distribution, and with their ability to interact with the surrounding microenvironment, influencing their toxicity potential. The relative state of dispersion influences the type of immune or pathological responses to nanomaterials. Thus, for the selection of doses, traditional methods of choosing doses such as LD50 (lethal dose 50%) may not be applicable (considering the fact that nanomaterials of similar composition may exhibit different LD50) *(34, 58)*. As a rule, a minimum of three doses in addition to controls is necessary. Where possible, the selection should be based on a pilot experiment.

6.4.4　Multiple exposure versus single exposure

In reality, exposure to low doses of respirable particles over a long period of time is the norm. However, most of the inhalation studies are limited to single exposure of a large dose of nanomaterial in a short period of time. This is especially true for intratracheal or pharyngeal aspiration methods, where a bolus amount of material is directly deposited in the lungs. Although it is recommended that the high bolus doses delivered by the intratracheal instillation or pharyngeal aspiration methods is the same as that deposited per unit alveolar surface area in humans exposed to occupational exposure levels over a 40-year working life, these studies do not take into account the rate at which these doses are delivered and cleared *(36)*. The inhalation method of deposition offers an opportunity to deliver doses at a low rate. Although this is an issue with intratracheal instillation and pharyngeal aspiration, the large bolus doses can be split into multiple smaller doses and administered over weeks. However, those techniques are invasive and require anaesthesia, which may predispose animals to accidental injury, and repeated anaesthesia can add to stress over time.

6.5　In vitro cell types, exposure methods, duration and levels

Special care should be taken when choosing the cell types and the species that they are derived from, passage numbers, duration, and level of exposure, and the way in which the cells are exposed in vitro should be noted. Guidelines for good cell culture practice are required and should be properly applied and documented, including the assessment of materials used in experiments (for example, cultured cells, culture medium, and culture substratum) *(59)*. Similar to chemicals, in vitro responses to nanomaterials will heavily depend on all of these factors *(60)*. While the cell types that are routinely used for in vitro testing of chemicals can be used, their sensitivity varies with each nanomaterial variant. Thus, the choice of cell types should also consider the possible exposure route, the type of nanomaterial being studied and the physical-chemical properties, along with the consideration of the types of effects being investigated.

Immortalized or primary cells extracted from tissues or organs are exposed under submerged conditions directly to nanomaterials

suspended in the appropriate medium (usually the serum-supplemented medium in which cells are grown). Depending on cell type, a specific medium is used to culture cells in vitro. For in vivo target tissues such as the lung or skin, exposure under submerged conditions is not realistic, since in vivo exposure of these tissues occurs at the air–liquid interface. While the exposure in submerged conditions is ideal for many chemicals that are soluble, for insoluble materials such as nanomaterials estimation of the exact dose of exposure becomes difficult as they aggregate in liquid suspension, and much of what is suspended may remain in suspension and never make contact with the cellular surface. Thus, more emphasis has been placed recently on characterizing the exposure to understand exactly what is experienced by cells in a Petri dish. It is suggested that generation of stable suspensions, characterization of agglomerates in suspension (size, density), and the modes of particle transport during exposure (which may be influenced by the agglomerate size, contents of the suspension medium and the exposure system used) must be included for an effective interpretation of dosimetry *(61)*. The movement of nanomaterials in liquids is mainly driven via diffusion (random motion). This, however, depends on size, material density, and possible agglomeration in the suspension media *(61)*. The characterization of nanomaterials in physiological fluids and the assessment of their colloidal behaviour are challenging due to the complex physical and chemical forces involved, the highly complex and different types of physiological fluids, the variety and complexity of analytical methods, and the various theories upon which these methods are based. The ultimate fate of nanomaterials in a fluid is then dictated by its mass density, that is, nanomaterials will settle if their mass density is greater than that of the fluid *(62)*. There is a steady increase in the number of research papers adopting the concept of particokinetics proposed by Teeguarden et al. *(63)* for interpreting dose–response curves *(64)* or the distorted grid model to estimate the deposited nanomaterial dose based on measuring the effective density of nano-agglomerates in suspension *(61, 65)*.

It is also routine practice to conduct in vitro testing using a single cell type (mono cell culture), which does not represent the complexity of a tissue consisting of multiple cell types. Alternative methods, such as use of more than one cell type (co-culture), ex vivo tissue slice cultures *(66)*, or co-cultures grown at the air–liquid interface,

are emerging *(67, 68)*. However, these methods are not completely validated even for assessing chemical-induced effects. Internationally harmonized protocols or guidance is lacking with respect to the emerging techniques. Some of the cell types and methods of exposure that are currently being used or tested for nanomaterials are discussed in the following sections.

6.6   Routinely used cell types of the respiratory system

A detailed description of individual cell types relevant to the respiratory system is provided in subsection 4.10.2. Here, specific cell types that are routinely used for assessing in vitro immunotoxicity of nanomaterials are listed. These include in vitro cell culture models of cells of the nasal, bronchial and alveolar regions. However, adequate characterization and validation of the models is necessary before their routine integration into immunological testing of nanomaterials.

6.6.1   *Cells of the innate immune system*

Epithelial cells

Although primary cultures of tracheal and bronchial epithelial cells or human primary small airway cells are technically feasible *(69, 70)*, a number of studies have been conducted using immortalized cell lines. The human airway epithelial cell lines Calu-3 (human origin), 16HBE14o- (a simian virus 40 (SV40) large T antigen-transformed, bronchial epithelial cell line derived from normal human airway epithelia), and BEAS-2B (normal human epithelial cells immortalized using the adenovirus 12-SV40 hybrid virus) are the other cell types regularly used to assess cellular interactions with nanomaterials and to investigate the potential of nanomaterial-induced respiratory toxicity in vivo *(69–75)*. The commercially available cell line A549, which originates from human lung carcinoma *(76)*, is one of the most well characterized and most widely studied in vitro models *(77, 78)*. It has been shown that the A549 cells have many important biological properties of alveolar epithelial type II cells. The A549 cells have already been used to assess both acute and long-term effects of exposure to ambient particles as well as occupational exposure to ENMs. A few studies have indicated that the A549 cell is more

representative of alveolar type I cells, which are the main targets of inhaled nanoparticles, accounting for 95% of the alveolar epithelial surface *(79, 80)*.

Phagocytes (neutrophils and macrophages)

Alveolar macrophages and neutrophils are the two most commonly used cell types for assessing immune responses of the lung. Monocytes can be isolated from human buffy coat and differentiated into monocyte-derived macrophages with the addition of monocyte colony-stimulating factor (M-CSF) *(81)*. This primary cell type can be produced in a very reproducible manner and represents a reliable in vitro system. J774.1A (mouse macrophages derived from ascites), THP-1 (human peripheral blood, derived from monocytic leukaemia patients), and RAW 264.7 (mouse macrophages, derived from ascites) are the frequently used phagocytic cell types for assessing nanomaterial-induced toxicity.

Other innate immune cells

Other innate immune cells are not directly linked to immunity against xenobiotics but still have an important role in lung homeostasis. Natural killer (NK) cells are innate lymphocytes that are critical to the defence against infections in the early phase. Upon ingestion of particles, phagocytes may activate NK cells in order to display effector functions *(82)*. NK cells kill infected cells by releasing granules containing perforin and granzymes by a mechanism similar to that in CD8+ T cells *(83)* and by producing IFN-γ, which induces macrophages to kill phagocytosed particles *(84)*. Mast cells, basophils and eosinophils have little role in particle uptake, but they play a central role as effector cells in allergies. In response to environmental allergens including nanomaterials, these cells produce Th2 cytokines, such as IL-4, IL-5 and IL-13 *(85, 86)*. Innate lymphoid cells are a newly described set of antigen-non-specific, non-T and non-B lymphocytes with conserved effector cell functions. These cells secrete a variety of cytokines, such as IFN-γ, IL-5, IL-9, IL-13, IL-17 and IL-22, and are involved in mucosal tissue homeostasis. Innate lymphoid cells may be involved in atopic diseases by interacting with other immune cells such as mast cells *(87)*.

6.6.2 **Specific cell types of the respiratory system: cells of the adaptive immune system**

Dendritic cells

Dendritic cells (DCs) are the most potent antigen-presenting cells in the respiratory tract. They are specialized in capturing, processing and presenting antigen. DCs also play an important role in bridging the innate and adaptive immune responses during infections *(88)*. It has been shown in several studies that there are different subtypes of DCs in the lungs and that their proportions and immune responsiveness depends on the respiratory compartment considered *(89, 90)*. It is common practice to isolate monocytes from human buffy coat and to differentiate them to monocyte-derived dendritic cells with the addition of growth factors such as granulocyte-monocyte colony-stimulating factor and IL-4 *(91)*. However, immortalized cell lines such as the histiocytic lymphoma cell type U-937, which exhibits monocytic characteristics *(92)*, or MUTZ-3 *(93)*, have also been used. Since distinct differences in the immunophenotypic characteristics are observed between primary DCs and the immortalized cell lines, these factors should be considered in interpreting the results.

T cells

The two major subsets of T lymphocytes are CD4+ helper T cells and CD8+ cytotoxic T cells, both expressing the αβ T cell antigen receptor. Another population of T cells, named the γδ T cells, expresses a distinct type of antigen receptor with more limited diversity. In the respiratory tract, antigen-specific T cell responses are initiated by DCs following sampling of airway antigen and presentation of antigen-derived peptides to naïve T cells in peribronchial and mediastinal lymph nodes. Both in vitro and in vivo studies have shown that T cell proliferation upon nanoparticle treatment is affected *(94–97)*. T cells can be generated from human buffy coat from the CD14 negative fraction. The CD14 negative fraction is further purified and the CD4+ fraction is then used to generate human T cells in vitro *(94)*. The most common way to measure T cell activation and proliferation in vitro is via radioactive labelled compounds (thymidine), as described in Blank et al. *(94)*.

B cells

Most mature B cells belong to the follicular B cell subset and are IgD+ IgM+. They have the ability to recirculate in lymphoid organs and reside in specialized niches known as B cell follicles, where they might encounter the antigen that they are specific to *(98)*. Similar to T cells, B cells can also be isolated from human buffy coat in vitro. Human B cells are characterized by CD19, CD20 surface marker expression.

Fibroblasts

Excessive activation and proliferation of fibroblasts can lead to excessive deposition of extracellular matrix components, including collagen, and lead to a fibrosis-like condition. At present it is not known if nanomaterials interact with fibroblasts directly to activate them or indirectly via inducing secretion of the extracellular matrix components. Primary mouse or human fibroblasts or cell lines such as MRC-5 (human lung fibroblasts) can be used to assess the proliferative activity upon exposure to nanomaterials *(99, 100)*.

6.6.3 Specific cell types of the respiratory system: co-culture systems

The lung responses to invading particles involve the interplay of several organ systems and multiple cell types. For efficient modelling of the more complex nature of the underlying physiology contributing to the response, it is necessary to develop more complex culture systems that model the interactions between different lung cell types. Co-culture models contain either primary or immortalized cell lines of epithelial origin cultured along with either primary or immortalized cells of other origin, including endothelial cells, fibroblasts and macrophages. A third cell type, usually of immune origin, is then added on top, mimicking the phagocytic environment of the lung. Though the feasibility of culturing multiple cell types permits investigation of cell–cell communication, an important feature of lung disease responses to nanomaterials, the model is technically challenging and requires specialized expertise. The in vitro co-cultures that are routinely employed include epithelial (A549) cells and endothelial cells to mimic the alveolar epithelial barrier *(101, 102)*. Recently, a

primary co-culture system to simulate the human alveolar–capillary barrier was constructed using primary cells of human pulmonary microvascular endothelial cells and primary human type II alveolar epithelial cells to study the impact of nanocarriers *(101)*. A triple cell culture in vitro model of the human airway wall was constructed to study the cellular interplay and intricate cellular responses of epithelial cells, human blood monocyte-derived macrophages and DCs exposed to nanomaterials *(103)*. In this model, monolayers of two different epithelial cell lines (A549 and 16HBE14o- epithelia) as well as primary epithelial type I cells were grown on a microporous membrane in a two-chamber system *(103)*. In addition, a quadruple culture containing epithelial, endothelial, macrophage and mast cells has been established *(104, 105)*, as well as co-cultures of epithelial cells with NK cells *(106)*, or epithelial cells, macrophages and fibroblasts *(107)*.

Though these models offer promising alternatives to exhaustive in vivo testing, experiments comparing the results from the two (in vivo versus in vitro) models are very scarce. Moreover, some studies suggest that the cellular responses following exposure to nanomaterials observed in the co-culture models are different from those observed following exposure of single cell types *(108, 109)*, and that the results obtained from the single cell type exposure are more reflective of the in vivo responses than the responses observed following stimulation of co-culture systems. Regardless, the time- and cost-effective in vitro systems can best be used to screen the potentially hazardous nanomaterials from the inert ones, and the results derived can be used as weight of evidence. It is important to note that by merely culturing two, three or four different types in a Petri dish, the responses occurring in humans cannot be mimicked. Thus, the architecture of the in vitro cell co-culture model in regard to the specific organ they represent is essential when nanomaterial effects are studied.

With regard to air–liquid cultures, the growth of epithelial cells on permeable supports enables the culture medium to be separated on either side of the cultured epithelium, leading to an increased differentiation of the cultured cells *(110)*. Furthermore, the medium can be removed from the upper side to expose the cells to air on one side, allowing the cells to "feed" from the medium in the chamber

underneath *(111)*. The air–liquid culture technique has been described in different cell culture models *(112–115)*. Air-exposed cell cultures allow studying the interaction of inhaled nanomaterials with cells in an environment that more closely mimics the in vivo situation. Of particular importance is that the cells are covered by a very thin liquid lining layer with a molecular surfactant film at the air–liquid interface, since surfactant plays an important role in particle displacement and retention *(116)*. It is demonstrated that A549 cells *(117)*, the bronchial epithelial cell line 16HBE14o- and Calu-3 cells can be exposed to air *(112)*. In these studies the air-exposed cultures exhibited a clear epithelial morphology and integrity, as in in situ conditions. Such in vitro cell systems combined with various air–liquid exposure systems that allow a dosimetrically accurate delivery of aerosolized nanomaterial offer a reliable method for the investigation of nanomaterial–cell interactions and possible cellular responses *(67)*.

6.6.4 *Ex vivo precision-cut lung slice method to investigate nanomaterial-induced tissue responses*

As outlined above, the establishment of in vitro systems that can accurately simulate the responses of an intact organ following exposure to toxicants continues to be a challenge. While monolayer culture or co-culture models have advanced our understanding of the cellular effects induced by nanomaterials, they lack the sophistication of the intact organ system. The lung is a multicellular organ and it is unlikely that the use of few cell types alone can accurately reflect the coordinated responses of several different cell types in the lungs following chemical insult. The precision-cut tissue slice is an organ mimic and the slices represent the organs from which they are prepared. In contrast to single cell type in vitro cell cultures or co-culture of select cell types, the method maintains the original tissue architecture with relevant structural and functional features, and cell–cell interactions. Precision-cut tissue slices can be prepared from different species. The ready application of this method to human tissues makes it specifically interesting and provides a link between animal-derived data and extrapolation to human relevance. This technique is being used to investigate the lung responses to nanomaterials *(118, 119)*. Similar to the above in vitro techniques, this method can be used for the purposes of screening potentially harmful nanomaterials.

6.7 Pre-testing considerations and methods applicable to inhalation route (respiratory system)

6.7.1 *In vivo pre-testing considerations*

Inhalation route of exposure applies to testing of immunostimulatory or immunosuppressive effects of nanomaterials on the respiratory tract. Inhalation is the primary route of exposure to the majority of nanomaterials and has been extensively characterized. The entire respiratory tract should be investigated, including nose, larynx, trachea, lymph nodes, lung lobes, regional lymph nodes and the pleura for different end-points. Post-exposure time points of 24 hours and 28 days are recommended for sampling, and standard OECD guidelines should be followed *(31, 32)*.

Mode of lung deposition

Experimental approaches for assessing hazards posed by airborne particles use several methods to deposit particles in the respiratory tract: whole-body or nose-only inhalation, intratracheal instillation or pharyngeal aspiration. While the inhalation method delivers the intended dose slowly over a period of time spanning hours, weeks or months, instillation or pharyngeal aspiration methods deliver the dose at a very high rate within half a second. This difference in time taken to deliver the dose alters the rate at which the dose is deposited, the total deposited dose and regional distribution, all of which influence the final outcome of the exposure *(4, 36)*. Where possible, it is recommended that information on lung deposition and distribution in the body be obtained and related to the observed toxicity. For example, a part of the tissue exposed to nanomaterial can be set aside for ICP-MS analysis for elemental detection. This enables effective evaluation of the dose–response, taking into account how much of the exposed material actually reaches the alveoli and other organs. However, there are limitations; ICP-MS is only suitable for materials for which the elements do not have a high background in the body. In addition, it is important to be aware that ICP-MS only analyses elements and not particles. New techniques, such as single particle ICP-MS, are being evaluated for their suitability to determine the delivered and retained dose.

Nanotubes have been shown to cause a spectrum of pulmonary effects following lung deposition. However, studies have shown

that these effects vary depending on the mode of exposure *(120)*. For example, pulmonary inflammation, lung tissue damage and granulomas are observed in rodents exposed to CNTs administered via intratracheal administration. A direct comparison of lung effects following exposure to the same dose and type of CNTs via intratracheal instillation or inhalation showed no lung lesions in mice exposed via inhalation *(121, 122)*. It is suggested that inhalation of uniformly dispersed CNTs causes systemic immune responses such as immune suppression observed in the spleen *(123)*.

Inhalation

Whole-body and nose-only exposure are the two ways by which animals are exposed to respirable substances. The former requires housing of animals individually, allowing free movement throughout the procedure. In the latter case, animals are restrained in a narrow tube with their noses protruding into the plenum, where they are exposed to aerosols. However, it should be noted that whole-body exposure will lead to exposure via other routes such as the gastrointestinal tract. On the other hand, nose-only exposure may lead to stress due to restraint of the animals, which may be particularly important for immune toxicity testing as immune responses are altered during stress. Increased stress levels and loss of body weight or slower gain in body weight are observed in long-term nose-only exposure to other substances *(124–128)*, but could be reduced by sufficient training of the animals prior to exposure to the substance, or selecting a different strain. Since the issues related to delivery of nanomaterials via either of the methods are common to chemicals as well, general recommendations outlined in OECD TG 403 and guidance document 39 should be followed.

Particle size determination for all aerosols is essential. It is important to be aware that it is not possible to produce aerosols consisting of single nanoparticles. Nanoparticles will agglomerate and hence the average size in the aerosol will be much larger. For chemicals, a mass median aerodynamic diameter (MMAD) in the range 1–4 µm with a geometric standard deviation of 1.5 to 3.0 is recommended. However, for nanomaterials, aerosol dispersion should be as small as possible. The aerosol preparation for nanomaterials should be < 3 µm MMAD. Use of a venturi for aerosolization of

powder and further deagglomeration of particles in the aerosol with a jet mill are recommended. Use of an electrospray generator or spark generator, atomizing a sufficiently diluted dispersion, and evaporation and subsequent condensation of metal nanoparticles are some other methods that can help keep the aggregation and agglomeration of nanomaterials to a minimum in the aerosol. Because the formation of aggregates and agglomerates directly correlates with particle resident time and the number of particles in air (per cubic centimetre), aerosol should be produced as close to the breathing zone of the animal as possible. Aerodynamic particle sizes in the range 0.3–3 µm are attainable. OECD guidance *(31)* on sample preparation and dosimetry for the safety testing of manufactured nanomaterials provides a list of techniques that can be used for generation of exposure aerosols. The exposure dose should consist of maximum tolerated dose on the high end and human-relevant doses on the low end of the dose spectrum. The maximum tolerated dose for nanomaterials may depend on the maximum dispersability attainable. Reporting should be based on the count median aerodynamic diameter, as mass-based dose metrics may not be suitable for nanomaterials. The scanning mobility particle sizer and electrical low-pressure impactor, which enable single particle counting, are suitable for the analysis (according to OECD guidelines for inhalation exposures).

In addition to particle counts and particle size, particle morphology in the aerosol should also be investigated. The particle collection equipment should enable collection and classification of the entire range of particle sizes present in the inhalation chamber. The actual concentration of nanomaterial in the breathing zones of the inhalation system has to be measured and reported. When mechanical processes are used to obtain a certain particle dispersion, the aerosol should be characterized to ensure that the particle has not been transformed during the process. Dry powders are preferred for aerosolization. In cases where nanomaterials are suspended in liquids prior to aerosolization, further characterization of particles in liquid suspension and aerosol is necessary to ensure that the primary particle properties are not altered. If a vehicle other than water is used for suspension, gas chromatography techniques should be used to determine the concentration of the vehicle in the atmosphere of the chamber. Detailed guidance on exposure chamber conditions is provided in the inhalation toxicity guidelines, including ISO10801

(2010), *Nanotechnologies: generation of metal nanoparticles for inhalation toxicity testing using the evaporation/condensation method (129)*. The exposure atmosphere should be maintained at a constant level to the extent possible, following the guidelines described in the OECD inhalation toxicity tests. Dry powders represent a physiological scenario of exposure to these materials in the environment or in the occupational setting, and thus the data derived from such studies are relevant to risk assessment. The results from studies using aerosols generated from the suspended nanomaterials can inform hazard-driven approaches.

Intratracheal instillation

While inhalation is the gold standard and constitutes a physiological route of delivery of nanomaterials to the respiratory system, because of the high aggregating or agglomerating behaviour of nanomaterials, uniform aerosol preparation is very difficult, and it is not easy to characterize the aerosol in the exposure chamber and estimate the deposited dose. In addition, the special expertise required to build and operate the inhalation system, the amount of test material required to generate sufficient concentrations of aerosols in the exposure chamber over a long span of time, and the highly toxic nature of the test material preclude the routine use of this method for lung exposure *(52)*.

Intratracheal instillation is used as an alternative to the cumbersome inhalation procedure. The method bypasses the upper respiratory system and administers a bolus amount of nanomaterials directly into the tracheobronchial and alveolar regions of the respiratory tract of animals under anaesthesia. Compared to inhalation, the dose delivered or deposited by this method is more uniform, precise and reproducible. While it has been argued that lung responses to nanomaterials administered via inhalation or instillation may be different, several studies have shown that lung responses to particles deposited via both methods are consistent for identical nanomaterials. Thus, results obtained by these techniques can be used to rank the pulmonary hazards of nanomaterials and allow qualitative risk assessment *(36)*. These results can also be used for proof of principle studies to generate hypotheses and to provide weight of evidence to support inhalation study results.

Pharyngeal or laryngeal aspiration

This involves deposition of suspended nanomaterials on the back of the tongue and pulling of the tongue, which results in gasping as part of the reflex mechanism leading to aspiration of deposited nanomaterials. In comparison to instillation, pharyngeal aspiration is supposed to aid in uniform distribution of nanomaterials in the lungs. However, comparisons of lung responses following exposure via instillation or aspiration are consistent.

6.7.2 In vivo testing methods applicable to inhalation route (respiratory system)

Body weights

Body weights should be monitored weekly; if they remain unchanged in the first week, bi-weekly monitoring should be carried out. If changes in the body weights are observed, food consumption should be monitored. Specific to inhalation exposures, loss of body weight or inability to gain body weight has been observed in some cases where the nose-only technique was used. The animals' weight has been linked to the immunological effects of nanomaterials *(130)*. The recommendations made by OECD TG 412 are applicable for nanomaterial testing; the individual animal weight is recorded on day 0 and at least once weekly thereafter for the duration of the study and before the necropsy.

Clinical pathology

For most of the respirable nanomaterials thus far investigated, the results of the standard clinical pathology parameters (OECD TG 412) have not revealed significant abnormalities. Thus, it needs to be discussed whether clinical pathology testing should be routine for all nanomaterials or only for those that show positive effects on the respiratory system.

Bronchoalveolar lavage fluid

OECD guidance document 39 provides details on the bronchoalveolar lavage (BAL) test *(48)*. The airway epithelium is the target surface for any inhaled substances, including nanomaterials. The

fluid lining the epithelium holds a wealth of information concerning the toxic potential of inhaled substances, and thus is routinely lavaged with isotonic buffer to examine its contents. Several biochemical and cytological constituents (including types of infiltrating cells, enzymatic activity, cytokines and chemokines) as markers of lung immune responses are quantified by examining the bronchoalveolar lavage fluid (BALF). Thus, BALF assessment can aid in developing dose–response models of a substance, ranking a substance's potency, and setting up a no observed effect level of exposure for regulatory purposes. Since all nanomaterials, due to their small size, are expected and in many cases demonstrated to be translocated to alveoli in the lower respiratory tract upon inhalation, BALF assessment is recommended as a mandatory test for nanomaterials (discussed in OECD guidance document 39 and in OECD inhalation test guidelines for nanomaterials). Temporal changes in BALF constituency can be prognostic of initiation, progression or severity of lung immune disease, and can potentially be indicative of the mechanisms involved *(131)*.

BAL total cell counts and differential cell counts

In normal situations, macrophages constitute 90% of the total cell population of BALF. Following exposure to lung toxicants, depending on the potency of a substance, influx of neutrophils, lymphocytes and eosinophils is observed, all of which imply different degrees of lung inflammation *(131)*. Thus, measurement of BAL differential cell counts provides information concerning the extent of pulmonary inflammation and possibly the type of inflammation induced by nanomaterials. Ideally, a separate set of animals with whole-lung lavage is recommended for BAL assessment. BAL total and differential cell counts are scored using an ordinary light microscope. Macrophages, polymorphic nuclear granulocytes, lymphocytes and other cell types, such as eosinophils and epithelial cells, are scored. The detailed methodology is provided in OECD guidance document 39.

BAL cytotoxicity assay

Traditionally, release of lactate dehydrogenase (LDH) into the surrounding medium is measured as an indication of loss of plasma membrane integrity and consequent cellular toxicity. The amount of cellular damage is directly proportional to the amount of LDH release.

This assay has been employed to investigate nanomaterial-induced cytotoxicity *(132)*. However, several nanomaterials, including TiO_2, silver, silica, Fe_2O_3, CeO_2, cadmium selenide, ZnO, quantum dots, polystyrene, copper, SWCNTs, carbon black, C60 and gold, are shown to either increase the absorbance, generating false positive results, or to quench the fluorescence, inhibit the LDH enzyme activity, or increase the enzyme adsorption, thereby generating false negative results *(133)*. Thus, the assay must be used with appropriate controls: the incubation of nanomaterials with the different components of the assay in the absence of cells, or the cells alone in the absence of any nanomaterials. Addition of bovine serum albumin to the nanomaterial preparation has been suggested to minimize enzyme adsorption. Interference with the assay components can differ based on the nanomaterial doses used; a series of nanomaterial doses have to be tested with assay components without the cells to eliminate the doses at which the interference occurs. For nanomaterials such as CNTs, carbon black and quantum dots the interference comes from their colour, which may be unavoidable. In such cases, multiple assays that use different detection methods are useful *(134)*.

Several studies have shown that the positive cytotoxicity results for BAL cells are transient, and thus the results must accompany histopathological assessment of lung sections *(135, 136)*. The measurement of total protein in BAL fluid (supernatant without cells), increases of which are a good indicator of the permeability of the lung alveolar–capillary barrier, can also be used to support the LDH cytotoxicity assay *(137)*. However, there is no consensus on whether this should be a routine test (OECD TG for nanomaterials). Several protein quantification methods are available and the choice of a specific type may depend on the material properties.

The other recommended measurements for determining the cytotoxicity in BAL include alkaline phosphatase (indicator of secretions from type II cells); gamma-glutamyltransferase (a membrane-bound surface enzyme that plays a role in the metabolism of xenobiotics and leukotrienes, and which is lost during lung injury); and N-acetyl-β-D-glucosaminidase (a lysosomal enzyme, secreted by activated macrophages that have phagocytosed particles, increased levels of which indicate activation or lysis of phagocytes and thus lung injury) *(135, 136)*.

