
**Nanotechnologies — Compilation and
description of sample preparation and
dosing methods for engineered and
manufactured nanomaterials**

*Nanotechnologies — Compilation et description de la préparation
des échantillons et des méthodes de dosage pour les nanomatériaux
d'ingénierie et manufacturés*



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

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

Introduction

This document provides guidance regarding the preparation of nanomaterials for toxicological, including eco-toxicological, testing. The goal of this document is to assist health and environmental scientists and scientists and experts from other disciplines to understand, plan, choose and address issues relevant to nanomaterials before and during conducting toxicological tests. These issues include the effects of the properties of the material on preparation methods and of the media into which the samples of nanomaterials will be added. Failure to consider these effects might lead to erroneous conclusions regarding the relationship between the nature of the nanomaterial and observed toxicological responses. In particular, the composition and other characteristics of test media can affect the dose to which an organism that is the subject of a test will be exposed. Information on preparation of the test material is necessary prior to any biological or ecological evaluation. Information such as this is consistent with other ISO documents. For example, ISO 10993-18^[1] specifically addresses the evaluation of the chemical characterization of materials used in medical devices, ISO 14971^[2] specifies that a toxicological risk analysis should take into account the chemical nature of the materials, ISO/TR 13014^[3] addresses issues pertaining to the materials themselves and ISO/TS 19337^[5] points out the need to clarify whether observed toxic effects come from tested nano-objects themselves or from other uncontrolled sources. Some examples are provided of methods that establish test conditions that are relatable to environmentally relevant conditions.

This document uses a number of technical terms which have been defined earlier in other documents. Some of these terms have been defined in multiple documents, in different areas of science and technology, providing potentially or seemingly conflicting definitions. This document does not provide new, authoritative definitions for the terms used herein. Instead, this clause provides short descriptions for the terms used. Where possible, reference is made to existing documents.

Nanotechnologies — Compilation and description of sample preparation and dosing methods for engineered and manufactured nanomaterials

1 Scope

This document provides guidance regarding the preparation of nanomaterials for eco- and bio-toxicological testing. It provides guidance regarding factors pertaining to sample preparation and dose determination that might be useful in toxicological, including ecotoxicological, testing of engineered and manufactured nanoscale materials.

The descriptions of sample preparation method factors for both *in vitro* and *in vivo* toxicological testing of engineered and manufactured nanoscale materials include considerations about physico-chemical properties, media, methods for transformation and accumulation studies, health effects and dosimetry. The document is not intended to be a literature review nor a thorough assessment of the quality of the methods or data generated. The document is intended to complement other international efforts.

The focus of this document is on factors that might lead to results that are not relevant to safety evaluations. When featured, referenced methods are considered for their general interest and potential applicability. It is likely that most of the described methods are not generally applicable to all nanomaterials but they do demonstrate important factors and limitations that are common for a variety of nanomaterials.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at <http://www.electropedia.org/>
- ISO Online browsing platform: available at <http://www.iso.org/obp>

3.1

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 80004-2:2015, 3.1]

3.2

structure

arrangement defined by four different aspects (crystallinity, crystal structure, molecular structure and microstructure)

3.2.1

crystallinity

presence or absence of crystalline structure in the arrangement of the atoms of which a material consists

3.2.2

crystal structure

lattice structure in which atoms of an individual crystal are arranged, using lattice parameters and lattice type, such as face-centred cubic, hexagonal close-packed, body-centred, cubic, etc.

3.2.3

molecular structure

arrangement of atoms of an individual molecule

3.2.4

microstructure

arrangement of individual crystals or amorphous phases in a polycrystalline or multiphase material

3.3

measurand

quantity intended to be measured or a quantity that is being determined by measurement

Note 1 to entry: The specification of a measurand requires knowledge of the kind of quantity, description of the state of the phenomenon, body, or substance carrying the quantity, including any relevant component, and the chemical entities involved. The measurement, including the measuring system and the conditions under which the measurement is carried out, might change the phenomenon, body, or substance so that the quantity being measured may differ from the measurand as defined.

[SOURCE: ISO/IEC Guide 99, 2007, 2.3 — modified]

3.4

nanomaterial

NM

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 *nanostuctured material*.

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

[SOURCE: ISO/TS 80004-1:2015, 2.4]

3.5

nano-object

discrete piece of material with one, two or three external dimensions in the *nanoscale*

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-2:2015, 2.2]

3.6

nanoparticle

NP

nano-object with all external dimensions in the *nanoscale* where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

Note 1 to entry: If the dimensions differ significantly (typically by more than three times), terms, such as *nanofibre* or *nanoplate*, may be preferred to the term nanoparticle.

[SOURCE: ISO/TS 80004-2:2015, 4.4]

3.7

nanoscale

length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from a larger size are predominantly exhibited in this length range.

