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Nanotechnologies — Compilation and description of toxicological screening methods for manufactured nanomaterials

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TECHNICAL REPORT

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Nanotechnologies — Compilation and description of toxicological screening methods for manufactured nanomaterials

Nanotechnologies — Compilation et description des méthodes de criblage toxicologiques pour les nanomatériaux manufacturés

Reference number
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Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
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Contents

Page

Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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The committee responsible for this document is ISO/TC 229, *Nanotechnologies*.

Introduction

This Technical Report provides a compilation of methods intended to aid the process of toxicological screening of engineered and manufactured nanomaterials prior to full-scale toxicological testing, analysis, and risk assessment. Toxicological screening methods focus on providing information and tools that can be used in decision-making processes. For instance, this Technical Report provides information on methods that can be used to screen nanomaterials in order to determine whether to continue development of a nanomaterial itself and/or a product containing a nanomaterial; determine whether to take on the cost of performing the remaining tiers within a complete tiered-testing strategy; or determine whether appropriate controls are in place to continue nanomaterial research in the laboratory.

This Technical Report is not intended to supplant or compete with any existing regulatory requirements regarding nanomaterial testing, use, and disposal, nor does it provide a list of validated tests for this purpose.

The information provided is consistent with other International Standards. For example, its sister document 'Compilation and Description of Sample Preparation and Dosing Methods for Manufactured NMs' is developed in concert and discusses methods used to prepare samples in various relevant media for toxicological studies. ISO 10993-18[1] specifically addresses the evaluation of the chemical characterization of materials used in medical devices, ISO 14971[2] points out that a toxicological risk analysis should take into account the chemical nature of the materials, and ISO/TR 13014[3] addresses issues pertaining to the materials themselves. ISO/TR 13121[4] describes a process for identifying, evaluating, and communicating the potential risks of manufactured nanomaterials and provides guidance on tiered nanomaterial toxicity testing.

TECHNICAL REPORT **ISO/TR 16197:2014(E)**

Nanotechnologies — Compilation and description of toxicological screening methods for manufactured nanomaterials

1 Scope

This Technical Report provides a compilation and description of *in vitro* and *in vivo* methods that can be useful for the toxicological, including ecotoxicological screening of engineered and manufactured nanomaterials. Toxicological screening tests included in this Technical Report can be used for such purposes as early decision-making in research and product development, rapid feedback on potential toxicological/safety concerns, or for the preliminary assessment of manufactured nanomaterials. This Technical Report is divided between screening assays related to humans and screening assays related to the environment. A screening test is a relatively simple, inexpensive test that can be administered easily and provides an indication of potential adverse outcomes and effects on human health or the environment.

The Technical Report is intended to complement other international efforts that address nanomaterial toxicology by focusing on screening methods suited for preliminary assessment and is not intended to duplicate similar efforts in other international organizations such as the Organization for Economic Cooperation and Development (OECD). If screening provides an early indication of hazard, the guidance will refer to other organizations' approaches for full-scale toxicological assessment or further tiered studies.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/TS 27687:2008, *Nanotechnologies — Terminology and definitions for nano-objects — Nanoparticle, nanofibre and nanoplate*

ISO/TS 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core terms*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 27687:2008 and ISO/TS 80004-1 and the following apply.

3.1
agglomerate
collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces, or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 27867:2008, 3.2]

3.2
aggregate
particle comprising strongly bonded or fused particles where the resulting external surface area can be significantly smaller than the sum of calculated surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent bods, or those resulting from sintering or complex physical entanglement.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 27867:2008, 3.3]

3.3
manufactured nanomaterial
nanomaterial intentionally produced for commercial purposes to have specific properties or specific composition

[SOURCE: ISO/TS 80004-1, 2.9]

3.4
nanofibre
nano-object with two external dimensions in the nanoscale and the third dimension significantly larger

Note 1 to entry: A nanofibre can be flexible or rigid.

Note 2 to entry: The two similar external dimensions are considered to differ in size by less than three times and the significantly larger external dimension is considered to differ from the other two by more than three times.

Note 3 to entry: The largest external dimension is not necessarily in the nanoscale.

[SOURCE: ISO/TS 27687:2008, 4.3]

3.5
nanomaterial
material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of nano-object and nanostructured material.

Note 2 to entry: See also engineered nanomaterial, manufactured nanomaterial, and incidental nanomaterial.

[SOURCE: ISO/TS 80004-1, 2.4]

3.6
nano-object
material with one, two, or three external dimensions in the nanoscale

Note 1 to entry: Generic term for all discrete nanoscale objects.

[SOURCE: ISO/TS 27687:2008, 2.2]

3.7
nanoparticle
nano-object with all three external dimensions in the nanoscale

Note 1 to entry: If the lengths of the longest to the shortest axes of the nano-object differ significantly (typically by more than three times), the terms nanorod or nanoplate are intended to be used instead of the term nanoparticle.

[SOURCE: ISO/TS 27687:2008, 4.1]

3.8
nanoscale
size range from approximately 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from a larger size will typically, but not exclusively, be exhibited in this size range. For such properties, the size limits are considered approximate.

Note 2 to entry: The lower limit in this definition (approximately 1 nm) is introduced to avoid single and small groups of atoms from being designated as nano-objects or elements of nanostructures, which might be implied by the absence of a lower limit.

[SOURCE: ISO/TS 27687:2008, 2.1]

3.9
nanotube
hollow nanofibre

[SOURCE: ISO/TS 27687:2008, 4.4]

3.10
particle
minute piece of matter with defined physical boundaries

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to nano-objects.

[SOURCE: ISO/TS 27687:2008, 3.1]

4 Symbols (and abbreviated terms)

ASTM American Society for Testing and Materials

ESR Electron Spin Resonance

IT Intracheal

ITS Integrated Testing Strategies (or in some cases, Intelligent Testing Strategies)

LTE Lymphoid Tissue Equivalent Module

NMs nanomaterials

OECD Organization for Economic Cooperation and Development

PBS phosphate buffer saline

PTE peripheral tissue equivalent

ROS reactive oxygen species

STS standard toxicity studies

5 Background

5.1 Role and relevance of toxicological screening for the safety evaluation of manufactured NMs

It is a challenge to keep up with the number of emerging NMs with novel properties and to toxicologically assay new materials prior to human exposure, including occupational and environmental exposure.

Because virtually every element on the periodic table could be explored in nanotechnology, the potential diversity of NMs makes it impractical to utilize our current testing paradigms to evaluate every new nanomaterial. High-throughput toxicological screening is essential to keeping pace with the rate of NMs currently emerging on the market. Such screening is typically conducted using cell culture or other *in vitro* techniques due to cost, infrastructure, and time constraints; such considerations prevent most whole animal studies. In addition, there is a worldwide effort to diminish the use of *in vivo* animal studies as presented in the 3R (replacement, reduction, refinement) principle.[5]

The purpose of a screening test is to provide an indicator of potential adverse outcomes and effects on human health or the environment. Although there are many definitions available for the term screening test, for the purposes of this Technical Report, a screening test can be generally defined as a relatively simple, inexpensive test that can be administered easily and provides rapid results.

A screening test might include the following:

— uses no (or a very limited number of) sentient animals;

— produces a quantifiable end point or a yes/no screen that is well-accepted and reliable;

— has demonstrated repeatability in multiple laboratories;

— is reproducible with appropriate positive and negative controls.

Screening tests often provide specific mechanistic data that should be used in the context of either an adverse outcome pathway framework or a chemical signature analysis. This Technical Report deals solely with screening tests that have been used for the purposes of assessing nanomaterial toxicity; therefore, results from this type of screening test could be used to determine whether or not to pursue the continued development of a particular nanomaterial product. For example, NMs predicted to be particularly hazardous can be removed from development.

When used in a tiered testing strategy, high throughput screening methods have the potential to eliminate further *in vivo* testing or to identify hazardous materials for targeted *in vitro* or *in vivo* investigation, thus streamlining the hazard identification process. The fact that screening assays can be conducted in a high-throughput fashion has especially important human health implications considering the complexity and the large number of NMs already on the market and currently in development.

However, there are also limitations of screening assays relative to more detailed, confirmatory assays; therefore, screening assays should be designed to be incorporated into an integrated testing strategy. Limitations of screening assays include

— screening assays often lack validated human predictability,

— extrapolation of dose-response relationships from screening assays to human exposure is complex, and

— prediction of human chronic exposure hazard from an acute exposure screen is difficult.