BAL cytokine and chemokine profile

In addition to cells and enzymes, the activated epithelium and macrophages secrete additional factors such as growth factors, chemokines and cytokines. Profiling of these factors can provide information concerning the sources of infiltrating cells and the underlying mechanisms of lung responses. Unique cytokine and chemokine patterns are associated with specific disease types and are helpful in discriminating the inflammatory, immunosuppressive and allergic responses to nanomaterials *(138)*. Multiplex enzyme-linked immunosorbent assay (ELISA), single chemokine- or cytokine-specific ELISA, single or multiplex reverse transcription polymerase chain reaction (RT-PCR) and western blot are the routinely used methods for assessing cytokines and chemokines *(137)*. Care must be taken during the preparation of protein or RNA preparation. Particles can interfere with the electrophoresis (western blot) and absorbance reading.

In conclusion, the BAL analysis following inhalation exposure to nanomaterials has recently become a mandatory assessment in OECD TG 412 and TG 413, and the existing methods for chemicals are readily applicable to nanomaterials, conditional on inclusion of appropriate controls.

Lung tissue cytokine and chemokine profiles

A number of nanomaterials have been shown to induce changes in expression of genes and proteins associated with inflammatory process in the lung tissue. These include several cytokines, chemokines and growth factors *(10, 136, 139, 140)*. The changes in the tissue expression of these molecules are directly correlated with the extent of lung inflammation observed. Moreover, studies have suggested that the magnitude of lung inflammation following exposure via the inhalation route is a good indication of the likelihood of occurrence of systemic effects *(11, 141)*. In some instances, elevated levels of cytokines and chemokines are observed in the absence of or receding infiltrating inflammatory cells, suggesting that tissue profiling for changes in inflammation-associated gene or protein expression may be a sensitive measure to monitor the long-term immune effects of nanomaterials *(10)*. Targeted single gene and protein, customized

low-throughput assays such as pathway-specific polymerase chain reaction (PCR) arrays and multiplex ELISA, or higher-throughput global analysis of gene expression *(13, 136, 139, 140)* and protein expression profiles using microarray or proteomic methods, are also applied to nanomaterials *(142)*. The high-throughput techniques have an advantage of revealing the unexpected or previously unknown immune effects of nanomaterials and the underlying mechanisms of such effects.

Cellular damage

Activated phagocytic cells release ROS upon internalization of pathogens or particles, resulting in a respiratory burst that can be measured in macrophages, monocytes and neutrophils derived from BALF cells *(143)*. A tiered approach for assessing oxidative stress is recommended *(143)*, including acellular assays such as electron spin resonance in the first tier *(144)*, followed by in vitro assays, including fluorimetric assay, which relies on the intracellular oxidation of 5- and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy H2DCFDA) *(145, 146)*. However, it is important to note that there are limitations to this method because of interference of nanomaterials with the assay. Protein carbonylation, which can be assessed by ELISA, is a high-throughput technique that can also be applied to screen a number of ENMs for their oxidative potential *(147)*, which helps identify the underlying mechanisms of oxidative stress. In the third tier, "omics" techniques involving mass spectrometry, which are both cost and time intensive, can be included to obtain a global view of the altered expression of proteins and the affected pathways. Comprehensive methods, such as in vitro and in vivo lipid peroxidation, protein oxidation, and protein carbonylation using proteomic techniques *(143, 148)*, have been used to assess nanomaterial-induced oxidative stress. Intracellular glutathione levels can be measured using the ThiolTracker™ Violet assay *(145, 149, 150)* and an assay that measures glutathionylation of proteins. Oxidative stress can also be measured by assessing the antioxidant pathways, involving measurement of relevant genes and proteins *(143)*. Other biomolecule modifications, such as nitrosylation, reflective of oxidative stress, can be assessed by measuring nitrosylated tissue proteins, or an increase in nitric oxide production and the nitrate–nitrite ratio in BAL. In addition to tissue analysis,

acellular glutathione levels, antioxidants and nitric oxide production in BAL supernatant can be used to assess ROS synthesis.

Cellular proliferation

Labelled nucleotide precursors, such as tritiated thymidine and 5-bromo-2'-deoxyuridine (BrdU), or via immunohistochemistry of lung tissue by staining for proliferating cell nuclear antigen, can be used to assess increases in cell division of epithelial cells, an indication of excessive cell growth or hyperplasia *(151)*. The methodology is the same as for chemicals, which includes pulsing of animals in the experimental groups with BrdU or tritiated thymidine. For immunohistochemistry, lung sections are reacted with antibodies against proliferating cell nuclear antigen. Other cellular proliferation indicators, such as Ki67, have also been used to assess proliferation of cells in the lungs of mice exposed to MWCNTs *(136)*.

Pleural lavage

Although not compulsory, for high aspect ratio nanomaterials such as fibres, tubes and plates, a pleural lavage should be conducted, as recommended by OECD guidance *(31)* and test guidelines on inhalation *(152, 153)*.

Tissue histopathology

Cellular influx, and consequent lung inflammation and immune responses induced by nanomaterials, are often transient, and depend on the exposure dose and inherent properties of nanomaterials. Thus, in addition to the detailed assessment of BAL fluid, a thorough histopathology should be conducted to determine the site of nanomaterial deposition, the type of inflammatory lesions and, more importantly, the sustainability of the effects. Histopathology should be conducted on a separate set of animals that are not used for BAL collection. A whole-lung assessment is recommended for biodistribution assessment, and a formalin-fixed, haematoxylin and eosin-stained left lung lobe is used for the histopathological analyses *(31)*. In addition to the morphological and pathological assessment, immunohistochemistry and special stains can be used to identify specific cell types, cellular apoptosis, alveolar thickening

and disease phenotypes (collagen deposition in fibrosis) *(136, 137)*. In the repeat dose study, histopathology of the parietal pleura and subpleural proliferation of lung tissue should be included for fibrelike nanomaterials.

It is important to note that particles when stained with haematoxylin and eosin appear darker under the microscope. Some particles may interfere with the fluorescence of antibodies, quenching or increasing the fluorescence. These factors must be kept in mind and appropriate controls (suggested in the BAL cytotoxicity subsection above) must be included in the study to avoid misinterpretation of the results.

Particles deposited in the lung are translocated to the regional and systemic lymph nodes and extrapulmonary immune-responsive tissues. Thus, a detailed analysis of particle deposition in the spleen, thymus, local lung lymph nodes and liver must be conducted, and this should be mandatory. Novel microscopic techniques, such as enhanced darkfield and hyperspectral imaging techniques *(3, 136, 137, 145)*, are highly sensitive and enable detection of very low levels of particles lodged in the tissue *(3, 154)*. This technique does not require any preprocessing of samples and can be applied to fresh, frozen, stained and unstained particles. Moreover, the technique does not require prelabelling of cells or particles with florescence or radiolabelling *(155)*. Consequent to finding particles in these organs, a detailed histopathological analysis of the tissues must be performed. Thus, the darkfield-enhanced and hyperspectral microscopic method of detection can aid in prioritizing lung samples for a detailed histopathological investigation.

Immunotoxicity-relevant pathology and organ weights

Nanomaterials are suggested to translocate to various organs from the target lung tissue exposed *(3–6)*. Since nanomaterials are mostly poorly soluble, as suggested in OECD TG 412, draining lymph nodes from the hilar regions of the lung should be examined with regard to weight, cellularity and cell composition. In addition, nasopharyngeal tissues should be investigated. Also, a detailed investigation of other tissues for translocation or associated immunotoxicity should be performed qualitatively using microscopic methods and quantitative analytical measures, and immunotoxicity end-points should be

performed. The lung, liver, spleen, thymus, bone marrow, heart, kidney and central nervous system should be weighed and examined.

While gross morphological and pathological changes in these tissues can be assessed by routine haemotoxylin and eosin staining *(135, 136)*, immunohistochemistry against cell surface markers (macrophages, neutrophils, Th cells) can be used for precise detection of the specific cell population that is enriched. Frozen tissue sections are preferred for detecting cell surface markers, expression of which is scarce. Antigen-specific antibodies can also be used to detect specific enrichment of a gene or enhanced activity of a protein *(136)*. Where the sample is abundantly available, tissue cellularity can also be assessed using flow cytometry. For example, flow cytometry with antigen-specific antibodies can be used to sort T and B lymphocytes or stem cell population of the bone marrow. Morphometric analysis of the histological tissue sections can also provide information on tissue distribution and localization of nanomaterial.

The relative effects on the T and B cell population in the spleen, atrophy of the spleen and lymph nodes in the case of immune suppression, and lymphoid tissue hypertrophy should be assessed for immune-stimulating nanomaterials. Due to the possibility of clearance of particles from the lung tissue to the intestine via mucociliary transport, cellular proliferation in Peyer's patches in the small intestine should also be investigated for potential indication of elevated immune response.

Morphological analysis of the bone marrow containing multipotent stem cells is capable of differentiating into B and T lymphocytes and macrophages. This can be conducted using bone marrow smears or by cytospin preparations of B cells. Cellular viability and bone marrow cellular differential counts are good indicators of chemical-induced effects on the immune system.

6.7.3   *In vitro testing methods applicable to inhalation route (respiratory system)*

Love et al. *(156)* and Fröhlich *(157)* summarize various in vitro toxicity testing methods available and experimental details to consider for assessing nanomaterial-induced toxic responses.

Immune cell damage

Cellular viability

Cytotoxicity to immune cells can be measured by the LDH assay, as discussed above. In addition, other cellular viability assays, such as MTT, WST-1, neutral red, alamar blue, and trypan blue exclusion methods, have been routinely employed to assess this end-point. However, as mentioned for the LDH assay, care should be taken to assess the potential interference of nanomaterials with the test reagents *(158)*. Appropriate assay and particle controls have to be included in the assay.

Oxidative damage is another type of cellular damage that is frequently assessed following exposures to nanomaterials. The methods and considerations are described in the subsection above on cellular damage. Fibrelike nanomaterials have been shown to affect the phagocytic function of macrophages. Depending on shape, aspect ratio and biopersistency, these materials can induce frustrated phagocytosis *(159, 160)*. For such nanomaterials, an ex vivo phagocytosis assay should be performed.

Antigen uptake, processing and presentation

The main function of DCs is to capture antigens (innate immune system), process them and present them to T cells via receptor mechanisms (adaptive immunity) in order to initiate an immune response. The potential of DCs to take up and process antigen following exposure to nanomaterial can be evaluated using OVA coupled to Alexa-647 and bovine serum albumin coupled to DQ-Red to analyse antigen uptake and antigen processing, respectively *(94, 161)*. Considering the co-stimulatory expression, the expression of surface phenotype markers (CD11c, CD11b) or activation markers (CD40, CD86 and MHCII), and the release of a broad range of cytokines or chemokines can give important information in terms of measuring the activation status of DCs *(90, 162)*.

Activation of T cells

Following interactions with DCs, effector T cells can be activated and differentiated towards different fates, depending on the signal

from the DCs (antigenic specificity). A different cytokine secretion pattern is a common characteristic of the selected differentiation pathways. Clonal expansion induced by a proliferation signal then follows *(163, 164)* and can be determined in vitro by utilizing an autologous CD4+ T cell stimulation assay *(94)*.

Ex vivo phagocyte function assay

There are no guidelines or validated methods for conducting a phagocyte function assay; however, various in vitro phagocyte function tests are currently available *(165)*, and their importance to screening potentially immunotoxic nanomaterials is reviewed in Fröhlich *(157)*. A panel of in vitro assays, including cytokine secretin, chemotaxis, phagocytosis and respiratory burst assays, can be used to measure phagocyte function. Recently, a rapid screening method to evaluate the interaction of nanomaterial with phagocytic cells has been proposed *(166)*. The conventional ex vivo phagocytic assays require fluorescence tagging of the test substance, which is not suitable for nanoparticles as it may influence cellular interaction and uptake of nanomaterials. A luminescence-based approach has been tested for nanomaterials. Although the method has only been tested using in vitro immortalized monoculture models, it can be applied to ex vivo models as well. BALF cells from animals are retrieved and are incubated with the test particles and luminol solution. The phagocytic activity is visualized with luminol, a dye that becomes luminescent only upon exposure to the low pH of the phagolysosome. BALF cells can also be incubated with particles, sorted using flow cytometry and then scored for specific cell types that contain particles using the luminol method.

For autofluorescing particles such as quantum dots, BALF cells from animals exposed to the vehicle alone or nanomaterials are retrieved, challenged with various doses of fluorescent particles, and allowed to internalize particles. Uptake of particles is then evaluated by confocal microscopy. As described above, cell type-specific phagocytosis can be scored by sorting the cells by flow cytometry prior to incubating with particles and visualizing by confocal microscope. For details on the various aspects of phagocyte function assay, see Fröhlich *(157)*.

Phagocytic activity of granulocytes, monocytes and phagocytic respiratory burst

Diluted human heparinized whole blood is reacted with nanomaterials for different durations. Following exposure, samples are reacted with hydroethidine solution for the assessment of respiratory burst, and fluorescein-labelled *Staphylococcus aureus* bacteria for assessment of the ability of nanomaterial-exposed phagocytes to ingest bacteria. Samples are tested by flow cytometry. This is a fairly simple assay but additional steps have to be added to make sure that the nanomaterials do not interfere with the fluorescein reading or the instrument *(157)*.

Lymphocyte proliferation

Leukocyte proliferation aids in the host's defence response to immunogenic substances and mitogens stimulating cell proliferation and division, respectively. Human blood cells are reacted with nanomaterials in vitro, or spleen or blood lymphocytes derived from animals exposed to nanomaterials are examined for proliferative activity of lymphocytes *(167)*. Cells in vitro or cells derived from animals exposed to nanomaterial are reacted with mitogens and cell-specific antigen before pulsing with tritiated thymidine. An increase in radioactivity incorporation is measured as an indication of cell division and differentiation of lymphocytes.

Other immune response related assays

Respiratory sensitization

With regard to respiratory sensitization, conventional substances are classified as respiratory sensitizers ad hoc, on the basis of human data. Nano forms of the known classified substances should also be regarded as respiratory sensitizers. At present, validated experimental models to determine the respiratory sensitizing potential of chemicals or nanomaterials do not exist. Some assumptions are exercised in evaluating the respiratory sensitization potential of nanomaterials and caution should be exercised in interpretation of the results, taking account of the following.

- The bulk counterpart of a nanomaterial that is classified as a respiratory sensitizer must be treated as a respiratory sensitizer and prioritized for further testing.
- Nanomaterials that are skin sensitizers should be considered respiratory sensitizers if they are bioavailable in the lung as nanomaterials. The nanomaterials that are positive for skin sensitizing tests, such as the LLNA and the guinea-pig maximization test (discussed below), should be further tested for their respiratory sensitization potential.
- Nanomaterials that are translocated from lungs to systemic circulation and other organs, and those that exhibit reactive properties, should be prioritized for sensitizer testing. As discussed above, low levels of systemic translocation have been demonstrated after inhalation or intratracheal instillation for a number of nanomaterials of various chemical compositions. In general, translocation potential seems to be higher for smaller size particles *(51)*. Thus, in the absence of evidence showing their translocation, particles with primary sizes < 30 nm and aerodynamic sizes between 10 and 100 nm can be assumed to translocate and should be prioritized for further testing. Some studies suggest that nanomaterials may act as adjuvants, aggravating respiratory sensitization in mice models of airway allergic diseases *(168)*. However, no OECD guidelines are currently available to test this, even for conventional substances, although it could be a very relevant end-point to consider in future.

Immune suppression

Immune suppression is a result of impaired T cell development and function leading to toxicity. The inhalation studies described above (and the other OECD standard toxicity studies on other exposure routes) focus mostly on direct immune activation. As such, nanomaterials have been more extensively characterized for their immunostimulation behaviours than for their immunosuppression. The immunosuppressive and anti-inflammatory properties of nanomaterials have been reviewed in Ilinskaya and Dobrovolskaia *(169)*. Spleen tissue is often investigated for markers of immune suppression following direct or systemic exposure. For systemic exposure, spleen is harvested in sterile tubes containing sterile Hanks'

balanced salt solution (HBSS) and homogenized on ice. Splenic lymphocyte subpopulation analysis, including CD4+ and CD8+ T lymphocytes, B lymphocytes and NK cells, is conducted. In non-immunized animals this will determine if exposure leads to a shift in the helper (CD4+) or cytotoxic (CD8+) thymus-derived lymphocytes or NK cells.

6.7.4 Testing considerations and methods applicable to dermal route (skin exposure)

Skin epithelium

The external surface of the skin consists of a keratinized squamous epithelium, known as the epidermis, which is supported and nourished by a thick underlying layer of connective tissue referred to as the dermis, which is highly vascular and contains many sensory receptors *(170)*. A major function of the skin, especially the stratum corneum, which is the outermost layer, is to provide a protective barrier against the hazardous external environment. The skin is relatively impenetrable to lipophilic particles larger than 600 Daltons in size, whereas lipophilic particles smaller than this may passively penetrate the skin *(171)*. In general, it is accepted that only compounds and drugs smaller than 500 Daltons in size can penetrate the skin readily *(172)*. Lipophilicity of the material also plays a role.

Nanomaterials have unique physical properties making them ideal for use in various skin care products currently on the market. Functionalized or surface-modified metal oxide nanomaterials, specifically ZnO (nano ZnO) and TiO_2 (nano TiO_2), are the primary nanomaterials used in sunscreen and skin care products as a UV adsorber *(173)*. Thus, the skin is exposed to nanomaterials present in cosmetic products such as moisturizers and sunscreens. The skin is also a potential target for drug delivery via nanocarriers *(174)*.

Nano TiO_2 and nano ZnO formulated in topically applied sunscreen products exist as aggregates of primary particles ranging from 30 to 150 nm in size. These aggregates are bonded in such a way that the force of sunscreen product application onto the healthy skin would have no impact on their structure or result in the release of primary particles. Many studies using skin tissue (which is easily

available from animal slaughterhouses) have also shown that under exaggerated test conditions neither nanostructured TiO_2 nor ZnO penetrate beyond the stratum corneum of skin using the "minipig" species *(175)*. Studies of the translocation of TiO_2 nanoparticles in histological skin sections suggest that these nanoparticles may only penetrate into the "horny" upper layers of the stratum corneum *(176)*. However, other studies have shown that nanosized particles can enter a small percentage of hair follicles and are stored in this location for a prolonged period compared to their location within the stratum corneum, a factor that may enhance drug delivery by this route, although it will also exacerbate any potential toxicity *(177)*. While such studies suggest little if any epidermal or dermal penetration of these nanoparticles, recent work using live mice and pigs indicates that topically applied nanosized TiO_2 particles (< 10 nm) may indeed pass through the stratum corneum *(178)*. In addition, stretched porcine skin was far more susceptible to dermal translocation of a C60 fullerene-substituted peptide, which could reach the intercellular spaces of the stratum granulosum in stretched skin *(179)*.

Preparation of nanomaterials for dermal exposure

ENMs can come into contact with skin in various forms, including as airborne pristine particles, as aerosolized particles in a liquid vehicle, or as ingredients of topical formulations. In order to clearly understand the dermal immune responses of nanomaterials, the role of the vehicle or other ingredients in the formulation in which nanomaterials are suspended must be investigated in parallel with a detailed characterization of the specific nanomaterial. Most of the dermal studies to date have been conducted using raw nanomaterials suspended in simple solvents; however, this method does not reflect the realistic skin application scenario. Thus, investigation of dermal absorption capacity of nanomaterials contained in the actual consumer products is preferred. However, simple suspensions of nanomaterials can still be used to provide information on the potential immunogenicity of these materials on the skin. In this respect, the United States Food and Drug Administration has recently endorsed new safety standards for testing sunscreens, where a lack of information about interactions between ingredients in sunscreen formulations was identified as a current shortfall *(180)*.

In vivo skin pre-testing considerations

OECD TG 427 is applicable to nanomaterial dermal testing *(181)*. Several species, including rodents, rabbits, monkeys and pigs, are recommended, though pig skin is the suggested choice if human studies are not possible, as it is the closest in mimicking the human dermal penetration rate *(182)*. Hairless mice or severe combined immunodeficient (SCID) mice have also been tested *(178)*. The anatomical origin of the skin plays an important role in substance penetration *(183)*. Skin from the forearm, leg, back, abdomen or breast are routinely used in the testing. As for chemicals, in vivo skin penetration studies for nanomaterials should consider age, sex, genetic background and skin condition of the test animal. The other important factor to consider is the presence of hair on skin, since deposition and potential penetration of nanomaterials has been found to be greatest around the hair follicles *(184)*. These parameters should be promptly recorded and reported, as they may influence nanomaterial penetration into the skin. Pretreatment of skin by shaving, depilation and clipping are routinely used for in vivo studies, and have been found to impact absorption through the skin *(185)*. Tape stripping is also used to remove layers in the stratum corneum, in cases where correlation with the physical barrier is desired. Although most in vivo skin studies are carried out in static conditions, skin massage, flexion of skin or animal movement during the exposure should be considered *(186)*. As suggested in OECD TG 427, animals should be housed separately to avoid consumption of nanomaterials through the oral route during grooming activities. Use of the skin on the back or on the shoulders alleviates the issue with personal grooming and hence is recommended by OECD TG 427.

6.7.5 In vitro/ex vivo and synthetic skin models

Ex vivo testing consists of applying a test nanomaterial onto the surface of a skin derived from animals (rodents, pig) or humans in an in vitro set-up that mimics the environment (donor chamber) and the systemic circulation (receptor chamber). Following the exposure to a nanomaterial for the required amount of time at the specific doses, the nanomaterial is wiped or washed off the skin surface and the receptor fluid in the receptor chamber is tested for nanomaterial penetration. Receptor fluid can also be sampled during the experiment at different

intervals of time to assess penetration. This is especially important if a nanomaterial is suspected to be unstable in the experimental conditions tested. Freshly derived skin or frozen skin is used in the testing. In addition, commercially available skin models, such as EpiDerm™ or EpiSkin™, have been evaluated for corrosivity testing of chemicals. Their potential for risk assessment for nanomaterials needs to be evaluated more thoroughly.

6.7.6   Skin cell cultures

Skin epithelial cell lines, such as the epidermal cell line A431 *(187)* and the immortalized human keratinocyte cell line HaCaT *(188)*, have been used to study the effects of silver *(189)*, TiO_2 *(190)* or SiO_2 nanoparticles *(179)*. Alternatively, primary human keratinocytes are physiologically relevant and readily available commercially *(191)*. However, genetic variations may need to be screened depending on experimental objectives.

Since the epidermis is composed of many different cell layers, the optimal in vitro skin model is still lacking *(179)* and further research needs to be performed. Since complete skin tissue can be easily obtained from the slaughterhouse, this might be a better tool to study dermal penetration and dermal immunogenic effects of nanomaterials.

In vitro three-dimensional organotypic skin cultures are available and serve as potential alternatives to using real skin *(192)*. These are either constituted from single cell cultures of keratinocytes and fibroblasts or bought commercially as products of tissue engineering (for example, EpiDerm, EpiSkin). Variations of such cultures are possible but they are essentially bilayered cultures consisting of a dermis and a stratified epidermis above. These cultures emulate the anatomy of real skin, including a proper stratum corneum, but are void of skin appendages and immune cells.

6.7.7   Skin test methods for immunotoxicity

Depending on the invasiveness of the technique used to test dermal absorption, special attention should be paid to potential artefacts as a result of techniques used to prepare the skin sample or the analysis

of samples. For example, cross-sections of the skin can be prepared by cutting from dermis to epidermis. This will prevent transfer of nanomaterials situated on the epidermis through the cutting knife.

In vivo skin sensitization

In order for the skin sensitization response to take place, nanomaterials will first have to be absorbed by the skin and reach the dendritic or other systemic immune cells involved in stimulating the sensitization responses. Not all nanomaterials are anticipated to be absorbed through the skin; some nanomaterial properties may influence their skin absorption potential, requiring a detailed characterization of nanomaterials before exposure. If a nanomaterial does not cross the skin barrier, it can be safely assumed that it is not a good candidate for skin sensitization tests.

Human repeated insult patch test

The human repeated insult patch test is the most relevant test for assessing the skin sensitizing potential of nanomaterials. A test article is applied repeatedly to the skin of healthy human volunteers and markers of skin sensitization such as skin redness are observed. However, for ethical reasons, this method cannot be used as a predictive tool to test substances with unknown dermal sensitization potential. These tests are usually only performed to confirm the safe use of potentially sensitizing substances in consumer products by confirming the dermal sensitization no observed effect level that has been obtained from dermal sensitization quantitative structure–activity relationships, animal preclinical data, and historical human data *(193)*. There is no validated regulatory guideline for the human repeated insult patch test, although a review of the test design and the critical factors that can affect the induction of dermal sensitization can be found in McNamee et al. *(194)*.

Guinea-pig maximization test and the Buehler test

A number of OECD guidelines exist to address skin sensitization for conventional chemicals. OECD TG 406 (adopted in 1981, revised in 1992) describes both the guinea-pig maximization test and the Buehler test *(195)*. In both tests, guinea-pigs are first exposed to

the test substance by intradermal injection or epidermal application (induction exposure). Following a rest period of 10 to 14 days (induction period) to allow for the development of possible immune responses, the animals are exposed to a challenge dose via topical application (challenge exposure). The degree of skin reaction, based on erythema and swelling, is recorded over time and compared with that in sham-treated control animals. The guinea-pig maximization test differs from the Buehler test in that the former uses adjuvant while the latter does not.

Local lymph node assay

The local lymph node assay (LLNA, OECD TG 429) is recommended to assess the potential of chemicals to induce sensitization as a function of lymphocyte proliferative responses in regional lymph nodes in mice *(196)*. It works on the basic principle that lymphocyte proliferation in the local lymph nodes will be increased proportionally to the dose and potency of the applied sensitizer. Briefly, the test substance is applied to the dorsum of each ear over the first three days. On day 6, the animals are injected with 20 microcuries (µCi) of 3H-methyl thymidine or 2 µCi of 125I-iododeoxyuridine and 10-5M fluorodeoxyuridine through the tail vein, and sacrificed 5 hours after injection. Single-cell suspensions of lymph node cells are then prepared and their proliferation quantified by 3H or 125I counting. A stimulation index, calculated as the ratio of the proliferation in treated groups to that in the concurrent vehicle control group, is used for comparing the extent of sensitization. Other observations such as ear erythema or ear thickening may be included. OECD guidelines for non-radioactive modifications of the LLNA were adopted in 2010. The LLNA:DA test (OECD 442A) *(197)* quantifies adenosine triphosphate content via bioluminescence as an indicator of lymphocyte proliferation, while the LLNA BrdU test (OECD 442B) *(198)* utilizes non-radiolabelled BrdU in an ELISA-based test system to measure lymphocyte proliferation. OECD recommends that LLNA (OECD TG 429) be used as the preferred method where an in vivo test is necessary, due to animal welfare benefits and the provision of more quantitative data, compared to the guinea-pig maximization test and Buehler test (OECD TG 406).

Since the absorption of nanomaterials through the skin and their systemic availability is essential for a dermal sensitization reaction

to occur, the standard experimental animal assays, such as the OECD guidelines for the guinea-pig maximization test, Buehler test and LLNA, become relevant only if it has been demonstrated that the nanomaterials are absorbed through the skin. However, precaution has to be taken not to underestimate the potential, since it is not entirely known if negative dermal absorption results from animal (mice, pigs) testing can be extrapolated directly to the human situation. For many nanomaterials it will be difficult to assess skin absorption in these tests, for example due to high background levels, which complicates the assessment of the sensitizing potential of nanomaterials with animal models.

