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Biological evaluation of medical devices —

Part 22: Guidance on nanomaterials

Évaluation biologique des dispositifs médicaux —

Partie 22: Lignes directrices sur les nanomatériaux

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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).

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).

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For an explanation on the voluntary nature of standards, 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.

This document was prepared by Technical Committee ISO/TC 194, *Biological and clinical evaluation of medical devices*.

A list of all parts in the ISO 10993 series can be found on the ISO website.

Introduction

This document is intended as guidance for the biological evaluation of medical devices that contain, generate or are composed of nanomaterials. Multiple definitions have been developed for the term nanomaterial. For the purposes of this document, the ISO definition will be used: A material is considered a nanomaterial when it has a size at the nanoscale including external and internal dimensions, i.e. when it has a size or is composed of structures with a length of approximately between 1 nm and 100 nm (ISO/TS 80004-1:2015). For regulatory purposes, it is advisable to check if specific national or regional regulatory definitions are applicable. It should be realized that other characteristics (e.g. nanospecific properties) might also be included in such definitions.

Morphological structures created on the surface of a medical device can also have sizes in the nanoscale. Therefore, possible effects of such structures on the biological response to the device also need to be considered.

Nano-objects having a length range from 1 nm to 100 nm can be generated during the life cycle of a medical device, so the evaluation of possible adverse effects due to the generation of nano-objects either from preparation, use, wear or degradation of medical devices needs to be addressed. This applies to medical devices manufactured using nanomaterials and medical devices that are manufactured not using nanomaterials but having the potential to generate nanoscale wear and/or degradation particles. For the biological evaluation of medical devices, knowledge on the potential generation and/or release of nano-objects from such materials is essential.

The procedures as described in the ISO 10993 series for the biological evaluation of medical devices can be used for the biological evaluation of those medical devices that contain nano-objects that are not released from such a device as they are an integrated part of the device. However, when release of the nano-objects is possible, a safety evaluation should also be performed on the released nano-objects. In addition to evaluating a medical device, nanomaterial components or constituents can also be separately evaluated.

This document provides trained professionals, in the context of medical device evaluation, a general approach to biological evaluation of nanomaterials and addresses how the other parts of the ISO 10993 series can be used when dealing with the evaluation of nanomaterials. It is likely that the various assays as described in the ISO 10993 series are not always appropriate as such in the testing of nanomaterials. Nanomaterials by themselves can be present as powders or colloid dispersions, but also can be present in medical devices while incorporated in a matrix, as nanostructured material or as surface structures on materials and/or medical devices. In general, nanomaterials themselves need to be evaluated instead of extracts as usually used when testing biomaterials or medical devices. Nanomaterials pose specific challenges when applying test systems commonly used for medical device evaluation and when interpreting test results.

The field of nanotechnology, development of nanomaterials and the evaluation of potential toxicity of such materials are emerging fields and this document represents only the knowledge at the time of writing. Although appropriate tools and methods for evaluation of nanomaterials are still under development, data on the characteristics and biological effects of nanomaterials should be provided in order to address safety issues in their application in the medical device field, taking into consideration a risk/benefit analysis.

This document provides guidance on how to perform a biological evaluation for those medical devices that contain, generate, or are composed of nanomaterials within a risk management process as described in ISO 10993.

Biological evaluation of medical devices —

Part 22:
Guidance on nanomaterials

1 Scope

This document describes considerations for the biological evaluation of medical devices that are composed of or contain nanomaterials. In addition, this guidance can also be used for the evaluation of nano-objects generated as products of degradation, wear, or from mechanical treatment processes (e.g. *in situ* grinding, polishing of medical devices) from (components of) medical devices that are manufactured not using nanomaterials.

This document includes considerations on the:

— characterization of nanomaterials;

— sample preparation for testing of nanomaterials;

— release of nano-objects from medical devices;

— toxicokinetics of nano-objects;

— biological evaluation of nanomaterials;

— presentation of results;

— risk assessment of nanomaterials in the context of medical device evaluation;

— biological evaluation report;

— nanostructures on the surface of a medical device, intentionally generated during the engineering, manufacturing or processing of a medical device.

The following are excluded from this document:

— natural and biological nanomaterials, as long as they have not been engineered, manufactured or processed for use in a medical device;

— intrinsic nanostructures in a bulk material;

— nanostructures on the surface of a medical device, generated as an unintentional by-product during the engineering, manufacturing or processing of a medical device.

NOTE Examples of unintentional nanostructures on the surface of a medical device are extrusion draw lines and machining/tool marks.

This document is intended to provide a general framework and highlights important aspects which need to be considered when assessing the safety of medical devices composed of, containing and/or generating nano-objects. Additionally, the document identifies several common pitfalls and obstacles which have been identified when testing nanomaterials compared to bulk materials or small molecule chemical species. As a technical report (TR), this document represents the current technical knowledge related to nanomaterials. No detailed testing protocols are outlined or provided. This document can serve as a basis for future documents containing detailed protocols with a focus on nanomaterial testing.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10993 (all parts), *Biological evaluation of medical devices*

ISO/TR 13014, *Nanotechnologies — Guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment*

ISO 14971, *Medical devices — Application of risk management to medical devices*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 10993 (all parts), ISO/TR 13014 and ISO 14971 and the following apply.