While screening tests are not meant to be used as stand-alone methods, they can eliminate further studies if the results suggest a NM is particularly toxic or non-toxic.

This means that screening assays in some instances will undoubtedly overestimate or underestimate human hazards.

5.2 Toxicological screening as part of tiered approaches to toxicological assessment of manufactured NMs

A tiered testing approach is based on a stepwise assessment, with each step of the assessment providing data/information that might be required for the subsequent step or will be of use for the whole testing approach.

Often screening tests are included in a tiered testing strategy, and they are generally carried out in one of the earliest tiers. This allows an efficient use of resources at several levels, e.g. both for identifying further testing needs and for decisions on product development in view of an early hazard profile.

Toxicological screening is part of most early tiers in weight-of-evidence-based, intelligent testing strategies (ITS).[6] ITS take into account the data available for the nanomaterial of interest and provide a rational testing strategy to understand the hazard properties of that nanomaterial without resorting to undue animal testing.

In addition to *in vitro* and *in vivo* methods, *in silico* methods could form part of ITS. In principle, the methods of Quantitative Structure–Activity Relationship (QSAR) analysis can be applied to nanoparticles, provided that suitable descriptors can be found to associate the structural and physicochemical characteristics of nanoparticles with their biological activity.[7][8] These could result in models that make qualitative (e.g. the potential for oxidative stress) or quantitative predictions (e.g. cytotoxic potency), depending on the data and modelling approach. To date, however, only a few studies have been published, probably due to a lack of suitable data sets.[9]-[17] The challenges and recent successes of developing QSARs for nanoparticles are further outlined elsewhere.[18][19]

5.3 Discussion of relevant dosing for toxicological screening

Humans and the environment are exposed to NMs via a limited number of routes, e.g. inhalation, ingestion, dermal contact, or, for the environment, water, air, and soil. The exposure concentrations for some of these exposure scenarios can be determined, e.g. airborne NMs in the workplace or particles per gram of oil-in-water emulsions applied to the skin. Currently, the exposure concentrations of engineered NMs for the environment are unknown. While certainty of these concentrations is required for quantitative risk assessment, it is not required for the hazard characterization typically associated with screening level assays. As with all studies, care should be employed by the investigator conducting screening assays and by the assessors evaluating the data so that the relationship between effect and dose is not overly interpreted. Whenever possible, investigators should use dose levels that approximate the estimated dose to which the species of concern might be exposed; thus, *in vitro* studies of cell cultures from the respiratory tract should use concentrations that relate to lung burden observed following inhalation, or *in vitro* studies of keratinocytes should use dose levels that are consistent with concentrations applied to the skin. Other dose levels could be used to demonstrate dose-related responses. For potential hazard identification, the dose-response is generally used. The lowest dose of such study might be in the range of expected human exposure. These additional dose levels are frequently exaggerated levels of real exposures, but they serve to stress the system investigated and to identify potential hazards.

There are many factors that need to be considered in sample preparation to ensure that the targeted doses are delivered. Methods should consider if dispersing agents are needed so that particles do not form agglomerates in media unless the agglomeration is intended to be considered in evaluations. If dispersing agents are used they need to be evaluated to ensure that their contribution to observed effects is understood. For example, if a nanoparticle is being evaluated for toxic effects the dispersing agent should not also contribute to the toxic effects. High energy methods such as ultrasonication are used to disperse particles. Such dispersion should be considered if the resulting particles are representative of particles in the environment.

As is noted at several locations in this Technical Report, sufficient physico-chemical information should be taken to allow investigators to consider the several dose-metrics (number, mass, and surface area) found in the literature. In the case of environmental studies, need should also be taken to differentiate among environmental concentration, exposure, and dose. Particular attention should be paid to situations where dispersion stability is shorter than the test duration and then consider alternative dosing stratagems.

By their nature, screening tests are intended for hazard identification and for range-finding when preparing for the more involved, exacting test protocols. Readers should be reminded that the dose levels (and environmental concentrations and exposure levels) used for risk assessment will likely differ from those appropriate to hazard identification.

5.4 Discussion of the relationship between this Technical Report and ISO/DTR 16196, Compilation and description of sample preparation and dosing methods for manufactured NMs

This Technical Report was developed in concert with a sister document, ISO/DTR 16196[1)].[20] ISO/DTR 16196 discusses methods used to prepare samples in various relevant media for toxicological studies, and also discusses issues of relevant dose metrics for toxicological testing considering the various routes of administration. This Technical Report complements ISO/DTR 16196 by moving from sample preparation and dosimetry into a more detailed discussion of the various methods used to perform toxicological screening. When using this Technical Report, it is important to consider ISO/DTR 16196, because difficulties associated with sample preparation and dosimetry are often problematic in performing a good toxicological assessment of NMs.

5.5 Discussion of the relationship between this Technical Report and ISO/TR 13014, Nanotechnologies — Guidance on physico-chemical characterization of engineered nanoscale materials for toxicological assessment

ISO/TR 13014 discusses the importance of NM characterization when performing toxicological testing and provides a list of parameters that are important to measure considering the current state of knowledge and the relationship of the nanomaterial parameter to a potentially adverse outcome.[3]

ISO/TR 13014 recommends rigorous characterization of the NM in order to better understand and interpret the results of any toxicological testing; therefore, it is excellent background material for this Technical Report.

5.6 Review of other relevant international activities and published documents

Other efforts to standardize nanotoxicity testing are led by OECD and ASTM International as well as several National Metrology Institutes.[21] OECD's Working Party on Manufactured Nanomaterials (WPMN) was established in 2006 and consists of nine steering groups (SG) as of 2011.

Table 1 — OECD Working Party on Manufactured Nanomaterials Steering Groups

Steering group	Steering group title
SG1/SG2	OECD Database on Manufactured NMs to Inform and Analyse EHS Research Activities
SG3	Safety Testing a Representative Set of Manufactured Nanomaterials (NMs)
SG4	Manufactured NMs and Test Guidelines
SG5	Co-operation on Voluntary Schemes and Regulatory Programmes
SG6	Co-operation on Risk Assessment
SG7	The Role of Alternative Methods in Nanotoxicology
SG8	Exposure Measurement and Exposure Mitigation
SG9	Environmentally Sustainable Use of Manufactured NMs

WPMN SG4 has conducted a comprehensive review of the OECD test guidelines for their applicability to manufactured NMs. The general finding was that in principle, existing OECD test methods are capable of detecting effects of NMs. The guidance notes on sample preparation and dosimetry for safety testing of NMs and risk assessment were published in 2012.[22]

In 2011, the European Food Safety Authority (EFSA) published a guidance document for the risk assessment of engineered nanomaterial (ENM) applications in food and feed. The guidance covers risk assessments for food and feed applications, including food additives, enzymes, flavourings, food contact materials, novel foods, feed additives, and pesticides (available at http://www.efsa.europa.eu/en/efsajournal/pub/2140.htm).

1) ISO/DTR 16196 is currently under development

Guidance on the safety assessment of nanomaterials in cosmetics was issued by the European Commission's Scientific Committee on Consumer Safety (SCCS) in 2012 (avalaible at http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_s_005.pdf).

ASTM international Technical Committee on Nanotechnology, E56, has developed and published three standards for nanomaterial toxicological testing (see Table 2).

Table 2 — List of published standards from ASTM E56 related to toxicological testing

Reference number	Title
E2524–08	Standard Test Method for Analysis of Haemolytic Properties of Nanoparticles
E2525–08	Standard Test Method for Evaluation of the Effect of Nanoparticulate Materials on the Formation of Mouse Granulocyte Macrophage Colonies
E2526–08	Standard Test Method for Evaluation of Cytotoxicity of Nanoparticulate Materials in Porcine Kidney Cells and Human Hepatocarcinoma Cells

A comprehensive database on the health, safety, and environmental impact of nanoparticles (NHECD) was recently established with funding from the EU 7th Framework (available at www.nhecd-fp7.eu). This information repository is open to automatic updates and critical evaluation by its users.

6 Methods for toxicological screening related to human health

6.1 General

In vitro screening methods can be either cellular or acellular (cell-free) assays or tests. Cell-free methods are used to examine the non-specific effects of NMs, such as interaction with proteins in cell culture media or plasma or the potential to generate free radicals.