In vivo skin irritation test

Human repeated insult patch test

The human repeated insult patch test, discussed earlier for skin sensitization, can also be used to assess skin irritation through repeated patching during the induction phase, but similar to sensitization, this test method cannot be used as a predictive tool.

OECD TG 404: acute dermal irritation/corrosion

In this test *(199)*, young adult albino rabbits are used. The substance to be tested is applied as a single dose over a shaved area of approximately 6 square centimetres (cm^2) on the dorsal area of the trunk of the test animals. The exposure period is normally kept at 4 hours. The degree of irritation or corrosion is scored for signs of erythema and oedema, at 60 minutes and then at 24, 48 and 72 hours after removal of the substance, in order to evaluate the reversibility or irreversibility of the effects observed. This time frame should be extended up to 14 days if there is damage to skin that cannot be identified as irritation or corrosion at 72 hours. The untreated skin areas of the test animal serve as the control. All local toxic effects (for example, skin defatting) and any systemic adverse effects (for example, loss of body weight) should also be recorded. Histopathological examination will be useful, especially for comparing cases that are ambiguous. Efforts should be made to harmonize grading of skin responses. The subjectivity of this test is a shortfall that needs to be taken into consideration.

In vitro skin sensitization tests

The need to conduct animal testing is being contested in many countries around the world and more emphasis is being placed on developing alternative in vitro testing methods to reduce, refine and even replace current reliance on animal testing. Although alternatives to animal testing are desired, unless they are mechanistically founded, their utility in quantitative or qualitative risk assessment and decision-making processes will be undermined. However, in the area of skin sensitization (allergic contact dermatitis in humans or contact hypersensitivity in rodents), the key events involved in the process of sensitization occurring at various levels of biological organization are identified through a well constructed skin sensitization adverse outcome pathway (AOP) *(200)*. Although the AOP can be generalized to many chemicals it does not include metal groups, as the underlying mechanism of metal-induced skin sensitization does not involve covalent binding to proteins. According to the AOP, the covalent binding of electrophilic substances to nucleophilic centres in skin proteins (molecular initiating event) initiates the sensitization cascade leading to inflammatory responses as well as gene expression associated with specific cell signalling pathways, such as the antioxidant/electrophile response element (ARE)-dependent pathways in keratinocytes, resulting in the activation of DCs and T cell proliferation. Based on the key events identified in this AOP, in vitro assays addressing the specific key events were developed and were adopted by OECD in 2015. These in vitro methods include the direct peptide reactivity assay, OECD TG 442C *(201)* for a molecular initiating event (protein–peptide binding); the ARE-Nrf2 luciferase test method, OECD TG 442D *(202)* for measuring activation of keratinocytes; and the human cell line activation test (h-CLAT), U-SENS and IL-8 Luc assay (OECD TG 442E) *(203)* for measuring the activation of DCs. Thus, skin sensitization is one toxicological area where validated and harmonized methods are available for in vitro assays that may also be applicable to nanomaterial testing with significant modifications.

Nevertheless, the individual tests cannot be used as stand-alone tests to predict the sensitizing potential of nanomaterials, as explained above. Rather, the data should be considered in the

context of integrated approaches, combining the results with other complementary information, such as the data derived from in vitro assays addressing other key events of the skin sensitization AOP, as well as non-testing methods, including read-across from chemical analogues or in silico tools. In general, it can be assumed that if the bulk counterpart of a nanomaterial is classified as a sensitizer, then the respective nanomaterials can be considered as sensitizers and should be prioritized for testing. Some nanomaterials, owing to their physical-chemical properties, can be more reactive and induce localized oxidative stress, which can then lead to skin sensitization. Photocatalytic nanomaterials, for example, may fall under this category. More research is required in this area that will result in the development of an intelligent testing strategy, similar to those recently evaluated by van der Veen et al. *(204, 205)* for conventional substances. Ideally, such a testing strategy should use a combination of in silico and in vitro methods specifically designed for nanomaterials. Additional in vitro methods are being validated and a few of them are at the OECD adoption stage. Moreover, an OECD proposal is under way to develop defined approaches that consider combinations of in vitro and in silico methods to provide information that is equivalent to or better than that generated by an in vivo animal test to assess substance-induced human skin sensitization. Thus, in the near future, additional in vitro methods that are validated, and guidance to use the information derived from each of these in vitro methods, is expected to become available. One other important consideration for in vitro testing of chemical-induced toxicity is the metabolic capacity of the cell types used, as many chemicals require metabolization for their activity. However, nanomaterials do not require metabolization and hence this limitation may not be applicable to in vitro testing of nanomaterials.

Direct peptide reactivity assay (OECD TG 442C)

This test is proposed to address the molecular initiating event of the skin sensitization AOP, namely protein reactivity, by quantifying the reactivity of test chemicals towards model synthetic heptapeptides containing either lysine or cysteine. Cysteine and lysine percentage peptide depletion values are then used to categorize a substance in one of four classes of reactivity for supporting discrimination between skin sensitizers and non-sensitizers. OECD TG 442C states that the method is not applicable for testing of metal compounds, since

they are known to react with proteins through mechanisms other than covalent binding. As discussed before, there is a high level of both covalent and non-covalent interaction between nanomaterials and proteins. Therefore, it is questionable whether this method is applicable to nanomaterials.

ARE-Nrf2 luciferase test method (KeratinoSens™) (OECD TG 442D)

The second in vitro test method, ARE-Nrf2 luciferase (OECD TG 442D), is proposed to address the second key event. Skin sensitizers have been reported to induce genes that are regulated by the antioxidant/electrophile response element (ARE). Small electrophilic substances such as skin sensitizers can act on the sensor protein KEAP1 (Kelch-like ECH-associated protein 1), for example covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes.

The TG states that the test method is applicable to soluble chemicals or chemicals that can form a stable dispersion (that is, a colloid or suspension in which the test chemical does not settle or separate from the solvent into different phases) either in water or DMSO. This method may therefore be applicable to nanomaterials, provided that the stable dispersion criteria can be fulfilled.

In vitro skin sensitization assays addressing AOP key event 3: DC activation

OECD TG 442E contains three individual test methods addressing the third key event of the AOP – dendritic cell (DC) activation. Two of the methods (h-CLAT and U-SENS) are based on the quantifiable change of the expression of cell surface markers linked to the DC activation (CD86 or CD54). The third method (IL-8 Luc assay) is based on the changes observed in cytokines that are linked to DC activation (IL-8).

Both the h-CLAT and the U-SENS assays use cell lines as surrogates to DCs: the h-CLAT assay uses the monocyte-derived human monocytic leukaemia cell line (THP-1) and the U-SENS assay uses the human myeloid U-937 cell line. Both assays follow in general

the same principle: h-CLAT measures the increase in the cell surface markers of CD86 and CD54, whereas the U-SENS assay uses only one cell surface marker (CD86) to predict whether a substance is a skin sensitizer or not. These test methods are applicable for chemicals soluble in suitable vehicles or that can form a stable dispersion. Therefore, the same considerations apply when considering the suitability to use these methods for ENMs, as explained above for the KeratinoSens assay.

The IL-8 Luc assay measures the increase in the induction of IL-8 mRNA by using the THP-1 cell line as a surrogate for DCs. In the assay, the increase in the IL-8 mRNA induction is quantitatively measured by luciferase expression (stable reporter cell line THP-G8, established by transfection of plasmid vectors into the THP-1 cell line). The test method is applicable to substances having water solubility of 100 μg/mL or more. However, negative results obtained for substances not dissolved at 20 mg/mL may produce false negative results and should not be considered in the assessment of skin sensitization potential. Substances interfering with the luciferase assay may also potentially affect the reliability of the measurements.

In vitro skin irritation

For conventional substances, the irritant and corrosive potential can be assessed using tests following OECD TGs 439, 431, 430 and 435 *(206–209)*. Reviewing the literature, very few nanospecific data are found on irritant and corrosive properties of nanomaterials. A number of studies have addressed skin and corrosion or irritation of nanoparticles using the above-mentioned OECD test guidelines or similar *(210–219)*. These studies reported no irritation or corrosion effects from 10 nm Ag-NPs *(211)*, 50 nm polystyrene nanoparticles, 21 nm TiO_2 *(216)*, 140 nm rutile/anatase TiO_2 nanoparticles *(219)*, 7 and 10–20 nm SiO_2 nanoparticles *(212)*, carbon nanohorns *(210)* and fullerenes *(213, 218)*. In studies investigating CNTs, one study did not find irritation from two forms of MWCNTs *(217)*. In another study, two products with SWCNTs and one with MWCNTs did not show any signs of skin irritation, while another with MWCNTs showed mild and reversible skin irritation *(214)*. One study investigated unwashed, washed and carbon-coated Ag-NPs of different sizes and did not see immediate signs of irritation, although focal points of inflammation

were observed in the skin upon closer examination *(215)*. More recently, anatase TiO_2 nanoparticles less than 25 nm in diameter were applied onto the ears of female BALB/c mice for three days *(220)*. Using the LLNA, it was found that auricular lymph node cell proliferation was not affected. However, skin irritation was observed with 5% and 10% TiO_2 exposure, based on measuring the percentage of ear thickness change.

In summary, most reported studies using OECD TGs to study corrosion and irritation in healthy skin do not find any effects of nanomaterials, and those that do only find very mild and reversible effects. This could indicate that nanomaterials are generally not irritant or corrosive, or it could mean that the OECD TGs are not appropriate to study these effects for nanomaterials, for example due to the recommended exposure and evaluation times being too short. This introduces some uncertainty into the evaluation of this end-point. As a rule of thumb, nanomaterials that are (partly) composed of corrosive or irritant chemicals can be assumed to cause corrosion and irritation themselves. In addition, nanomaterial suspensions with an extreme pH (< 2 or > 11.5) can be assumed to cause corrosion to the skin. One could also consider that nanomaterials with reactive properties are at risk for causing irritation or corrosion, although this would be based mostly on speculative assumptions. However, the term "reactive properties" would need a better definition.

Skin responses in susceptible populations

There is interest in extending skin exposure studies to models of skin diseases, because the defective physical barriers presented in some skin conditions could allow greater penetration of nanomaterials. There are limited reports of such studies but the transgenic NC/Nga mice to model atopic dermatitis has been used. Yanagisawa et al. *(221)* found that repeated exposure of TiO_2 nanoparticles (15, 50, or 100 nm) on the ear skin of NC/Nga mice, through intradermal injections over a 17-day period, resulted in significant increase in histamine levels in blood serum and IL-13 expression in the ear. Interestingly, coexposure of the TiO_2 nanoparticles, together with mite allergen extracts, aggravated epidermis thickening and elevated levels of IL-4, IgE and blood serum histamine, suggesting that atopic dermatitis symptoms were exacerbated through Th2-biased immune

responses. A similar study was conducted by Hirai et al. *(222)* using topical application of 30 nm silica nanoparticles, together with mite allergen extracts, on the ears and upper backs of NC/Nga mice. Atopic dermatitis-like lesions in the form of increased ear thickness were recorded. Allergen-specific Th1-related IgG2a and Th2-related IgG1 levels were detected, which were absent when the mice were exposed to silica nanoparticles applied separately from the allergen or to well dispersed silica nanoparticles. The presence of allergen-adsorbed agglomerates of silica nanoparticles led to a low IgG–IgE ratio, which is a key risk factor in human atopic allergies.

With the interest in understanding sensitization of diseased skin to nanomaterials, Ilves et al. *(223)* sensitized the back skin on BALB/c mice with OVA/staphylococcal enterotoxin B as the allergen/superantigen cocktail to evoke local inflammation and allergy to model atopic dermatitis. Exposure of this skin to ZnO particles showed that nanosized ZnO (< 50 nm) is able to reach the dermis layer in atopic dermatitis skin. In addition, ZnO nanoparticles induced systemic production of IgE more significantly than larger ZnO particles, suggesting the allergy-promoting potential of ZnO nanoparticles. Smulders et al. *(224)* exposed mouse ear skin with TiO_2, silver and SiO_2 nanoparticles for a day before applying DNCB for three days, and found that dermal exposure to silver or SiO_2 nanoparticles prior to DNCB sensitization did not influence the stimulation index after six days of exposure. In contrast, the stimulation index was increased with the application of 4 mg/mL TiO_2 nanoparticles prior to DNCB sensitization. Other studies reported no skin sensitization from 50 nm polystyrene nanoparticles or 21 nm TiO_2 *(216)*, 140 nm anatase/rutile TiO_2 nanoparticles *(219)*, fullerenes *(213)*, carbon nanohorns *(210)*, and SWCNTs and MWCNTs *(214)*. Thus, the responses to nanomaterial exposure in the normal population and susceptible population may be entirely different; it is important to consider these differences in the final decision-making processes.

6.8 Testing considerations and methods applicable to oral route

The OECD has published guidelines for assessing toxicokinetics in acute, repeated dose (28- or 90-day) toxicity studies following oral exposure. According to these guidelines, administration of the

test material via gavage, drinking water or food is recommended. However, it is important to note that some nanomaterials precipitate in water and form aggregates in water; when incorporated in the food matrix they can be transformed, which may potentially affect their uptake, bioavailability, interaction with surrounding biological milieu and toxicological activity. Characterization of the physicochemical properties of the nanomaterial in the matrix used for the study is therefore important. Caution is needed when applying the results obtained from an oral study with a specific matrix for the safety assessment of nanomaterials contained in a different matrix.

During their passage through the gastrointestinal tract, nanomaterials may be exposed to a range of different physiological environments that may affect their physicochemical properties, which will in turn affect their potential local toxicity and absorption of the particles *(225)*. In this light, when conducting oral studies, it is important to be aware of the species-specific differences in the physiology of the gastrointestinal tract. For example, the pH of the gastrointestinal tract in rodents and humans is different *(226)*. Outcomes of oral studies in rodents with particles that undergo large pH-dependent changes in their physicochemical properties may not be relevant for humans. It is therefore recommended to characterize nanoparticles at various ranges of pH prior to starting oral studies.

The majority of oral biokinetic studies have demonstrated that most nanomaterials remain in the gut lumen and are excreted via faeces. Little is known of the local effects of nanomaterials in the gastrointestinal tract. Effects of nanomaterials with antibacterial activity on gut microbiota may need to be investigated, since an imbalance in gut microbiota has been linked to a number of immune-related diseases, such as inflammatory bowel disease, type 1 diabetes and spondyloarthropathies. Although no validated methods exist to address this issue, improvements in sequencing technologies, coupled with a renaissance in 16S ribosomal RNA gene-based community profiling, have enabled the characterization of microbiomes *(227)*.

Since for most nanomaterials absorption through the gastrointestinal tract is limited, acute systemic effects of nanomaterials are not likely to be encountered frequently after oral exposure. However, the clearance rate of some systemically available

nanomaterials was found to be dose dependent and very low, resulting in accumulation in the body *(228, 229)*, which could potentially result in long-term effects. Therefore, investigating the distribution and clearance of nanomaterials in the body after oral exposure is recommended prior to conducting oral toxicity studies.

OECD TG 417 *(230)* offers a number of considerations for conducting such toxicokinetic studies that are also applicable to nanomaterials. Where possible, nanomaterials should be labelled radioactively with C14 to allow for an adequate time course study. Alternatively, ICP-MS or other analytical techniques can be used for elemental analysis of the materials in different organs at different time points. The suitable application of ICP-MS for this purpose and other relevant analytical techniques have been discussed in detail by Krystek et al. *(231)*.

In time course studies, part of the collected organs from toxicokinetic studies could be set aside for later use to investigate systemic (immune) toxicity parameters in cases where the results indicate that significant absorption has taken place. However, most of the tissue material will often be needed to detect the presence of nanomaterials, since their absorption is frequently low.

Once the absorption and clearance of the nanomaterial has been determined, the appropriate duration of subsequent toxicity studies can be selected. Technical guidelines and guidance documents for conducting acute and (sub)chronic repeated dose toxicity studies can be found in the library of the OECD.

If significant absorption of nanomaterials occurs, it will probably occur primarily via the M cells of the Peyer's patches in the small intestine, where they are taken up by the gut-associated lymphoid tissue *(232)*. From there systemic distribution to various organs may occur, most probably to the liver, spleen and other tissues of the mononuclear phagocytic system. These tissues therefore deserve thorough histopathological investigation in subsequent studies, paying special attention to markers of inflammation responses. Apart from directly affecting immune parameters locally or systemically, it has also been suggested that nanomaterials can act as a "Trojan horse" by taking along toxins or other components that are normally not

absorbed by the gastrointestinal tract *(226, 233)*. In addition, they may act as adjuvants to cellular immune responses *(234)*, implying that nanomaterials may exacerbate inflammatory bowel diseases *(235)*. However, studies investigating this link have produced contradicting results, and so far a causal relationship has not been established.

6.9 Systemic exposure and translocation of nanomaterials into the bloodstream

The human blood is composed of a cellular fraction (45%), comprising red blood cells, white cells and platelets; and the blood plasma (55%), consisting of proteins, glucose, amino acids, fatty acids and other components such as clotting proteins (fibrinogens). The term "blood serum" refers to plasma from which fibrinogen has been removed. Blood contains more than 1000 types of proteins, and over 50 of them have been found adsorbed onto nanomaterial surfaces *(236, 237)*. Since blood composition is highly complex, analysis of the nanomaterial behaviour in complete (human) blood is very challenging. Studies are routinely conducted ex vivo using defined suspension conditions or using in vivo experiments *(238)*. Indeed, most research is focused on the identification of surface-adsorbed proteins on nanomaterials with respect to their physicochemical properties *(239)*.

Recently the use of human blood cell models has been described to study the immunotoxic potential upon exposure to nanomaterials *(240)*. This approach includes the isolation of lymphocytes, NK cells, granulocytes and monocytes from fresh peripheral whole-blood cultures and subsequent assessment using specific ex vivo assays, such as proliferation activity of lymphocytes, killing activity of NK cells, and phagocytic activity of granulocytes.

Information on immunotoxicity of nanomaterials that become systemically available can be obtained from standard repeated dose in vivo toxicity studies, for which various technical guidelines and guidance documents are available in the library of the OECD or ICH. Standard toxicity assays include haematological changes, such as leukocytopenia or leukocytosis, granulocytopenia or granulocytosis, and lymphopenia or lymphocytosis; alterations in immune system organ weights or histology (for example, changes in thymus, spleen, lymph

nodes, or bone marrow); changes in serum globulins not explained by other factors; changes in serum immunoglobulins; enhanced infection incidence; and augmented tumour rate without a different possible explanation. The necessity for additional immunotoxicity studies can be based on the weight of evidence of the outcome of the initial immunotoxicity parameters. Additional studies that may be recommended include immune function studies in rodent or non-rodent species, such as T cell-dependent antibody response in affected cell types. If such additional tests provide sufficient data to conclude on a risk of immunotoxicity that is considered acceptable, no extra animal testing might be called for. If it is unknown what specific cell type is affected and should be used to perform a T cell-dependent antibody response assay, then other cell-specific assays need to be selected. An acceptable study design of the additional rodent studies represents a 28-day study with daily dosing, which includes any of the following immunotoxicity assays: immunophenotyping (flow cytometry or immunohistochemistry); NK cell activity assays (performed if immunophenotyping studies demonstrate a change in number or if standard toxicity studies display high viral infectious rates); host resistance studies (testing if the test compound can modulate the host resistance); macrophage or neutrophil function (macrophage or neutrophil function assessment); and assays to measure cell-mediated immunity (in vivo assays where antigens are used for sensitization). For nanomaterials demonstrating high absorption and low clearance rates, repeated dose toxicity studies should be combined with a developmental toxicity study including a wide range of immunological parameters, since the developing immune system has been shown to be very susceptible to disruption by chemicals *(241)*.

6.10 Conclusions

The majority of assays that are mentioned in this chapter are focused on the detection of direct immunotoxicity, and the recommended tests are thus limited to evaluating the potential for inadvertent immunosuppression and immunostimulation. Both are possible scenarios for nanomaterials, but other immunotoxic effects may also be encountered, such as hypersensitivity and autoimmunity. The ICH S8 guideline for immunotoxicity testing of pharmaceuticals states that testing for respiratory or systemic allergenicity or

autoimmunity is not based on standard testing approaches, since no (validated) models are available. To adequately assess the immunosafety of nanomaterials, safety evaluation guidelines need to be expanded with a testing strategy consisting of standardized and predictive immunotoxicity tests tailored to the unique properties of nanomaterials. Figure 6.1 serves as preliminary guidance for the types of questions to be asked before deciding on the testing strategy and prioritization of the types of end-points.

It is obvious that for safety testing, not all assays mentioned could be applied for a specific test material. Many of the tests mentioned are explorative tests. For the purpose of risk assessment, human data would be most relevant, but these are scarce, and usually lack proper exposure assessment. Animal data are helpful, but it is not always possible to extrapolate animal data to humans. In addition, there is a strong movement to reduce animal testing. In vitro testing often lacks the complexity of the entire system or organism. Hence, specific guidance on what could be used for hazard assessment and which risk assessment framework could be employed is not available. Thus, such decisions should be driven by the nature of the test material and the purpose of the eventual risk assessment. The case studies in Annex 1 demonstrate how the existing data derived from the application of currently available methods can be used to assess the immunotoxic hazards of nanomaterials.

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7. APPROACHES FOR RISK ASSESSMENT

7.1   Introduction

Risk assessment is a decision support tool that enables regulators to derive decisions on the safety of exposure to a substance, the nature of potential hazards elicited by the substance, the affected receptors or population, and how best to manage the risks. As discussed in detail in the previous chapters, nanomaterials exhibiting distinct physicochemical properties are increasingly found in a variety of products and, as a consequence, exposure to these materials in our surroundings (home, at work and in the environment) is inevitable. It is accepted that in the nano form, many materials behave differently than their bulk counterparts, implying that they have novel toxicological properties that are different from the same chemicals in their bulk form. This also suggests that knowing the risks posed by the bulk form is not sufficient and that separate safety testing and risk assessment frameworks are needed for nanomaterials.

Studies conducted so far on the human health effects of nanomaterials reveal that the potential for exposure to most of these substances in occupational settings does occur; however, exposure via consumer products or in the environment is likely but limited. Furthermore, it is noted that the mechanisms by which nanomaterials are internalized and transported in the environment or in the human body may be different *(1–3)*, but so far toxicological effects unique to nanomaterials have not been revealed. Thus, human health and environmental safety assessment and regulation of nanomaterials may follow a conventional risk assessment framework that is established for bulk substances. However, some physicochemical properties of nanomaterials may influence their toxic potency and thus, to be certain, a risk assessment strategy involving investigation of potential exposure and hazard should be tailored to each property and application of nanomaterials.

Although the steps involved in human health risk assessment (HHRA) of chemicals in general may vary from one regulatory organization to the other, as in the case of chemicals, HHRA

of nanomaterials could consist of four major steps: (a) hazard identification, (b) dose–response, (c) exposure assessment, and (d) risk characterization *(4, 5)*. Similar to chemicals, basic questions concerning the specific nanomaterial being assessed must be addressed before the process of risk assessment is initiated, including:

- Who are the receptors or who (population) is at risk?
- What are the pathways of exposure – air, water, soil, food, consumer products, pharmaceuticals – and routes of exposure – inhalation, ingestion, skin contact?
- What are potential exposure (doses) based on use scenarios?
- What are the potential health effects?
- What information is known regarding biodistribution, absorption, metabolism and excretion?
- What is the duration of exposure required to elicit an adverse health effect?

These are indicated in Figure 7.1 in the context of the four general steps of risk assessment, as applicable to nanomaterials.

Hazard identification
Is the identity of a nanomaterial known? Is the nanomaterial potentially hazardous to humans? Is the material known to cause hazard in its bulk form?

Exposure assessment
Are the pathways of exposure identified? Are the routes of exposure known? Is the amount and the duration of exposure known? What metrics are appropriate for characterizing the exposure? (Traditional use of mass or mass per unit volume is not appropriate for nanomaterials. Surface area, particle number per volume in addition to mass should be considered.) Are the analytical techniques required to characterize physicochemical properties of nanomaterials available and are they sensitive?

Hazard characterization
Dose–response – How much of it is required to trigger a toxic response? Physicochemical properties – What properties contribute to potential health effects? (Particle size, size distribution, shape, surface area, surface charge, surface chemistry, and agglomerate status and stability in the exposure media are some of the important parameters to be considered.) Are the test models appropriate?

Risk characterization
What are the probabilities that a nanomaterial will induce an adverse health effect? What is the type or nature/severity of effects? In what form does the nanomaterial pose risk (pre or post inclusion in a product, in its raw form during production)? At what life cycle stage is it hazardous? Are the effects reversible?

Figure 7.1 Putative risk assessment framework for nanomaterials
Source: Adapted from United States Environmental Protection Agency *(4)*.

7.1.1   Hazard identification

The hazard identification step determines if exposure to a substance results in an increased incidence of a specific adverse health effect and if that adverse effect is likely to occur in humans. As part of this first step, the available scientific literature concerning the substance of interest is examined and an understanding of how exposure to substances leads to an adverse effect (mode of action) is established. Mode of action is a sequence of key biological events and processes that are initiated when a substance interacts with a cellular component leading to a cascade of changes at the biochemical, cellular and organ levels, eventually resulting in an adverse effect. It was originally described for carcinogenic substances but is now commonly applied to any substance-induced adverse effect *(6)*. More recently, the adverse outcome pathway (AOP) framework, another mechanism to assemble and organize biological information in a chemically agnostic manner, has been proposed *(7, 8)*. The mode of action approach is used to support the weight of evidence for the observed deleterious effects induced by the substances under consideration, which is a component of the subsequent hazard characterization process. However, the application of the AOP framework in the context of risk assessment is yet to be demonstrated. More often, a single substance may be responsible for multiple adverse effects. Importantly, the type of adverse end-points that are affected (for example, cancer versus non-cancer) will determine the risk assessment guidelines employed. Although human clinical studies or epidemiological studies involving evaluation of large human populations to confirm the association between exposure to a substance and a health effect are most desired, such studies are expensive and scarce. Thus, most of the HHRA studies conducted on chemicals rely on data collected from animal studies, with due consideration given to the uncertainties involved in extrapolating information derived from such models to humans. Understanding how a substance is absorbed, distributed, metabolized or eliminated in or from the body (toxicokinetics), and how exposure to a substance leads to negative health effects (toxicodynamics), is very important; however, such information is not often available for consideration.

7.1.2   *Dose–response relationship*

Once the potential hazard of a specific substance is identified, the level of exposure required to initiate the effects described above has to be determined, and the specific conditions under which exposure is likely have to be described. These data then become the basis for establishing concentration/dose–response or exposure–response relationships. It is commonly accepted that an increase in adverse effect is directly proportional to the dose of the substance administered. This relationship (dose–response) is influenced by various factors, such as the type of substance, the target (humans or animals), the recipient age and sex, and the exposure route. For risk assessment purposes, in addition to knowing the dose at which an adverse effect is observed, determining a dose at which the substance does not induce any effects, the lowest dose at which an effect is observed, and a dose beyond which the response plateaus is critical. The lowest dose at which the first observed changes in a predetermined adverse response are observed is then used as a critical effect dose for risk assessment. Factors are applied to that value to calculate the regulatory exposure limits imposed on that substance. Although epidemiology studies involving humans are desired in obtaining the dose–response relationship, for a majority of chemicals such data are non-existent. There are other challenges, including the following: (a) animal studies, which are routinely used, are very expensive and as a result many studies do not investigate the response over a range of doses; (b) many animal studies are conducted using very high concentrations of substances and non-realistic exposure methods, which may lack relevance to a human scenario; and (c) extrapolation from animal species to humans often involves several uncertainties. Nonetheless, using high doses can provide information on the absence of adverse effects at concentrations relevant to human exposure.

Two types of dose–response assessments exist: non-linear dose–response assessment and linear dose–response assessment.