[SOURCE: ISO/TS 80004-2:2015, 2.1]

4 Abbreviated terms

BET Brunauer–Emmett–Teller isotherm

CNT carbon nanotube

DLS dynamic light scattering

ICP-MS inductively coupled plasma mass spectrometry

NOAA nano-objects, and their aggregates and agglomerates greater than 100 nm

NOM natural organic material

TEM transmission electron microscopy

5 Background

5.1 Discussion of the importance of sample preparation and dosing

Nanomaterials are diverse, being based on endless combinations of composition, particle size and distribution, surface chemistry and many other key properties. With this diversity, nanomaterials cannot be treated as a single class of substances. Just as in other areas of toxicology, the assessment of biological effects should consider how samples and doses are prepared and dosimetry is assured so that the observed effects are meaningful and test results can be used in a realistic way such as in safety assessments.

Screening tests are used for rapid evaluations and are typically conducted using cell culture or other *in vitro* techniques due to fast response time, cost, infrastructure and time constraints, factors that limit most whole animal studies. 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 document, a screening test can be generally defined as a relatively simple, inexpensive test that can be administered easily and provides rapid results. The screening tests should reflect the compromise between simplicity, rapidity and low-cost while still providing results that have meaning to safety-relevant situations, the way that samples are prepared and the doses administered will ideally be relatable to realistic situations. Therefore, the points considered in this document also apply to screening tests and should also be taken into account in tiered testing to ensure consistent conditions with each step of the tier.

5.2 Discussion of specific issues when testing the toxicology of manufactured nanomaterials

5.2.1 Physical properties

5.2.1.1 General

The subject that is sometimes referred to as “nanotoxicology” considers the same issues as the broader subject of toxicology with additional scrutiny paid to elements based on a very small size. These include, but are not limited to, physical properties that are described in detail below in [5.2.1.2](#) to [5.2.1.5](#).

5.2.1.2 Size

Smaller particles can have the ability to reach target sites that larger forms cannot. In addition, smaller particles present larger surface areas possibly increasing their chemical reactivity. An example might be inhaled particles (<100 nm) that can reach the alveolar region of the lungs.

5.2.1.3 Size distribution

Most nanoparticles are not of a single size, therefore there is a distribution of sizes. For some materials, there might be nano-sized fractions and aggregated larger particles present of the same composition. The toxicological contributions of each should be distinguished where possible.

5.2.1.4 Dissolution

Nanomaterials have a greater surface to mass ratio so sparingly soluble nanomaterials more readily dissolve in solutions than the same material in bulk form. Examples include amorphous silicon dioxide, zinc oxide and silver. When a material has any appreciable solubility, the relative contributions of toxicological effects due to particulates versus dissolved species should be considered. In some cases where dissolution is complete, observed effects might be due to the ionized/dissolved fraction rather than the nanoparticles, even though the initial test substance was a nanoparticle.

5.2.1.5 Transformation

Because nanomaterials are not a single class of materials there are an enormous number of uses for them. Some of these uses might involve intentional and unintentional transformations. An example of an intentional transformation is the use of aluminium nanomaterials in energetic applications where at least some of the aluminium is converted to alumina^[4]. An example of an unintentional transformation is the release of CNTs from matrices into environmental settings in which they would be subject to photochemical processes, oxidation, biotransformation, etc. CNTs might be subject to combustion processes if they are part of matrices that are burned. With each transformation it is important to consider if changes have occurred that require recharacterization of the test nanomaterial.

5.2.2 Sample preparation

Toxicological studies are generally performed to evaluate the potential hazards of materials. For sample preparation, care should be taken to ensure that the material is prepared in a way that is appropriate for the toxicological evaluation that will be applied. The OECD “Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials”^[5] provides information regarding the critical aspects that should be considered for preparation of nanomaterial samples for testing and the use of relevant dose and dose metrics. It is important to prepare and administer nanomaterials in a way that is representative of a potential exposure, i.e. which can be linked to an exposure scenario. As with assays in general, potential interferences should be considered^{[6][7][8][9]}.

5.2.3 Administration of doses

After sample preparation it is important to quantify and characterize the administered dose and the received dose.

5.2.4 Discussion of relationship between this document and ISO/TR 13014

ISO/TR 13014 provides essential guidance to all researchers who attempt to assess relationships between nanomaterial exposures and linked biological responses. However, as of the time of writing, the practices described in ISO/TR 13014 are not widely practiced. It is not possible to find many references in literature where materials are characterized as described in ISO/TR 13014. ISO/TR 13014 was used as a benchmark in considering whether the studies cited in the references contained in this document should be considered.

5.3 Discussion of relevant dosing for toxicological screening

Humans and the environment may be exposed to nanomaterials via a number of routes, e.g. inhalation, ingestion, dermal for humans, or environment via water. The exposure concentrations for some of these scenarios can be determined, e.g. airborne nanomaterials in the workplace or particles per mass unit of oil-in-water emulsions applied to the skin. Currently, the exposure concentrations of engineered and manufactured nanomaterials for the environment is 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.