Cellular assays can be employed to examine the ability of the NMs to interfere with the biological processes essential for maintenance of cellular homoeostasis. These processes include cell survival, proliferation, DNA replication, and cellular division, among others. *In vitro* screening methods have also been successfully used to determine deleterious effects of NMs, such as DNA damage, mutagenicity, apoptosis, or necrosis. Although individual, end point-based *in vitro* tests are not intended to measure effects at the level of the whole organism, batteries of *in vitro* tests can be potentially predictive of *in vivo* effects (e.g. specific organ function or tissue specific pathways or processes). High-throughput *in vitro* screening strategy is also a component of the 'Toxicity Testing in the 21st Century' initiative issued by the United States National Research Council.[23] In a tiered testing approach, screening methods can serve as a first tier for hazard identification and prioritization of NMs that require further testing and validation through confirmatory assays, if required by the relevant regulatory framework or use context. Alternate screening methodologies can also be integrated into a tiered testing approach. The techniques include high-content, but medium-throughput, platforms, such as genomics, proteomics, and metabolomics. These approaches can provide information on all biological processes that can be affected in response to nanomaterial exposure in a single experiment for a given sample. It is recommended to perform multiple screening tests, if possible, to avoid false positives or false negatives.

6.2 Positive and negative controls for nanomaterial toxicity testing

In order to be meaningful, the screening experiment should incorporate the positive and negative controls.

Typically, the positive control is used to validate the experimental procedure. A positive control should be known from previous experience to induce an adverse response when exposing the test system to it. In order to be relevant, the positive control has to share common physicochemical characteristics with a NM, at least, represent a similar class, for example metal oxides, metals, polymers etc. That way, the NM of unknown toxicity could be benchmarked with a reference material whose toxicity has been well characterized, and, preferably, follows a known biological mechanism. Moreover, a positive control dosing range should reflect real-world exposure conditions. Crystalline silica (nanoUsil) has been used

as positive control for particle toxicology in case of intratracheal instillation studies. The crystalline silica has been known to induce inflammatory response *in vivo*. Similarly asbestos fibres also have been used as positive control for fibre toxicology *in vivo* and *in vitro*. The users of this Technical Report should always consider proper positive control particles and fibres when designing screening tests.

A negative control, on the other hand, should produce a negligible or low level effect that could further be treated as a background level toxicity. If a positive effect is observed following exposure to a negative control, it usually indicates that some other factors are at play and experiment results should be discarded.

The established toxicity screening protocols were developed for chemical substances and larger scale particulates, therefore, current nanotoxicology screening assays mostly rely on chemical or larger particulate substances as assay controls. It is expected that nanomaterial-based controls will gradually replace traditional test benchmarks when the dedicated nanoparticle toxicology screening assays are developed. Along with the emergence of the other nanoparticle reference materials types, this should facilitate the creation of the nanotoxicology measurement infrastructure, intended to streamline the screening process and reduce the end result inconsistency among different laboratories. Recently the term Representative Test Material (RTM) was introduced for a set of well characterized NMs that are available for researchers from European Union - the Joint Research Center in Ispra, Italy NM repository.[24] These NMs are widely used so a lot of basic information is available for these nanomaterials.

6.3 Relevant methods for *in vitro* toxicological screening of manufactured NMs

6.3.1 General

The first step in building an *in vitro* screening set is identifying the relevant *in vitro* assays to include. In general, *in vitro* assays for testing of NMs should incorporate positive and negative controls and acceptance criteria to ensure assay performance. The positive and negative controls ideally should be nanoparticles associated with (or unassociated, in the case of the negative control) a human hazard, the mechanistic basis of which is evaluated by the assay. In cases where such nanoparticle controls have not been identified, small molecule controls are suitable. Assays should also include methods to identify nanoparticle interference, as there are many instances of interference as a result of nanoparticle spectral, catalytic, or reagent absorption properties.[25][26][27] Furthermore, it is prudent to use multiple assays to measure the same mechanism, in order to identify potential false positive and negative results.

Ideally, the *in vitro* assays incorporated into a nanomaterial hazard screening set should be validated for their ability to predict actual human hazards, except local effects, and include appropriate positive control NMs. The utility of these *in vitro* methods for screening purposes, and eventual triaging of the positive materials into higher tier bioassays, depends upon this predictability. Encouragingly, recent data suggest that combining *in vitro* oxidative stress responses with surface area dosimetric conversions holds great promise for prediction of acute pulmonary inflammation.[28] The results of many *in vitro* assays have been predictive of the human response, and researchers are encouraged to use (and further develop) carefully controlled and well-designed *in vitro* assays that are predictive of human health effects.[28][29][30][31][32][33][34] However, in some cases, different mechanisms are responsible for the observed toxicities than the ones being measured, or the *in vitro* assays themselves do not accurately measure these mechanistic targets. Either way, this represents an enormous problem for screening of NMs for hazard by *in vitro* assays. For several assays (e.g. cytotoxicity, genotoxicity) nanoparticle uptake and contact in cells needs to be demonstrated, because the absence of the effect might be due to lack of exposure of the target organelles in the cells.

The toxicology of engineered NMs is still a very young science in comparison to small molecule toxicology, and even for small molecules, developing sets of *in vitro* assays that are both predictive and inclusive of all potential hazards has been challenging and incomplete.[35] Separating known toxicants into recognized classes makes the problem of identifying relevant assays more tractable; small molecule toxicant classes, e.g. endocrine disrupters, are associated with defined toxicities, mechanisms of action, structure-activity-relationships, and, most importantly for screening purposes, validated *in vitro* assays. The same will likely be true of nanomaterial toxicology in the future, with segmentation of nanomaterial toxins into classes based on characteristic physicochemical properties, with well-defined toxicities and mechanisms of action.

Unfortunately, as a science, nanomaterial toxicology has not reached this level of sophistication. Similar to NMs, in many cases, the mechanistic basis of small molecule toxicities are unknown. In these cases, programs such as the United States Environmental Protection Agency's (EPA) ToxCast[35] are using bioactivity profiling of known toxicants across large *in vitro* assay matrices to identify characteristic signatures for predicting the hazards of new chemical entities.[35] Matrix profiling of known nanomaterial toxicants across large sets of bioassays for the purpose of identifying toxicity signatures could also hold promise for nanomaterial hazard prediction.

Oxidative stress-mediated inflammation is currently the most well established mechanistic paradigm in nanoparticle toxicology.[36][37] However, it should be noted that this paradigm has been developed almost exclusively by researchers studying pulmonary toxicities arising from inhalation exposure to NMs and might not be relevant for toxicities affecting alternate target organs and/or by alternate exposure scenarios. This fact could limit the utility of a screening incorporating a base set of oxidative stress and inflammatory assays to strictly evaluate the NMs for environmental or occupational hazard, where inhalation exposure is the primary concern. For other purposes in which inhalation exposure is not the primary concern, such as biomedical applications, these assays might not be relevant. For example, screening sets incorporating haematological assays could be more relevant for biomedical applications that utilize systemic administration.[38] Similarly, cutaneous phototoxicity testing and barrier function could be more appropriate for screening nanoparticles intended for topical applications, such as those used in sunscreen lotions. Therefore, it is important that researchers and others using any *in vitro* assay as part of a screening set understand the context for which the individual assay was developed, such as the intended route of exposure and target organ. Indeed, as the field of nanomaterial toxicology evolves, new mechanistic assays will likely be identified and validated for not only prediction of specific toxicities arising from specific exposure scenarios, but also for specific NM classes.

6.3.2 Cytotoxicity screening methods

The cytotoxicity test is the most basic and common *in vitro* assay and corresponds to the acute high-dose *in vivo* test when appropriate cells or cell lines are used. Usually the cytotoxicity is evaluated by 50 % lethal concentration (LC_{50}) or 50 % effective concentration (EC_{50}) 24 h after exposure. Since the cytotoxicity assay is often carried out in the presence of fetal bovine serum (FBS) or in growth condition, EC_{50} is a more proper term than LC_{50}, because the cell number would increase during 24 h of culture.