Non-linear dose–response assessment. This is based on the threshold hypothesis, which refers to the minimum dose of a substance required to initiate a toxic response; below this concentration, a toxic effect is not observed. Based on this assessment approach, two exposure levels are determined. A no observed adverse effect level

(NOAEL) is the exposure level at which no statistically significant biological change or adverse outcome is observed in experimental subjects following exposure to a substance compared to the non-exposed controls. A lowest observed adverse effect level (LOAEL) is the lowest dose at which the first statistically significant biological change or the adverse outcome itself is observed and is used when a NOAEL has not been identified. An alternative to the NOAEL can also be used, such as the benchmark dose (BMD). The BMD incorporates mathematical modelling of responses over several doses to determine a critical dose at which a predetermined change in response or adverse effect occurs (referred to as the benchmark response, which is in the range of 1–10% depending on the statistical power of the study and the type of data). The benchmark dose lower confidence limit (BMDL) refers to the statistical lower confidence limit on a dose that is observed to produce the predetermined response. In a non-linear approach, the LOAEL, NOAEL or BMDL is used as the point of departure.

From the NOAEL, LOAEL or BMDL a safe daily intake level can be derived. This can be referred to as one of the following: (a) a reference dose (RfD, from an oral or dermal study); (b) a reference concentration (RfC, from an inhalation study); or (c) a derived no effect level (DNEL, from all studies). RfD, RfC or DNEL derivation requires application of several uncertainty factors to take into account the variability and uncertainty reflecting possible differences between the human and animal population, as well as variability within the human population.

An RfD is an estimate of a daily human oral exposure (in milligrams per kilogram of body weight per day) that is not likely to induce significant increases in risk of developing negative health effects during a lifetime. An RfC is used to assess safety of exposure to substances via the inhalation route. Here, concentration refers to milligrams of substance in air per cubic metre of air. Similar to an RfD, a DNEL is defined as the level of exposure beyond which humans should not be exposed. It is applicable to threshold effects (and is not applicable to non-threshold effects).

Linear dose–response assessment. This is used for cancer-inducing substances. When mode of action information does not exist or when it suggests that there is no threshold to a toxicity observed,

a linear dose–response assessment is applied *(9)*. In this type of assessment, there is no safe dose level. Extrapolation from animals to humans is based on a straight line that is drawn from the point of departure for the observed effect to the dose showing zero response. The slope of this straight line – referred to as the slope factor or cancer slope factor – is used to estimate risk at dose levels that fall along this line. Usually, BMDL values are used as points of departure *(9)*.

7.1.3   *Exposure assessment*

Exposure assessment determines whether people are, or will be, in contact with potentially hazardous chemicals from specific conditions of use *(9)*. It measures the life cycle of a substance in the environment from its origin to the target use and the route of exposure (inhalation, skin, oral). Direct exposure data are often missing, and thus the exposure assessment is conducted using available data on the environmental concentrations of the substance. Then the estimates of human exposure over time, and mathematical models to calculate transport and fate of the chemical in the environment, are applied. While it is important to know the levels of exposure to a substance in the environment, it is equally critical to know the doses that are biologically available, which determine the bioactivity potential of a substance *(2)*. For example, the exposure assessment must consider the amount of a substance that is present in the environment, the amount that can be inhaled or ingested or may be applied to the skin, and the amount that is internalized and available for interaction with biological material.

7.1.4   *Risk characterization*

The last step of the HHRA takes into account all of the information gathered from the above three steps and informs the risk management exercise on the likelihood that a substance will induce risk and the nature of that risk. It documents the various steps involved in risk assessment, including the assumptions made and the uncertainties recognized, and considers the possible policy implications.

Although the four steps that form the basis of HHRA of chemicals can be applied to nanomaterials, several additional factors unique to nanomaterials (described below) must be taken into consideration *(10)*.

7.1.5 Major issues with applying the existing HHRA paradigm to nanomaterials

One of the major hurdles in applying the HHRA to nanomaterials is that there is little nanospecific regulation at present. Although a handful of attempts have been made to conduct risk assessment using existing HHRA approaches for some nanomaterials, they are not complete due to the lack of quantitative data. While individual regulatory agencies have developed definitions of what constitutes a nanomaterial, an internationally harmonized definition or agreement in this respect is lacking. There are no specific triggers (the criteria used to support a regulatory decision to review a new material or a product for human health and environmental impacts before its release into commerce) assigned to nanomaterials that will initiate their risk assessment *(11)*. Various limitations associated with regulating nanomaterials have been reviewed and summarized by Hansen *(12)*.

In Europe, at least for cosmetics and food, industries are obliged to register products containing nanomaterials and are asked to add the term "nano" on the label when nanomaterials are used in the products. Although this is not harmonized across Europe, some European Union Member States have implemented national laws that oblige industries to register nanomaterial-containing products *(13–15)*. However, this is not a common practice globally. In the majority of European Union countries there are no mandatory reporting systems for industries when their products contain nanomaterials. As a result, it is very difficult to track these materials in products and determine the extent of their use. This in turn makes it difficult to accurately monitor workers or consumers for actual exposure. Moreover, there is no consensus on what type of information is required for risk assessment (for example, what properties should be considered in asking for information from the notifier), and there is no binding legislation that obliges industry to register the use of nanomaterials in their products *(16, 17)*. Recently, the European Union announced that it would not administer a mandatory European Union-wide harmonized registry for nanomaterials; however, a voluntary European Union Observatory for Nanomaterials (EUON) has been launched, hosted by the European Chemicals Agency *(18)*. The EUON is expected to contribute to increased transparency of uses of nanomaterials in

products on the European Union market. Whether the EUON can influence risk assessment and management strategies is debatable, given the voluntary nature of the EUON and its limited access to publicly available sources.

Another technological limitation that has further hampered progress in this area is the lack of validated methodologies to effectively detect nanomaterials in products, matrices and biological materials, characterize their properties, and assess their safety *(19)*. Although existing regulatory acts and other legislation are not specific to nanomaterials, they can be used to assess and manage the potential risks they may pose *(20)*. Thus, similar to chemicals, the risk assessment approach for nanomaterials in general will depend on the type of nanomaterial and its intended use, which will determine the legislation under which they should be assessed and regulated *(21)*. This in turn will depend on how a nanomaterial is defined under that legislation.

Legislative provisions that explicitly address nanomaterials would ideally refer to a definition to identify and distinguish them from other materials *(21)*. On 18 October 2011, the European Commission published its "Recommendation on the definition of a nanomaterial" to promote consistency in the interpretation of the term "nanomaterial" for legislative and policy purposes in the European Union *(22)*. This definition is not legally binding but serves as a reference that is broadly applicable across different regulatory sectors and can be adapted to specific product legislation (Table 7.1). The impact of the European Commission definition on different legislative frameworks is extensively discussed by Bleeker et al. *(23)*. Table 7.1 summarizes which regulatory frameworks in the European Union have a nanomaterial definition, whether a label (on the product) is required, whether there are specific provisions in place, and any further developments that are anticipated *(23)*. The European Commission definition, which was reviewed in 2014 and 2015 *(24–26)*, applies to all particulate nanomaterials irrespective of their origin – natural, incidental or manufactured – whereas some jurisdictions limit the definition to manufactured nanomaterials only.

The differences between the European Union, Canada and the United States of America with respect to global considerations concerning regulations on nanocosmetics are shown in Table 7.2. In

addition, differences between various countries on this subject were presented in a recent Organisation for Economic Co-operation and Development (OECD) document *(33)*, which describes the results of a survey on the different types of risk assessment, tools available for risk management measures, and uncertainties that guide additional nanospecific data needs in various OECD member countries (Australia, Canada, Denmark, Germany, Japan, Republic of Korea, the Netherlands, Switzerland, the United Kingdom, and the United States).

Since nanomaterials are extensively used in various products, a definition of "nanomaterial" is implemented by many regulatory agencies when evaluating whether a substance or a product can be categorized as a nanomaterial. Along with the size (1–100 nm) parameter, particle size distribution is also specified. The nanomaterial definitions and particle size distribution thresholds recommended by selected jurisdictions are summarized in Table 2.1, Chapter 2 of this document.

However, the analytical techniques available to measure precisely the amount of ENMs have limitations, for example with respect to measurements in complex matrices, and need further development and validation *(34)*. Thus, several deficiencies (discussed above) in the fundamental risk assessment framework as it applies to nanomaterials have led to a current situation where these materials are largely unregulated or regulated as conventional chemicals without due consideration of their nano properties. The collective technical impediments have resulted in insufficient hazard data to perform a detailed risk assessment in support of regulatory decision-making. Specific challenges in the context of the four risk assessment steps are described below.

Hazard identification process: challenges

Hazards posed by nanomaterials will depend on their diverse physicochemical properties *(35)*. For some nanomaterials, toxicity is shown to increase with decreasing particle size and consequent increase in relative surface area. For others, surface properties, including surface charge, chemical functional group and shape, seem to be important *(36)*. In addition, although nanomaterials are defined as materials in the size range 1–100 nm, exposure often involves

Table 7.1 Overview of the European Union legal frameworks governing nanomaterials

Legislation	Regulation	Regulation-specific definition available	Label required	Specific provisions	Anticipated discussions or developments
Biocides	Regulation (EU) No. 528/2012 (27)	Yes	Yes	Separate assessment	Guidance
Plant protection products	Regulation (EC) No. 1107/2009 (28)	No	No	None	Guidance
Cosmetics	Regulation (EC) No. 1223/2009 (14)	Yes	Yes	Separate assessment	Guidance
Food information to consumers	Regulation (EU) No. 1169/2011 (15)	Yes	Yes	None	None
Contact materials	Regulation (EU) No. 10/2011 (29)	No	No	Separate assessment	Guidance
Novel foods	Regulation (EU) No. 2015/2283 (30)	Yes	Yes	Separate assessment	Guidance
Additives	Regulation (EC) No. 1331/2008 (31)	No	No	Separate assessment	Re-evaluation of authorized food additives; guidance
Medicinal products		No[a]	No	None	Definition: guidance. The European Commission New and Emerging Technologies Working Group recommended the addition of "all devices incorporating or consisting of particles, components or devices at the nanoscale" in the highest risk class (32). Currently this is being revised

Table 7.1 (Contd)

Legislation	Regulation	Regulation-specific definition available	Label required	Specific provisions	Anticipated discussions or developments
Medical devices		Yes	No	None	Definition, risk classification, specific provisions, guidance
Registration, Evaluation, Authorization and Restriction of Chemicals (REACH)		No	No	None	Guidance
Classification, labelling and packaging		No	No	None	Additional or adaptation of legislation and guidance
Occupational health and safety		No	No	None	Guidance and occupational exposure limits

a. The legislation does not include a definition, but the European Medicines Agency provides a definition on its website.

Source: Adapted from Bleeker et al. (23).

Table 7.2 Global considerations concerning regulations on nanocosmetics

Global considerations	European Commission	Canada	United States (Food and Drug Administration)
Regulatory definition of nanomaterials	Regulation (EC) No. 1223/2009 *(14)*	None	None
Notification of cosmetic ingredients	Mandatory	Mandatory	Voluntary
"Nano" on product label	Mandatory	Voluntary	Voluntary
Notification of nanomaterial cosmetic ingredients	Mandatory six months pre-market, including identity and foreseeable uses; guidance, 2012	Voluntary (~ 50 notified since 2016 versus 40 000 total notifications per year)	Voluntary guidance, 2014
Catalogue of nanoma-terials in cosmetics	Published 31 December 2016	None	None

aggregates or agglomerates ranging from a few nanometres to several micrometres. This may in turn influence their ability to interact with biological materials, their uptake by living cells, and their biological activity or toxic effects *(37)*. Thus, in order to determine if a nanomaterial can pose a risk to humans, it is important to demonstrate that it comes into contact with biological material in the nano form and that the observed toxicity following exposure is influenced by its nanoenabled properties. Although at present exposure to nanomaterials is considered highest for workers at production sites, with several products containing nanomaterials now in use, exposure via consumer products is exponentially increasing. This suggests that the hazard identification step of the risk assessment must include a detailed characterization of exposure, including the amount of materials released in the environment and their changing properties as they move through the life cycle chain (synthesis to disposal). Other factors that need to be carefully considered are dose metrics and target tissue. By virtue of their small size, it is demonstrated that many nanomaterials freely translocate to deeper regions within tissues and cells, accessing vesicles or organelles that are not accessed by bulk particles. Although translocation to other organs is expected to be low, several recent studies have shown that particles deposited in

lungs or the gastrointestinal tract are capable of translocating to other distant organs, where they may induce biological effects *(38–42)*. However, limitations in the methodologies currently available to track biodistribution and translocation make it nearly impossible to generate such information.

To summarize, the available data concerning the hazard potential of nanomaterials are inconclusive and often questionable, and the appropriate test methods or benchmarks for assessing the potential risks are not established. As discussed earlier, an integral part of assessment of the potential toxicological effects of nanomaterials is adequate characterization of nanomaterials through various life cycle stages from synthesis to exposure. Significant efforts have been made internationally to identify and validate measurement and characterization tools for nanomaterials *(43)*; however, at present, standardized or internationally harmonized protocols to carry out such measurements are not available, making it difficult to directly relate a specific biological response to a specific nanomaterial or its properties.

Dose–response metrics: challenges

Mass per volume or per body weight is a relevant dose metric for chemicals. However, for most nanomaterials, this seems to be a poor predictor of toxicity, owing to their diverse physicochemical properties and unique behaviour patterns *(37, 44)*. Total surface area and total particle number are suggested as appropriate dose metrics for most nanomaterials. For example, particle surface area and particle volume have been shown to better predict lung responses in rats or mice exposed to several nanomaterials of varying sizes. However, surface area or particle number cannot be used as universal parameters to report dose metrics since biological effects induced by some nanomaterials are not directly correlated to the total surface area or particle volume *(37, 44)*. At present, most studies report only the administered dose, ignoring important cellular uptake processes such as diffusion or sedimentation, which define the rate at which the nanomaterials become available for cellular uptake, their fate and transport within cells. To add complexity, the uptake, fate and transport of nanomaterials is heavily influenced by the particle and exposure medium characteristics, which must be accounted for in the dosimetry

report *(45–48)*. Traditionally, dose–response assessments have been conducted in animal models (mainly rodents). The extrapolation of the data thus derived to human scenarios is then carried out using several untested assumptions and applying uncertainty factors. However, the numbers of nanomaterials that require immediate assessment make it impossible to test each one of them using in vivo models. In vitro alternatives are available, but are not readily applicable to nanomaterials due to the issues discussed earlier, including dynamic and changing particle properties in different exposure systems, dose metrics, and their relationships with observed effects. Thus, a standardized approach to measuring nanomaterial properties is crucial in order to compare the results derived from various studies, which in turn will help identify material-specific dose metrics and allow read-across prediction of effects. Standardized parameters that can be applied to nanomaterials, a standardized classification system, and generation of toxicological data for a set of reference nanomaterials using standardized measurement methods may help tackle some of these issues.

Exposure assessment: challenges

Issues concerning the exposure assessment of nanomaterials relate back to the lack of appropriate metrics for quantifying occupational or consumer exposures, which stems from issues with finding appropriate exposure monitoring techniques *(49, 50)*. Adding to the complexity is the lack of sensitive methodologies to detect and differentiate nanomaterials from background noise and the lack of analytical characterization methodologies. A single nanomaterial may have several potential routes of exposure as it traverses through the environment and interacts with different media. Thus, a thorough investigation of exposure throughout the life cycle of a nanomaterial is needed. For consumer exposure, another problem is that there is hardly any obligation to label or register nanomaterials in consumer products. Manufacturers can label "nano" on the product, but this does not necessarily mean that nanomaterials are actually present in that product; also, a product without a "nano" label can contain nanomaterials. Information on concentrations of nanomaterials in products is also lacking. Furthermore, there are few human exposure models available that are validated for nanomaterials. Some, such as the worker exposure model Stoffenmanager nano and the consumer

exposure model ConsExpo nano, are currently under development. For the latter, a new version has recently been made publicly available.[7] Thus, several attempts are being made to accurately measure exposure; however, in its present state, lack of accurate exposure assessment poses the largest hurdle in HHRA of nanomaterials.

Risk characterization: challenges

Since there are very few mandatory requirements for labelling products that contain nanomaterials, it presents a significant challenge to measure realistic exposure levels and to relate actual exposure levels to adverse effects. Although comparative approaches are commonly used to characterize the risk posed by chemicals (comparison of nanomaterial properties and behaviour with those known for bulk materials of similar composition), these can only provide qualitative information, since derivation of quantitative risk estimates is hindered by gaps in the data on the distinct size-related properties attributed to nanomaterials.

While the issues related to nanomaterials are daunting, the scientific community is hopeful that the novel techniques that are being developed will permit quantitative measurement of the relationships between nanomaterial properties and associated toxicity. However, in the interim, less quantitative or qualitative ranking approaches can be applied to existing nanomaterials. To this end, several strategies have been proposed, which are outlined in the following sections.

Before implementing any strategy, basic questions must be asked as part of the problem formulation aspect of HHRA of nanomaterials. These include the following.

- What is the chemical composition of the nanomaterial?
- Are the nanomaterials sufficiently characterized; that is, are there sufficient data regarding particle size, surface coatings, and other physicochemical properties of nanomaterials?
- Has the nanomaterial been characterized in the exposure medium (for example, in relation to aggregation or agglomeration)?
- Are the nanomaterials readily internalized by living cells?

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[7] ConsExpo nano: www.consexponano.nl.

- Is there information available regarding their absorption, distribution, metabolism and excretion?
- Is there information regarding dose metrics?
- Who are the populations or individuals at risk?
- What further information is needed regarding product volume, potential application and uses, or behaviours through the life cycle? (For example, if a nanomaterial is coated, masking nanomaterial contact with biological samples, how durable is the coating?)
- What are the most plausible routes of exposure?
- What are the toxic effects and threshold dose?

Current major knowledge gaps with regard to both human health and environmental risk assessment are presented in Figure 7.2 *(51)*.

Human health risk assessment		Environmental risk assessment
Use and occurrence	**Definition: what are nano-materials?**	Use and occurrence
Exposure		Distribution in air, water and soil
	Properties: which physico-chemical characteristics are important?	
Uptake (inhalation, ingestion, skin)		Uptake in target species (inhalation, ingestion, skin)
	Techniques and tools: how are nanomaterials measured?	
Tissue distribution		Tissue and/or species distribution
	Behaviour: how do nanomaterials interact with their surroundings?	
Concentration at target organ		Concentration at target organ
	Transformation: how do nano-materials transform during the life cycle through different media?	
Dose with toxic effects		Toxic effect–correlation with exposure
Risk assessment		Risk assessment

Figure 7.2 Summary of current major knowledge gaps for risk assessment of nanomaterials
Source: Adapted from RIVM *(51)*.

Once these questions are answered satisfactorily, additional questions regarding the likelihood of exposure to these materials, their potential for adverse effects at those exposure levels, the nature or severity of risk, and the use of safety controls (personal protection equipment) to mitigate exposure and reduce hazard while handling the nanomaterials should be answered before recommending risk management measures. In the meantime, a precautionary approach should be exercised.

7.1.6 Conclusions: application of conventional risk assessment approach to nanomaterials

Attempts have been made to conduct risk assessments for nanomaterials (listed below) using approaches used for chemicals. These have revealed that a complete and quantitative risk assessment is not possible at present due to lack of or insufficient exposure and hazard data *(52–56)*. Moreover, hazard information currently available is generated from methodologies that are not validated for nanomaterials *(57)*. Although definitive conclusions cannot be reached, in the interim the available results are being used to support decision-making. The following are some examples of authoritative nanomaterial-specific risk assessments conducted, which are not complete due to lack of qualitative and quantitative data *(51)*. The examples are limited to a few first-generation nanomaterials.

- The Scientific Committee on Consumer Safety assessed a number of cosmetic ingredients that are in the nanoscale, such as carbon black (CI 77266), titanium dioxide (TiO_2) and zinc oxide. It was found that uptake of these ingredients through the dermal route was minimal and hence internal exposure was negligible. However, use of these ingredients in cosmetic products dispensed through spray bottles, potentially leading to exposure via the inhalation route, was not recommended *(58–64)*.
- The Scientific Committee on Existing and Newly Identified Health Risks (SCENIHR) reviewed the available information for nanosilver in the context of life cycle analysis, consumer and occupational exposure, absorption, distribution, metabolism and excretion (toxicokinetics), and toxicity to humans and environmental species. The results concluded that with the available information, an adverse effect associated with nanosilver exposure could not be identified, and a quantitative risk assessment could not therefore be conducted *(65)*.
- Nanosilver and TiO_2 are currently undergoing substance evaluation under REACH. Other nanomaterials such as silica (SiO_2) are widely used and the potential for human exposure to these materials is increasing. As a result, SiO_2 has been evaluated by the Scientific Committee on Consumer Safety *(66)* and is undergoing a substance evaluation under REACH.

- The European Food Safety Authority strategy for 2020 includes re-evaluation of the safety of established food additives, which will include nanoforms of the additives. This evaluation process is scheduled to be completed in 2020.

- The United States National Institute for Occupational Safety and Health *(67)* completed an HHRA for lung fibrosis induced by exposure to carbon nanotubes (CNTs) and carbon nanofibres. The study recommended an exposure limit of $1\mu g/m^3$ elemental carbon as a respirable mass 8-hour time-weighted average concentration. The study also noted that this concentration reflected the analytical limit of quantification, and thus exposure levels to CNTs should be kept below $1\mu g/m^3$. This study highlights additional limitations to quantitative risk assessment, which include the availability of sensitive analytical tools to detect nanomaterials.

Thus, for now, the challenges and knowledge gaps faced by risk assessors with regard to nanomaterials have been clearly identified *(50, 57, 68)*. There is also consensus among the agencies, researchers and regulators *(51, 52)* that the conventional risk assessment framework currently available for chemical substances should be critically evaluated and eventually adapted for application to nanomaterials.

7.2   Recent initiatives developing risk assessment approaches for nanomaterials

7.2.1   Introduction

As discussed in the previous chapters, nanomaterials exhibit versatile properties that influence their biological behaviour and their ultimate fate. At present, the relationship between those properties and the observed toxicity is not clear. Moreover, it is not clear if some of the physicochemical properties are more important than others. Therefore, a systematic evaluation of the toxicological potential of each nanomaterial on a case-by-case basis is encouraged. However, the number of nanomaterials that require immediate risk assessment, and the cost, time and experimental animals associated with extensive testing of each nanomaterial variant, make the case-by-case approach impractical. In the interim, the available information concerning physicochemical properties, exposure, and hazard potential can be used to inform prioritization, read-across or grouping approaches,

which will help guide the testing strategy. In this process of prioritization and grouping, fundamental questions – such as "How do we prioritize nanomaterials?" and "What is the basis for their grouping?" – also need to be addressed before they can be put into practice. Recently, the Government of Canada, as part of its ongoing assessments for phase 3 of its Chemicals Management Plan, has been developing an approach to address the legacy of nanomaterials already in commerce in Canada. In this approach, Health Canada and Environment and Climate Change Canada were tasked with assessing the risks of nanoforms of substances that are listed in the Domestic Substance List (existing substances). The nanoforms of these existing substances are not subject to risk assessments under the Canadian Environmental Protection Act, 1999 *(69)*. The approach taken by Health Canada and Environment and Climate Change Canada for prioritizing nanomaterials is shown in Figure 7.3. In brief, the approach consisted of three phases:

1. establishment of a list of existing nanomaterials in Canada;
2. from the phase 1 list, prioritization of existing nanomaterials for action;
3. action on substances identified for further work.

Phase 1 of the Chemicals Management Plan, which was initiated in 2015, involved a mandatory survey of a list of 206 substances on the Domestic Substance List with unique Chemical Abstracts Service (CAS) numbers that are expected to have a nano counterpart in commerce. Of the 206 substances, 53 were identified to be in commerce in Canada in the nano form (since 2014). These 53 nanomaterials were then prioritized for phase 3 action, and a report is being prepared giving consideration to these 53 substances and the prioritization factors applied for human health and environmental risk assessment *(70)*.

Thus, effective strategies to even prioritize nanomaterials for further consideration are urgently required. In addition, alternative risk screening tools, such as the Swiss precautionary matrix for synthetic nanomaterials and Stoffenmanager nano, are available. At present these tools are designed for industrial use, and the development of similar tools to assist regulators in their decision-making is on the horizon.
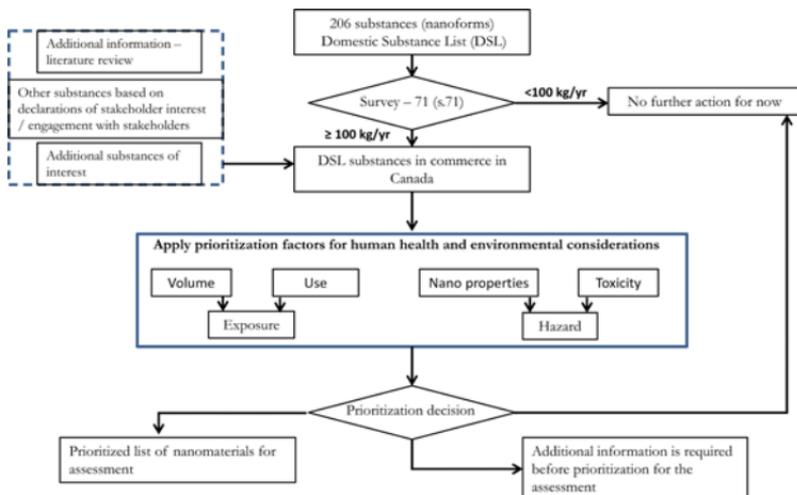
Figure 7.3 Overview of prioritization approach of Health Canada and Environment and Climate Change Canada

The suitability of risk assessment approaches for nanomaterials has been assessed by various international scientific organizations and committees, such as SCENIHR *(71–73)*, the United States Environmental Protection Agency *(4)* and the United Kingdom Department for Environment, Food and Rural Affairs (Defra) *(74)*. A summary of these analyses has been reported by Defra *(75)* and an updated list of various recent risk assessment initiatives and other supporting information is included in Table 7.3.

In a recent paper by Oomen et al. *(76)*, risk assessment frameworks for nanomaterials with respect to scope, link to regulations, applicability and outline for future directions have been reviewed. Some of the cited strategies will be explained in more detail in subsections 7.2.3 and 7.2.4.

7.2.2   *Risk assessment approach by SCENIHR*

SCENIHR *(72)* was the first body to describe a potential risk assessment approach for nanomaterials. SCENIHR suggested that identification of nanomaterials of interest and their detailed characterization were prerequisites for the success of an effective risk

Table 7.3 Summary of recent developments in risk assessment approaches for nanomaterials

Sources	Risk assessment strategy	Read-across and grouping approaches	Other supporting information
MARINA (Managing Risks of Nanomaterials)	Bos et al. *(77)*	Oomen et al. *(78)*	
GUIDEnano	S.W.P. Wijnhoven and P. Van Kesteren, personal communication	M. Park and G. Janer, personal communication	
Intelligent Testing Strategy (ITS)-Nano	Stone et al. *(79)*	Stone et al. *(79)*	Stone et al. *(79)*
NANoREG	Dekkers et al. *(80)*		
SUN (Sustainable Nanotechnologies)	Malsch et al. *(81)*		
ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals)		Arts et al. *(82–84)*	
European Chemicals Agency		European Chemicals Agency *(85)* European Chemicals Agency, Joint Research Centre, and National Institute for Public Health and the Environment (RIVM) *(86)*	European Commission *(87)* Hankin et al. *(88)* Aitken et al. *(89)* European Chemicals Agency *(90–92)*
OECD	OECD *(10)*	OECD *(93)*	OECD *(94)*
SCENIHR	SCENIHR *(73)*		SCENIHR *(72)*
RIVM and Arcadis		Sellers et al. *(95)*	
Health Canada	Labib et al. *(96)*		AOP and mechanism-driven approaches

Source: Based on Dekkers et al. *(80)*.