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

— ISO Online browsing platform: available at http://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
aggregate
particle comprising strongly bonded or fused particles where the resulting external surface area is 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 bonds, 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 80004-2:2015, 3.5, modified — definition and Note 1 to entry changed]

3.2
agglomerate
collection of weakly bound particles or *aggregates* (3.1) 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 80004-2:2015, 3.4]

3.3
engineered nanomaterial
nanomaterial (3.7) designed for a specific purpose or function

[SOURCE: ISO/TS 80004-1:2015, 2.8]

3.4
incidental nanomaterial
nanomaterial (3.7) generated as an unintentional by-product of a process

Note 1 to entry: The process includes manufacturing, bio-technological or other processes.

Note 2 to entry: See “ultrafine particle” in ISO/TR 27628:2007, 2.21.

[SOURCE: ISO/TS 80004-1:2015, 2.10]

3.5
manufactured nanomaterial
nanomaterial (3.7) intentionally produced to have selected properties or composition

[SOURCE: ISO/TS 80004-1:2015, 2.9]

3.6
nanofibre
nano-object (3.8) with two similar external dimensions in the *nanoscale* (3.12) 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 80004-6:2013, 2.6]

3.7
nanomaterial
material with any external dimension in the *nanoscale* (3.12) or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of *nano-object* (3.8) and *nanostructured material* (3.17).

Note 2 to entry: See also 3.3 to 3.5.

Note 3 to entry: For regulatory purposes, it is advisable to check if specific national or regional regulatory definitions are applicable. It should be realized that different size ranges or other properties might be included in such definitions.

[SOURCE: ISO/TS 80004-1:2015, 2.4, modified — Note 2 to entry modified and Note 3 to entry added]

3.8
nano-object
discrete piece of material with one, two or three external dimensions in the *nanoscale* (3.12)

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

[SOURCE: ISO/TS 80004-1:2015, 2.5]

3.9
nanoparticle
nano-object (3.8) with all external dimensions at the *nanoscale* (3.12) where the length 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 3 times), terms such as *nanofibre* or *nanoplate* may be preferred to the term nanoparticle.

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

3.10
nanoplate
nano-object (3.8) with one external dimension in the *nanoscale* (3.12) and the two other external dimensions significantly larger

Note 1 to entry: The smallest external dimension is the thickness of the nanoplate.

Note 2 to entry: The two significantly larger dimensions are considered to differ from the nanoscale dimension by more than three times.

Note 3 to entry: The larger external dimensions are not necessarily in the nanoscale.

[SOURCE: ISO/TS 80004-6:2013, 2.4]

3.11
nanorod
solid *nanofibre* (3.6)

[SOURCE: ISO/TS 80004-2:2015, 4.7]

3.12
nanoscale
length range approximately from 1 nm to 100 nm

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

Note 2 to entry: Properties impacting biocompatibility can also occur at larger sizes, e.g. between 100 nm and 1 um.

[SOURCE: ISO/TS 80004-1:2015, 2.1, modified — Note 2 to entry was added]

3.13
nanoscale phenomenon
effect attributable to the presence of *nano-objects* (3.8) or *nanoscale* (3.12) regions

[SOURCE: ISO/TS 80004-1:2015, 2.13]

3.14
nanoscale property
characteristic of a *nano-object* (3.8) or *nanoscale* (3.12) region

[SOURCE: ISO/TS 80004-1:2015, 2.14]

3.15
nanoscience
study, discovery and understanding of matter, where size- and structure-dependent properties and phenomena manifest, predominantly in the *nanoscale* (3.12), distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material

[SOURCE: ISO/TS 80004-1:2015, 2.2]

3.16
nanostructure
composition of inter-related constituent parts in which one or more of those parts is a *nanoscale* (3.12) region

Note 1 to entry: A region is defined by a boundary representing a discontinuity in properties.

[SOURCE: ISO/TS 80004-1:2015, 2.6]

3.17
nanostructured material
material having internal *nanostructure* (3.16) or surface nanostructure

Note 1 to entry: This definition does not exclude the possibility for a *nano-object* (3.8) to have internal structure or surface structure. If external dimension(s) are in the *nanoscale* (3.12), the term nano-object is recommended.

[SOURCE: ISO/TS 80004-1:2015, 2.7]

3.18
nanotechnology
application of scientific knowledge to manipulate and control matter predominantly in the *nanoscale* (3.12) to make use of size- and structure-dependent properties and phenomena distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material

Note 1 to entry: Manipulation and control includes material synthesis.

[SOURCE: ISO/TS 80004-1:2015, 2.3]

3.19
nanotube
hollow *nanofibre* (3.6)

[SOURCE: ISO/TS 80004-2:2015, 4.8]

3.20
representative test material
RTM
material, which is sufficiently homogenous and stable with respect to one or more specified properties, and is implicitly assumed to be fit for its intended use in the development of measurement and test methods that target properties other than those for which homogeneity and stability have been demonstrated

[SOURCE: ISO/TS 16195:2013; 3.1, modified — Notes 1 and 2 to entry deleted]

4 General principles

4.1 General considerations

Nanomaterials are manufactured and used because of the specific properties that can be associated with the decrease in size accompanied by an increase of surface area. Also, materials with dimensions in the size range >100 nm or <1 micron can elicit properties different from those in the macroscale (>1 micron). For these types of particulate materials, it might be considered to perform an assessment similar to nanomaterials in the size range between 1 nm and 100 nm.