The assay methods commonly used for the cytotoxicity measurement of chemicals can be applied to cytotoxicity assay of NMs. The most popular method is 3-(4,5-Dimethyl-2-thiazolyl) –2,5 -diphenyl-2H-tetrazolium bromide (MTT) or modified MTT. Alamar blue, LDH release, and the cell number counting with trypan blue dye exclusion assay methods have been routinely used as well. The presence of non-transparent NMs in the measurement samples might disturb the colourimetric or fluorometric value in cytotoxicity assays. This problem can be avoided by transferring the supernatant of the assay solution carefully to another well or cuvette before the measurement. However, the ability of the tested nanoparticles to adsorb the final product of the test system and/or their ability to interfere in their fluorescense or UV-visible light absorption should also be investigated. If such adsorption or interference is confirmed, these tests might not produce reliable results on the toxicity of the tested NMs. Proper controls for such interferences need to be included in the assays.

When hydrophobic NMs are tested in the cytotoxicity assay, the dispersability of test materials is one of the major determinants, since agglomerated hydrophobic materials might not even reach to the cell monolayer. It should be also noted that the polarity of the zeta-potential, one of the most important characteristics of particles that determine the cellular uptake, can be changed by some additives and/or coatings.[39] Therefore, it is preferable to measure uptake rate of NMs by cells before cytotoxicity measurements.[40]

6.3.3 Inflammatory and immune response screening methods

Immunotoxicity evaluation of NMs might face different challenges from conventional pharmaceutical and chemical immunotoxicity testing. NMs could have physicochemical properties different from non-nanoscale materials. The physicochemical properties originating from this nanoscale size could then be a major consideration for immunotoxicity evaluation of NMs. The particle size and distribution in relation to the surface area have also been identified as critical parameters in assessing the environmental, health,

and safety aspects of NMs. The size-specificity with regard to the toxicity of a material has already been discussed in relation to its surface area.[41][42] Chemical reactions take place on surfaces; therefore, a material with a large surface area can be expected to have a higher reactivity on a mass basis than the same material with a low surface area to volume ratio. Aggregation/agglomeration might also affect particle ingestion by alveolar macrophages. The particles inhaled to the lung are usually recognized and cleared by macrophages. Some research demonstrated that macrophages recognize more easily the aggregated or agglomerated particles than individual nanoparticles and clear the aggregated or agglomerated particles at a much faster rate than individual nanoscale particles. The effects of the shape on the toxicity of NMs have not been fully investigated, yet recent publications indicated that high aspect ratio nanofibres (HARN) have been shown to have the potential to cause an asbestos-like response in animal studies.[43] In 2007 a comprehensive issue of methods was published dedicated to the evaluation of immunotoxicty in animal models.[44]

For skin sensitization (delayed type hypersensitivity), three *in vivo* assays are currently available being the guinea-pig maximization test (GPMT), Beuhler test (BT), and local lymph node assay (LLNA).[45] The latter two assays depend on skin penetration of the agent to be tested before sensitization can occur. However, for NMs skin penetration is generally considered to be low or absent.[46]-[49] Currently, for chemicals, *in vitro* alternative tests are under development for skin sensitization testing. Whether these are applicable to NMs is yet unknown.

A well-defined and standardized set of methods for assessing immunotoxicity is carried out by the Modular Immune *In vitro* Construct System. This system is a proprietary set of human cell-based, *in vitro* cultures and assays that mimic the human immune system.

The system comprises modules termed the Peripheral Tissue Equivalent (PTE), the Lymphoid Tissue Equivalent Module (LTE), as well as functional assays and additional disease models. The PTE works in an analogous way as both the innate immune response and the adaptive immune response in peripheral tissues such as the skin, lungs, and additional mucosal tissues. The PTE can predict adjuvant or vaccine potency, toxicity, and the immunostimulatory potential of various biological and chemical compounds. There are models specific for the subcutaneous or intramuscular exposure, intravenous exposure, and mucosal tissue.

The LTE module uses relevant cell types and interactions to mimic conditions of the lymph nodes where dendritic cells activate T-cells, which then activate B-cells and begin the antibody-production process. The LTE module is capable of producing activated T-cells, antibodies, and cytokines and has been shown to be superior to peripheral blood mononuclear cell assays. This technology is relevant not only to NMs but to drugs and traditional chemicals, as well.

Although it is more oriented to pharmaceuticals and not specific to NMs, one internationally accepted and widely used guideline for immunotoxicity studies is the International Conference on Harmonization harmonized tripartite guideline.[50] The purposes are to provide

a) recommendations on nonclinical testing approaches to identify compounds which have the potential to be immunotoxic, and

b) guidance on a weight-of-evidence decision making approach for immunotoxicity testing.

Immunotoxicity is, for the purpose of this guideline, defined as unintended immunosuppression or enhancement. Drug-induced hypersensitivity and autoimmunity are excluded. The general principles that apply to this guideline are the following:

a) All new human pharmaceuticals should be evaluated for the potential to produce immunotoxicity.

b) Methods include standard toxicity studies (STS) and additional immunotoxicity studies conducted as appropriate.

Whether additional immunotoxicity studies are appropriate should be determined by a weight-of-evidence review. The data from STS should be evaluated for signs of immunotoxic potential. Signs that should be taken into consideration are the following:

a) haematological changes (such as leukocytopenia/leukocytosis, granulocytopenia/granulocytosis, or lymphopenia/lymphocytosis);

b) alterations in immune system organ weights and/or histology (e.g. changes in thymus, spleen, lymph nodes, and/or bone marrow);

c) changes in serum globulins that occur without a plausible explanation, such as effects on the liver or kidney, can be an indication that there are changes in serum immunoglobulins;

d) increased incidence of infections;

e) increased occurrence of tumours can be viewed as a sign of immunosuppression in the absence of other plausible causes such as genotoxicity, hormonal effects, or liver enzyme induction.

When systematically available most nanoparticles end up in organs of the re reticuloendothelial system (RES) like liver and spleen which are rich in phagocytizing cells. The adherence of serum proteins and the formation of a protein corona are suggested to enhance recognition and uptake by cells of the RES. [51][52][53]

Exposure to small particles can elicit immune responses in the blood.[54] These effects on blood are not universal to all small particles,[55][56] probably reflecting the surface properties of the particle. Likewise, immune responses following inhalation exposure to NMs range from immunosuppression to immunostimulation, and again are not consistent for all small particles, probably reflecting the surface chemistry of the particle.

Human immune responses are difficult to evaluate in model systems, because the humoral milieu in the body is difficult to replicate. However, Ryan et al.[57] demonstrated that fullerenes suppressed immune response when incubated with mast cells or peripheral blood basophils. Furthermore, fullerenes inhibited anaphylaxis in a mouse model primed with IgE. Schöler et al.[47][58] studied the release of cytokines from peritoneal macrophages incubated with solid lipid NMs. While these particles do not represent the same particle that is of environmental interest, because they are typically 200 nm in diameter and are not insoluble, crystalline particles, the method might have value. In their study, these authors have harvested peritoneal macrophages and incubated them with the nanomaterial. After 3 h, 24 h, and 48 h, the levels of released IL-6, IL-10, IL-12, and TNF-α were measured in the supernatant. The results indicate that solid lipid NMs did not elicit secretion of cytokines outside of cytotoxicity. Validation with other NMs is necessary to understand the value of this study design.

Similar to the assessment of toxicological risk in other organ systems, the assessment of immunotoxicity should include the following:

— statistical and biological significance of the changes;

— severity of the effects;

— dose/exposure relationship;

— safety factor above the expected clinical dose;

— treatment duration;

— number of species and end points affected;

— changes that might occur secondarily to others;

— possible cellular targets and/or mechanisms of action;

— doses which produce these changes in relation to doses which produce other toxicities;

— reversibility of effect(s).

Table 3 — Immunotoxicity studies

Parameter	Specific component
T-cell dependent antibody response	Using a recognized T-cell dependent antigen [e.g. sheep red blood cells (SRBC) or keyhole limpet haemocyanin (KLH)] that results in a robust antibody response.
Immunophenotyping[a]	Identification and/or enumeration of leukocyte subsets using antibodies with flow cytometric analysis or by immunohistochemistry.
Natural killer (NK) cell activity assays[a]	Conducted if immunophenotyping studies demonstrate a change in number, or if STS studies demonstrate increased viral infection rates, or in response to other factors.
Host resistance studies	Involve challenging groups of mice or rats treated with the different doses of test compound with varying concentrations of a pathogen (bacterial, fungal, viral and parasitic) or tumour cells.
Macrophage and neutrophil function assays	Assessing macrophage/neutrophil function of cells exposed to the test compound *in vitro*, *in vivo*, or obtained from animals treated with the test compound (*ex vivo* assay).