Stage 1	Stage 2	Stage 3	Stage 4
Life cycle analysis (synthesis to disposal and during recycling), evaluation of potential exposure to human or environmental receptors at different life cycle stages	Characterize the nature, level and duration of any exposure; identify potential for uptake by human or environmental receptors	Identify the hazardous properties of any form of nanomaterial (including the transformed forms) to which significant exposure is likely using tiered in vitro/in vivo models	Characterization of dose response; assessment of risk

Figure 7.4 SCENIHR four-stage process
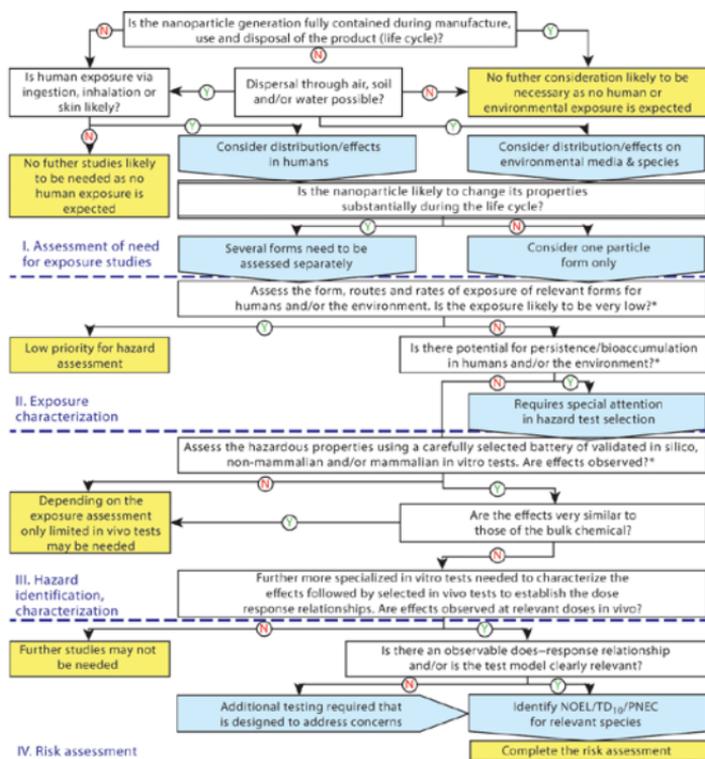
*Compare against the risk assessment for appropriate well studied nanomaterials. Appropriate for benchmarking purposes.

Figure 7.5 Stage approach for identification of the human and environmental risks from nanomaterials
Source: Adapted from SCENIHR *(72)*.

assessment paradigm for nanomaterials. Human and environmental risks from exposure to the specific nanomaterial would then be evaluated in a four-stage process (Figures 7.4 and 7.5).

In a tiered or incremental nanomaterial risk assessment process, the four stages as suggested by SCENIHR refer to the following. In stages 1 and 2, the focus is on nanomaterial exposure. If no human or environmental exposure is expected or if exposure is expected to be very low, the process stops or the nanomaterial is considered a low priority for hazard assessment. Stage 3 focuses on careful selection of in silico and in vitro models for hazard testing. If the results are positive, either limited in vivo testing (when effects are very similar to those of the bulk chemical) or in-depth specialized in vitro testing followed by in vivo testing (if effects are different from those of the bulk chemical or if an effect is observed that was previously not known) is recommended. The data generated from the first three stages are used to derive exposure and dose–response and to assess the risk. SCENIHR, however, does not provide in this proposal details on the kind of in vitro or in vivo testing to be performed. It is implied that the route of exposure and potential application may guide the choice of toxicity models for testing. In 2009, SCENIHR concluded, in its opinion on the risk assessment of products of nanotechnologies, that "this framework remains appropriate although a few further details can be added in the light of recent publications" *(73)*.

7.2.3 Summary of risk assessment approaches developed in the recent European Union FP7 projects

Under the European Union FP7 MARINA project, a hypothetical framework for risk assessment of nanomaterials has been developed *(77)*. The strategy involves data generation in two phases: phase 1, problem framing; and phase 2, risk assessment (Figure 7.6). Identification of the relevant exposure scenario at different stages of the life cycle takes the central stage in this strategy, driving the prioritization, ranking and risk characterization decisions**.**

The main goal of the problem framing phase is to set the scope of the risk assessment and to identify the relevant exposure scenario for each step in the life cycle of a nanomaterial. The risk assessment phase (phase 2) is a stepwise, iterative process comprising identification of data gaps from the perspective of risk assessment, choosing the appropriate
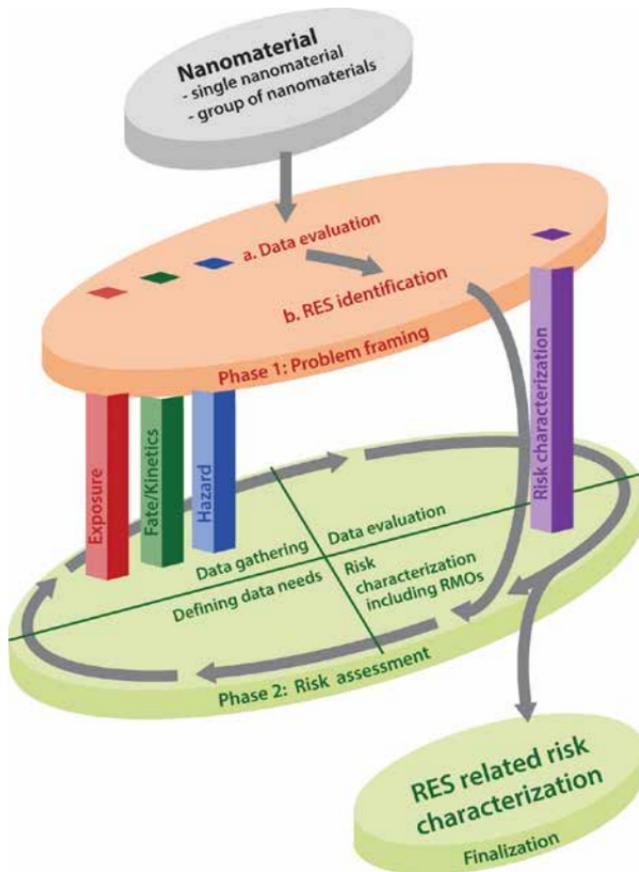
Figure 7.6 MARINA framework for risk assessment of nanomaterials
RES = relevant exposure scenarios.
RMOs = risk management options.
Source: Adapted from Bos et al. *(77)*.

tools for data generation to fill in the gaps, generation and collection of the data, and performance of a risk characterization (human health or ecological, according to the user's objectives) *(76, 77)*.

In the Intelligent Testing Strategy (ITS)-Nano project, a survey was conducted to collect opinions from more than 80 stakeholders to identify research priorities that would enable effective intelligent

risk evaluation strategies for nanomaterials. The main overarching, cross-cutting priorities identified were as follows: availability of standardized nanomaterials for testing; standardization of methods for characterizing nanomaterials throughout the life cycle and as pertinent to different risk assessment steps; defining important properties that are likely to change over the life cycle of a nanomaterial and those that are detrimental; and characterizing the exposure in the context of dose delivered and bioavailable dose. This framework highlights not only priority research needs, but also how each of those priorities can be addressed *(79)*.

For NANoREG, the focus was to develop harmonized approaches to conduct risk assessment of nanomaterials. Taking into consideration the questions and needs of regulatory communities and legislators, NANoREG proposes a strategy that identifies the situations under which the nanospecific grouping, read-across and quantitative structure–activity relationship (QSAR) tools are applicable, or where data gaps exist. This is the first project to recommend the types of data or end-points to consider for any nanomaterial requiring risk assessment. In the proposed flowchart for risk assessment of nanomaterials *(80)*, six overlapping elements have been identified as the most important nanospecific determinants: exposure potential, dissolution, nanomaterial transformation, accumulation, genotoxicity and immunotoxicity.

The main objectives of the proposed strategy *(80)* are as follows:

- to prioritize those applications that have the highest potential to cause human health effects;
- to identify the most important information needed to address the nanospecific issues within the risk assessment.

In another European project – GUIDEnano – a web-based tool is being developed, with a primary focus on providing guidance to developers of nanoenabled products on safe design, product-specific risk assessment, and risk mitigation. This approach will also develop tools to identify hot spots for release of nanomaterials and decision trees to provide guidance on the uses of (computational) exposure models, and, when necessary, support design of cost-effective strategies for

experimental exposure assessment.[8] Specific publications on this tool are currently in preparation and will be published soon.

In addition to these projects, two recent reports conducted risk assessment of TiO_2 and SiO_2 in food, based on internal organ concentrations (instead of external concentrations) and using a kinetic model taking into account accumulation of nanomaterials in the body over time *(97, 98)*.

7.2.4 *Read-across approaches for identifying the risk of nanomaterials*

In general, application of grouping of substances and read-across between substances is recognized as a valuable approach in regulatory frameworks, for example to fill potential data gaps in the hazard characterization, based on availability of adequate data from similar substances. It is expected that for nanomaterials, grouping and read-across approaches will be an important means of addressing identified data gaps. Due to the numerous possible nanoforms with the same chemical identity (that is, covered by the same registration dossier or CAS number) but with differences regarding other physicochemical properties (surface modification, size distribution or particle shape), there is a need for alternative approaches that would allow predicting hazardous properties by implementing read-across between nanoforms or from non-nanoforms to nanoforms of the same substance to minimize testing, in particular on animals.

For nanomaterials, industry (ECETOC) *(82–84)* and Member States *(95)*, as well as several research projects in FP7 (for example MARINA *(78)*, NANoREG and GUIDEnano) and individual research groups *(99, 100)*, have proposed methodologies for grouping and read-across of nanomaterials through case studies. Also, the OECD guidance on grouping of chemicals *(101, 102)* mentions nanomaterials, though no concrete guidance is provided for nanomaterials in that document.

Based on the ideas developed in the former approaches, the European Chemicals Agency, together with RIVM and the Joint Research Centre, has recently developed a new approach for

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[8] GUIDEnano: http://www.guidenano.eu/.
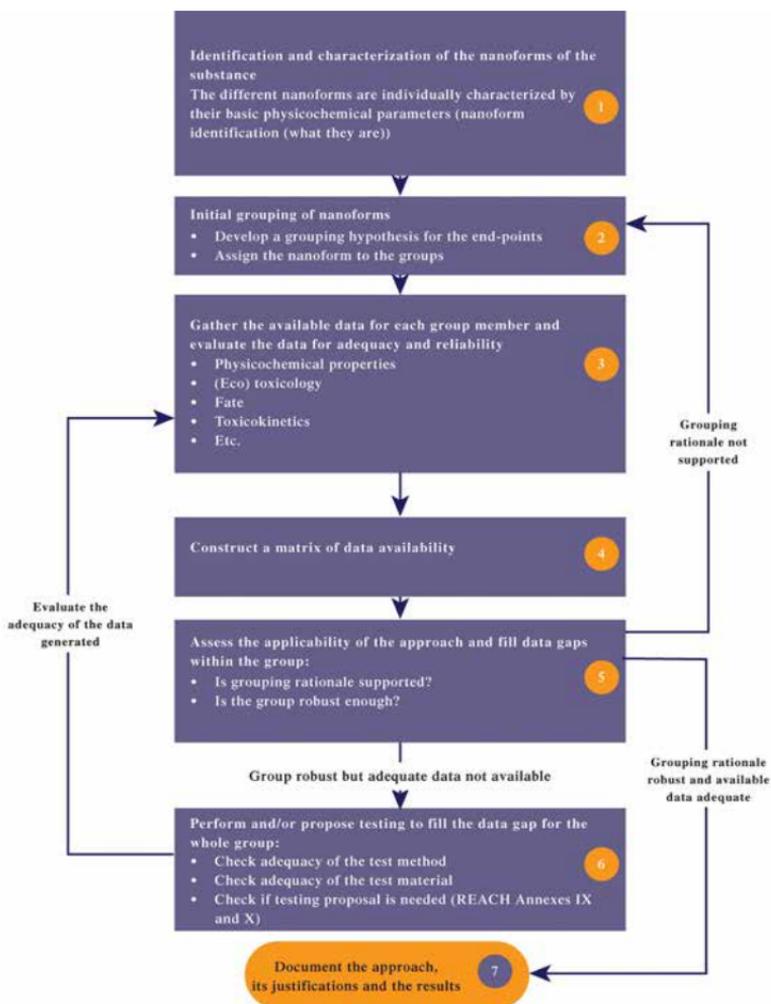
Figure 7.7 Proposed strategy or stepwise procedure for using data between (nano)forms
Source: Adapted from European Chemicals Agency, Joint Research Centre, and RIVM *(86)*.

read-across that aims to illustrate a number of core principles, or elements to consider, which are based on the current, albeit incomplete, scientific understanding *(86)* (Figure 7.7). This has led to a structured approach to guide registrants and regulators on how to apply grouping and read-across concepts to nanoforms within regulatory frameworks such as REACH. Based on this working document, the European Chemicals Agency has recently published guidance on QSARs and grouping of chemicals *(92)*.

Apart from the above, approaches to classification of nanomaterials in order to estimate their potential risks have been proposed. These include stochastic multicriteria acceptability analysis *(103)*, weight of evidence *(104)*, grouping *(82, 84)*, and Bayesian networks *(105, 106)*. In addition, an approach has been outlined to combine the heterogeneous data available from all the above methods and various sources *(19)*. Thus, tremendous efforts are being made to determine the best risk assessment and management strategies. Elements emphasized across the various initiatives include the need for detailed physicochemical characterization of materials in their dry and aqueous states, before and after exposure, at different stages of the life cycle, supported by analysis of bioavailable dose and material kinetics. However, there is no harmonized agreement on the types of toxicity end-points to be included or the toxicity models used.

7.3   Nanomaterial immunotoxicity risk assessment

7.3.1   Summary of existing immunotoxicity risk assessment approaches for chemicals and pharmaceuticals

As described in the previous chapters, nanomaterials that gain access to the body through various portals of entry can interact with immune cells and tissues and have the potential to induce a variety of immunotoxic effects, as demonstrated via testing in animal and cell culture models. Collectively, a growing body of research supports the conclusion that adverse effects on the immune system are a relevant consideration in nanomaterial risk assessment (as exemplified in the previous chapters). Immunotoxicity testing guidelines and methodologies developed for pharmaceuticals, cosmetics, agrochemicals, food additives, industrial chemicals and environmental chemical contaminants can inform approaches for nanomaterial immunotoxicity risk assessment. Table 7.4 summarizes guidances and

Table 7.4 Immunotoxicity guidances and testing approaches for chemical and pharmaceutical risk assessment

Document and reference	Description
Preclinical safety evaluation of biotechnology-derived pharmaceuticals: S6(R1). ICH harmonised tripartite guideline, 2011 *(110)*	Guidance for assessing immunostimulation and immunotoxicity assessment of biotechnology-derived pharmaceuticals. Recommendations include immunogenicity assessment (measurement of antibodies) when conducting repeated dose studies; immunotoxicity, including stimulation or suppression of cellular and humoral immunity, inflammatory reactions at the site of administration, and surface antigen expression on target cells; routine tiered testing or standardized batteries not recommended.
Immunotoxicity studies for human pharmaceuticals: S8. ICH harmonised tripartite guideline, 2005 *(107)*	Guidance for assessing immunosuppression or enhancement by human pharmaceuticals. Recommendations on nonclinical (rodent) testing to identify potential immunotoxins and guidance on a "weight of evidence decision-making approach" for immunotoxicity testing; recommends consideration of data from standard toxicity studies; if warranted additional immunotoxicity studies may include a T cell-dependent antibody response (TDAR) assay, immunophenotyping, NK cell activity, host resistance, macrophage/neutrophil function, or assays of cell-mediated immunity; standardized approaches for respiratory or systemic allergenicity or drug-specific autoimmunity not recommended due to lack of availability; well-standardized methods for skin sensitization testing not included in this guidance.
Principles and methods for assessing direct immunotoxicity associated with exposure to chemicals. International Programme on Chemical Safety (IPCS) Environmental Health Criteria monograph 180 *(111)*	Overview of pre-1996 methods for assessing immune status, primarily immunosuppression, inflammation and autoimmunity. The document also summarizes early studies validating immunosuppression assays for immunotoxicity risk assessment.
Principles and methods for assessing allergic hypersensitization associated with exposure to chemicals. IPCS Environmental Health Criteria monograph 212 *(112)*	Overview of allergic disease, clinical aspects, epidemiology and experimental models. Highlights lack of exposure and hazard data for use in assessing allergic disease risks associated with chemical exposure.

Table 7.4 (Contd)

Document and reference	Description
Principles and methods for assessing autoimmunity associated with exposure to chemicals. IPCS Environmental Health Criteria monograph 236 *(113)*	Overview of autoimmune disease, clinical aspects, epidemiology and experimental models. The document also highlights exposure, mode of action, susceptibility and other issues, concluding that much of the information needed to address the risk of chemical-induced autoimmune diseases is not available.
Guidance for immunotoxicity risk assessment for chemicals. IPCS Harmonization Project Document No. 10 *(108)*	Framework for chemical immunotoxicity risk assessment, with schematics for weight of evidence approaches, for the major categories of potential immune system–chemical interactions: direct immunosuppression, direct immunostimulation, immune sensitization and allergy, and autoimmunity and autoimmune disease.
OECD guidelines for the testing of chemicals, section 4: health effects *(114)* Test No. 443: extended one-generation reproductive toxicity study *(115)* Test No. 407: repeated dose 28-day oral toxicity study in rodents *(116)* Test No. 409: repeated dose 90-day oral toxicity study in non-rodents *(117)*	Test guidelines for assessing chemical toxicity using animal models, primarily rats and mice, including oral, inhalation and dermal exposure routes. Most guidelines for repeated dose studies (28 days or longer) incorporate descriptive end-points relevant to immunotoxicity, including total and differential leucocyte counts, serum albumin and globulin levels, spleen and thymus weights and immune tissue histopathology. Test guideline No. 443 includes an optional F1 cohort for a TDAR assay.
EPA Harmonized Test Guideline: OPPTS 870.7800. Immunotoxicity *(109)* A retrospective analysis of the immunotoxicity studies (OCSPP Test Guideline No. 870.7800) *(118)* Boverhof et al. *(119)*	Test guideline for assessing immunosuppression due to repeated dose chemical (pesticide) exposure using rodents; includes descriptive parameters (haematology, lymphoid organ weights, histopathology) from standard rodent toxicology studies; includes functional assay (TDAR), lymphocyte phenotypic analyses.
EPA Series 870: health effects test guidelines *(120)*	Test guidelines for oral, dermal, inhalation, developmental and other toxicity studies, ranging from acute to chronic. Most incorporate basic immune parameters, including haematology, clinical end-points and optional histopathology of immune tissues that may provide support for more focused immunotoxicity studies (OPPTS 870.7800).

Document and reference	Description
United States Food and Drug Administration. Guidance for industry and other stakeholders: toxicological principles for the safety assessment of food ingredients (Redbook 2000) *(121)*	Review of toxicological information submitted in support of the safety of food ingredients. Includes guidelines for immunotoxicity studies.
United States National Toxicology Program. General Methods for Immunotoxicology Studies *(122)*	Outlines general methods for immunomodulation testing in mice, and methods for hypersensitivity testing.

methodologies that are widely used for immunotoxicity testing. While some focus specifically on immunotoxicity assessment *(107–109)*, others incorporate selected immune parameters in protocols designed to characterize general toxicity for various routes and durations of exposure. These include OECD test guidelines under Section 4 (health effects) and the United States Environmental Protection Agency test guidelines in Series 870 (health effects). Basic immune parameters incorporated in these protocols usually include total and differential leucocyte counts, serum albumin and globulin levels, spleen and thymus weight, and optional immune tissue histopathological assessment, which may include spleen, thymus, and selected lymph nodes relevant to the route of exposure. Significant changes in such end-points, which are largely observational, may serve as triggers for further characterization of immunotoxicity using a functional immune assay such as a T cell-dependent antibody response (TDAR).

7.3.2 Levels of evidence for concluding on immune system toxicity

Based on the results of individual immunotoxicity studies of chemicals and other test substances, the United States National Toxicology Program *(123)* established a level of evidence system that was adopted by the World Health Organization *(108)* to accurately interpret and conclude whether a test substance should be considered immunotoxic or not. Five categories (clear evidence, some evidence, equivocal evidence, no evidence, inadequate evidence of toxicity to the immune system) are defined by the type and magnitude of effects on immune parameters. According to these criteria, dose-related effects on functional immune parameters provide the strongest

evidence of immunotoxicity and are required to demonstrate positive (clear or some) evidence of immunotoxicity. In the context of nanomaterials, a requirement for evidence of a functional change in immunity to indicate clear evidence of immunotoxicity hazard is applicable, even if the means by which this is established diverges from those used for other test substances as methods for nanomaterial hazard characterization evolve.

7.3.3   *Nanomaterial immunotoxicity risk assessment*

The adverse effects of any regulated chemical or product on the immune system are assessed as part of hazard identification according to the guidelines and regulations that govern the specific chemical or product. Immunotoxicity is one potential hazard that has been addressed, to varying degrees, by international scientific organizations and committees in their considerations of nanomaterial risk assessment approaches and methodologies (see Table 7.3 for a summary of various nanomaterial risk assessment initiatives). Furthermore, immunotoxicity is identified as one of the six elements considered in the test strategy for the identification of potential risk of a nanomaterial *(80)*. Although there is no formal consensus, the overall HHRA concepts for chemicals are generally considered to be applicable for nanomaterials. Likewise, within current risk assessment frameworks, hazard characterization methodologies and approaches are generally considered to be appropriate for nanomaterials. Implicit in this statement is the recognition that issues relevant to nanomaterial risk assessment in general are also relevant to nanomaterial immunotoxicity risk assessment. These include uncertainties related to detecting nanomaterials and characterizing their physicochemical characteristics in biological matrices, during transport and uptake into tissues, and at their active sites in cells, and other issues related to conducting and interpreting nanomaterial animal studies.

Similar to chemicals, prior to initiating risk assessment for nanomaterial-induced immunotoxicity, it is important to understand whether or not immunotoxicity tests should be performed and the types of immunotoxicity (immunostimulation, immunosuppression, autoimmunity, sensitization) to be considered. The list of entry points suggested for chemicals (IPCS) are applicable to nanomaterials. However, only a small number of effects, such as changed cytokine levels, increased incidences of inflammation or increased

inflammatory markers, have been extensively characterized for nanomaterials. Studies reporting on other immunotoxic observations, including blood counts, changes in organ weights or histopathology of immune-related organs, are scarce.

Human clinical studies on nanomaterials

While human epidemiology studies, clinical studies and case studies demonstrating immune suppression, immune stimulation, sensitization or autoimmunity due to nanomaterial exposure provide critical evidence for nanomaterial risk assessment, there is currently limited availability of such studies. As outlined above, the limitations in the availability of these studies for nanomaterial immunotoxicity risk assessment are similar to their limitations for use in establishing toxicity to any other organ system, namely their small number, use of descriptive parameters to assess functionality, and experimental design limitations that challenge the discernment of association and causality.

Animal-based and alternative studies

Animal model-based regulatory guidelines for assessing immunotoxicity, summarized in Table 7.4, have not yet been widely applied or validated for nanomaterials, though the OECD health effects test guidelines have been assessed for applicability in nanomaterial hazard characterization *(124)*. Test guidelines for dermal, oral and inhalation routes of exposure, all applicable to nanomaterials, were considered. Some gaps and issues identified were relevant to nanomaterial immunotoxicity hazard characterization. The extent to which immune parameters are assessed in repeated dose 28- and 90-day rodent toxicity studies varies according to the route of exposure. For the oral exposure route, test guidelines 407 (28-day) and 409 (90-day) have been updated to include additional observational immune end-points (as described in Table 7.4 above). Test guidelines 412 and 413 for repeated dose inhalation studies of similar durations *(125, 126)* have recently been revised to accommodate testing of nanomaterials, including some further immune parameters. Since inhalation is a potential route of human exposure to nanomaterials, the OECD Working Party on Manufactured Nanomaterials separately considered inhalation toxicity testing protocols for nanomaterials

(127). A key recommendation of the Working Party on Manufactured Nanomaterials with relevance to nanomaterial immunotoxicity was mandatory bronchoalveolar lavage fluid (BALF) analysis to assess changes in total and percentage inflammatory cell populations (see subsection 6.7.2).

An earlier review of nanomaterial risk assessment methodologies by SCENIHR *(72)* also identified nanomaterial-specific concerns related to inflammation and immunotoxicity end-points in animal studies. Although inflammation is a protective response to cellular insult and not necessarily indicative of a direct effect on adaptive immunity, inflammasome activation due to particulate exposure has been linked to adjuvanticity and exacerbation of responses to allergens in atopic individuals. The potential for inappropriate immune responses due to airway, as well as dermal, nanomaterial exposure was raised by SCENIHR as a question under immunotoxicity hazard assessment. Similar to the OECD test guideline review, BALF assessment was recommended for airway exposure guidelines as a means of detecting inflammatory responses to nanomaterials *(72)*. Among the OECD guidelines available for skin and eye, the local lymph node assay (test guideline 429) was considered to be appropriate for investigating nanomaterial skin sensitization potential, as well as advantageous with respect to animal welfare considerations and quantity of well characterized test chemical required *(128)*.

Few studies have examined nanomaterials using functional assays that have been widely used, standardized or validated for immunotoxicity risk assessment. TDAR assays have been used to demonstrate significant dose-dependent adverse or beneficial effects of nanomaterials on immune function. More often, a TDAR assay is used to demonstrate that a substance in nano form can modulate immunotoxicity due to a primary chemical treatment. For example, nanocurcumin ameliorated arsenic-induced suppression of rat IgG responses to keyhole limpet haemocyanin (KLH), and chromium(III) nanoparticles enhanced anti-sheep red blood cell IgG responses in heat-stressed rats *(129, 130)*. Rarely to date has a TDAR been used to demonstrate a direct adverse effect of nanomaterials on immune responses. In one study, intravenous exposure to nanosilver for 28 days increased KLH-specific IgG and significantly altered a number of descriptive parameters, providing support for the plausibility

of applying "standard" regulatory immunotoxicity models to nanomaterial hazard characterization *(131)*. Other functional assays used for regulatory purposes include host resistance assays and sensitization assays *(132, 133)*.

7.4 New risk assessment approaches for nanomaterials and links with immunotoxic substances: conclusions

There appears to be general agreement that parameters indicative of immunotoxicity should be included in nanomaterial hazard characterization, at the very least by assessing observational parameters that are already incorporated into OECD health effects and other standardized test guidelines. Savolainen et al. *(50)* proposed a tiered risk assessment approach for well characterized engineered nanomaterials in which physicochemical and acellular testing would be conducted in tier I, in vitro testing in tier II, in vivo testing in tier III, and carcinogenicity and reproduction studies in tier IV, in which immunotoxicity assessment is incorporated as a tier II and a tier III end-point.

In the proposed MARINA risk assessment strategy for nanomaterials, immunotoxicity data are considered part of the minimum information required for data evaluation in phase 1, which was described as the problem framing phase consisting of data evaluation and relevant exposure scenario identification *(77)*. Furthermore, in the recently developed risk assessment strategy of the NANoREG project, which is based on six different nanospecific elements, immunotoxicity is identified as one of these six elements. These elements are the basis of the test strategy for the identification of potential risk of a nanomaterial *(80)*.