The biological evaluation of any material or medical device intended for use in humans should form part of a structured biological evaluation program within a risk management process in accordance with ISO 14971 and ISO 10993-1. The risk management process is applicable to devices that contain or are composed of nanomaterials. The risk management process is also applicable to devices that generate nano-objects as products of degradation, wear, or from mechanical treatment processes (e.g. *in situ* grinding, polishing of medical devices). Similarly, if there is release of nano-objects, there are specific challenges in the safety evaluation of such products. The safety evaluation and risk assessment of nanomaterials requires a special focus as various nanomaterials consisting of the same chemical substance can have a different toxicological risk profile depending on a number of variables, including size, surface chemistry, physicochemical properties and intended application. For medical devices that are composed of or that contain nanomaterials, the safety evaluation program should specifically address issues related to the safety evaluation of nanomaterials. The ISO 10993 series, ISO/TR 13014, ISO 14971, and References [5],[14],[15],[16],[21],[23],[24],[28],[46],[47] and [49] deal with biological evaluation of medical devices and various aspects of nanomaterials.

Nanomaterials have sizes similar to structures at subcellular levels including DNA, and thus (theoretically) can reach and interact with such structures. Also, medical devices utilizing materials with nanoscale internal structures or with surface nanoscale features associated with coatings, functionalization, or with other topographical features on the nanoscale, that are intended as part of the functionality of the device, can have specific and unique properties that might need to be addressed in the biological evaluation. For example, it has been shown that nanoscale surface topography can influence cell alignment, cell morphology, cell signalling, gene expression and extracellular matrix [52] [53] [54].

The release of nano-objects and the use of free nanomaterials are considered to pose the highest potential for risk in view of the potential internal exposure that can occur.

4.2 Biological evaluation of nanomaterials

ISO 10993-1:2009, Annex A, provides a framework for the development of an assessment program of the biological risks that should be considered depending upon a device's type and duration of body contact. This framework is also generally applicable to devices that contain, generate or are composed of nanomaterials. Such testing should be based on each device's merits. Special considerations apply to the ISO 10993 series of tests due to the presence of nanomaterials, as outlined in this document.

ISO 10993-1 provides guidance on the risk management process, which includes hazard identification, exposure assessment and risk estimation. This process is generally sufficiently robust and flexible to provide a basis for evaluation of nanomaterials, even though they can have properties that can be different from conventional ones. This process, including biological evaluation strategy, program content and acceptance criteria of the risk related to the nanomaterials as required by ISO 10993-1, should be planned, carried out and documented by knowledgeable and experienced professionals. The initial step in the biological evaluation of nanomaterials is to gather existing information on that particular nanomaterial according to the general approach as described in ISO 10993-1. Literature review of clinical and non-clinical data should be carried out according to ISO 10993-1:2009, Annex C to provide a rigorous and objective summary of available information about the nanomaterial and its intended application. Reference [55] has summarized several places where information about nanomaterials can be found. Following the logic of ISO 14971 and ISO 10993-1, if the biological safety assessment concludes from existing data that the identified risks are acceptable, no further testing is needed. Otherwise, additional information should be obtained. In order to use existing data for the biological evaluation, demonstration of nanomaterial equivalence is necessary (see 4.4).

4.3 Categorization of nanomaterials

The exposure assessment and hazard identification should be based on the characteristics of the finished medical device and the intended use. Hazard identification should consider the physicochemical and toxicological properties of the nanomaterial, including additives and processing aids. Exposure assessment should consider the concentration of nanomaterial used in the medical device, intended use and exposure route, and the rate and pattern of release and estimated patient exposure. The manner in which the nanomaterial is incorporated into the finished medical device can significantly alter the exposure characteristics[56]. General considerations for different categories of nanomaterials and medical devices are presented in Table 1. Certain devices might fall into more than one category, in which case evaluation appropriate to each category should be considered. The evaluation of any device that does not fall into one of the categories described should follow the general principles contained in ISO 10993-1, along with any special considerations outlined within this document.

Table 1 — Considerations for biological evaluation of medical devices that contain, generate, or are composed of nanomaterials

Category[a]	Type of nanomaterial in the medical device	Considerations in addition to the biological evaluation according to ISO 10993-1
1	Surface nanostructures	— Consider potential cellular or tissue effects due to direct interaction with surface nanostructures (beneficial or adverse). — Consider potential of structures to be released (break off) from the surface. — Consider potential of nano-objects to be generated by degradation, wear or mechanical treatment processes. — Consider characterization of nanostructures (see Clause 5).
2	Nano-objects bound to or incorporated within a medical device; without intention to be released	— Consider potential cellular or tissue effects due to direct interaction with surface-bound nano-objects/nano-materials (beneficial or adverse). — Consider potential of nano-objects to be released from the device. — Consider potential of nano-objects to be generated by degradation, wear or mechanical treatment processes. — Consider characterization of physicochemical properties of the nano-objects (see Clause 5).
3	Nano-objects/nanostructures on the surface of or within a medical device; with intentional/expected release from the device	— Consider release kinetics (rate and quantity) of the nano-objects and contact duration of the medical device. — Consider potential cellular or tissue effects due to direct interaction with nano-objects/nanomaterials (beneficial or adverse). — Consider characterization of physicochemical properties of the released nano-objects (see Clause 5). — Consider toxicokinetics and tissue distribution of the nano-objects (see Clause 8). — Consider biological evaluation of the nano-objects (see Clause 9). — Consider potential of nano-objects to be generated by degradation, wear or mechanical treatment processes.
4	Nano-object medical device	— Consider characterization of physicochemical properties of the nano-objects (see Clause 5). — Consider toxicokinetics and tissue distribution of the nano-objects (see Clause 8). — Consider biological evaluation of the nano-objects (see Clause 9).