However, 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 over-interpreted. Whenever possible, investigators should use dose levels that approximate the estimated concentrations to realistic exposure dose; therefore, *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 concentration levels that are consistent with dose applied to the skin. Other dose levels might and should be used to demonstrate dose-related responses. These additional dose levels are frequently exaggerated levels of real exposures, but they serve to stress the system investigated. The use of single dose level studies is discouraged.

5.4 Discussion of the relationship between this document and ISO/TR 16197

This document was developed in concert with a sister document ISO/TR 16197. ISO/TR 16197 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. ISO/TR 16197 complements this document by moving from sample preparation and dosimetry into a more detailed discussion of the various methods used to perform toxicological screening. When using ISO/TR 16197, it is important to consider this document since difficulties associated with sample preparation and dosimetry are often the bottleneck in performing a good toxicological assessment of nanomaterials.

5.5 Review of other relevant international activities and documents

Readers/users of this document should also take note of an OECD document, "Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials"^[5]. This document provides a perspective based on a detailed review of the literature at the time of the document's publication including potential issues to be addressed.

6 Physico-chemical properties

6.1 Particle size, shape, size distribution and degree of agglomeration

The size of a particle can change once it is put into a new medium. For example, some particles might form homo- or hetero-agglomerates and others might de-agglomerate. A study was performed using micro- and nano-sized carboxylated polystyrene beads to assess the effect on size using *daphnia* as the target organism^[10]. It was observed that the larger particles were taken up more readily but were also more readily cleared.

6.2 Chemical description — Composition and identification

The possibility that the chemical composition of a nanomaterial, where the composition considers the chemical constituents primarily on the surface, might change due to reaction with the medium should not be overlooked. Also, the chemical description should be considered critically to be certain that surface chemistry and surface modifications are accounted for. Impurities should also be considered. For example, some carbon-based nanomaterials may include polycyclic aromatic hydrocarbons produced during the manufacture of the nanomaterials.

6.3 Specific surface area

The effective surface area of individual particles can change due to the addition of surface species from the medium, aggregation, agglomeration and other factors. It is important to recognize that most surface area measurement methods are approximations under specified conditions. It is not uncommon for gas adsorption methods to not agree with liquid based methodologies. The variations are partly due to assumptions but in most cases largely due to differences in medium-surface interactions. Specific surface areas can be normalized by a material's mass, or alternatively by a material's volume.

6.4 Surface chemistry

Surface chemistry describes the chemistry and chemical structure of the exposed outermost layers of a particle. Surface chemistry also refers to the type and area density of potentially reactive surface groups or active adsorption sites. When the surface has been modified to facilitate subsequent chemical or physical reactions, the surface is often noted as being “functionalized”.

When dealing with surface chemistry there are typically at least two important layers: (1) the outermost layer consisting of adsorbed molecules that are often exchangeable and dynamic; (2) the underlying surface representative of the pristine particle that is often not exchangeable but responsive to environmental conditions, influencing the adsorption and adsorbed configuration of the molecules that constitute the outermost layer of the particle.

Observed effects can be greatly impacted by surface chemistry and surface treatments can change these effects. For example, a reactive surface can be passivated and a passive surface can be made more reactive. The composition of the treated surfaces should be described as part of the chemical composition of particles. There are many examples of describing particles as having a composition based on the core of the particle but the treating chemical is not described. It is important to note that techniques used to probe surface chemistry tend to have different surface sampling depths and therefore do not always represent the outermost atomic layers. Many surface sensitive methods are also ultrahigh vacuum methods and therefore might not provide an adequate description of the surface as exposed to different environmental mediums.

It is important to describe both the pristine particle surface chemistry and the resulting surface chemistry of the particle due to adsorption from environmental media. Although the outermost layer is the primary contact to the external environment, it cannot be assumed that the underlying surface is not a factor in interactions with the external environment. For example, the medium might contain components that can collect on particle surfaces forming partial or full coronas. The presence of the corona might change the properties of the surface to be more like the coronal surface and less like the original particle surface. Many authors have noted examples^[11], such as titanium dioxide in cement and

silver in membranes. In cases where nanomaterials are incorporated in a matrix and then are released, the surface of the released particles can be modified by the new exposure medium such as by redox processes. It might also be the case that the coronal surface is transient where observed effects are due to a combination of the coronal surface and the underlying particle surface.

6.5 Isoelectric point, zeta potential and Hamaker constant

In many situations, the dispersion, agglomeration, and deposition of nanomaterials are influenced by pertinent ion electrostatic and van der Waals forces. These forces also influence the adsorption of molecules from the environment and their adsorbed conformation. The isoelectric point and zeta potential are measurands that provide information on surface charge or the mode and extent of ion electrostatic interactions within a medium, whereas the Hamaker constant provides information on van der Waals interactions.