Given the complexity associated with risk assessment of nanomaterials, a weight of evidence approach may be better suited, which generally refers to a method or methods for summarizing and interpreting all available scientific evidence on health effects, including associated uncertainties, in the hazard identification step. The process is adaptable, includes expert opinion and professional judgement, and is often used when assessing complicated systems that require integrating diverse evidence *(134, 135)*. Despite the ubiquity of the term "weight of evidence" in the regulatory context,

it may be used to refer to distinctly different methodological approaches, ranging from simple evidential summary to complex frameworks for quantitative weighting and scoring of the available evidence. Examples of the latter have been described for ranking and screening nanomaterial hazard *(135–137)*. In the context of chemical immunotoxicity risk assessment, weight of evidence criteria for immunotoxicity, immunostimulation, sensitization and autoimmunity have been described *(108)*. Taken together, a quantitative weight of evidence approach for nanomaterial immunotoxicity risk assessment within the context of nanomaterial hazard assessment is feasible.

In conclusion, a validated risk assessment framework for immunotoxicity by nanomaterials does not exist; however, with the vast amount of data available and the ongoing risk assessment initiatives, the gaps in knowledge and limitations in the approaches have been identified. Research priorities that can generate the types of missing data have also been identified. Considering the potential nanomaterial variants and lack of data, difficulties are to be expected in categorization, grouping or binning tasks. Use of standardized, well characterized materials, standardized test methods and biological end-points may help compare toxicity across nanomaterials. An intelligent, mechanism-based, tiered testing strategy, taking into account various questions related to the four fundamental steps of risk assessment, will expedite the process without hampering the safe use of nanomaterials in many applications.

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8. FUTURE RESEARCH

There have been important developments in the fields of molecular biology, bioinformatics and toxicology in general, which nanotoxicology stands to benefit from. Some of the advances made, including new technologies and novel toxicity testing paradigms, are discussed below in the context of their applicability to nanotoxicology.

8.1   Emerging toxicology paradigm

As exemplified in the previous chapters, toxicology relies heavily on animal-based bioassays to evaluate toxicity induced by chemicals, including nanomaterials. While animal testing is a gold standard for regulatory acceptance, the approach lacks the capacity to generate the quantitative toxicity data required for the assessment of several classes of nanomaterials and thousands of variants of each nanomaterial class that require immediate testing. The issues associated with the use of assays based on single end-points apply to other areas of regulatory toxicology as well; for example, it is evident that animal-reliant approaches to generate data for the risk assessment of thousands of chemicals already in commerce, and thousands that are added to the registry every year in a timely manner, are largely ineffective due to the laboriousness of the approach. Thus, a call for a transformative shift in the toxicity testing paradigm was made in 2007 by the National Research Council of the National Academies, United States of America *(1)*. In the paradigm-shifting report *Toxicity testing in the 21st century: a vision and a strategy*, the National Research Council called for reduced whole-animal testing by transitioning from animal-based toxicity assessment methods to mechanistically based in vitro human cell culture assays. It was hoped that these targeted in vitro assays, apart from being rapid, affordable and aligned with the 3R principles of ethical use of animals in testing (replacement, reduction and refinement), would enable development of predictive toxicological tools leading to rapid screening of a large number of chemicals, including nanomaterials, for their toxic potential. This would enable prioritization and categorization of the chemicals for further toxicological evaluation. However, it was soon realized that such a paradigm shift would require thorough knowledge of the mode of action of the chemicals being tested. This knowledge is not

available at present for all substances including nanomaterials. High-content technology and global-scale screening of genes, proteins and metabolites using omics tools has considerably increased the knowledge of diverse pathways or processes perturbed by the chemicals that were previously unknown. However, the application of this vast data repertoire to derive meaningful information to support targeted mechanism-based assays for regulatory decision-making has not been realized so far.

8.2 Global molecular screening to identify perturbed toxicity pathways, biological functions and processes

Regardless of the approach taken, the important question of what toxicity end-points should be prioritized forms the basis of the hazard assessment of nanomaterials. The development of a comprehensive array of in vitro toxicity tests will require a detailed understanding of the intricate networking and interactions occurring at the molecular and cellular level, toxicity pathway responses, and molecular perturbations relevant to human biology. Such understanding cannot be derived from assays based on single end-points. A systems biology approach (Figure 8.1) combining computational modelling, bioinformatics tools and quantitative molecular biology techniques will play an important role in revealing those complex biological interactions *(2)*. The ability already exists to systematically and quantitatively measure the genome-wide changes occurring in a specific biological system (genomics, transcriptomics, proteomics, metabolomics, lipidomics, metagenomics), leading to a vast knowledge of the changes in the expression of molecular entities (genes, proteins, small biomolecules and associated pathways) *(2, 3)*. With advances in bioinformatics, the relationships shared between the molecular perturbations and the toxicological effects are beginning to be unravelled *(4, 5)*. Although this approach is widely used in medical and pharmaceutical research, its use in toxicology and specifically in nanotoxicology is still modest. It will require a large data repertoire, an efficient data visualization and analysis framework, and extensive computational power to reduce and synthesize the data in a biologically meaningful manner for its application to predictive science. In silico tools for data analysis, data transformation, machine learning and computational modelling are an absolute prerequisite for the successful implementation of this new

toxicology paradigm. The resulting details on the perturbed system at the different levels of biological organization can then be used to inform the specific in vitro or in chemico models, and end-points to be used in the assessment.

As illustrated in Figure 8.1, development of an effective safety assessment strategy for nanomaterials will require cross-disciplinary collaboration and integration of data from various sources, enabling a comprehensive understanding of material properties and behaviours at different levels of biological organization.
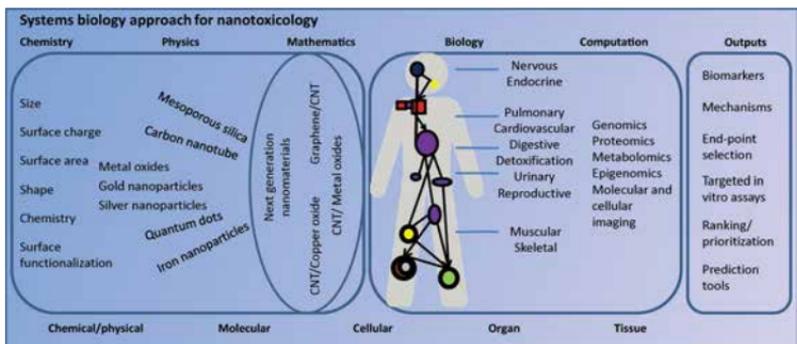
Figure 8.1 Systems biology approach for nanotoxicology
Source: Modified from Halappanavar et al. *(2)*.

Of the many omics systems, genome-scale gene expression analysis and mapping of biological pathways have been the mainstays, and associated methodologies are well established and validated for research. Although limited in the context of nanotoxicology, the studies that have used genomics tools have shown that gene expression data can be used in the following areas:

- to qualitatively relate changes elicited by a single ENM or different classes of ENM *(6)*;
- to characterize the mechanistic nature of the response to individual ENMs *(7–11)*;
- to identify sets of genes or gene signatures as qualitative markers of exposure to or adverse effects from exposure to ENMs *(12, 13)*;

- to identify and prioritize perturbated biological pathways and processes that correlate with adverse effects *(5)*;
- to identify those ENMs that are harmful, as opposed to those that are innocuous *(6, 14)*.

Some of these advances have obtained regulatory acceptance for incorporation in the routine testing of chemicals as weight of evidence tools. In addition, recent progress in gene expression analysis has enabled derivation of gene- or pathway-based dose–response curves, mathematical modelling *(5)*, and points of departure calculation for the purpose of conducting human health risk assessment *(15–18)*.

While multiplex microarrays are the predominant platforms used in this type of global-scale analysis, pathway-specific quantitative PCR arrays querying a targeted subset of genes are also extensively used to assess chemical-induced hazards. These methods have been applied to understanding nanomaterial-induced toxicity in both in vitro and in vivo models *(19)*. So far, there have been no reports concerning the interference of nanomaterials with RNA isolation, complementary DNA preparation, dye labelling, microarray hybridization or quantification of dye signal intensity. However, care should be taken in isolating RNA from cells or tissues. In general, the established methodologies currently in use for RNA isolation are capable of efficiently removing the nanomaterials and thus do not face interference from nanomaterials at the subsequent steps leading to dye labelling and microarray hybridization.

There are very few studies that have used proteomics *(20)*, another widely used method in toxicology to catalogue global expression changes at the protein level. Although the approach is used to investigate the mode of action of nanomaterials *(21)*, compared to the genome, the chemical composition of the proteome is highly dynamic, and standardized protocols for protein separation and isolation, identification, and quantification are still emerging. Moreover, the additional layers of regulation involving post-translation modifications of proteins make it difficult to effectively interpret the results. Other high-throughput but low-density platforms, such as bead arrays, that investigate several cytokines, chemokines and growth factors have become popular and have been applied to investigating the inflammatory potential of nanomaterials *(22)* and

determining the specific mechanisms involved in the inflammation *(23)*. Nanomaterials have been shown to interfere with colorimetric and optometric methods involved in quantification of proteins (Bradford assay) and ELISA reading. Centrifugation of protein lysates to remove left-over nanomaterials has been shown to reduce the assay interference.[9] Until the proteomic methods are further validated, the proteomics results can only be used to support the gene expression results. Other global analysis platforms include microRNA profiling and methylation profiling. However, very few studies have applied these tools to understanding the toxicity induced by nanomaterials *(11, 24, 25)*, and the resulting data are used to generate research hypotheses or to support the findings of the apical end-points and understand the gene expression results.

Thus, global profiling of biomolecule expression following exposure to nanomaterials will play an important role in determining the toxicity mechanisms, pathways and processes involved, and will enable correct selection of in vitro cell systems, identification of important toxicity end-points, and development of novel toxicity assays (see Figure 8.1).

8.3   Mode of action and adverse outcome pathways

Through the use of omics technology, hundreds of toxicity pathways have been identified for a variety of nanomaterials in experimental animals or in vitro cell culture models *(3–6, 12)*. However, the relationships among these various pathways or networks of pathways that eventually become toxicity pathways leading to adverse outcomes or disease progression are yet to be firmly established. In this context, the adverse outcome pathway (AOP) tool was developed by the OECD to support the new paradigm *(1)* that is reliant on the underlying mechanisms rather than the direct toxicity observations. AOP is a conceptual framework consisting of sequential biological events that occur with a molecular initiating event – an initial interaction of a toxicant or a chemical substance with cellular biomolecules – and progressing through several key biological events that are interdependent and occur at various levels of biological organization, eventually resulting in a disease or adverse

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[9] Halappanavar et al., unpublished data.

outcome *(4, 26)*. The molecular initiating events and key biological events describe the toxicologically relevant events that are essential for the disease progression and are measurable. Such organized representation of the perturbed biology enables development of integrated testing strategies consisting of a combination of in vitro cell culture models and in silico computational approaches to predict in vivo toxicity and support risk assessment *(27)*. While only a handful of AOPs have been developed so far that demonstrate their potential application in chemical risk assessment, the AOP for skin sensitization initiated by covalent binding to proteins, the first AOP to be developed, reviewed and approved by the OECD *(27)*, has successfully shown that well constructed and quantitative AOPs can enable development of mechanistically driven in vitro methods that can reduce reliance on animal testing and help screen a vast number of substances for their potential to induce toxicity. As a result of this AOP, a defined approach has been proposed involving a combination of in vitro, in chemico and in silico methods in an integrated assessment and testing approaches framework to assessing the risk of skin sensitization without animal testing.

While putative AOPs for nanomaterial-induced toxicity are beginning to emerge *(5, 28, 29)*, quantitative AOPs are yet to be established for nanomaterials. Hence their implementation in the mechanism-based ENM screening strategy has not yet been achieved. In the context of nanomaterial-induced immunotoxicity testing, the systematic organization of the existing scientific information concerning the known immunotoxicity potential of nanomaterials in an AOP framework will help to identify data gaps, formulate novel and appropriate research questions, and design alternative in vitro predictive assays and testing strategies. Although still in its formative stages, incorporation of this concept in the routine investigation of nanomaterial-induced toxicity may be essential for the effectiveness of the predictive and quantitative strategies of chemical risk assessment.

8.4   High-throughput screening

High-throughput screening refers to industrial scale screening for "positive hits", the chemical compounds that display significant biological activity in a specific toxicity screen. Originally designed for drug target identification, high-throughput screening has turned into

a field of its own dedicated to the design of toxicological assays that are optimized for speed, efficiency, high throughput, high sensitivity and reproducibility. In recent years, several attempts have been made in the field of nanotoxicology to develop assays that rapidly assess the toxicity induced by nanomaterials. Nel et al. *(30)*, Meng et al. *(31)*, and Damoiseaux et al. *(32)* have developed multiparametric high-throughput screening assays to explore oxidative stress, inflammation and resulting cytotoxicity induced by several well characterized nanomaterials. This is in agreement with the trend-setting 2007 report from the National Research Council of the National Academies of the United States, which advocated rapid and efficient toxicity testing by transitioning from qualitative and descriptive animal-based testing to quantitative, mechanism-based testing using in vitro human cells *(1)*. However, at present, the meaning of positive hits from the high-throughput oxidative stress or inflammation panel of assays to adverse outcomes observed in whole animals or in humans following nanomaterial exposure is not clear *(31)*. Although many nanomaterials are shown to induce oxidative stress and inflammatory mediators in both in vitro and in vivo models, both of which can be mounted as the organism's defence mechanism, under what circumstances the observed oxidative stress or modulation of expression of inflammatory mediators will induce toxicity is not known. Therefore, it is important that detailed mechanisms underlying toxicity induced by each class of nanomaterial be first sought before establishing the high-throughput screening assays.

It is also important to note that a single high-throughput screening assay or data derived from a single toxicological end-point using a single cell type will not be sufficient to accurately reflect the affected biology. Rather, integration of multivariate data (incorporating dose and concentration, post-exposure time points, nanomaterial properties, and biological activity) derived from different types of test systems using the systems biology approach as described above is required *(2)*.

These newer techniques with a focus on a predictive approach can help test a number of nanomaterials and aid in hazard ranking in a manner that is proportionate to the ever-growing list of novel nanomaterials being commercialized. Rigorous efforts are being made in toxicology to deliver on the promises made by National Research

Council's landmark document. However, its acceptance by the regulatory community, who historically have based their regulatory decisions on observing an adverse phenotype in animals, is something that is going to be debated for a long time to come.

8.5   Microbiome and impacts on immunotoxicity

The microbiome serves as a gatekeeper between the organism and the environment, and defines the levels of an organism's exposure to the environment. It is estimated that, by cell numbers, humans are around 90% microbial. In the recent past, increasing emphasis has been laid on understanding the relationship between the altered microbiome and human health. It has now been shown that the composition of the microbiome impacts the toxicity of xenobiotics. Based on this increasing awareness, a proposal for a new model of health risk assessment that incorporates the link between the microbiome and environmental exposure is suggested. This is especially important in the context of immunotoxicity, as the microbiome plays an important role in the maturation and function of the immune system, impacting the various immune processes. Thus, future immunotoxicity assessments must take into consideration the entire microbiome in order to fully understand xenobiotic or nanomaterial-induced responses.

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9. CONCLUSIONS AND RECOMMENDATIONS

9.1　Conclusions

- Numerous studies in recent years using in vitro or in vivo model systems have demonstrated that ENMs or certain classes of ENMs may be associated with harmful effects on human health.

- A major challenge in assessing the human health risks of ENMs to ensure safety is the lack of systematic knowledge about exposure to these materials. There is also a paucity of studies on possible long-term effects.

- The particle nature of ENMs predicts their uptake by phagocytic cells, including professional phagocytes such as macrophages, hence the intimate interaction of ENMs with the immune system. There is considerable concern that some ENMs with fibrelike dimensions may exert asbestos-like toxicity due to the inability of the innate arm of the immune system to handle long fibres. In addition to size and shape (aspect ratio), the chemical composition, surface charge and degree of solubility have all been shown to be important determinants of ENM toxicity.

- The immune system consists of an innate arm, which is able to directly respond to foreign agents and serves as the first line of defence; and an adaptive arm, which needs some time to mount an immune response and is endowed with immunological "memory" (that is, responses are specific for certain pathogens and responses are stronger upon subsequent encounters with the same pathogen). Both arms of the immune system may be affected by foreign agents. This interaction may result in immunosuppression, immunostimulation, or hypersensitivity and autoimmunity. Immunosuppression may lead to reduced resistance to infectious agents and defective immune surveillance against malignant cells. Recently, numerous reports have identified ENMs as potential triggers of immunotoxicity.

- There are several different types of nanoparticles, such as carbon-based nanomaterials (SWCNTs, MWCNTs, graphenes, fullerenes); metal-based nanomaterials (silver, gold, iron, aluminium); oxides (SiO_2, TiO_2, CeO_2); semiconductors; organic polymeric nanomaterials (dendrimers, nanocellulose);

bioinspired nanomaterials; and lipid-based nanomaterials. There are also composite nanomaterials. All these materials have specific life cycles, from synthesis to incorporation in products to disposal or recycling. They will interact with physiological fluids in different compartments upon entering the body, and this may endow ENMs with a biocorona of adsorbed biomolecules. They have specific biological effects on different organ systems, including the immune system.

- Exposure to ENMs may occur in various different settings, for example occupational exposure, consumer exposure, or exposure through environmental contamination. In addition, deliberate exposure to specific ENMs may occur in the clinical setting (nanomedicine). Exposure may occur through inhalation, oral intake, or through the skin, and could also occur through direct administration into the bloodstream (in the clinical setting). ENMs may also reach the bloodstream following translocation across biological barriers, such as the air–blood barrier in the lungs.

- Organs at risk can be identified by toxicokinetic studies. There are currently no guidelines for assessing the toxicokinetics of nanomaterials. It is therefore advisable, whenever possible, to carefully study the absorption, distribution, metabolism and excretion of ENMs on a case-by-case basis.

- For potential immunotoxic effects, besides the composition of the nanomaterial, other physical and chemical properties of nanomaterials, such as size, surface area, shape, crystallinity, charge, and aggregation, are of significant relevance.

- The immunotoxic effects described for other chemicals may in principle be applicable also to ENMs. However, while ENMs may be viewed as chemical entities, they are more complex than traditional chemicals due to the variations of multiple different material properties as well as composition. In addition, the so-called biocorona may impact the immune response to ENMs.

- Challenges for toxicity studies of nanomaterials include the dispersion of nanomaterial in biological systems and the identification of the appropriate dose, in terms of mass, number and surface area.

- For hazard identification a variety of methods are available, which in principle have all been used for classical toxicity assessment of chemicals, including immunotoxicity. Given the multitude of nanomaterials, and the drive to minimize the use

of laboratory animals for safety testing, emphasis has been on in vitro methodologies as an alternative to assessing immune effects in vivo. However, many of these have not yet been validated for use in testing of ENMs. Moreover, structured (tiered) approaches for immunotoxicity testing are so far lacking.

- Risk assessment of ENMs should follow the risk assessment paradigm for chemicals, namely hazard identification, hazard characterization, exposure assessment, and risk characterization. A risk assessment framework for immunotoxicity of nanomaterials has not yet been fully developed.

9.2   Recommendations

Based on the preceding analysis, the following recommendations are put forward:

- include nanoimmunotoxicity risk assessment in general nanotoxicity risk assessment;
- define the minimal requirements for characterization of test materials before, during and after the experiments, and control the dispersion of ENMs for both in vivo and in vitro testing;
- define reference materials for further testing, standardization and validation of test systems;
- advance testing for effects of nanomaterials by implementing developments in the fields of molecular biology, systems biology, bioinformatics, high-throughput screening, and in silico modelling;
- validate test methods for ENMs by ensuring that the test material itself does not interfere with the assay, and establish validated protocols for endotoxin testing of ENMs;
- advance approaches to relevant exposure regimens, both in vivo and in vitro, and define the most appropriate dose metrics (particle mass, number, surface area);
- identify positive controls for various bioassays;
- explore absorption, distribution, metabolism, and excretion of ENMs on a case-by-case basis, and investigate how interactions with the immune system affect the toxicokinetics of ENMs;
- develop more advanced nanomaterial-relevant models to study immunotoxicity, including more advanced in vitro model systems to study the immunotoxicity of ENMs, taking into

account the interplay between immune cell populations and other cells (for example, lung epithelial cells), as well as the role of the biocorona on the surface of ENMs;

- unravel mechanisms of ENM immunotoxicity and develop adverse outcome pathways, and define patterns of responses applying to different groups of ENMs to facilitate risk assessment of ENMs;
- develop an intelligent, mechanism-based, tiered testing strategy for ENMs taking into account the four fundamental steps of traditional risk assessment (hazard identification, hazard characterization, exposure assessment and risk characterization);
- identify susceptible populations who are at greatest risk for potential adverse effects of nanomaterials on immune-mediated disease (taking account of such criteria as sex, age, ethnicity, pre-existing condition);
- develop approaches to immunotoxicology of complex mixtures of ENMs and other compounds;
- establish acceptable uncertainty associated with exposure to nanomaterials;
- adapt OECD test guidelines related to immune toxicity to include the assessment of nanomaterials.

ANNEX 1. CASE STUDIES

The following case studies illustrate how the information derived from the application of currently available methods can be structured to assess the hazards in terms of the immunotoxic properties of two extensively studied nanomaterials.

Case study 1. Carbon nanotubes

Introduction

Immunotoxic responses to carbon nanotubes (CNTs), such as allergic sensitization and immunosuppression, have been documented in experimental animals (primarily rodents) following inhalation exposure or other pulmonary delivery routes, such as oropharyngeal aspiration or intranasal aspiration *(1)*. However, to date no epidemiological evidence is available to support immunotoxicity or any adverse outcome in humans exposed occupationally, through unanticipated environmental contact or through exposure to consumer products containing CNTs. This is probably due to the infancy of the nanotechnology industry, the relatively recent development of bulk manufacturing of CNTs over the past decade, and the time required for completing an epidemiological evaluation of adverse health effects in a human population. Based on data from experimental animals, there has been guidance for occupational exposure limits that consider CNTs as a single type of material *(2)*. However, CNTs represent a diverse class of nanomaterials that have variable physicochemical characteristics, for example SWCNTs versus MWCNTs, different aspect ratios, rigid versus flexible CNTs, or modified forms after CNT synthesis by surface functionalization. The case study for CNTs includes a survey of the available data from rodent studies in vivo that document immunotoxicity in response to well characterized CNTs. In addition to experimental evidence, recent risk assessment and hazard evaluation of CNTs has become available with specific emphasis on cancer *(3, 4)*. Since cancer involves immune cell dysregulation, at least in part, such risk assessment has important implications for this case study. Moreover, evidence documenting occupational levels of CNTs measured in the

workplace has important implications for dose–response relationships used in studies with experimental animals *(5, 6)*. Finally, experimental studies of fibrotic lung disease following CNT exposure indicate the relevance of an adverse outcome pathway (AOP) approach to the evaluation of immunotoxic responses to CNTs, since the mechanism of fibrosis involves dysregulation or changes the immune system *(7, 8)*.

Background on immune effects induced by CNTs

Immunotoxicity is defined as any adverse effect on the immune system following toxicant exposure that results in immune stimulation or immune suppression *(9, 10)*. Immunostimulation increases the incidence of allergic reactions, proinflammatory responses, or autoimmunity, while immunosuppression suppresses the maturation and proliferation of immune cells, resulting in increased susceptibility to infectious diseases or tumour growth. In addition, immune responses against the CNT itself (for example, allergy) may occur. The available literature on CNTs indicates both immunostimulatory and immunosuppressive effects in experimental animals or cultured cells. CNTs are capable of affecting both innate and acquired immune responses.

CNTs primarily impact innate immune function by affecting the function of macrophages (professional phagocytic cells). For example, SWCNTs form bridge-like structures between macrophages that could impair macrophage functions, such as migration or particle/ fibre clearance *(11)*. Long, rigid and biopersistent MWCNTs behave like asbestos fibres to cause frustrated phagocytosis (the rupture of lysosomal or cell membranes to cause leakage of proteases and cytokines that cause inflammation) *(12, 13)*. Lysosomal membrane damage by MWCNTs is a key factor in releasing cathepsin B into the cytoplasm, which plays a role in inflammasome activation, resulting in the secretion of IL-1β and IL-18 as a primary innate immune response *(14, 15)*. IL-1β signalling through its cognate receptor IL-1R is necessary for acute lung inflammation caused by CNTs *(16)*. In addition, there is evidence that CNTs impair macrophage clearance mechanisms. For example, SWCNT pre-exposure impairs macrophage phagocytosis and clearance of *Listeria monocytogenes* from the lungs of mice, resulting in greater bacterial infectivity *(17)*. Other innate responses could involve effects of CNTs on the systemic levels of cytokines and potential effects on the coagulation cascade *(18)*.

The acquired immune response is also affected by pulmonary CNT exposure. For example, there is evidence in mouse models of allergic asthma that "challenges" exposure to either SWCNTs or MWCNTs after allergen sensitization exacerbates airway remodelling (fibrosis, mucous cell metaplasia and mucus hypersecretion, eosinophilic inflammation, increased levels of Th2 cytokines such as IL-13) and elevates levels of allergen-induced serum IgE *(19, 20)*. While exacerbation of allergen-induced airway disease has been reported with SWCNTs or tangled flexible MWCNTs, most of these CNTs do not directly elicit allergic Th2 responses in the lungs of mice upon a single inhalation or oropharyngeal exposure. Instead, the typical pulmonary immune response to SWCNTs or tangled MWCNTs is a Th1 immune response characterized by acute neutrophilic inflammation that progresses to pulmonary fibrosis or granuloma formation over the course of several weeks *(21)*. However, more recent work has demonstrated that rigid, rodlike MWCNTs can directly mediate asthma-like immunological effects (Th2 cytokines), pathological effects (eosinophilia, mucus hypersecretion, fibrosis) and physiological effects (airway hyperresponsiveness) after inhalation exposure in the absence of any allergen coexposure *(22)*. Mast cells were found to partly regulate allergic inflammation caused by rodlike MWCNTs. However, mast cells are not solely linked to allergic inflammatory responses, as these inflammatory cells have been demonstrated to play a key role in pulmonary and cardiovascular response to tangled MWCNTs *(23)*. An emerging concept is that different types of MWCNTs with different physical and chemical characteristics can cause widely different acquired immune responses (for example, Th1 versus Th2 lymphocyte polarization). Even when different types of CNTs produce seemingly similar inflammatory responses in the lungs of mice, they may elicit differences in transcriptional and protein biomarkers of fibrosis or innate immune responses of macrophages *(24, 25)*.

Application of the weight of evidence approach for assessment of immunotoxicity

A series of questions is presented that is intended to aid in organizing and characterizing immunotoxicity data for CNTs from the strongest and most predictive data to the least predictive evidence supporting human risk for immunostimulatory diseases (allergy,

autoimmunity, hypersensitivity) or immunosuppression. The weight of evidence conclusions developed by answering these questions summarize the hazard identification for immunotoxicity and should describe the database in terms of consistency and biological plausibility, including strengths, weaknesses, uncertainties and data gaps.

> *Are there epidemiological studies, clinical studies or case studies that provide human data on end-points relevant to immunostimulation (that is, unintended stimulation of cellular or humoral immune function, autoimmunity or allergy)?*

Yes. Recently, Shvedova and colleagues conducted a workplace exposure assessment in an MWCNT manufacturing facility in Russia *(26)*. They found an average inhalable elemental carbon concentration of 14 mg/m³ in harvesting and packaging areas of the facility, which is clearly higher than the National Institute for Occupational Safety and Health recommended exposure limit of 1 mg/m³ for an 8-hour time-weighted average of elemental carbon. The mRNA and non-coding RNA (ncRNA) expression profiles in the blood of exposed workers (with at least six months direct contact with aerosolized MWCNTs) and non-exposed employees of the same production facility were compared. Results revealed significant changes in the expression of genes involved in lung inflammation and immunosuppression in MWCNT-exposed humans. The limitation of this study is that there was no direct evidence of exposure (for example, CNTs in BALF or nasal washes), although detection of CNTs in the workplace suggests that human exposure occurred. Other studies documenting workplace levels of CNTs have been conducted and recently reviewed *(6, 27)*. However, these studies did not address whether workers were exposed or if workers had changes in biological responses. The most abundant information on workplace concentrations is for MWCNT composites, where the expected release scenarios may include free-standing CNTs, although agglomerated CNTs and composite fragments with and without protruding CNTs are the most commonly released entities. The amounts of CNTs detected in the workplace are commonly related to the recommended exposure limit *(2)*. While there is emerging evidence of occupational CNT exposure in humans, it is important to note that no chronic immune diseases have yet to be linked to exposure.