[a] A device can contain nanomaterials in more than one category.

[b] Nano-objects can be generated from a medical device that does not contain nano-objects.

[c] Degradation, wear or treatment of a medical device containing nano-objects can generate new or unintended nano-objects.

Table 1 *(continued)*

Category[a]	Type of nanomaterial in the medical device	Considerations in addition to the biological evaluation according to ISO 10993-1
5	Nano-objects[b] released from a medical device as product of degradation, wear, or from mechanical treatment[c] processes (e.g. *in situ* grinding or polishing)	— Consider characterization of physicochemical properties of the nano-objects (see Clause 5). — Clause 7 describes additional considerations for nano-objects released by wear or generated by in situ processing. — Consider toxicokinetics and tissue distribution of the nano-objects (see Clause 8). — Consider biological evaluation of the generated nano-objects (see Clause 9). — Consider contact duration and release kinetics (rate and quantity).

[a] A device can contain nanomaterials in more than one category.

[b] Nano-objects can be generated from a medical device that does not contain nano-objects.

[c] Degradation, wear or treatment of a medical device containing nano-objects can generate new or unintended nano-objects.

4.4 Nanomaterial equivalence

Proper identification and characterization of the nanomaterial is essential. For nanomaterials, equivalence is dependent on multiple factors. Chemical composition alone is not sufficient to demonstrate equivalence as nanomaterial-specific properties can also be influenced by a number of other factors such as size, shape and surface properties of the nanomaterial and/or the source (manufacturer) of these nanomaterials, manufacturing process and storage conditions. Equivalence can only be claimed if properly demonstrated and justified by accompanying data.

In general, extrapolation of results by using existing data from other products using/containing similar nanomaterials, or from the corresponding parent compound of the same substance is not applicable, although such products can give an indication of possible safety concerns. If testing is considered necessary, it should be performed on the actual product and/or any nanomaterials which can come into contact with patients.

5 Characterization of nanomaterials

5.1 General considerations

Knowledge of the physicochemical properties of nanomaterials is essential to understanding their behaviour in biological systems. The physicochemical characterization is necessary for the identification of a specific nanomaterial. Characterization of the physicochemical properties of nanomaterials/nano-objects incorporated in a device and/or created by degradation, wear or mechanical treatment processes of the device is thus an important step in completing its biological evaluation. Physicochemical characterization can also be useful in the screening of potential new nanomaterials for suitability in a medical device for a proposed application. In addition, a proper characterization is necessary to establish or confirm the specifications of a nanomaterial in a defined medium and conditions.

Physicochemical characterization addresses three fundamental questions about a nanomaterial used in or released from a medical device.

— **Chemical composition:** What is it made of?

— **Physical description:** What does it look like?

— **Extrinsic properties:** How does it interact with the surrounding environment?

The physicochemical properties associated with these questions encompass a wide range of nano-object characteristics. In keeping with guidance for evaluation of conventional materials used in medical devices, the physicochemical characterization of nano-scaled materials is targeted to properties that are relevant to the biological evaluation and the intended use of the device (clinical application and duration of use). General principles for chemical, physicochemical, morphological and topographical characterization of materials used in medical devices are covered in ISO 10993-18 and ISO/TS 10993-19. The identification and quantification of degradation products in medical devices are addressed in ISO 10993-9, ISO 10993-13, ISO 10993-14 and ISO 10993-15. Detailed guidance specifically for the physicochemical characterization of nanomaterials is emerging. Recently published guidance includes ISO/TR 13014 and ISO/TR 14187 and guidances published by the European Commission for food and feed, cosmetic ingredients and medical devices[57] [58] [59].

ISO/TR 13014 lists the following as properties of engineered nanomaterials to be characterized in the context of toxicological testing:

— chemical composition;

— purity;

— object size and size distribution;

— aggregation and agglomeration state;

— shape;

— surface area;

— surface chemistry;

— surface charge;

— solubility;

— dispersibility.

These properties should be viewed as a starting point for the evaluation of nanomaterials used in a device; characterization of additional properties might be indicated depending on the design, intended use and wear characteristics of the device. Examples of other physicochemical properties that might be considered on a case-by-case basis include:

— crystallinity;

— porosity;

— redox potential;

— (photo)catalysis;

— radical formation potential;

— octanol/water partition coefficient (might not be applicable for solid materials).

In order to obtain the required data as indicated above, multidisciplinary collaborations among toxicologists, physical chemists, engineers and other subject-area experts are necessary in developing a relevant and reliable characterization program for a specific nanomaterial containing device.