The zeta potential of a particle is a calculated charge based on the electrical mobility of a particle in a fluid and is used as a proxy for surface charge density. The zeta potential is calculated at the shear plane or the outermost fluid layer that travels with the particle as it moves in an electrical field. A number of different theoretical derivations of zeta potential exist and each has its own assumptions and related limitations. When reporting zeta potential, the method applied, the associated theory, fluid conditions (including ionic strength, ion speciation and pH) should be reported. Because a vast number of factors, including adsorption from solution, can influence zeta potential values, changes in zeta potential are often more valuable in terms of understanding a material's behaviour in complex media than a single zeta potential value^[12].

To compare different materials, it is common practice to measure the zeta potential of a material in water as a function of pH with a finite amount of a monovalent background electrolyte. Key variables include the determination of the isoelectric point and the plateau zeta potential values for the given materials. The isoelectric point of a nanomaterial is the pH at which that material has a zeta potential of zero. This is also the point of zero charge for the particle. When characterizing a nano-object, a recommended base set of measurands for describing the state of "surface charge" should include the determination of the nano-object's isoelectric point in a monovalent electrolyte in its as received condition, as well as its zeta potential in the medium to which the nano-object is to be exposed and at the same pH in the monovalent electrolyte system. Additional information on zeta potential and isoelectric point measurements, their limitations and utility can be found in Reference ^[13].

The Hamaker constant is a measure of a material's dielectric susceptibility and is applied in macroscopic continuum approaches to van der Waals interactions. Though the Hamaker constant is denoted a "constant", for historical reasons it is important to realize that the effective Hamaker constant can vary with separation distance due to the effects of retardation.

To calculate the Hamaker constant of a system one should first either derive or obtain the dielectric susceptibility spectra of the interacting bodies and the intervening media. Details on how to obtain these parameters are described in detail elsewhere^{[14][15]}. A simple approach to estimating the Hamaker constant of a system is to use the Tabor–Winterton approximation that only requires the refractive index and dielectric constant of the interacting particles, separately. Typically, the dispersion term (induced-dipole/induced-dipole interactions) is dominant and the extent of van der Waals attraction in a system can be inferred simply by differences in refractive indices. The Hamaker constant can be derived to represent a single material in a vacuum, a material interacting across a medium, or two different materials interacting across a medium. It is important that the form of the Hamaker constant is noted or understood. Since van der Waals forces are often the predominant attractive forces in colloidal systems, the Hamaker constant provides an indication of the relative theoretical dispersibility of a given material within a matrix. The Hamaker constants of particle systems are often but not always similar to their bulk counterparts. The overall van der Waals force is dependent on the Hamaker constant but also on the geometry and size of the particles, as well as the presence or absence of coating, coronas or transition layers. The calculation of van der Waals interaction forces from Hamaker constants is extensively described in Reference ^[14].

6.6 Influence of water chemistry on nanomaterial properties and dispersion behavior

Factors such as pH, hardness, ionic strength, natural organic matter (NOM), etc. (collectively described as “water chemistry”) can affect nanomaterial properties. For nanomaterials that might be released into the environment, agglomeration can be encouraged due to adsorption of NOM onto particle surfaces[11]. Since this adsorption may be an equilibrium effect, desorption of NOM (or other adsorbed species) can occur if the concentration of the contaminating chemical in the medium is reduced, such as through dilution or if a competing contaminant becomes available at a higher concentration.

6.7 Preparation of liquid dispersions

The presence of species that encourage dispersion can also change other properties such as surface chemistry[1]. These might be naturally present or added to the medium used for the evaluation, or might be added separately to the test material as part of its preparation.

The dispersion of nano-objects within a medium is not only dependent on the chemistry of the materials involved, but also on the extent of shear force exerted on the sample. In many scenarios, it is often more difficult to reproduce the shear energy within a protocol rather than the applied chemistry. Detailed dispersion protocols are now being developed to better address this energy input variable[16]. It is also important to note that the theoretical and practical amount of dispersion energy to disperse a sample is dependent on particle size. It is not uncommon for nano-objects to require orders of magnitude more shear energy to reach their smallest dispersible size without fracture than macroscopic materials. This is due to the much greater number of associations that need to be broken for nano-objects and the increased influence of surface forces on their association.

6.8 Crystal structure

Some nanomaterials will have different crystalline forms and there can be differences in the effects of each of the forms. If different crystal structures are possible it should be determined which one(s) is (are) present. For many materials, crystal defects and impurities/dopants also significantly modify the behaviour of nanomaterials. Crystallite grain size and apparent domain strain are other factors that are important to consider. The methods and assumptions used to obtain these values need to be carefully reported, since device resolution, analysis method and assumptions can significantly influence outcomes.

6.9 Surface energy or interfacial tension

The differences between the surface energies of a test material and the media to which it is added will influence its wettability and therefore its dispersibility or absorbability. There are a number of methods that can be used to infer the surface energy or critical surface tension distribution for nano-objects. However, each method has its caveats. Common methods include vapour absorption, heat of immersion microcalorimetry experiments, Washburn capillary rise experiments, pellet formation followed by traditional contact angle measurements, film flotation experiment, liquid-liquid partitioning, and agglomeration potential experiments. To determine surface energy or critical surface tension, the nanomaterial powder in question should be interrogated with multiple liquids of at least different surface tension and of different polar and dispersive wetting contributions. As with surface energy determinations for macroscopic interfaces, different method and measurement approaches often lack agreement.