[a] Could be used as standard toxicity tests.

6.3.4 Stress response (including oxidative and protein production) screening methods

6.3.4.1 General aspects

The role of oxidative stress in the toxicity of ambient NMs and the pathways involved in a cellular oxidative stress response have been reviewed previously.[36][59][60] Reactive Oxygen Species (ROS) are generated by cells as by-products of normal cellular activity that can be neutralized by cellular antioxidant defenses. Oxidative stress occurs if the production of ROS exceeds the neutralizing capacity of the cellular antioxidant defence. The cellular response during oxidative stress includes the induction of a number of redox-sensitive signalling cascades according to the following proposed three tiered model.[59] At low levels of oxidative stress, the tier 1 protective response induces antioxidant enzymes such as glutathione S-transferase isoenzymes to restore redox homoeostasis. A further increase in ROS production or a failure of the initial antioxidant response can result in proinflammatory (tier 2) and eventually in cytotoxic (tier 3) effects. The induction of oxidative stress and resulting inflammation is thought to be a plausible mechanism by which NMs might induce toxicity, similar to fine and ultrafine particulate matter.[59][60] NMs could induce oxidative stress via a number of pathways. For example, generation of $O_2^{\bullet-}$ and $OH^{\bullet}$ radicals could result from the formation of electron hole pairs by photoactivation of TiO_2 or from an electron jumping from the conduction band of semiconductor NMs.[61] In addition, dissolution of NMs releasing metal ions and the presence of transition metals such as Fe, Ni, Cu, Co, and Cr on the nanomaterial surface can generate $OH^{\bullet}$ via the Fenton reaction. Finally, even inert NMs could give rise to ROS production by lodging in mitochondria and perturbing their function.[59]

Many *in vitro* studies with NMs have therefore focused on assays measuring markers of oxidative stress and inflammation.

6.3.4.2 Acellular redox activity of nanoparticles

The acellular redox activity of nanoparticles can be evaluated using the dithiothreitol (DTT) assay.[62] The DTT assay determines the general redox activity of a sample by measuring the stimulus (e.g. nanoparticles) dependent oxidation of DTT, a dithiol. The assay measures the formation of ROS by a known amount of DTT. During this reaction, DTT is consumed, followed by its reaction with 5,5'-dithiobis-(2-) nitrobenzoic acid (DTNB) to form 2-nitro-5-mercaptobenzoic acid, which can be measured by a spectrophotometer. Nanoparticles can be incubated for various time periods, after which the reaction

is terminated by adding trichloroacetic acid solution. Aliquots are then incubated with a tris-HCl buffer (pH 8,9) containing DTNB and the absorbance can be read at 412 nm on a spectrophotometer.

A more sophisticated method involves using Electron Spin Resonance (ESR) spectroscopy in combination with the spin trapping technique,[63][64] which offers the advantage that ROS and similar kinds of free radical species generated by the nanoparticles can be identified. For detection of ROS formation, the principle is based on the trapping of radicals with 5,5-dimethyl-1-pyrolline N-oxide (DPMO). ESR spectra are recorded after incubation of the nanoparticles with DPMO in the presence or absence of H_2O_2. The identification and quantification of characteristic DPMO-trapped ROS signals can be performed by integration of so-called peak surface measurements. The resulting data are an identification of type of radical and ROS generated by the nanoparticles and a quantification based on peak surfaces of, for example, the DPMO-OH• in arbitrary units.

6.3.4.3 Generation of ROS in cells

6.3.4.3.1 General

ROS can be generated by cells as by-products of normal cellular activity, but an increase in ROS in response to stress factors might exceed the antioxidant capacity and could result in cell damage. However, not all cell lines produce ROS after exposure to nanoparticles.

For induction of oxidative stress in cells, the two most commonly used assays in nanotoxicology research are measurement of intracellular ROS generation and reduced glutathione (GSH). ROS is measured by means of a fluorometric assay based on intracellular oxidation of 2,7-dichlorofluoroscein diacetate, while glutathione (GSH) is mostly measured with assays based on the production of a fluorescent or coloured dye upon reaction with GSH. The results of ROS and GSH assays correlated remarkably well. [65][66][67][68]

6.3.4.3.2 ROS determination

Nonfluorescent 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) can be used to measure the generation of ROS. Upon entering the cell, the acetate groups are cleaved off, and the remaining compound H2DCF remains inside the cell where it can be oxidized by ROS to the fluorescent compound DCF. After incubation of cells with nanoparticles, the tissue culture medium is removed and the culture is washed. Cells are incubated with freshly prepared 10 mM H2DCF-DA probe in PBS for 45 min at appropriate tissue culture conditions, while protected from light. After incubation, the probe that is not taken up by the cells is removed by washing the cells with warm PBS. A final aliquot of 100 ml of PBS is added to cells in the tissue culture well and fluorescence can be measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm using a fluorescence spectrophotometer. Cells exposed to the vehicle serve as negative or solvent control. For a positive control, cells such as macrophages, can be exposed to cell culture medium containing 10 mg/ml lipopolysaccharide (LPS) and/or 10 mg/mL phorbol myristate acetate (PMA), if differentiation is required. A nanoparticle only control should be included in the assay to exclude interference of the nanoparticles under investigation with the fluorescent read out system. If such interference is detected, false-positive results might be produced.

6.3.4.3.3 GSH measurement

It is suggested that loss of GSH compromises antioxidant defenses, thus resulting in an increase in ROS in the cell and eventually cell death.[66] Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent to other unstable molecules, such as ROS. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). Through this mechanism, GSH acts as antioxidant and can protect cells against damage by radical oxygen species. An increased GSSG-to-GSH ratio is considered indicative of oxidative stress. GSH levels in cells can be determined using commercially available kits. The determination of the GSH content in cell lysates can be based on a chemical agent that specifically binds to GSH, after which a second reaction transforms the complex to a chromophoric compound that can be measured in a spectrophotometer. Also, antibody kits are available that use a specific anti-GSH antibody in a so called Enzyme Linked Immuno Sorbant

Assay (ELISA) for the measurement of GSH levels. In kits for each type of assay, samples are included for preparing standard curves. Once again, interference by the tested nanoparticles with the absorbance of the final reaction product should be checked.

6.3.5 Blood components (including clotting factors, haemolysis) screening methods

6.3.5.1 General

Studies of the interaction of particles with blood components fall into three general categories.

a) one that focuses on effects on the cardiovascular system and blood as a target;

b) one that looks at how blood and blood proteins interact with nano-objects;

c) one that uses effects on blood as an indicator of a biological property.

Each of these will be discussed.

6.3.5.2 Effects on cardiovascular system or blood

It is known that inhalation exposure to small particles can affect the cardiovascular system, an increased risk of heart attack has been observed following exposure to ambient particles[69] including diesel exhaust. In addition, exposure to ultrafine particles such as volcanic ash[70] can affect haemolysis and blood clotting.[71] The following section summarizes screening studies that focused on either blood or the cardiovascular system as the target. Helfenstein et al.[72] evaluated the effect of NMs directly on cardiac muscle, *in vitro*. In their study, neonatal rat ventricular myocytes were incubated with varying concentrations of single-wall carbon nanotubes, diesel emission particles, or TiO_2 and conduction velocity and ROS production were measured. While the results suggest differing effects on heart cell function of ROS production, the investigators never confirmed the physical state of their particles in the culture medium. Carbon nanotubes are known to adsorb nutrients from culture medium, and serum proteins can alter toxicity. Therefore, this study design requires a number of appropriate assay controls (e.g. occurrence of interference, protein depletion), and extensive validation.

Li and coworkers[73] studied the effects of nanoparticles directly on the erythrocyte; erythrocyte sedimentation, and morphology were altered by nanoscale TiO_2 relative to micron-scale material. Further, haemolysis and ROS production were more extensive with nanoscale material relative to micron-scale material. Evaluation with different particles will be necessary to ascertain the predictive value of this test.

6.3.5.3 Interaction with blood that modifies biological response

It has been recognized that blood, specifically proteins, can interact with NMs by coating the surface and forming a corona.[74] In some cases, protein-coated NMs have altered toxicity, generally resulting in lower toxicity compared with the 'naked' particle; for example, The protein corona has been suggested to both facilitate and reduce cellular uptake,[51]-[53] and alteration in biological activity.[75] While these interactions could be important for understanding the effects of particles on biological systems, it is not yet possible to predict the biological effect from that interaction. Therefore, these phenomena will not be discussed further.