Specific guidance for characterization is available for certain nanomaterials, e.g. single and multiwall carbon nanotubes (SWCNT, MWCNT), nanoscale calcium carbonate powder, nanoscale titanium dioxide powder, gold and silver nanoparticles, and nanoclays[1)].

1) For details, see http://www.iso.org/iso/home/store/catalogue_tc/catalogue_tc_browse.htm?commid=381983.

Devices incorporating nanostructured surfaces can require characterization of morphological features in addition to characterization of the physicochemical properties listed above. Modification of medical device surface structure at the nanoscale continues to be explored with the objective of modifying cell and microbial interactions with devices. The measurement parameters required for the adequate characterization of surface architecture depend on the specific application[60] [61]. For example, Webb et al.[42] propose the use of a minimum of three statistical parameters as a standard for describing the vertical and horizontal nanoarchitecture of surfaces in the context of bacterial adhesion.

— Average surface roughness (*Ra*): the average deviation of surface height values from the mean plane.

— Surface area difference (*R*sa): the increase in surface area caused by roughness, i.e. the percentage difference between the actual surface area and the projected surface area. This parameter can be used if it is possible to precisely measure the surface area.

— Peak count (*R*pc): the number of peaks in the measured profile.

In addition, peak height and peak-to-peak distance can be useful spatial parameters.

Nanoporous materials are being developed for use in device applications including drug delivery and tissue scaffolding. Useful information for characterization of porous materials includes:

— size and structure of pores or voids;

— density of pores or voids;

— distribution of the pores or voids.

Nanomaterials should be characterized in the form they will be delivered to the end-user, i.e. as the final device. Representative samples from the final device or materials processed in the same manner as the final device can also be characterized, in order to directly evaluate a nanomaterial component. In addition, characterization of the nanomaterial as manufactured might be necessary when conducting toxicological studies and biocompatibility testing. For biological safety evaluation, the above referred physicochemical characteristics of the nanomaterials should be determined also in the biological medium used in the test system. The interaction between these media and the nanomaterials can have a profound influence on the behaviour of the nanomaterials in the test system. This factor should be taken into account during the test procedures and during the evaluation of the test results.

The contact with body fluids can alter the surface characteristics of nanomaterials and thus their biological behaviour which can have an impact on the hazard caused by a nanomaterial (see 8.2.2).

The isolation and characterization of nano-objects generated by wear of medical devices remains a challenge. *In vitro* methods for simulating the *in situ* generation of nanomaterials/nano-objects should be representative of the clinical use.

5.2 Characterization parameters and methods

Table 2 summarizes fundamental parameters that are useful as a starting point for characterization of nanomaterials used in medical devices. Detailed information on the relevance of these parameters to biological evaluation is provided in ISO/TR 13014.

Table 2 also provides examples of methods that can be used to provide quantitative and/or qualitative data for each of these parameters, based on information provided in ISO/TR 13014. This list should be considered a dynamic compilation, as development of best practices for nanomaterial characterization is an area of ongoing research and discussion. The listed methods include some developed for conventional particle analysis, as well as others developed specifically for nanoscaled materials.

Multiple methods are often available for characterization of a specific physicochemical parameter. A single characterization method might not provide an accurate assessment of the parameter (e.g. size distribution, surface composition). In such cases, a complementary method, if available, might be necessary to provide an adequate assessment of the property to be characterized; two independent methods for characterization might be needed. It is important to note that the results obtained

for a particular property using different methods might not be directly comparable and that there are currently few harmonized methods for physicochemical assessment of nanomaterials to aid development of a robust test plan. The method(s) selected for characterization should be justified on the basis of the nanomaterial type and form, and proposed use of the device. For example, for nano-object size determination, at least one microscopy technique [e.g. transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM)] should be used as indicated in several guidance documents[57] [58] [59]. A report was published by the EU-JRC describing possibilities and pitfalls on nano-object size measurements[62].

Because characterization of nanomaterials is often scientifically and technically challenging, a quality assurance programme and laboratory best practices should be considered. The selection of techniques, analysis of nanomaterials and interpretation of nanomaterial properties should be performed by professionals appropriately qualified by training and experience. In performing the analyses, careful consideration should be given to sample preparation procedures to ensure that the data obtained are representative of the material used in the device. All aspects of the characterization process should be carefully documented to ensure transparency and reliability of results. Methodology used should be demonstrated to be appropriate for the nanomaterials investigated.