6.10 Solubility

According to 5.2.1.4, dissolved species (or free species) may have significantly different toxicological profile from the particulates, as pointed out in 5.2.1.3. In addition, dissolution profile is dependent on the media, as well as concentration and should be characterized under relevant conditions. This is particularly relevant to some of the nanomaterials where the active pharmaceutical ingredients are incorporated to decrease toxicity (by decrease exposure of health organs and tissue to the API).

7 Considerations for preparing samples of nanomaterials in exposure media in ecotoxicity studies

7.1 General

7.1.1 Introduction to existing knowledge

Ecotoxicity studies are performed to assess potential effects of releasing the nanomaterial into the environment. The connection of these studies to a realistic environmental exposure should be made clear and the relevance assured. For example, if a study is attempting to model the exposure to zinc oxide of a certain particle size used in a sunscreen formulation that is released into surface water and results in an exposure of fish, it should be assured that the particle size used in the test is representative of the nanomaterial in the environment and not necessarily the particle size used in the sunscreen, as the size might change once introduced into environmental media. An example is given for nanoparticles of aluminium being transported through soil and sand matrices^[4]. The evaluation matrix was designed to simulate an actual soil where aluminium was present due to an environmental exposure. Additional measures were taken to simulate potential variations in the matrix due to the addition of surface waters. For some of the ecotoxicity tests the information in [8.1](#) may be useful.

7.1.2 Environmental behaviour

The behaviour of nanoparticles is affected by the medium in which they are surrounded. For example, CNT in pure water will have different effects on test organisms compared to CNT in media, such as serum or waters containing NOM. Examples include: (1) CNTs tested in Suwannee River water^[17] and (2) aluminium used in a model study of dispersed nanoparticles in soil where using BET, TEM and DLS, the materials were shown to start as aggregates and further agglomerated throughout testing^[4]. Due to the complexity of environmental media and potential transformations, test systems will often need to be set up using model systems or reduced complexity to emulate specific situations to answer narrow questions. Since many systems are very sensitive and can be relevant only to one nanomaterial, the conditions suitable to answer one question might not be appropriate for another question due to changes in factors such as pH, ionic strength, NOM, sunlight, shifting equilibria, etc. For example, nano-silver has an equilibrium between the solid and ionic forms so performing a size measurement in liquid media could require well-defined conditions (initial mass, temperature, amount of liquid) for the measurement and the result might only be valid for those conditions^[18].

7.1.3 Degradation and transformation

Some nanomaterials can change upon exposure to the environmental/biological medium. Examples include nano-iron which can quickly oxidize to iron oxides, and slightly soluble materials, such as zinc oxide and silver. In the example of nano-silver, a recent study^[19] shows that particles of silver can dissolve and then form particles of nano-silver after a longer period of time. There are literature reports showing that nano-silver might ultimately form particles of nano-silver sulfide^[19]. In a study with aluminium, the transformation of the test substance resulted in an effective increase in particle size due to agglomeration, reducing the mobility of the nanomaterial in the test medium^[4].

7.1.4 Bioaccumulation

Bioaccumulation is defined as accumulation of a substance, such as a toxic chemical, in various tissues of a living organism. Bioaccumulation should also be considered for engineered and manufactured nanomaterials.

Some nanomaterials might not degrade readily, e.g. some metal oxides. Other nanomaterials are not persistent, such as amorphous silicon dioxide, zinc oxide and silver, which have appreciable solubility under many environmental conditions, and would therefore not bioaccumulate.

7.2 Test method applicability and dosimetry

7.2.1 Environmental distribution

7.2.1.1 General

Many nanomaterials are used in very small quantities. Detecting them after release to the environment is difficult and could require very sophisticated equipment and analytical techniques. If the elements of these nanomaterials are naturally present in the environment, the interference from the natural background concentration should be evaluated.

7.2.1.2 Methods

A careful strategy is needed to determine many nanomaterials in the environment due to their low concentrations and many potential interferences. Methods that might be useful in evaluating environmental distribution include isotopic fingerprinting, though this requires highly specialized equipment and may not be readily available. Ideally, the method chosen should be highly selective for the nanomaterials of interest even within matrices. An example is mass spectrometry (e.g. ICP-MS) methods which are capable of detecting many metals in trace concentrations. In some cases, it might be possible to prepare concentrated samples to overcome environmentally dilute concentration though it will then need to be considered if the concentration process affected key properties^[18]. Detection of carbon-based nanomaterials in biological and environmental matrices is very difficult because of the other carbon that is naturally present but in some cases it can be determined indirectly if there are trace amounts of metallic catalysts present at consistent concentration. A good perspective on this issue can be found in Reference ^[18]. Comparable care needs to be taken when preparing samples of nanomaterials as test materials for exposure to organisms.