6.3.5.4 Interaction with blood as indicator of toxic property

Interactions of NMs with blood have been used to predict biological properties, specifically surface reactivity. These studies will be presented and summarized. In general, such assays suffer from a lack of validation.

Blood, particularly haemolysis, has been used as an indicator of surface reactivity. Warheit et al.[76] used haemolysis as an indicator of surface reactivity and correlated that with pulmonary responses following *in vivo* exposure. In their study, the authors have incubated nano-sized or micron-sized quartz with erythrocytes from fresh whole human blood and measured haemolysis spectrophotometrically. In

addition, animals were exposed by IT instillation to these same materials and the pulmonary toxicity evaluated using histopathology and broncho-alveolar lavage. The extent of haemolysis was correlated better with lung inflammation and lung histopathology than did physical characteristics such as size, crystallinity, surface area, or radical formation as measured by ESR.

Aisaka et al.[77] also correlated the extent of haemolysis to pulmonary toxicity, but not in the same experiment. Using erythrocytes from fresh human blood, the haemolysis of anatase and rutile nano-sized and micron-sized TiO_2 was compared with the pulmonary effects as reported by Sayes et al.[29] However, the characterization of the particles used by Sayes et al. was not adequate to allow direct comparisons to the particles used by Aisaka et al.[77]

Li et al.[73] have reported the haemolytic activity of titanium dioxide nanoparticles. Lin and Haynes[78] also used haemolysis as an indicator of cytotoxicity. Erythrocytes were washed and incubated with porous and non-porous silica nanoparticles. Concentration-dependent increases in haemolysis were observed, and haemolysis was correlated with surface area. The response curves were very steep for porous silica, but not for the non-porous particle. There was no attempt to correlate haemolytic activity with other assays for cytotoxicity.

Using the same principle, Rogers et al.[79] modified the 'Ferric reducing ability of plasma' assay (FRAP) by using serum (FRAS) rather than plasma. Serum was incubated with varying concentrations of different NMs and the degree of oxidative stress was measured. Converting the raw data to Trolox equivalent units, the different NMs could be ranked for their ability to reduce the anti-oxidant capacity of serum. The results are generally what one might expect – nano-sized carbon black reduced anti-oxidant capacity more than the 220 nm carbon black did; fullerene soot reduced anti-oxidant capacity more than refined fullerenes; and anatase TiO_2 reduced capacity more than the rutile did.

6.3.6 Genotoxicity screening methods

The testing of NMs for genotoxicity *in vitro* as a predictor of potential carcinogenic or developmental hazard is a subject of great debate among toxicologists, and there have been many thorough reviews on the subject.[80][81][82][83][84] The primary issues are

1) the suitability of *in vitro* genotoxicity assays originally developed for testing of small molecules,

2) mechanistic relevance of *in vitro* genotoxicity end points to nanomaterial carcinogenesis,

3) the lack of availability of nanomaterial positive controls for evaluation of assay performance, sensitivity and potency comparison, and

4) the relevance of *in vitro* concentrations to *in vivo* exposure scenarios.

The current battery of *in vitro* assays, many of which are available in standardized formats, include single cell gel electrophoresis assay (comet assay),[85] the bacterial reverse mutation test (Ames assay),[86] mammalian chromosome aberration test in Chinese hamster ovary cells,[87] the HPRT forward mutation assay,[88] micronucleus test (MNvit),[89] unscheduled DNA synthesis test in mammalian cell *in vitro*,[90] and *in vitro* Syrian hamster embryo cell transformation assay.[83]

Surveys of the nanomaterial genotoxicity testing literature have identified some common trends.[81][82] For instance, the majority of nanomaterial bacterial gene mutation assays, e.g. Ames assay, results have been negative, possibly due to a lack of nanomaterial penetration of the bacterial cell wall.[81][82] By contrast, the majority of comet assays for DNA damage and mammalian gene mutation assays have been positive, but this might also be skewed by a reporting bias for positive results.[82] As genotoxicity is primarily based on direct DNA effects (although indirect effects via ROS can occur) for any genotoxicity assay internal cellular exposure should be demonstrated. Particles positive in the comet assay include carbon-based NMs, such as fullerene and carbon nanotube, as well as metal nanoparticles, such as cobalt and TiO_2.[82] Within NMs classes, e.g. NMs with common physicochemical characteristics and/or chemical composition, and genotoxicity assay classes, e.g. chromosome aberration and gene mutation, there has been a lack of consistent findings.[81] Furthermore, inconsistencies in assay design and nanomaterial physicochemical characterization and identification have been a common problem when attempting to compare studies and draw conclusions regarding the genotoxic nature of specific NMs

and the performance of individual assays[81] and, as with all studies, show the importance of developing standardized protocols.

Another issue to consider is how standardized genotoxicity assays might need to be modified in order to accommodate evaluation of NMs. As described above, mammalian cell systems could be more suitable than bacterial strains, due to differences in nanomaterial cell uptake.[83] The standard *in vitro* micronucleus assay commonly utilizes media containing serum and the actin polymerization inhibitor cytochalasin-B, and both these components can also interfere with cell uptake of NMs.[83] [88] This could require alteration of the serum concentration or addition of cytochalasin-B following nanomaterial treatment. Additionally, the issue of how *in vitro* concentration relates to *in vivo* exposure is especially problematic for NMs, as not only is the nanomaterial concentration an important issue, but also the nanomaterial state (e.g. aggregation/agglomeration state, proteins bound to the surface, etc.), as this can affect both cell exposure and biological activity. Thus, it is important to characterize the nanomaterial in the treatment media and relate these physicochemical properties to those following actual nanomaterial exposure.

Ultimately, *in vitro* genotoxicity testing of NMs is only useful if the mechanistic end points relate to human mechanisms of carcinogenesis or reproductive hazard. At this time, the prevailing understanding of nanomaterial-induced pulmonary carcinogenesis is that underlying mechanisms do not involve direct nanomaterial interaction with the genome, but rather occur through secondary or indirect mechanisms, e.g. inflammation and oxidative stress.[90][91] While an oxidative mechanism could potentially be evaluated in an *in vitro* system if the oxidative stress is not secondary to inflammation, an indirect inflammatory mechanism cannot be evaluated. Thus, the nanomaterial pulmonary carcinogenesis literature does not provide a strong mechanistic rationale for employing *in vitro* genotoxicity assays at this time. This mechanistic rationale could come from future studies of nanomaterial carcinogenesis, especially in alternate organs following alternate routes of exposure. Predictive batteries of genotoxicity assays need to be developed, standardized, and validated for their relevance to human health. The most appropriate methods might include those that are already OECD-accepted or, if necessary, those that are altered to more specifically address NMs.

6.3.7 *In vitro* barrier tests and appropriate testing systems to assess toxicity to biological barriers

6.3.7.1 General

Adverse effects of NMs on human health might be linked to their ability to cross biological barriers in the body. The route of exposure (pulmonary, gastro-intestinal, dermal) and, hence, the portal of entry of nanoparticles into the body, is of critical importance. The barriers identified below are two that are considered important for NMs at this time.

6.3.7.2 Pulmonary

Inhalation toxicity studies conducted in the early 1990s prompted focus to a developing area of toxicological research, that of nanotoxicology and especially to the potential pulmonary effects of NMs. These often cited preliminary papers focused on the pulmonary effects of NMs and have continued to influence pulmonary nanotoxicology research decades later. Examples of early studies include those conducted by Ferin et al.[92] and Oberdorster et al.,[93] among others. Subsequent to the publications of these papers, concepts such as surface area, particle number, delivered dose, delivered dose rate, and potential translocation of particles to the pulmonary interstitium became of keen interest. Later, characteristics such as the generation of reactive oxygen species, agglomeration/aggregation state, composition, purity, heterogeneity, specific surface area, surface chemistry, zeta potential, catalytic properties, size distribution, absorption properties, surface charge, crystalline phase, grain size, hydrodynamic size/particle size, and shape became characteristics of interest. In addition, details such as dustiness and fat solubility might also aid in predicting the potential toxicity of nanoparticles.