Table 2 — Key physicochemical characteristics and examples of measurement methods

Characteristic	Measurand	Example measurement methods	Relevant ISO guidance on methodology
Chemical composition and purity	The number and identity of elements alone or in molecules (can be expressed as a chemical formula Level or concentration of unintended constituents (impurities)	X-ray fluorescence X-ray photoelectron spectroscopy Auger electron spectroscopy Fourier X-ray diffraction (XRD transform infrared spectroscopy) (FTIR), Raman and other molecular spectroscopies Thermogravimetric analysis UV/visible spectrometry Scanning electron microscopy + XRD or energy dispersive x-ray spectroscopy (EDS) Nuclear magnetic resonance (Single particle) Inductively coupled plasma- mass spectrometry (spICP-MS)	ISO 22309 ISO 22489 ISO 24173 ISO 13084 ISO 18144

Table 2 *(continued)*

Characteristic	Measurand	Example measurement methods	Relevant ISO guidance on methodology
Particle size and particle size distribution	**Particle size**: Equivalent spherical diameter for particles displaying a regular geometry Length of one or several specific aspects of the particle geometry **Particle size distribution**: Graphical representation, e.g. histogram, and/or values for statistical parameters such as mean, median, and/or mode	Dynamic light scattering Small angle X-ray scattering Size exclusion chromatography Analysis of images of scanning electron microscopy (SEM), transmission electron microscopy (TEM), or scanning probe microscopy (SPM) Differential mobility analysis Centrifugal liquid sedimentation Nanoparticle tracking analysis Raman spectroscopy Laser-induced incandescence Confocal laser scanning microscope (CLSM) Single particle ICP-MS Tangential flow filtration for nanomaterial separation followed by appropriate detection, e.g. ICP-MS (ISO 10993-14)	ISO 9276 series ISO 9277 ISO 13318 series ISO 13320 ISO 22412 ISO 13322 series ISO 14488 ISO 14887 ISO 15900 ISO 16700 ISO/TS 19590 ISO 20998-1 ISO 21501 series ISO 22412
Aggregation/ agglomeration state	Particle size Number of aggregate/ agglomerate particles in comparison to the total number of primary particles Number of primary particles in the aggregate/ agglomerate Distribution of number of primary particles per aggregate/agglomerate	Analysis of (cryo-)SEM or (cryo-)TEM image Angle dependent scattering at different wavelengths Static light scattering Small angle X-ray scattering X-ray diffraction (XRD) X-ray absorption spectroscopy (XAS) Small angle neutron scattering Rheology methods Centrifugal liquid sedimentation Laser diffraction Nanoparticle tracking analysis	See guidance for particle size. ISO/TR 13097 ISO/TS 12025 ISO 13322-1
Shape	Size-independent descriptors of shape Distribution of values of the size-independent shape descriptors	Analysis of SEM, TEM, atomic force microscopy (AFM), or SPM images Scattering techniques spICP-MS	ISO 16700 ISO 13322-1
Surface area	Volume- and/or mass-specific surface area	Methods based on gas or liquid adsorption isotherms [Brunauer-Emmett-Teller (BET) theory] Liquid porosimetry Image analysis Laser-induced incandescence	ISO 15901-1 ISO 15901-2 ISO 15901-3 ISO 18757 ISO 13322-1 ISO 9277

Table 2 *(continued)*

Characteristic	Measurand	Example measurement methods	Relevant ISO guidance on methodology
Surface nanostructures	Size and geometry	Interferometry Reflectometry Scanning probe microscopy (SPM) and atomic force microscopy (AFM) Scanning tunnelling microscopy (STM) Contact profilometry Non-contact profilometry	ISO 25178
Surface chemistry	Elemental and molecular abundance Reactivity (chemical reaction rate)	Auger electron spectroscopy X-ray photoelectron spectroscopy (XPS) Secondary ion mass spectrometry (SIMS) 3D atom probe tomography Energy dispersive X-ray spectrometry Electron energy loss spectrometry (EELS) Low energy ion spectroscopy Raman and other molecular spectroscopies	ISO/TR 14187 ISO 18115 ISO 24236 ISO 15471 ISO 18118 ISO/TR 19319 ISO 17973
Surface charge	Net number of positive and negative charges per unit particle surface area Zeta potential	Isoelectric point Electrophoretic light scattering Electrophoresis Electro-osmosis Electric sonic amplitude Colloidal vibration current	ISO 20998 ISO 13099
Solubility/ dispersibility	**Solubility:** Maximum mass or concentration of the solute that can be dissolved in a unit mass or volume of a solvent at specified (or standard) temperature or pressure **Dispersibility:** Maximum mass or concentration of the dispersed phase present in a unit mass of the dispersing medium (solvent) or unit volume of the dispersion (solvent plus dispersed phase) at specified (or standard) temperature and pressure	There are no specific methods for assessing the solubility of nano-objects. Tangential flow filtration for nanomaterial separation followed by appropriate detection, e.g. ICP-MS (ISO 10993-14) Methods to assess dispersibility of nano-objects are based on particle size/ size distribution and aggregation/ agglomeration state (see particle size above).	ISO 20998 ISO 13099

The information given in Table 2 is based on ISO/TR 13014. There are other documents available providing recommendations on nanomaterial/nano-object characterization (e.g. ISO/TS 17200).

Most of these parameters and methods listed are relevant for the characterization of nanoparticles. It should be noted that other forms/shapes of nanomaterials can be used, e.g. nanofibres and nanoplates,

for the production of medical devices. Some parameters or methods listed might not be applicable to other forms/shapes of nanomaterials.

The methods listed can each have their limitations; therefore, the methodology used should be demonstrated to be appropriate for the nanomaterials investigated.

For certain nanomaterials (e.g. nanosilver, nanoscale zinc oxide), dissolution or ion shedding can occur. The dissolution of nanomaterials in different physiological environments can be assessed using tangential flow filtration which is a method to separate nanomaterials from their solubilized counterparts. The separated fractions can then be quantified using inductively coupled plasma mass spectrometry (ICP-MS)[63].