7.2.1.3 Dosage and sample preparation for physico-chemical fate studies

Many factors need to be considered during sample preparation to ensure that the targeted doses are delivered in a form that is representative of particles in the environment. Methods such as sonication and the use of dispersing agents are needed to ensure uniform dispersion of the nanomaterials in the media unless agglomeration is intended to be considered in evaluations. If dispersing agents are used, then dispersant-only controls should be included in the studies to evaluate the potential of the dispersant to cause the observed effect. For example, if a nanoparticle is being evaluated for toxic effects, the dispersing agent should not also contribute to the toxic effects.

To study nanomaterials in a more dispersed form the materials should be dispersed preferably using only constituents in the environmental media or reasonable compounds that the nanomaterials might have on their surface. When possible, the materials should be dispersed in simple solutions (e.g. water and pH adjustment) at a higher concentration then dosed into the environmental matrix to meet the desired exposure concentration without changing the intended solution chemistry. If a dispersed material is dosed into a matrix and agglomerates then the solution conditions of that environmental matrix favours agglomerations. The investigators should consider that if this is the case, the nanomaterials might not be practically available in a dispersed form under the designed environmental conditions.

7.2.2 Degradation and transformation

7.2.2.1 Methods for degradation

Methods will vary with the material being studied. The chemistry of each nanomaterial should be considered for how it can influence degradation. For example, it is known that iron can readily oxidize under certain conditions. If nano-iron is being evaluated, its potential oxidation to iron oxide(s) should be considered and addressed.

7.2.2.2 Dosage and sample preparation for degradation studies

Samples shall be prepared in a manner that is representative of what can occur in the environment. This will include an assessment of the form of the test material as it will enter the environment, which might be different than the as-manufactured or as-used forms. A common issue for many nanomaterials is the propensity to rapidly aggregate or agglomerate. Many scientists have used energy and dispersing agents to create stable suspensions of nanomaterials, however, it was not always recognized that these conditions could create forms of nanomaterials not found under normal environmental conditions or that some dispersing agents contributed to toxic effects^[20]. When dispersing agents are used, it is essential to ensure that they do not affect the results of assessing biological impacts. In the reference cited here, the least impact for carbon-based nanomaterials were dispersions with non-ionic surfactants though the results were target organism dependent.

7.2.3 Bioaccumulation

7.2.3.1 Methods for bioconcentration and bioaccumulation

Methods used should be representative of realistic scenarios and the relationship explained. For example, if organisms are to be part of a test, it should be assured that the uptake pathway is a reasonable model for how uptake will occur in the environment.

7.2.3.2 Dosage, exposure and sample preparation for bioaccumulation studies

Test concentrations used should be similar to environmental exposures. Exposure methods should be relatable to realistic scenarios and samples prepared in a form that will result in a dose and exposure that is characteristic of the described scenario. In a study of titanium dioxide^[21], a series of aquatic test matrices were created using a variety of pHs, electrolytes and NOM in synthetic test water and a series of waters from natural and municipal sources. Not surprisingly, there was a significant variability in the particle diameter and this could have an impact on determinations of size-dependent effects.

8 Methods for preparing nanomaterials for toxicological studies in mammals that ensure correct dosing

8.1 Issues to consider for stock dispersion preparation

In vivo studies are thought by some scientists to better predict potential effects of chemicals (including nanomaterials) than *in vitro* models, giving data that are closer biologically to the potential effects on humans. In the studies, realistic concentrations and materials should be applied and the relevance of the exposure level assured. Using the same example described in 7.1.1, if a study is attempting to model the exposure to zinc oxide of a certain particle size used in a sunscreen, it should be assured that the particle size used in the test is representative of the nanomaterial in “real world” use after application of the sunscreen. The particle size of the nanomaterial before addition to the sunscreen (or even the size in the sunscreen) is not necessarily the most relevant one as the size might change once the sunscreen is applied. The dispersion has to mimic the most relevant of these sizes.

8.2 Importance of monitoring stability of test dispersions during experiments

In order to better assure uniform dosing, dispersions should be assessed for their stability throughout the entire dosing period. Some nanomaterials that had been dispersed uniformly throughout a medium might separate into a layer of particles on top of the medium that is no longer dispersed or the particles settle onto surfaces such as those of the walls of a container effectively reducing the dose to which the test animal is ultimately exposed. Transmission electron microscopy (TEM) analysis of the tissues from the test animals can add relevant information on internal exposure to nanomaterials and their agglomeration in target organs.

8.3 Special considerations for physiological salines used in mammalian studies

Physiological salines have different properties to water and the manner in which each saline represents mammals in general or specific mammals needs to be addressed. For example, metal oxide nanoparticles in a phosphate buffered saline can lead to unintentional passivation of the surface with phosphate and, for some materials, lead to dissolution of the particles through coordination interactions.