For screening purposes, there are higher throughput and lower cost methods that show data concordance when compared to *in vivo* results. Due to initiatives such as Tox21,[94] there is a concerted effort to begin using human cell-based co-cultures to assay potential toxicity for this exposure route.

In vitro models using cell cultures and co-cultures have proven to be informative.[95][96] Some three-dimensional models, using glass-bottom cell culture dishes and *in vitro* cell models of the human airway epithelium can be maintained for several months.

A study on quantum dots in murine macrophage-like cells (J774.A1 cells) has also helped to delve into the mechanistic reasoning behind the toxicity of nanoparticles. Clift et al. hypothesized that the intracellular localization of a given NM could in fact directly determine its toxic potential.[97] While this study focused on quantum dots, other studies illustrate that this might be a worthwhile hypothesis.

Alfaro-Moreno et al.[98] found that bi-cultures and tri-cultures of human lung cells released granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-1beta, interleukin (IL)-1 β, IL-6, tumour necrosis factor α, and MIP-1 α. The authors go on to state that these effects are consistent with those systemic effects described for particulate matter and correspond to inflammation, endothelial dysfunction, and bone marrow cell mobilization.

Rothen-Rutishauser et al.[99] developed a triple co-culture comprised of epithelial cells, macrophages, and dendritic cells stating that it 'simulates the most important barrier functions of the epithelial airway.' With this model, the authors have shown measurement of cellular responses to titanium dioxide, including reactive oxygen species and the release of tumour necrosis factor, and also have illustrated the intracellular localization of the nanoparticles by energy filtering electron microscopy. This model is presented as a tool to study the interaction of nanoparticles and lung cells and a method for the investigation of the toxic potential of NMs. This model system has been compared to *in vivo* results and good data concordance was observed. Subsequently, toxicological responses resulting from exposure to (nano)cellulose, multi-walled carbon nanotubes, and crocidolite asbestos fibres were discerned from one another in the tri-culture system.[97] Human alveolar epithelial-like type II cells (A-549) were exposed to magnetite ranging in size from 20 nm to 10 µm, separated into four different size-based fractions and tested for their propensity to cause ROS, genotoxic effects, and the stimulation of cytokine pathways. c-Jun pathway activation appeared to be ROS-dependent and ROS formation appeared to also play a role in genotoxicity of the magnetite.[100] In addition, co-culture systems can also be used for exposure in a so called air-liquid-interface to mimick the inhalation exposure via an air flow even better, in contrast to submerged cell culture exposure.[101]

While many of the published cell culture or co-culture experiments produce promising results, very few standardized protocols are used by multiple laboratories. The exception to this is the commercially available *in vitro* human airway tissue model that has been tested in NMs by many collaborating laboratories and is the method cited in multiple publications.

6.3.7.3 Dermal

The issue of skin penetration with regard to nanoparticles is still largely unresolved. There is limited evidence to suggest NMs have the ability to penetrate through the skin or reside in hair follicles;[102][103] however, there is a fundamental knowledge gap surrounding the behaviour of nanomaterials on broken or flexed skin. Additionally, it is known that nanoparticles behave differently according to the chosen dispersant; therefore, when analysing dermal toxicity data, the dispersant used should be taken into consideration.

Rat and pig skin have been the most common proxies for human skin for use in permeability experiments. However, the permeability barrier of skin from laboratory animals is known to be relatively weak, due to significant follicular transport and physiological differences. Morphological differences between skin types, mainly comprising the density of hair follicles along with the concentration of free fatty acids and triglycerides, represent a huge factor in the differences amongst skin barriers in different species. The inter-individual variability of human skin samples also needs to be addressed, which is achievable using current *in vitro* methods.

The many variables involved in dermal exposure using animals make these methods much less efficient and potentially irrelevant to human safety than if *in vitro* human models are used.

OECD TG 428[104] uses human skin samples as a component of the skin absorption testing strategy. This *in vitro* technique is already being applied to NMs using human skin *in vitro* and *in vivo*.[105][106][107] The skin samples are used *in vitro* to assess nanoparticle absorption or the diffusion of substances through

the skin by using Franz-type diffusion cells or the Saarbruecken penetration model. Other dermal toxicity testing protocols, validated by OECD include EpiSkin corrosion assay,[108] Corrositex for Skin Corrosivity,[109] Rat TER skin corrosivity[99][110] and *in vitro* skin irritation OECD TG 439.[111] Dermal penetration of nanoparticles is generally considered to be low or absent.[46][47][48][49] Skin sensitization screening tests are described in 6.3.3.

Accessible, commercially available models have proven reliable and relevant for NMs. Both glass-bottom cell culture dish and 3D *in vitro* cell model based technologies address dermal irritation and permeability concerns and have been validated for chemicals.[112] These methods have been used by industry and academia and are reproducible across laboratories.

6.3.8 Omics measurements

The toxicogenomic approach has been applied for the mechanistic assessment NMs. Omics measurements, such as genomics, proteomics, transcriptomics, metabolomics, and other omics systems are used regularly for pharmaceutical development and ecotoxicology assessment, and are often applied to NMs in a similar manner. The use of cell culture in conjunction with omics for assaying NMs for human health effects is seen often in the literature, although these techniques are not typically performed in a standardized manner. Nonetheless, the resulting data prove helpful and could provide valuable information on potential nanomaterial toxicity in a relatively quick and cost-effective experiment. In addition, the use of base-level trophic species (e.g. bacteria, algae, *Daphnia*, and *C. elegans*) for nano-ecotoxicology screening have also been assayed in combination with omics analysis.

Lee et al.[113] have illustrated the application of functional genomics and report the effects of nanoceria (known to impart redox properties, yet the genome-wide impact was previously not understood) on the global gene transcription in mouse neuronal cells. Findings such as these indicate that genomics and transcriptomics are likely useful in identifying disease-specific genes and can be applied to analyses of nanomaterial-specific health effects.[114]

An example of a commonly applied methodology for ecotoxicity screening is seen in Roh et al.,[115] where silver nanoparticles are tested using base-level organisms, such as *C. elegans*. The same type of genomics and transcriptomics can be done using bacteria found in soil, algal species found in water, or *Daphnia*, the water flea. In the *C. elegans* study, whole genome microarray was used to qualitatively assess transcription profiles, while specific profiles were monitored in a quantitative manner.

6.3.9 Other toxicities

Additional toxicological screening assays, not covered above, include tests for neurotoxicity, developmental toxicity, embryotoxicity, and effects on particular organs.

Neurotoxicity: Cell co-cultures that model the blood-brain barrier and are capable of predicting nanoparticle transcytosis and toxicity have been developed. Comparison between the *in vitro* results and results from *in vivo* tests in rats showed data concordance.[116]

Developmental Toxicity: *Ex vivo* human placental perfusion models have proven useful in determining whether small molecules and nanoparticles are able to cross the placental barrier and can therefore help ascertain whether a given nanoparticle might put a developing foetus at risk.[117]

Embryotoxicity: Partial replacements for the *in vivo* developmental toxicity test exist and include tests such as: embryonic stem cell test, rat limb bud test, and micromass test.[118]

Organ Effects: To assess the effects of pharmaceuticals on particular organs or the ability of drug or molecular delivery devices to target specific cell types, a novel *in vitro* multi-chambered microfluidic microchip of various human cell types has been developed. Sin et al.[119] have developed one such device, the HuREL (Human Relevance), which is particularly relevant for metabolomics, toxicogenomics, multi-organ toxicity, and the evaluation of compound efficacy and metabolites. Walker et al.[120] developed a nano-capable lab-on-a-chip for cytotoxicity testing, which is able to test nine linear dilutions in parallel in human cell lines. Gottwald et al.[121] have developed a chip-based platform for the *in vitro* generation of three-dimensional tissue organization that is compatible with automation. Similar lung-on-a-chip and

gut-on-a-chip devices have been developed.[122][123][124] These lab-on-a-chip advances allow researchers to test for both toxicity as well as cell targeting using a variety of human cells.

6.4 Relevant methods for *in vivo* toxicological screening of manufactured NMs

6.4.1 General

New *in vivo* model systems such as the embryonic zebrafish, can be easily and rapidly interrogated at the cellular and molecular levels, in addition to the whole animal level, to rapidly advance our understanding of the biological consequences of nanomaterial exposure.