5.3 Use of reference materials

The availability of positive and negative control samples of nanomaterials is of key importance for reproducibility and reliability of nanomaterial safety testing. However, positive and negative control samples for nanomaterials are lacking. Therefore, the use of "representative test materials" has been proposed[65]. ISO/TC 229 has defined a representative test material as "material which is sufficiently homogenous and stable with respect to one or more specified properties, and is implicitly assumed to be fit for its intended use in the development of measurement and test methods that target properties other than those for which homogeneity and stability have been demonstrated" (ISO/TS 16195). This approach is currently applied in the OECD Working Party on Manufactured Nanomaterials collaborative project for nanomaterial safety testing using materials from the European Commission Joint Research Centre repository of representative nanomaterials. Guidance on generic requirements for representative test materials for development of methods for characteristic testing, performance testing and safety testing of nanoparticle and nanofibre powders is provided in ISO/TS 16195.

General guidance for the use and preparation of reference materials in the biological evaluation of medical devices is provided in ISO 10993-12. However, there are currently few, reference materials available for nanomaterial properties other than size. Reference materials for nanomaterial particle size assessments are increasingly available from national and community agencies, including the National Institute of Standards and Technology (NIST, United States), the Joint Research Centre of the European Commission (JRC, European Union) and the Federal Institute for Materials Research and Testing (BAM; Germany). BAM, in cooperation with ISO/TC 229, maintains a database of all currently available nanoscale reference materials worldwide, available at http://www.nano-refmat.bam.de/en/. Currently available nanoscaled reference materials include titanium dioxide, colloidal silica, gold and single walled carbon nanotubes. A number of new reference materials are reported to be under development by these organizations, as well as commercial sources, but the initial focus of development has been on nanoscale reference materials for calibration of instrumentation, rather than on development of reference materials for benchmarking biological responses.

It should be noted that the use of appropriate standard reference materials (SRM) is significant only in the context of standardized protocols for size and shape metrology. Protocols should work across a range of imaging technologies and require high statistical certainty with minimal human bias. In addition, reference materials can also be used as internal and/or external reference. For example external standards can be used to calibrate the length scale of imaging tools for subsequent imaging, while internal standards allow the inclusion of a size standard within the unknown.

Development of a set of commonly accepted reference materials, including consensus on suitable positive and negative control nanoparticles for different testing systems, has been identified as a critical need for the risk assessment of nanomaterials[65]. Although the need for such reference materials is well recognized, development continues to be slow as a result of practical difficulties including lack of consensus on which materials and properties to characterize, significant technical challenges, and economic factors[64] [65] [66].

6 Sample preparation

6.1 General considerations

Sample preparation is a critical process in the characterization and biological testing of medical devices and the materials used in their fabrication. Important considerations for testing of medical devices in their final product form include representative sampling of the device; preparation of product extracts, and storage and stability of the prepared test material. General guidance on these topics is provided in ISO 10993-12.

Special considerations may be necessary for the sample preparation of medical devices containing or consisting of nanomaterials (e.g. the use of nano-object dispersions instead of extracts).

NOTE Specific guidance for nanomaterials on sample preparation and dosing methods is available in ISO/TR 16196.

6.2 Special considerations for nanostructured materials

ISO 10993-12 is applicable to medical devices having internal nanostructures and/or surface nanoscale structures. A nanostructured surface increases the total surface area of a device. The use of the recommended surface areas in ISO 10993-12 underestimates the actual surface area of a nanostructured device, if measured at the external dimensions. However, this could be considered as a conservative approach of the extraction process used for sample preparation, but it should be realized that the patient is exposed to the total surface.

In ISO 10993-12, it is stated that the standard surface area can be used to determine the volume of extraction vehicle needed, and that this area includes the combined area of both sides of the sample and excludes indeterminate surface irregularities. The same approach should be used for material with nanostructured surfaces.

In line with ISO 10993-12, other surface-area-to-volume extraction ratios of nanoporous materials can be used if they simulate the conditions during clinical use or result in a measure of the hazard potential.

The extraction techniques as described in ISO 10993-12 will probably not result in the release of embedded nanomaterials and/or nanomaterials on the surface of a material due to the fixation in the matrix or on the surface. Only when there is a relatively weak bonding on the surface extraction of the nano-objects can be expected.

6.3 Special considerations for nano-objects

For nano-objects, some of the sample preparation requirements described in ISO 10993-12 might not be applicable and additional aspects (e.g. particle aggregation/agglomeration) might need to be considered. For example, it might be that by using extracts, only the effects of residues/contaminants are evaluated. In addition, the nanoscale size allows the nanomaterials to be dispersed in a liquid for most assays needed to be performed for a biological evaluation and risk assessment of nanomaterials. Nano-objects may be dispersed in a liquid allowing most biological evaluations to be performed, thereby allowing a direct risk assessment of the nano-object. Polar and non-polar media are generally considered for extraction per ISO 10993-12. However, a nano-object dispersion should be selected using media based on clinical relevance and dispersability. The dispersion is optimized to deliver the nano-object in a specific test system and most nanodispersions are prepared and used in a polar solution.