8.4 Routes of delivery and behaviour of nanomaterial dispersions in mammalian studies

8.4.1 Respiratory tract exposures

For risk assessment of inhaled nanomaterials, testing by inhalation in rodents is expensive, technically difficult and associated with animal welfare concerns. This method should only be considered as a last resort once all available *in vitro* methods have been exhausted. Whole body exposure is a more 'natural' exposure method compared to nose-only exposure^[22]. When using whole body exposure, delivery of nanomaterials by other routes (dermal and oral) can be considered. Nose-only exposure requires animals to be restrained in tubes which results in an inability to eat or drink during the exposure time, possible overheating of the animal, and possible suffocation if the animal tries to turn around in the tube. The stress experienced by the animal under these conditions is likely to confound the study results.

Where the exposure is done through normal breathing processes, the exposure is likely to be to dry nanoparticles and the test method should ensure that particle size changes are considered and addressed as the particles might transform as they move from a dry environment to a moist environment in respiratory passages.

Many respiratory exposures in mammals have also been done by instillation, which is carried out using high local doses and results in nanomaterial overload as compared to inhalation^[23]. In such studies, the relevance of exposure routes and concentrations should be considered and explained. Instillation has also been demonstrated to produce a less even particle deposition pattern in the lung than the inhalation method and efforts need to be made to disaggregate the nanomaterial suspended in vehicle. However, the defence system of the lung is likely to be affected by the addition of particles suspended in a liquid to the lung surface^[24]. The dose rate and format (wet suspension rather than dry cloud) are also very different to inhalation, therefore caution should be taken when interpreting data generated from instillation studies.

8.4.2 Oral exposure

Oral exposures are typically applied to gain an understanding of the implications of the direct ingestion of nanomaterials as part of a food source or indirectly from lung clearance or simple external transfer from surfaces to the oral tract. Each of these scenarios can realistically reflect nanomaterials at very different states of agglomeration and effective concentrations. The vehicles applied to deliver nanomaterials should be carefully considered to realistically reflect the exposure scenario and to ensure that they will meet the requirements to address the specific question to be answered. It is important to note that as a particulate material transfers through the gastro-intestinal tract it will likely transform (e.g. agglomerate, dissolve and possibly re-precipitate) due to the varying environments encountered. Care should be taken in the interpretation of the results to ensure that the effects are the result of nanomaterial and not ionic forms of the material.

It should be considered and addressed how particle size can change and the relevance to the realistic scenarios.

The use of gavage can lead to underestimates in the dosing due to the nanomaterial sticking to the side of the tubing. Furthermore, this exposure method is not comparable to any expected route of human exposure and can be associated with adverse effects, such as reflux, which can result in serious respiratory effects and mortality.

8.4.3 Dermal exposures

Dermal exposures are of particular concern in certain applications, for instance when nanoparticles are dispersed in liquid matrices. Methods should address how the nanoparticles are dispersed in a way that is uniform (if not uniform additional explanation will be needed) and that can be correlated to pertinent realistic exposures. In a study on zinc oxide, researchers used human skin to test a commercial sunscreen as the source of nanoparticles^[25]. This approach clearly attempted to develop a realistic exposure scenario that closely modelled human exposure and nanomaterials in commercial use.

8.4.4 Injection routes

As with other liquid media, it should be considered how nanoparticles can transform from dry particles to particles in a liquid medium. If dispersing practices are used, it should be addressed how the dispersing method is relevant in the realistic scenario and potential biological effects from any dispersing agents should be considered and addressed. Exposure scenarios should be described so it is clear whether a scenario is to represent potential exposures in the use of a nanomaterial or if the exposure is an overexposure intended to elicit a biological response. Administration by injection can result in particle deposition at the injection site, resulting in the exposed dose being less than the administered dose.

Because injections of any material, and solids in particular, into an organism can lead to significant interactions, the basis for performing such experiments should be well explained, as well as how potentially lethal impacts are mitigated and how the results will be used.

9 Methods for preparing samples of nanomaterials for use in cell cultures

9.1 Cell cultures and dispersion of nanomaterials in culture media

9.1.1 General

Cell culture studies should involve methods to disperse nanomaterials so that cells are exposed equally. Nanomaterials have been shown to agglomerate in physiological fluids leading to uneven deposition of nanomaterial aggregates on the cells. The nanomaterials are deposited onto the cells through a combination of sedimentation (i.e. deposition due to the force of gravity on an object with mass) and Brownian forces (i.e. deposition due to random Brownian motion)^{[26][27]}. The sedimentation force is a function of both agglomerate size and density, while the Brownian force is a function of the agglomerate size only^{[28][29]}. The deposition of nanomaterials is therefore dependent on their size, shape, density and agglomeration, and these properties should be accounted for in dosing^[26].