Is there evidence that exposure to the substance is associated with exacerbation of hypersensitivity responses, allergy or induction of autoimmune disease, or alters the outcome of host resistance assays?

Yes. There is evidence that CNTs exacerbate allergen-induced airway inflammation and chronic airway remodelling in mice. MWCNTs or SWCNTs delivered by oropharyngeal aspiration to ICR mice after OVA allergen pre-exposure (sensitization) exacerbate allergic airway inflammation *(19, 28)*. OVA pre-sensitization is also exacerbated by tangled, flexible MWCNTs delivered by nose-only inhalation exposure in C57BL/6 mice *(20)*. These same MWCNTs were shown to exacerbate house dust mite allergen-induced airway inflammation and fibrosis in C57BL/6 mice *(29)*. CNTs have also been reported to directly stimulate allergic airway inflammation. For example, MWCNTs delivered by oropharyngeal aspiration to ICR mice caused increased distribution of B cells in the spleen and in blood *(30)*. Whether or not CNTs directly cause allergic lung inflammation or exacerbate lung inflammation may depend on mouse strain differences. However, the ability of some CNTs to directly promote allergic lung inflammation is also influenced by physical or chemical characteristics. For example, a comparison of tangled, flexible MWCNTs and rigid, rodlike MWCNTs delivered by occupationally relevant doses via inhalation exposure showed that rodlike MWCNTs possessed antigenicity and caused allergic airway inflammation characterized by increased numbers of eosinophils and mucus hypersecretion, along with other cytokine and immunoglobulin biomarkers of allergic inflammation discussed below *(22)*. In contrast, tangled MWCNTs caused more conventional neutrophilic lung inflammation with no mucous cell metaplasia or mucus hypersecretion.

There is also evidence that specific genes play a role in susceptibility to MWCNT-induced exacerbation of allergic lung disease. For example, COX-2 knockout mice have exaggerated lung inflammation when exposed sequentially to OVA and then MWCNTs. These mice also exhibit elevated levels of serum IgE and increased IL-13 mRNA *(31)*. STAT1 knockout mice are also susceptible to lung inflammation and fibrosis induced by OVA pre-sensitization followed by MWCNT exposure by oropharyngeal aspiration and have increased biomarkers of Th2 inflammation *(32)*.

There is thus far no direct evidence of hypersensitivity reactions or autoimmunity caused by CNTs, although studies have not yet been conducted in mouse models of autoimmunity and there is a paucity of information on dermal exposure to CNTs that might give clues to hypersensitivity reactions.

Is there evidence that exposure to the substance is associated with unintended stimulation of immune function (antibody production) or alters the balance of immunoregulatory cytokines?

Yes. Levels of IgE, associated with allergic inflammation, are increased by MWCNTs in ICR mice along with elevated levels of Th2 cytokines such as IL-4 and IL-5 *(30)*. ICR mice to which MWCNTs were delivered by oropharyngeal aspiration developed allergic lung inflammation and also had increased serum IgE levels *(19, 28)*. Rodlike MWCNTs directly stimulated allergic lung inflammation after a single or repeated inhalation exposure, and induced increased lung mRNA levels of the Th2 cytokines IL-5 and IL-13 in healthy mice *(22)*. In addition, rodlike MWCNTs increased the Th2-promoting cytokine IL-33, and eosinophil-attracting chemokine CCL17 was expressed in BAL cells as well as in other cell types present in lung tissue *(22)*. Tangled MWCNTs exacerbated OVA allergen-induced airway inflammation in C57BL/6 mice, which showed increased levels of the Th2 cytokine IL-5 *(20)*.

Immune stimulation after exposure to microbial products is also increased by CNTs. For example, the mycobacterial antigen ESAT-6, a T cell activator associated with tuberculosis and sarcoidosis, caused increased CD3(+) lymphocyte infiltration in the lungs of C57BL/6 mice and, as discussed below, also exacerbated associated lung pathology *(33)*. MWCNTs also enhanced ESAT-6-induced production of the immunoregulatory cytokines osteopontin and CCL2 in mouse lung tissue. Bacterial LPS pre-exposure enhanced MWCNT-induced lung fibrosis in Sprague-Dawley rats and was associated with increased levels of PDGF produced by alveolar macrophages *(34)*. These studies highlight the overall concept that CNTs exacerbate microbial immune responses.

Is there evidence that the substance causes immunosuppression and reduces immune function (for example, antibody production, T cell proliferation, macrophage function, and NK cell function)?

Yes. There is some evidence that MWCNTs cause systemic immunosuppression following inhalation exposure in mice. Mice exposed to MWCNTs by whole-body inhalation exposure exhibited systemic immunosuppression *(35)*. The mechanism was shown to be mediated by the release of TGF-β1 from the lungs, which enters the bloodstream to signal COX-2-mediated increases in prostaglandin E2 and IL-10 in the spleen, both of which function to suppress T cell proliferation *(36)*. Moreover, T cell proliferation was not suppressed in COX2 knockout mice, further confirming the importance of this enzyme in immunosuppression.

There is evidence that CNTs reduce or suppress macrophage function. SWCNT pre-exposure impairs macrophage phagocytosis and clearance of *Listeria monocytogenes* from the lungs of mice, resulting in greater bacterial infectivity *(37)*. Helical CNTs inhibit macrophage-mediated phagocytosis of *Pseudomonas aeruginosa* *(38)*. Furthermore, SWCNTs delivered to the lungs of rats by intratracheal instillation form bridge-like formations that link two or more macrophages, which presumably would impair macrophage functions, such as migration or particle/fibre clearance *(11)*. Long, rigid MWCNTs cause frustrated phagocytosis in macrophages, resulting in leakage of proteases and cytokines that cause inflammation *(13)*.

There is also evidence that MWCNTs reduce allergen-induced IgE production in mice when delivered by inhalation exposure prior to house dust mite allergen exposure. This contrasts with increased levels of IgE observed following OVA or house dust mite allergen pre-exposure followed by MWCNT exposure.

> *Is there histopathological evidence, haematological changes or increases in immune organ weight that suggest that the substance causes immunostimulation or modulates autoimmunity or allergy?*

Yes. SWCNTs or MWCNTs delivered to the lungs of rats or mice cause pulmonary inflammation and fibrotic lesions *(11, 39)*. MWCNTs delivered to the lungs by inhalation or aspiration migrate to the subpleural tissue; more tangled flexible MWCNTs are found within subpleural macrophages while long, rigid MWCNTs penetrate the mesothelial lining of the pleura *(20, 39)*. The inhalation of MWCNTs

in mice has been shown to produce proinflammatory lesions on the pleural surface that have been referred to as mononuclear cell aggregates *(40)*. Granulomas are a major pathological feature in the lungs of rodents exposed to MWCNTs. MWCNT-induced granuloma formation with infiltration of CD3(+) lymphocytes in the lungs of mice is exacerbated by the mycobacterial antigen ESAT-6, a T cell activator associated with tuberculosis and sarcoidosis *(33)*. Lung fibrosis is increased in the lungs of rats exposed to MWCNTs and MWCNT-induced fibrosis is increased by pre-exposure to LPS *(34)*. Mucous cell metaplasia (also described as airway goblet cell hyperplasia) and mucus hypersecretion are pathological features in mice exposed to rodlike MWCNTs but not tangled MWCNTs *(22)*. Mucous cell metaplasia in mice exposed to OVA allergen or house dust mite allergen is enhanced by exposure to tangled MWCNTs *(29, 32)*. Some types of functionalized MWCNTs (for example, ZnO-coated MWCNTs) increase lung inflammation *(41)*, while other types of functionalized MWCNTs (for example, COOH-MWCNTs, aluminium oxide-coated MWCNTs) reduce inflammation or fibrosis *(21, 42)*.

Conclusion

CNTs were selected because of the strong database for animal studies, although gaps in our knowledge of CNTs include lack of information on the potential for autoimmune disease and systemic effects of CNTs. Also, a limitation is that there is currently no information on immunotoxic effects of CNTs in humans. The case study also illustrates an example of the kind of variation seen with multiple types of CNTs, which vary in physicochemical properties. It should be noted that this case study on CNTs is provided for the purpose of illustration – it does not represent a comprehensive assessment, nor does it represent a final regulatory position.

Case study 2. Silver nanoparticles

Introduction

Nanomaterials in consumer products are already widely present in our society. According to the Nanotechnology Consumer Products Inventory, created in 2005 by the Woodrow Wilson International Center for Scholars, and the Project on Emerging

Nanotechnologies, more than 1800 consumer products have a claim to contain nanomaterials *(43)*. Silver nanoparticles (Ag-NPs) are among the most used nanomaterials in consumer products. In fact, according to the product inventory mentioned above, nanosilver is associated with 441 products, making it the most used nanomaterial. Ag-NPs are widely used in medicine as an antimicrobial agent due to their antibacterial, antiviral and antifungal properties. Specifically, Ag-NPs are used in cosmetics, food packaging, dietary supplements, clothes, toys and especially in medical devices such as bandages, wound and burn dressings, surgical instruments and implants *(44, 45)*. Recent developments in printed electronics have been enabled by applications based on usage of Ag-NP-based inks, including manufacture of semiconductors, radiofrequency identification systems, flexible printed circuit boards and solar cells *(46)*.

However, the widespread use of Ag-NPs has given rise to concerns about their toxicity. Inflammatory, oxidative, genotoxic, and cytotoxic effects are associated with, and may be inherently linked to, Ag-NP exposure *(47)*. For Ag-NPs a complicating factor is their capacity for (partial) dissolution. This makes it difficult to attribute the resulting positive (antimicrobial) or negative (toxic) effect to the nanoparticles, as such activity was also demonstrated for silver ions *(48, 49)*. An additional complicating factor for silver ions is that soluble silver salts can be deposited as metallic silver, silver sulphide, and silver chloride nanoparticles *(50–52)*.

Ag-NPs can be fabricated using physical, chemical, and biological (or green) syntheses *(53)*. For risk assessment the characterization of the Ag-NPs is essential, as different effects have been observed depending on size, coating and dissolution rate *(54–56)*.

Background to immune effects induced by silver nanoparticles

Immunotoxicity is defined as any adverse effect on the immune system following toxicant exposure that results in immune stimulation or immune suppression *(9, 10)*. Immunostimulation increases the incidence of allergic reactions, inflammatory responses, or autoimmunity, while immunosuppression suppresses the maturation and proliferation of immune cells, resulting in increased susceptibility

to infectious diseases or tumour growth. In addition, immune responses against nanosilver itself (such as allergy) may occur. Toxicity of Ag-NPs was noted for several cells of the immune system, including human neutrophils *(56)*. Cells and organs composing the mononuclear phagocytic system are a major target of Ag-NPs, as indicated by their organ localization after intravenous administration *(54, 57)*.

Immunotoxicity determined by cellular effects in vitro

In vitro Ag-NPs of various sizes were cytotoxic for both fibroblasts and macrophage cell lines, with the smaller Ag-NPs of 20 nm being more toxic *(55)*. The effective concentration inducing a 20% reduced metabolic cell activity (EC20) was 2.7 and 7 µg/mL, respectively, for L929 fibroblasts and RAW 264.7 macrophages. For the RAW 264.7 macrophages the EC20 value of the 20 nm Ag-NPs was similar to that of ionic silver, while for the fibroblasts the metabolic activity was affected more by the 20 nm Ag-NP (EC20 = 2.8 µg/mL) than for ionic silver (EC20 = 7.1 µg/mL). Macrophages were less sensitive for membrane damage, as indicated by a lack of LDH release, while for the fibroblasts clear membrane damage was noted. Larger Ag-NPs of 80 and 113 nm were less effective in reducing metabolic activity when compared to ionic silver. The 20 nm Ag-NPs induced ROS in the RAW 264.7 macrophages. Exposure of RAW 264.7 macrophages to Ag-NPs of all sizes tested (20 nm, 80 nm, 113 nm) resulted in the release of a variety of inflammatory markers, although the reaction varied widely from a low (< fivefold) increase for IL-1β and IL-10 to a high (> 500-fold) induction for granulocyte colony-stimulating factor. Comparing the effect on both cell lines, the results indicate that macrophages may not be the most sensitive cell type for Ag-NP toxicity *(55)*. In another study, Pratsinis et al. *(58)* showed that smaller Ag-NPs exerted higher cytotoxicity in RAW 264.7 macrophages. When compared with ions released from the Ag-NPs, the ions dominated the cytotoxicity for the smaller Ag-NPs (< 10 nm), whereas for the larger Ag-NPs cellular interactions with the nanoparticles were found to be the dominant factor for cytotoxicity.

THP-1-derived human macrophages were less sensitive than HepG2 and A549 cells for cytotoxic effects of nanosilver. Nanosilver decreased metabolic activation, as well as increasing cell death *(59)*.

Also, Carlson et al. *(60)* showed that Ag-NPs induced ROS in a lung macrophage cell line, which ultimately lead to cytotoxicity for the lung macrophages. In addition, an inflammatory response was indicated by the production of TNF-α, macrophage inflammatory protein (MIP-2), and IL-1β. Barbasz, Oćwieja and Barbasz *(61)* showed that differentiated monocytes and macrophages were more resistant to Ag-NP cytotoxicity than their undifferentiated parent cells. For both undifferentiated and differentiated monocytes and macrophages, nitric oxide levels were increased.

In an in vitro study with murine peritoneal macrophages, a decrease in viability and in nitric oxide production was observed at concentrations as low as 0.4 ppm *(62)*. Furthermore, a significant decrease in viability was noted in another study for peritoneal macrophages at 10 µg/mL *(63)*.

In conclusion, as indicated by a decreased viability and induction of ROS and cytokines, Ag-NPs affect macrophage functionality. Nevertheless, macrophages may be less sensitive for Ag-NP toxic effects compared to other cell types. These effects on macrophages warrant careful evaluation of Ag-NPs.

Ag-NPs show haemolytic activity when incubated in vitro with human blood *(64)*. Two silver nanoparticle preparations (obtained from two different suppliers) showed a higher haemolytic activity for red blood cells when compared to micron-sized silver particles at equal mass concentrations (dose > 220 µg/mL or > 10 cm²/mL). The increased haemolysis was attributed to an increase in ion release for the nanoformulations when compared to the micron-sized particles. For the Ag-NP preparation, considerable size changes were noted when incubated with phosphate-buffered saline and media components. The increased haemolysis was related to their greater surface area, increased silver ion release, and direct interaction with red blood cells *(64)*. When nanosilver was incorporated in a nanocomposite polymeric material (polyhedral-oligomeric-silsesquioxane-poly(carbonate-urea)urethane) intended to be used in medical devices for cardiovascular applications, higher silver levels in the polymeric material (> 0.75% by weight) demonstrated a haemolytic tendency, whereas lower levels (< 0.40% by weight) did not show haemolysis *(65)*. The mechanisms by which Ag-NPs exert

their toxic activity for erythrocytes can be due to the release of silver ions, interaction of the Ag-NPs themselves with the erythrocyte membrane, or a combination thereof *(66)*. In contrast, in vivo exposure by inhalation or intravenous injection did not show long-term effects of Ag-NPs on red blood cells *(67–69)*. Nevertheless, at early time intervals after administration, red blood cell toxicity was indicated by reduced haemoglobin, red blood cell and haematocrit levels in blood *(69)*. After repeated intravenous administrations for 28 days, several red blood cell parameters (haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration) were decreased, while the red blood cell number itself was unaffected *(70)*. In a two-week oral study in mice with Ag-NPs, the values of red blood cells, haemoglobin, and haematocrit did not vary significantly in the control and Ag-NP-treated (size range 35–45 nm) animals. Liver enzyme determination in blood indicated liver toxicity *(71)*.

Ultra-small silver nanoclusters (size 1.8 ± 0.3 nm as determined by TEM) were cytotoxic for PBMCs at concentrations as low as 1 µg/mL with incubation times up to 24 hours *(72)*. In addition, intracellular ROS have been induced with a similar level of ROS activation independent of the exposure dose (in the range 0.1–5 µg/mL). No effect of incubation time was present over a time period ranging from 3 to 12 hours.

Immunotoxicity determined by effects in vivo

It is well known that particulates, including nanoparticles, induce lung inflammation after inhalation. For Ag-NPs, a series of inhalation studies were performed by a research group from the Republic of Korea *(67, 68, 73)*. Sung et al. *(68)* reported that dose-dependent increases in lesions related to Ag-NP exposure were indicated by histopathological examinations, including mixed inflammatory cell infiltrate, chronic alveolar inflammation, and small granulomatous lesions. In male rats the lung inflammation persisted after a 12-week recovery period following exposure, while female rats showed a gradual recovery *(73)*. In a 10-day inhalation exposure study in mice, numbers of both macrophages and neutrophils were increased in the BALF *(74)*. The inflammation persisted for at least three weeks following the last exposure.

One of the characteristics of particle-induced lung inflammation is the attraction of neutrophils as demonstrated in the BALF *(75)*. Both similar and distinct effects were shown in a study with human neutrophils exposed to 20 nm and 70 nm Ag-NPs *(76)*. Both sizes of Ag-NPs acted as inhibitors of de novo protein synthesis, but showed opposing effects on neutrophil apoptosis. Opposite effects were found with regard to neutrophil apoptosis; Ag-NPs of 20 nm induced apoptosis whereas the 70 nm Ag-NPs delayed apoptosis. Furthermore, Ag-NPs of 20 nm increased the cell production of CXCL8 chemokine (IL-8), and induced the release of albumin and metalloproteinase-9 levels into the culture supernatants. Both forms of Ag-NPs did not induce ROS formation in the neutrophils.

In addition, an effect of Ag-NPs (18–20 nm) on the development of the fetus was observed after repeated nose-only inhalation exposure of pregnant female mice *(77)*. Pregnant females were exposed from gestation day 0.5 to gestation day 14.5, and the effects on reproduction were determined. After Ag-NP inhalation exposure fetal resorption was increased, and gene expression of inflammatory cytokines was increased in both the lung and placental tissue. In fetal tissues electron-dense particles were observed by TEM, which were confirmed to contain silver by energy-dispersive X-ray spectrum analysis.

Two in vivo 28-day repeated dose toxicity studies showed immunotoxicity as the most sensitive parameter *(70, 78)*. Two different sizes of Ag-NPs (20 nm and 100 nm) were intravenously administered in male and female Wistar-derived rats *(70)*. The doses evaluated ranged from 0.0082 mg/kg body weight per day to 6 mg/kg body weight per day for the 20 nm Ag-NPs. For the 100 nm Ag-NPs, only the highest dose of 6 mg/kg body weight per day was evaluated. General toxicity and immunotoxicity was evaluated one day after the last administration. Treatment with a maximum dose of 6 mg/kg body weight was well tolerated by the animals. However, for both 20 nm and 100 nm Ag-NPs growth retardation was observed during the treatment. There was a decrease in body weight and thymus weight, and an increase in liver and spleen weight. Both thymus and spleen effects may be indications for possible immunotoxicity. This increase in spleen weight was due to an absolute increase in both T and B cell numbers, whereas the relative cell numbers remained constant. Brown

and black pigment indicating Ag-NP accumulation was observed by histopathology in the liver, spleen, and lymph nodes. Clinical chemistry indicated liver damage (increased alkaline phosphatase, alanine transaminase, and aspartate transaminase) that could not be confirmed by histopathology. Haematology showed a decrease in several red blood cell parameters. The most striking toxic effect was the almost complete suppression of NK cell activity in the spleen at high doses for both the 20 nm and 100 nm Ag-NPs. After treatment of 20 nm Ag-NPs for mitogen-stimulated spleen cells, a decrease in IFN-γ and IL-10 production after concanavalin A stimulation was noted, while after LPS stimulation decreased IL-6 and IL-10 levels and TNF-α production were present, as well as increased IL-1β production. After the 100 nm Ag-NP treatment, only IL-10 production decreased. In addition, an increase in serum IgM and IgE antibodies and an increase in blood neutrophilic granulocytes were observed. For the spleen weight, a critical effect dose (CED) of 0.37 mg/kg body weight could be established. The lowest CED for a 5% change compared to control animals was observed for thymus weight (CED05 0.01 mg/kg body weight) and for functional immune parameters, namely a decrease in NK cell activity (CED05 0.06 mg/kg body weight) and LPS stimulation of spleen cells (CED05 0.04 mg/kg body weight). These results showed that for nanosilver effects on the immune system the most sensitive parameters were for potential adverse responses.

In a follow-up study by Vandebriel et al. *(78)*, male rats were treated for 28 days with a similar dose range of 20 nm Ag-NPs to investigate functional activity of the immune system. This was performed by measuring the T cell-dependent antibody response to keyhole limpet haemocyanin (KLH). A reduction in KLH-specific IgG was observed, with a lowest 5% lower confidence limit of the benchmark dose (BMDL) of 0.40 mg/kg body weight per day. This suggests that Ag-NPs induce suppression of the functional immune system. Other parameters sensitive to Ag-NP exposure were in line with the previous study: a reduced thymus weight with a BMDL of 0.76 mg/kg body weight per day, and an increased spleen weight, spleen cell number, and spleen cell subsets, with BMDLs between 0.36 and 1.11 mg/kg body weight per day. Both studies show that the systemic intravenous exposure of the immune system resulted in immunotoxicity, as indicated by the almost complete suppression of NK cell activity and a reduced IgG antibody production.

A 28-day subacute oral toxicity evaluation of 60 nm Ag-NPs in male and female rats was performed by oral administration of doses of 30, 300, and 1000 mg/kg of body weight. No significant changes in body weight were observed, while significant dose-dependent changes were found in alkaline phosphatases and cholesterol values in both the male and female rats, indicating that exposure to more than 300 mg/kg body weight of Ag-NPs may result in liver damage with an increased incidence of bile duct hyperplasia *(79)*. An increase in some red blood cell parameters (red blood cells, haemoglobin, and haematocrit) was observed in female rats, whereas the increase present in male rats was not significantly different from controls. The no observed adverse effect level reported by this study was 30 mg/kg body weight (being the lowest tested dose). In this study, a dose-dependent increased accumulation of Ag-NPs was observed in the lamina propria in the small and large intestines and in the tip of the upper villi in the ileum and protruding surface of the fold in the colon. The Ag-NP-treated rats showed higher numbers of goblet cells releasing mucus granules in the crypt and ileal lumen. Lower amounts of neutral and acidic mucins were found in the goblet cells and the amount of sialomucins was increased, while the amount of sulphomucins was decreased. In the colon of the Ag-NP-treated rats, sialylated mucins were detected in the lamina propria. This study suggested that Ag-NPs were a powerful intestinal secretagogue and induced an abnormal mucin composition in the intestinal mucosa *(80)*.

A 13-week subchronic oral toxicity study of Ag-NPs (56 nm) was conducted at doses of 30, 125, and 500 mg/kg body weight *(81)*. A significant decrease in the body weight of the male rats ($P < 0.05$) was observed after four weeks of exposure. Consistent with the 28-day oral toxicity study by Kim et al. *(79)*, significant dose-dependent changes of alkaline phosphatase and cholesterol were found, indicating liver damage. Histopathology of the liver indicated a higher incidence of bile duct hyperplasia in exposed male and female rats compared with controls. There were no effects on red or white blood cells. The no observed adverse effect level reported from this study was 30 mg/kg body weight (being the lowest tested dose).

In a 13-week oral repeated dose toxicity study with Ag-NPs of 10 nm, 75 nm, and 110 nm from the same supplier (nanoComposix, San Diego, United States), as in the studies above, no meaningful

effects of Ag-NPs on the absolute organ weights and relative organ weights (ratio of organ weight to body weight) were observed *(82)*. An effect of particle size on tissue distribution was observed, as the silver concentrations were higher in the blood and bone marrow of rats exposed to 10 nm Ag-NPs when compared to 75 nm or 110 nm. Brown pigment was observed by histopathology in various organs, which was considered a measure of silver mobility rather than toxicity. The study demonstrated for the 10 nm Ag-NPs that the translocation, and thus systemic availability, from the gastrointestinal tract to organs and tissues was mostly as intact particles. Histopathology and other parameters did not indicate toxicity after the 13-week oral exposure to the Ag-NPs.

In older literature it has been reported that percutaneous exposure to powdered silver, silver solutions, and dental amalgams can induce allergic contact dermatitis *(83–85)*. Tests for acute eye and dermal irritation and corrosion using rabbits were conducted with Ag-NPs (average 10 nm) and revealed no significant clinical signs or mortality and no acute irritation or corrosion reaction for the eyes and skin *(86)*. A skin sensitization test using guinea-pigs reported Ag-NPs as a weak skin sensitizer, showing discrete or patchy erythema *(86)*. However, in an evaluation of the Scientific Committee on Consumer Safety, the study was judged negative, as only one out of 20 guinea-pigs showed a positive response *(87)*. Silver is considered to be a non-sensitizer.

In contrast to macrophage toxicity observed in vitro (see above), Xu et al. *(63)* also demonstrated that Ag-NPs can exert adjuvant activity by stimulating antigen-processing cells such as macrophages. When administered by intraperitoneal and subcutaneous immunization with OVA or bovine serum albumin as antigen, serum antigen-specific IgG and IgE levels were significantly increased. The serum levels were lower than those obtained with Freund's complete adjuvant but similar to the levels obtained with alum adjuvant. In these studies, Ag-NPs were mixed with either OVA or bovine serum albumin antigen. Peritoneal macrophages were activated by the intraperitoneally administered Ag-NPs, as indicated by the increase in the number harvested and increase in TNF-α and IFN-γ in the peritoneal lavage fluid. Supporting in vitro studies performed with a low Ag-NP concentration (10 µg/mL) using peritoneal exudate macrophages showed that there was no effect of Ag-NP exposure on

antigen uptake by the peritoneal macrophages. An increased IgG1–IgG2a ratio and IgE response indicated a Th2-mediated immune response. The authors attributed the adjuvant activity of the Ag-NPs to the recruitment and activation of local leukocytes *(63)*. Also, Chuang et al. *(88)* demonstrated the adjuvant activity of Ag-NPs after inhalation exposure before an antigen challenge in the lung in intraperitoneally OVA-sensitized mice. The Ag-NP inhalation had allergic and inflammatory effects in both healthy and allergic mice.

More recently, an in vivo study in mice evaluated the use of green synthesized Ag-NPs as an adjuvant in rabies veterinary vaccine *(89)*. Different amounts of Ag-NPs were mixed with inactivated rabies virus and administered intraperitoneally twice with a one-week interval (day 1 and day 7) in mice. Animals were intracerebrally challenged at day 14 with rabies virus and survival and neutralizing antibodies were determined at day 35. Adjuvant activity of the Ag-NPs was demonstrated, as survival and induction of virus-neutralizing antibodies were similar to the positive alum control vaccine used *(89)*.

Epidemiology

Colloidal silver has been used already for a long time *(90)*. Silver and silver compounds are considered to have low toxicity in humans *(91)*. The most well known clinical condition is argyria, which is characterized by a striking bluish-grey colouring of the skin and the eye *(92)*. The discoloration is caused by silver salt deposition in the skin and eye. No adverse immune effects were observed in these workers.