6.4.2 Whole animal screening models (including measurements of metabolomics, proteomics, transcriptomics, and genotoxicity)

Whole animal screening approaches using model systems employing non-sentient species are emerging as possible tools to assess the toxicological impacts of NMs for environmental and human health. For instance, the embryonic zebrafish model has emerged as a useful and valuable model of vertebrate biology and concomitantly, human toxicology.[125][126][127][128][129][130][131][132] This model system offers the power of whole-animal investigations (e.g. intact organism, functional homoeostatic feedback mechanisms, and intercellular signalling) with the convenience of cell culture (e.g. cost-efficient and time-efficient, minimal infrastructure, small quantities of nanomaterial solutions required). It should be realized that toxic effects might also occur when the gills of the fish are clogged by nanoparticles. So, there is a physical cause of death instead of toxicological cause.

The embryonic zebrafish model can be employed to rapidly provide information on the biological impacts of nanomaterial exposure on whole animal systems. The test for nanomaterial toxicity provides a sensitive indicator of integrated system effects, because vertebrates at the earliest life stages are often more responsive to perturbation. Fundamental processes of development are highly conserved across species, enabling broad translation of assay results. Since highly coordinated cell-to-cell communications and molecular signalling are required for normal development, if NMs perturb these interactions, development would be expected to be disrupted. Perturbed development can manifest as morphological malformations, behavioural abnormalities or death of the embryos. A suite of morphological, developmental, and behavioural analyses are combined to provide a measure of integrated system effects.

6.4.3 Other relevant *in vivo* screening tests

In whole animal systems, toxicity can also be induced as a secondary effect of exposure to the nanomaterial; for instance, downstream effects of the intact nanomaterial or by-products, dissolved ions or reactive species could be seen on downstream organs after uptake. Additionally, secondary effects can be seen when NMs accumulate in organs or organ systems. For instance, accumulation of carbon nanotubes in the gut tract of *Daphnia magna*[133][134] induces mortality by apparent physical overloading and not due to direct effect of the size of the material. The importance of a weight-of evidence framework for determining no adverse effect levels was highlighted in the carbon nanofibre exposed rat study.[135]

One study design can be considered ‘screening’ for thrombogenicity. Silva and coworkers[136] evaluated the use of the rat ear vein as a screen for effects on thrombosis. In this model, a thrombus is triggered by intravenous (IV) infusion of Rose Bengal and illumination of the vessel with a laser light. The effect of the nanomaterial on clot formation can then be studied by simultaneous IV infusion of the particle, or intratracheal (IT) administration prior to infusion of Rose Bengal, because clots form within 10 min of activation of the Rose Bengal in the vein. Their results indicate that IV administered polystyrene beads significantly decreased the clotting time compared with the untreated group, but IT administered beads decreased the clotting time even beyond the IV group. Interestingly, coating of the polystyrene with an amine was more effective than carboxylate-coated beads; furthermore, amine-coated beads demonstrated a U-shaped dose response. Much work needs to be done to validate this method relative to other particles and particle sizes, but it does hold the possibility of a screening assay for thrombogenicity. Intratracheal administration tests are proposed as screening methods for inhalation toxicity.

7 Methods for toxicological screening related to the environment

7.1 Introduction

Important considerations for environmental toxicology testing include:

— choosing the concentration range of nanoparticles to which the organisms are exposed,

— quantification and characterization (aggregation, surface coatings, etc. and to what extent these characteristics change during the test) of NMs of interest in the exposure media,

— selecting the exposure pathway(s) (i.e. ingestion, inhalation, or contact with the outer surface of the organism),

— characteristics of the exposure medium, feeding conditions (presence or absence of food, and how much and what types of food are available),

— end points investigated (acute toxicity, reproductive toxicity, oxidative stress, etc.), and

— sensitivity of the organisms exposed.

The end points or effects to be tested are similar to those that would be tested for typical environmental pollutants, such as those commonly measured (mortality, weight loss, and population level or reproductive effects), as well as more novel toxicological end points, such as changes to gene or protein expression or oxidative damage to biomolecules.

The extent to which current standard test methods can be applied to NMs without modification, and what modifications if any are needed to ensure reliable and reproducible results, are topics for ongoing research. The applicability of current methods without modification or the modifications needed will probably vary based upon the organism and medium (soil, water, or sediment) in which the test occurs and the properties of the NMs. Nevertheless, the current standard test methods[137][138] are a recommended starting point for the testing of ecological effects from NMs. Some examples of potential methods for rapid screening of NPs include: Algae growth inhibition test,[139] *Daphnia* sp. Acute immobilization test,[140] Water quality-determination of the toxic effect of sediment and soil samples on growth, fertility, and reproduction of *Caenorhabditis elegans* (Nematoda), (4 d for growth test),[141] and the Standard Guide for Conducting Laboratory Soil Toxicity Tests with the Nematode *Caenorhabditis elegans*, (1 d or 2 d for acute mortality test).[142][143] Another promising opportunity for a rapid screening method is bacteria toxicity using 96 well plates and automated plate readers. One example for this is called community-level physiological profiling and has been demonstrated to be effective for distinguished spatial and temporal changes in microbial communities. In applied ecological research, the 96 well platform can be used to measure the metabolism of 31 carbon sources per assay.[144] While there are no standard methods developed for this approach, there is a short (4 h) method for activated sludge respiration inhibition.[145]

Specific methodological considerations for the testing of carbon nanoparticles were recently issued,[143] and a review of the ecological effects of carbon nanotubes in different matrices was also published.[146] Additionally, the preparation method for the NM suspension and the environmental relevance of this approach or lack thereof need to be carefully considered. For example, some early experiments with fullerenes[147] used fullerenes suspended with tetrohydrofuran (THF), which caused toxic effects related to THF byproducts and not the fullerenes themselves.[148] Another important factor related to nanoparticle suspension procedures is ultrasonication and reproducibility of this procedure among laboratories.[149]

7.2 Environmental fate and distribution

The environmental fate and distribution of NMs depend on a large number of factors, such as the release pathway through which NMs initially enter the environment (e.g. in biosolids from a wastewater treatment plant for land application or degradation of consumer products). After release, NM mobility depends on environmental characteristics of the media, such as soil or sediment properties or the aqueous chemistry of a water body. NM characteristics are typically also highly relevant, such as their

surface coatings or lack thereof, shape and morphology (rods versus spheres), particle composition, and affinity for other molecules in the environment, such as natural organic matter.[150][151]

7.3 Environmental degradation and transformation

Potential degradation and transformations differ for carbon-based and inorganic NMs. Carbon nanoparticles (e.g. fullerenes and carbon nanotubes) might ultimately be mineralized into carbon dioxide through biotic (biodegradation) or abiotic pathways.[152] Carbonaceous nanoparticles could also be modified, such as through oxidation and changes to the surface chemistry of the particles. Inorganic NMs can also be transformed through dissolution into their characteristic ions, oxidation processes (e.g. zero-valent iron to iron oxides), and chemical reactions, which otherwise change the composition of the NMs (e.g. silver nanoparticles being transformed to silver chloride or silver sulfide). Another relevant topic for some NMs concerns changes to their surface coatings, such as biodegradation, exchange of the surface coating (e.g. natural organic matter for citrate), or modification of surface coatings by biotic or abiotic processes (e.g. photolysis).

7.4 Environmental biopersistence and bioaccumulation

The biopersistence and bioaccumulation of NMs depend on the NM characteristics (e.g. size, nanoparticle composition, surface coatings), characteristics of the environmental media (with different factors being most important for aquatic media, terrestrial media, or sediment), and characteristics of the organisms. For NM exposure through ingestion of food, water, soil, or sediment, a critical factor is to what extent the material is absorbed into the organisms' tissues rather than remaining in the digestive system. The capability of the organism to excrete NMs is another critical component of their biopersistence. Thus far, absorption into the organisms' tissues has usually not been observed in ecologically relevant exposures, with NMs remaining in the digestive system.[133][134][151][152] However, absorption of gold NMs into earthworms [153] and absorption of gold and fullerene nanoparticles and carbon nanotubes into plants[151][152][154] have been observed. Additionally, food chain transfer has been observed on a number of occasions,[155][156][157][158] but biomagnifications (i.e. an increase from one trophic level to the next) is much less common.[158][159][160] One of the substantial challenges in this field is the lack of NM-specific detection and extraction procedures. Another is the lack of analytical methods to assess the distribution and modifications of the nanoparticles within the organism.

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