In addition to nanomaterial properties, the organization of cells in the *in vitro* culture has a bearing on the overall exposure to the nanomaterial. For instance, a recent study comparing cellular orientation (upright versus inverted) in an *in vitro* culture suggested that nanomaterials deposit onto cells by sedimentation in an upright position and by Brownian force only in an inverted orientation^[29]. Deposition of nanomaterials to the bottom of the cell culture wells might result in overload effects that could be misinterpreted as cytotoxicity^[30]. Therefore, modifications to the dosimetry paradigm are required to produce uniform nanomaterial deposition to allow for better prediction and correlation of the treatment dose to response. These changes in dosimetry include: i) considerations for nanomaterial dispersion in the dosing solution, ii) consideration of the area of exposure in the dosing regimen (may be better than volume in some cases) and iii) consideration of relevant exposure scenarios.

The potential impact of endotoxins should be considered. Additional details on how to do so can be found in ISO 29701^[31]. Also, the impacts of the media can be important, such as uncontrolled sources of impurities. An approach on how to do so is described in ISO 19337^[6].

9.1.2 Considerations for nanomaterial dispersion in the dosing solution

If the nanomaterial is dispersible in water, agglomeration can be reduced by making stock dispersions in ultrapure water and then dispersing a small volume of this stock into cell culture medium or another

buffer[32]. Alternatively, if smaller volumes are used, the nanomaterial can be dosed in ultrapure water[5][33]. If necessary, additional methods to improve the dispersion of the nanomaterial include the use of dispersants, for example, protein such as albumin, surfactants such as polysorbates, or solvents such as dimethyl sulfoxide. Mechanical and physical methods, such as sonication can also be used for manipulation of ion strength and pH of the dispersion medium[34]. Relevant controls should be added to the assay to ensure that added peptides or other organic ligands do not affect how the nanomaterial interacts with the cell surface or the adhesion of the cells to the culture plate.

The serum often added to culture medium contains bovine serum albumin (BSA), which is a useful dispersant. BSA stabilizes the nanomaterial suspension by forming a coating on its surface, referred to as the corona, leading to reduction in aggregation. Using BSA as a dispersant has little effect on the balance of protein in a cell culture and helps prevent false positive cytotoxicity, which can result from nutrient depletion caused by adsorption of nutrients from culture medium onto nanomaterial surfaces[6][35][36][37][38]. In addition, the presence of serum within nanomaterial dispersions is more relevant to the physiological condition and its presence has been shown to enhance the uptake of nanoparticles by hepatocytes[39]. Since serum and BSA inevitably contain a number of unknown and variable ingredients, the use of high purity BSA, or a chemically defined medium that has been manufactured from non-animal sources should be considered[5][33]. Furthermore, it is important to consider the relevance of the medium formulation to the relevant exposure and context.

9.1.3 Consideration of relevant exposure scenarios

Following human exposure, nanomaterials become coated with a number of biomolecules, often referred to as the protein corona or bio-corona. The nature of the corona depends on the surface properties of the nanomaterial in addition to the route of exposure[40]. Therefore, the likely route of human exposure should be considered prior to conducting nanotoxicity testing. For example, prior to exposure of hepatocytes *in vitro*, a nanomaterial could be coated with lung lining fluid and then with serum proteins in order to represent inhalation and subsequent transit to the liver[41]. In another example, nanomaterials were coated with a surfactant lipid found in the lung lining fluid, dipalmitoylphosphatidylcholine (DPPC), in addition to BSA or serum before being added to lung epithelial cells[42]. The addition of BSA and DPPC to the culture medium enhanced and stabilized the dispersion of ultrafine carbon black particles, leading to increased reactive oxygen species production as compared to particles suspended in saline solution[43]. This method is more physiologically relevant for studies modelling the lung.

Likewise, dermal exposure to nanomaterials can be modelled by dispersing nanomaterials in an aqueous sweat/oily sebum mixture[44]. Because the state of agglomeration is not easily studied in the assessment of skin absorption *in vivo*, it is preferable to use OECD TG 428[45] which can involve the use of human skin samples[5][33]. This *in vitro* human skin technique has already been used for the testing of nanomaterials[46][47][48][49][50][51].

Whichever dispersion method is used, a balance between a “well dispersed” nanomaterial suspension and a relevant human exposure should be maintained. In addition, the stability and life cycle changes of any nanomaterial suspension over time should be determined using techniques, such as dynamic light scattering, field flow fractionation, differential centrifugal sedimentation, modified light microscopy, and ultraviolet-visible spectroscopy.

9.2 Cell cultures

Air-liquid interface (ALI) culture systems provide a more physiologically relevant alternative to conventional cell culture for studies modelling the lung[52][53][54]. ALI systems employ aerosol exposure of lung cell cultures to prevent interactions between nanomaterials and media components, providing more realistic dosing and better control of particle agglomeration. In one study, an integrated system capable of generating and depositing airborne nanoparticles directly onto cells cultured in an ALI system was shown to provide uniform and controlled dosing of particles with high efficiency[55][56]. Exposure to copper nanoparticles in this study decreased cell viability and significantly elevated levels of lactate dehydrogenase, intracellular reactive oxygen species and interleukin-8, similar to results obtained with inhalation studies *in vivo*. In another study, cytotoxicity of amine-functionalized polystyrene nanoparticles was significantly higher when applied as an aerosol to cells cultured in