A surveillance case study of workers involved in the manufacture of Ag-NPs reported by Lee et al. *(93)* identified two male workers who had worked for seven years manufacturing silver nanomaterials and who were exposed to silver concentrations of 0.35 and 1.35 µg/m^3, based on personal air sampling. These two workers showed silver blood levels of 0.034 and 0.0135 µg/dL and urinary silver levels of 0.043 µg/dL and not detected, respectively. No health effects were observed and blood haematology data were in the normal range. One case report identified a 27-year-old man involved in plating mobile telephone subunits with aerosolized silver for four years. The man showed general argyria and hallmark blue-grey skin and mucosa

pigmentation with elevated serum silver levels of 15.44 µg/dL (normal range 1.1–2.5 µg/dL) and a urinary silver concentration of 243.2 µg/L (normal range 0.4–1.4 µg/L). No other adverse physical or organ effects were observed *(94)*. Although the exact use of Ag-NPs in the workplace was uncertain, exposure to silver particles, whether nanoscale or non-nanoscale, appeared to induce the general argyria within four years.

Application of the weight of evidence approach for assessment of immunotoxicity

A series of questions is presented that is intended to aid in organizing and characterizing immunotoxicity data for nanosilver from the strongest and most predictive data to the least predictive evidence supporting human risk for immunostimulatory diseases (allergy, autoimmunity) or immunosuppression. The weight of evidence conclusions developed by answering these questions summarize the hazard identification for immunotoxicity and should describe the database in terms of consistency and biological plausibility, including strengths, weaknesses, uncertainties and data gaps.

> *Are there epidemiological studies, clinical studies or case studies that provide human data on end-points relevant to immunostimulation (for example, unintended stimulation of cellular or humoral immune function, autoimmunity or allergy)?*

No. In general, epidemiological studies are limited to case studies identifying an increase in silver concentration in workers without clinical signs of immune effects.

> *Is there evidence that exposure to the substance is associated with exacerbation of hypersensitivity responses, allergy or induction of autoimmune disease, or alters the outcome of host resistance assays?*

Yes. Intraperitoneal and subcutaneous immunization with OVA or bovine serum albumin as antigen resulted in increased serum antigen-specific IgG and IgE levels when Ag-NPs were administered together with the antigen *(63)*. Also, inhalation of Ag-NPs before

a nasal OVA challenge in OVA-immunized mice resulted in an increased allergic response *(88)*.

> *Is there evidence that exposure to the substance is associated with unintended stimulation of immune function (antibody production) or alters the balance of immunoregulatory cytokines?*

Yes. Macrophages were activated after in vivo exposure to Ag-NPs after intraperitoneal and subcutaneous administration, as indicated by the increase in the number harvested and increase in TNF-α and IFN-γ in the peritoneal lavage fluid *(63)*. Intravenously administered Ag-NPs altered the cytokine pattern secreted by mitogen-stimulated spleen cells *(70)*.

> *Is there evidence that the substance causes immunosuppression and reduces immune function (such as antibody production, T cell proliferation, macrophage function, NK cell function)?*

Yes. The study of De Jong et al. *(70)* showed after intravenous administration of Ag-NPs (20 nm) an almost complete disappearance of NK cell activity in spleen cells. In addition, a reduction of T and B cell mitogen responses by spleen cells was demonstrated. For T cells, a decrease in IL-10 and INF-γ was observed. For B cells, a decrease in IL-6, IL-10 levels and TNF-α production was present, while IL-1β production was increased. In the in vivo follow-up study by Vandebriel et al. *(78)*, a reduction of KLH-specific IgG was observed after Ag-NP exposure in KLH-immunized animals.

> *Is there histopathological evidence, haematological changes or increases in immune organ weight that suggest that the substance causes immunostimulation or modulates autoimmunity or allergy?*

Yes. Ag-NPs show haemolytic activity *(64, 65)*. The mechanisms by which Ag-NPs exert their toxic activity for erythrocytes can be due to the release of silver ions, interaction of the Ag-NPs themselves with the erythrocyte membrane, or a combination thereof *(66)*. Ultra-small silver nanoclusters were found to be cytotoxic for PBMC *(72)*.

An increase in spleen weight was demonstrated by the studies of De Jong et al. *(70)* and Vandebriel et al. *(78)* after intravenous administration of Ag-NPs. In several oral exposure studies, effects on spleen weight were not observed *(79, 81, 82)*. Changes in haematology parameters were observed in both intravenous and oral toxicity studies *(70, 78, 79)*.

Conclusion

Silver nanoparticles (Ag-NPs) show immunotoxic properties that are dependent on the size of the nanoparticles, with smaller Ag-NPs apparently having a larger effect when based on a dose expressed in mass. The evidence for immunotoxic activity of Ag-NPs is based on both in vivo animal studies and in vitro studies for various cells of the immune system. The mechanism of the toxic activity is not known but is partially caused by silver ion release. Also, interactions of Ag-NPs with cells can lead to cytotoxicity. The immunosuppressive effect of silver particles seems to be dependent on the route of exposure. The oral exposure studies reveal no effects on immune organs. Hence, distribution is an important factor in immunotoxicity. In view of the antimicrobial activity of silver particles, their effect on the microbiota and interaction with the immune system should be considered. A limitation is the current lack of information on the immunotoxic effects of Ag-NPs in humans. It should be noted that this case study on Ag-NPs is provided with the purpose of illustrating how the risk assessment guidance can be used for immunotoxicity, but it does not represent a comprehensive risk assessment, nor does it represent a final regulatory position.

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ANNEX 2. GLOSSARY OF TERMS

Acute exposure. Exposure occurring over a short time, generally less than one day.

Adverse effect. Any change in the morphology, physiology, growth, development, reproduction or lifespan of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences.

Aerosol. Mixture of small particles (solid, liquid or a mixed variety) and a carrier gas (usually air).

Allergen. An antigen that induces an allergic or hypersensitivity reaction, resulting in immune-mediated or non-immune-mediated tissue damage; restricted mainly to immediate hypersensitivity or anaphylactic reactions.

Allergic contact dermatitis. An inflammatory skin disease resulting from allergic sensitization.

Allergic response. Adverse response of an allergic individual to the specific allergen.

Allergy. Hypersensitivity caused by exposure to an exogenous antigen (allergen) resulting in a marked increase in reactivity and responsiveness to that antigen on subsequent exposure, resulting in adverse health effects.

Antibody. Immunoglobulin molecule produced in response to immunization or sensitization, which specifically reacts with antigen.

Antigen. Any substance that induces a specific immunological response.

Apoptosis. A form of regulated or programmed cell death that transpires according to a regulated sequence of morphological and biochemical changes, including cell blebbing, chromatin condensation, nuclear DNA fragmentation, and the formation of so-called apoptotic bodies. Apoptosis is initiated mainly through one

of two pathways: the mitochondria-dependent or intrinsic pathway, and the death receptor-dependent or extrinsic pathway.

Autoimmune disease. A disease involving immune responses against self-antigens, resulting in pathological change.

Autoimmunity. Inappropriate reaction of the immune system against the organism's own antigens (autoantigens) that may be either destructive or non-destructive. Destructive autoimmunity is associated with the development of autoimmune diseases.

Biomarker. Indicator of changes or events in biological systems. Biological markers of exposure refer to cellular, biochemical, analytical or molecular measures that are obtained from biological media such as tissues, cells or fluids and are indicative of exposure to an agent. Biomarkers of effect refer to biological changes that represent an alteration in endogenous body constituents (e.g. depression of cholinesterase levels as an indicator of exposure to pesticides).

Breathing zone. The area immediately surrounding a worker's nose and mouth from where the majority of air is drawn into their lungs.

Bulk material. The larger counterpart of a nanomaterial not confined to the nanoscale in any dimension, e.g. gold as the bulk material and nano-gold as the nano-form material.

Carbon nanofibres. Cylindrical nanostructures with graphene layers arranged as stacked cones, cups or plates.

Carbon nanotubes. Hollow nano-objects with two similar external dimensions in the nanoscale and the third dimension significantly larger, composed of carbon (ISO/TS 80004-3:2010).

Chronic exposure. Exposure over a long period, for humans over years.

Control banding. A risk management approach to identify and recommend exposure control measures for potentially hazardous substances for which toxicological information is limited.

Dose–response relationship. Relationship between the amount of an agent administered to, taken up by or absorbed by an organism, system or (sub)population and the change developed in that organism, system or (sub)population in reaction to the agent.

Exposure assessment. Evaluation of the exposure of an organism, system or (sub)population to an agent (and its derivatives). Exposure assessment is the third step in the process of risk assessment.

Hazard. The inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub)population is exposed to that agent.

Hazard characterization. The qualitative and, wherever possible, quantitative description of the inherent property of an agent or situation having the potential to cause adverse effects. This should, where possible, include a dose–response assessment and its attendant uncertainties. Hazard characterization is the second of four steps in risk assessment.

Hazard identification. The identification of the type and nature of adverse effects that an agent has an inherent capacity to cause in an organism, system or (sub)population. Hazard identification is the first of four steps in risk assessment.

Hypersensitivity. Increased reactivity or sensitivity; in immunological reactions, often associated with tissue destruction.

Immunostimulation. Unintended stimulation of the immune system.

Immunosuppression. Dominant immunological tolerance, a phenomenon that plays an active role in regulating T and B cell responses to both foreign antigens and autoantigens (suppressor T lymphocyte). The downregulation of responses to autoantigens is a major regulatory mechanism involved in the induction and maintenance of self-tolerance.

Immunotoxicity. Any adverse effect on the immune system that can result from exposure to a range of environmental agents, including chemicals.

Inflammation. Process whereby blood proteins or leukocytes enter tissue in response to or in association with infection or tissue injury.

Manufactured nanomaterials. Solid, particulate substances intentionally manufactured at the nanoscale, consisting of nano-objects with at least one dimension between 1 and 100 nm, and their aggregates and agglomerates.

Mechanism of action. The specific biochemical interaction through which a substance produces an effect on a living organism or in a biochemical system.

Mode of action. A biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data.

Multi-walled carbon nanotubes. Tubes of multiple concentric cylindrical one-atom-thick layers of graphene, as opposed to single-walled nanotubes.

Nano-object. A material with one, two or three external dimensions in the nanoscale.

Nanoparticle. Nano-object with all three external dimensions in the nanoscale (< 100 nm diameter).

Nanoscale. Size range from approximately 1 nm to 100 nm.

Occupational exposure limit. Maximum concentration of airborne contaminants deemed to be acceptable, as defined by the authority having jurisdiction (ISO 16972:2010).

Particulate matter. A mixture of solid particles and liquid droplets suspended in the air.

Risk. The probability of an adverse effect in an organism, system or (sub)population caused under specified circumstances by exposure to an agent.

Risk assessment. A process intended to calculate or estimate the risk to a given target organism, system or (sub)population, including the identification of attendant uncertainties, following exposure to a particular agent, taking into account the inherent characteristics of the agent of concern as well as the characteristics of the specific target system. The risk assessment process includes four steps: hazard identification, hazard characterization (dose–response assessment), exposure assessment and risk characterization.

Risk characterization. The qualitative and, wherever possible, quantitative determination, including attendant uncertainties, of the probability of occurrence of known and potential adverse effects of an

agent in a given organism, system or (sub)population, under defined exposure conditions. Risk characterization is the fourth step in the risk assessment process.

Sensitization. Induction of specialized immunological memory in an individual by exposure to antigen.

Single-walled carbon nanotubes. A cylindrical one-atom-thick layer of graphite called graphene, as opposed to multi-walled nanotubes.

Solubility. The ability of a material to release ions in water or in another liquid. Solubility may be expressed by the dissolution rate of the material and may also be described using words such as insoluble, very soluble or poorly soluble.

Threshold. Dose or exposure concentration of an agent below which a stated effect is not observed or expected to occur.

Tiered approach. A stepwise approach in which each step has an increased level of complexity; here it refers to a risk-based approach for conducting an exposure or release assessment to determine whether exposure to manufactured nanomaterials may occur and to determine if there is a need for further risk management steps to be taken.

Time-weighted average. An average concentration of an airborne contaminant that workers may be exposed to over a period of time such as an 8-hour day or 40-hour week (an average work shift).

Tolerance. Persistent condition of specific immunological unresponsiveness, resulting from previous non-sensitizing exposure to the antigen.

Sources

The definitions in this glossary are derived from the following sources:

IPCS guidance for immunotoxicity risk assessment for chemicals: http://www.who.int/ipcs/methods/harmonization/areas/guidance_immunotoxicity.pdf?ua=1.

WHO guidelines on protecting workers from potential risks of manufactured nanomaterials: http://www.who.int/occupational_health/publications/manufactured-nanomaterials/en/.

Note: See Chapter 2 for further definitions of, and information on, types of nanoparticles.

RÉSUMÉ D'ORIENTATION

Les nanomatériaux d'ingénierie (NMI) peuvent présenter des dangers pour l'environnement et la santé humaine et nuire à la santé de l'homme. Plusieurs types de NMI existent : les NMI à base de carbone (dont les nanotubes de carbone, le graphène et les fullerènes) ; les NMI à base de métaux (notamment les formes nano de dioxyde de titane, d'or, d'argent et les points quantiques) ; les dendrimères (des polymères principalement utilisés pour la libération de médicaments) ; et les composites (des associations de NMI, incluant des nanotubes de carbone enrobés de nano-oxydes métalliques). Ces matériaux trouvent de nombreuses applications dans divers produits nanotechnologiques, qui peuvent être classés en quatre générations : (a) les nanoproduits de première génération, qui incluent les NMI à base d'un seul matériau utilisés dans divers produits de consommation, comme les produits cosmétiques et alimentaires ; (b) les nanoproduits de deuxième génération, formés de nanostructures plus complexes, comme les engrais ; (c) les nanoproduits de troisième génération, utilisant à la fois des nanostructures de première et de deuxième génération pour élaborer des nanosystèmes, comme dans la création d'organes artificiels ou de microbes d'ingénierie, ou des matériaux auto-assemblés qui forment de nouvelles structures dans l'organisme une fois libérés ; et (d) les nanoproduits de quatrième génération, encore en phase de développement, comportant des nanosystèmes moléculaires conçus pour une fonction spécifique, comme les dispositifs moléculaires utilisés dans la thérapie génétique. Bien que la technologie elle-même ait fait des progrès rapides et que des milliers de produits de consommation contenant des NMI soient apparus sur le marché, les questions liées à l'exposition et à la toxicologie de ces matériaux ne sont pas suffisamment bien comprises. Le manque de connaissances concernant les modalités et l'étendue de l'exposition du grand public à ces NMI, ainsi que les dangers potentiels qui y sont associés, représente un obstacle majeur à la mise en œuvre de pratiques spécifiques en matière de santé et de sécurité des nanomatériaux.

D'importantes recherches menées au cours des 20 dernières années ont montré que l'interaction des NMI avec les systèmes biologiques

dépend non seulement des propriétés chimiques des nanomatériaux, mais aussi de leur taille, de leur forme et de leurs caractéristiques de surface. Par conséquent, les toxicologues et les spécialistes de l'évaluation des risques ont beaucoup de difficultés à rester en phase avec l'évolution rapide des technologies, la multiplicité des NMI aux propriétés diverses et les contraintes associées aux méthodes conventionnelles d'évaluation de la toxicité. Afin de relever les défis présentés par les NMI, de nouvelles approches sont nécessaires, en plus des méthodologies conventionnelles, pour estimer l'exposition, identifier les dangers et évaluer les risques, intégrant les domaines de la physique, de la chimie et de la biologie.

Le système immunitaire est formé d'une composante innée, capable de répondre directement aux agents étrangers immédiatement après l'exposition, indépendamment du type de stimulus, et d'une composante adaptative, qui renforce l'immunité au cours du temps. L'interaction avec des agents exogènes, notamment les NMI, peut mener à une immunodépression, une stimulation du système immunitaire, une hypersensibilité ou une réaction auto-immunitaire. Selon de récents rapports, les NMI sont des stimulateurs potentiels de la réponse immunitaire, ce qui peut ultimement provoquer une immunotoxicité. Bien qu'aucune méthodologie validée n'existe pour évaluer l'immunotoxicité des NMI, ce document décrit plusieurs essais généralement utilisés pour examiner l'immunotoxicité induite par les produits chimiques, qui pourraient être compatibles avec l'analyse des nanomatériaux. Il n'est pas réaliste de s'attendre à ce que chaque NMI soit évalué à l'aide de toutes les méthodes disponibles ; toutefois, quelques règles simples peuvent être suivies, qui sont décrites ci-dessous.

Même si l'exposition aux NMI peut se faire selon toutes les voies possibles d'exposition aux produits chimiques, l'organe cible le plus souvent étudié jusqu'à présent est le poumon, et l'inflammation pulmonaire est l'effet le plus souvent signalé à la suite d'une exposition aux NMI. Les autres systèmes organiques ont été moins souvent étudiés à ce jour. L'inflammation pulmonaire ne peut pas en elle-même être considérée comme une immunotoxicité. Cependant, une stimulation prolongée des divers constituants du système inflammatoire, notamment l'opsonisation et l'activation du complément, peut favoriser des pathologies telles que l'asthme.

Pour cette raison, les NMI dotés de propriétés immunostimulantes doivent être examinés avec soin en vue de déterminer leur capacité potentielle à induire une immunotoxicité. Plusieurs études ont indiqué que les NMI peuvent migrer des poumons vers d'autres organes impliqués dans la réponse immunitaire, comme la rate, le foie et les ganglions lymphatiques, selon les propriétés des NMI. Ainsi, s'il s'avère que l'exposition à certains NMI provoque une inflammation pulmonaire et que ces NMI se sont acheminés vers d'autres organes impliqués dans la réponse immunitaire, ils doivent faire l'objet d'une évaluation prioritaire complète à l'aide d'autres essais spécifiques d'immunotoxicité.

En outre, même si on ne s'attend pas à ce que les NMI soient absorbés par les couches de la peau ou qu'ils y pénètrent, tout NMI provoquant une hypersensibilité respiratoire doit être évalué en vue de déterminer s'il risque également d'induire une irritation cutanée. Si l'équivalent en vrac d'un nanomatériau est connu pour être immunotoxique, sa forme nano doit être considérée comme présentant un risque élevé et évaluée selon les méthodes appropriées. Bien que le choix des méthodes de test doive principalement reposer sur l'application potentielle, dans le cas des sprays pour la peau, la toxicité pulmonaire doit être étudiée en plus des tests d'irritation et d'absorption cutanée, car une exposition fortuite par inhalation des brumes de vaporisation est prévisible. Ainsi, il convient de tenir compte de plusieurs facteurs pour choisir les méthodes et l'étendue de l'évaluation, notamment les propriétés des NMI, leur application potentielle et la voie d'exposition. En outre, la stratégie doit inclure plus d'une méthode de test examinant le même critère, afin d'accroître la fiabilité des résultats.

À l'heure actuelle, il n'existe aucune ligne directrice pour l'évaluation des conséquences immunotoxicologiques de l'exposition aux NMI. Diverses méthodes sont disponibles pour l'identification des dangers qui, en principe, ont toutes été utilisées pour l'évaluation conventionnelle de la toxicité des produits chimiques, y compris l'immunotoxicité. Étant donné le grand nombre de NMI et la nécessité de réduire au minimum l'utilisation des animaux de laboratoire pour les analyses de sécurité, les méthodes *in vitro* ont été privilégiées. Toutefois, nombre de ces méthodes n'ont pas encore été standardisées ou validées pour l'évaluation des NMI. De plus, il est difficile de

reproduire la complexité du système immunitaire, et en particulier de simuler la réponse en aval, à l'aide d'expériences sur des cultures cellulaires. En termes généraux, l'évaluation des risques liés aux NMI doit suivre la stratégie d'évaluation des risques liés aux produits chimiques, à savoir : identification des dangers, caractérisation des dangers, évaluation de l'exposition et caractérisation des risques. La recommandation actuelle est de réaliser les analyses des risques avec une certaine souplesse, au cas par cas, en incluant les éléments les plus pertinents selon le matériau et son utilisation proposée.

Ce document sur les critères de santé environnementale présente les connaissances actuelles concernant les principes et méthodes d'évaluation des risques d'immunotoxicité associés à l'exposition aux NMI. L'évaluation de l'immunotoxicité doit être intégrée dans un contexte élargi d'évaluation des dangers et des risques posés par les NMI. Par conséquent, même si ce document porte principalement sur l'immunotoxicité, il aborde également des questions courantes spécifiques aux NMI, notamment leur caractérisation, la préparation des échantillons et la dosimétrie.

DOCUMENTO DE SÍNTESIS

Los nanomateriales de ingeniería (NMI) pueden entrañar riesgos para el medio ambiente y el ser humano y afectar a la salud de las personas. Estos materiales se pueden clasificar en distintos tipos: a base de carbono (como los nanotubos de carbono, grafeno y fulerenos); a base de metales (por ejemplo, las nanoformas de dióxido de titanio, oro, plata y los puntos cuánticos); dendrímeros (polímeros que se utilizan principalmente para administrar fármacos), y NMI compuestos (es decir, materiales que combinan más de un NMI, como los nanotubos de carbono recubiertos con nanopartículas de óxidos de metales). Estos materiales son de fácil utilización en distintos productos de nanotecnología, de los que se distinguen cuatro generaciones: *a*) la primera generación de nanoproductos, entre ellos los NMI de un solo material que se utilizan en distintos productos de consumo, como los cosméticos y los alimentos; *b*) los NMI de segunda generación, que consisten en nanoestructuras más complejas, como los fertilizantes; *c*) los NMI de tercera generación, en los que se usan nanoestructuras de primera y de segunda generaciones para obtener nanosistemas, para fabricar, por ejemplo, órganos artificiales y microorganismos de ingeniería, o materiales capaces de ensamblarse por sí mismos para formar nuevas estructuras tras ser liberadas en el organismo, y *d*) los NMI de cuarta generación, que se encuentran todavía en fase de desarrollo y que pueden consistir en nanosistemas moleculares con una función específica, como los dispositivos moleculares que se usan en la terapia génica. Podemos decir que, gracias a los rápidos avances de la tecnología, están apareciendo en el mercado miles de productos de consumo que contienen NMI, pero no se conocen suficientemente los efectos tóxicos de estos materiales y la exposición a ellos. De hecho, la falta de claridad en relación con el modo y el grado de exposición de la población general a estos materiales y con los riesgos que entraña la exposición ha resultado ser un obstáculo importante para las prácticas específicas para ellos en las esferas de la salud y la seguridad.

En numerosos estudios llevados a cabo en los últimos 20 años se ha puesto de manifiesto que la interacción de los NMI con los sistemas biológicos no depende solamente de su composición química, sino

también de su tamaño, su forma y las características de su superficie. Por esta razón, los toxicólogos y los expertos en evaluación de riesgos no han sido capaces de seguir el ritmo de los rápidos avances de la tecnología, el abrumador número de NMI, cada uno con propiedades específicas, y las limitaciones de los métodos convencionales de evaluación de la toxicidad. Por ello, los NMI obligan no solo a utilizar métodos convencionales convenientes sino nuevas formas de estimar la exposición, detectar peligros y evaluar riesgos, que integren la física, la química y la biología.

El sistema inmunitario consiste en un componente innato capaz de responder de forma directa a los cuerpos extraños inmediatamente después de la exposición a ellos, con independencia del tipo de estímulo, y en un componente adaptativo que confiere inmunidad con el tiempo. La interacción con agentes exógenos, entre ellos los NMI, puede ocasionar depresión del sistema inmunitario, estimulación de este, hipersensibilidad y reacciones autoinmunes. En informes recientes se ha señalado que los NMI podrían desencadenar una respuesta inmunitaria que, en último término, cause inmunotoxicidad. En vista de que no se dispone todavía de métodos homologados que permitan evaluar la inmunotoxicidad de los NMI, presentamos aquí varios ensayos utilizados habitualmente para determinar la inmunotoxicidad inducida por sustancias químicas que pueden servir también para los nanomateriales. Aunque no cabe esperar que se puedan analizar todos y cada uno de los NMI con todos los métodos de ensayo disponibles, se pueden seguir unas reglas sencillas, como se indica a continuación.

Si bien las personas están expuestas a los NMI por las mismas vías que lo están a las sustancias químicas en general, el órgano que ha sido más estudiado a este respecto son los pulmones y, de hecho, el efecto más notificado tras la exposición a los NMI es la inflamación pulmonar. Por el momento, se han realizado pocos estudios sobre otros sistemas de órganos. Es cierto que la inflamación de los pulmones no puede considerarse, por sí misma, inmunotoxicidad, la estimulación prolongada de varios elementos del proceso inflamatorio, mediante fenómenos como la opsonización y la activación del sistema del complemento, pueden provocar afecciones como el asma. Por consiguiente, es necesario estudiar exhaustivamente los NMI que estimulan el sistema inmunitario a

fin de determinar su capacidad para causar inmunotoxicidad. Se ha demostrado en varios estudios que algunos NMI poseen propiedades que les permiten migrar a otros órganos inmunorreactivos como el bazo, el hígado o los ganglios linfáticos. Por tanto, debe concederse prioridad al estudio más detallado, mediante ensayos específicos para detectar inmunotoxicidad, de los NMI que, por exposición, inflamen los pulmones y puedan desplazarse a otros órganos inmunorreactivos.

Del mismo modo, no se prevé que los NMI sean absorbidos por las capas de la piel o penetren a través de ellas, pero, si se comprueba que alguno de ellos causa hipersensibilidad respiratoria, se debe estudiar la posibilidad de que ocasione también irritación cutánea. Si se sabe que el equivalente de mayor tamaño de un nanomaterial es inmunotóxico, el nanomaterial también se debe considerar de alto riesgo y ser objeto de estudio por los métodos convenientes. Aunque la elección de los métodos de ensayo se debe basar principalmente en sus posibles usos, en el caso de los nebulizadores cutáneos se debe estudiar, además de las pruebas de absorción e irritación cutáneas, la toxicidad pulmonar, puesto que el aerosol puede difundirse y ser inhalado por otras personas. En consecuencia, al elegir los métodos y definir el alcance de los estudios se deben considerar detenidamente varios factores, como las propiedades del NMI, sus posibles usos y la vía de exposición. Además, la estrategia debe incluir más de un método de ensayo para el mismo criterio de valoración, con el fin de mejorar la fiabilidad de los resultados.

No se dispone todavía de orientaciones que permitan evaluar la toxicidad para el sistema inmunitario de la exposición a los NMI. Existen distintos métodos para detectar los peligros que entrañan, que, en principio, se han utilizado todos para evaluar la toxicidad de las sustancias químicas, incluida la inmunológica. No obstante, habida cuenta de la diversidad de NMI y de la tendencia a no utilizar animales de laboratorio en los ensayos de seguridad, se concede prioridad a los métodos *in vitro*. Con todo, todavía no se han normalizado u homologado muchos de estos métodos *in vitro* para analizar NMI. Además, no resulta fácil reproducir la complejidad del sistema inmunitario —por ejemplo, la activación en cascada— mediante experimentos en cultivos celulares. En términos generales, la evaluación del riesgo de los NMI debe seguir la estrategia habitual para analizar los riesgos de las sustancias químicas, es decir, detectar

los peligros, caracterizarlos, evaluar la exposición y caracterizar los riesgos. Actualmente se recomienda evaluar los riesgos con cierta flexibilidad, caso por caso, mediante los elementos más pertinentes en función del material y de su uso propuesto.

En el presente documento sobre criterios de salubridad ambiental se presentan los conocimientos actuales sobre los principios y los métodos empleados para evaluar los riesgos de inmunotoxicidad asociados con la exposición a los NMI. Esta evaluación debe integrarse en el contexto más amplio de la evaluación de los peligros y los riesgos que plantean estos materiales. Por tanto, aunque aquí nos centremos principalmente en la inmunotoxicidad, también abordamos cuestiones comunes específicas de los NMI, como la caracterización, la preparación de muestras y la dosimetría.