The small size and potentially altered physicochemical characteristics of nano-objects present significant challenges for sample preparation when compared to testing of bulk (non-nanoscaled) materials or chemicals. Contributing factors include surface properties of nano-objects that increase their reactivity; formation of aggregated or agglomerated objects; transformation of nano-objects in dispersions via hydration, partial dissolution or other processes and the potentially strong impact of low-level contaminants on the physicochemical properties and toxicological properties of nano-objects. As with other types of test articles, there is a possibility for nano-objects to adsorb to container surfaces.

Also, the rate of delivery to cells in culture can be affected by diffusion and gravitational settling (see 9.2). It is important to verify number concentrations of the nano-objects used in test samples.

Sample preparation protocols have to be developed in tandem with metrology methods (Table 2). Aggregation and agglomeration limit the capability of determining small changes in particle size distribution with high throughput automated metrology. At the same time, it is metrology of large populations of particles or fibers that allows for the determination of the extent of agglomeration and aggregation. An awareness of these issues is necessary for development of reliable sample preparation protocols for nano-objects, devices that contain nano-objects, or devices generating or releasing nano-objects. Resolving these issues can require significantly increased effort directed toward development of sample preparation and handling strategies compared to devices using conventional materials.

An important consideration in the sample preparation of nanomaterials is the distinction between solubility and dispersibility of a test item or test item component. Some nanoparticles are partly or slowly soluble. The distinction between solubility and dispersibility is important because a dispersion of particulate material can elicit a response that differs from the molecular or elemental toxicity predicted from the chemical composition[67]. A nano-object that is soluble or partly slowly soluble in biological media is likely to present to the test system in molecular form. Sparingly soluble or insoluble nanomaterials will likely be presented to the test system as a dispersion of nano-objects. In practice, careful analysis can be required to determine whether a particular nano-object is fully dispersed, partially dissolved (e.g. in the case of some metals) or fully dissolved under specified laboratory conditions. These dissolved nano-objects can elicit a response similar to that of non-nanoscale solubilized materials. When applicable, the dissolution rate should also be considered in addition to the solubility itself.

Nano-objects can be very sensitive to techniques used in sample preparation as a result of their unique surface properties. The dispersion of nano-objects is influenced by interactions between nano-objects and by interactions of nano-objects with their environment. Dispersed nano-objects are not necessarily only in the primary form. Secondary objects in the form of aggregates (objects comprising strongly bonded or fused objects) and agglomerates (collections of weakly bound objects, aggregates or mixtures of the two) can form in solution, powder and aerosol forms of nano-objects unless stabilized by surface charge or steric effects. As a result, the state of dispersion and object size distribution of nano-objects in a sample can change over time. This behaviour has important implications for preparation of extracts and/or stock and dosing dispersions, where slight modifications of pH, ionic strength or presence of molecular constituents can significantly alter nano-object dispersion. For this reason, determination of test article stability is a critical factor in obtaining representative and reproducible results during biological evaluation.

Sample preparation of nano-objects can encompass characterization of as-produced or vendor-supplied material and preparation of stock and dosing solutions for animal or *in vitro* studies. The details of preparation can vary depending on the dosing route and method of delivery. Common issues in test sample preparation and administration include:

— identity, storage and stability of test materials including batch-to-batch variability;

— chemical composition of the test medium;

— selection of appropriate dose metric;

— characterization of samples prepared from stock dispersions prior to dose administration.

Additional information on these topics is presented below. The sample preparation protocols and rationale should be carefully documented to enable replication of test samples as needed.

6.4 Identity, storage and stability of stock nanomaterials

Following are some of the factors that should be considered for dispersion and storage of nanomaterial preparations.

— Stock dispersions should be prepared according to the processes used to prepare the nanomaterials in the final product. Alternative preparation techniques may be appropriate to prepare dispersions for biological evaluation. Stock dispersions are ideally characterized by uniform object size and predictable polydispersity[68].

— The methods used to disperse nano-objects are based on their applications and include sonication, stirring, use of solvents and use of stabilizing agents such as surfactants. For instance, chemical moieties such as polyvinylpyrrolidone, citrate or tannic acid can be placed on nano-objects to prevent agglomeration/aggregation or increase dispersion in solution. Small chain polymers such as polyethylene glycol can be conjugated to nano-objects to prevent recognition by the mononuclear phagocyte system (MPS) and thus increase circulation time within the body[69] [70]. Proteins or carbohydrates can be added to nano-objects to target certain cells or tissues within the body. One should be cognizant of the chemicals/coatings which are attached to the nanomaterial of interest since they are a key to identifying biological effects, and can also be a source of contamination.

— The procedures used to disperse nano-objects should be carefully chosen as they have the potential to alter the physicochemical characteristics and toxicity of nano-objects. Possible mechanisms for altered toxicity include fragmentation of nano-objects by grinding and alteration of surface properties by use of dispersing agents, desorption of surface coatings, and changes in oxidation state, among others. Dispersion methods should be evaluated for such effects on a case-by-case basis. Effects observed during evaluation of the dispersion method should be controlled or quantified when there is evidence that the toxicity of the nanomaterial and/or nano-object preparation is altered.

— Nanomaterials should be stored per an appropriate defined, controlled procedure. General considerations include avoiding extremes of temperature, light, and moisture.

— Storage and handling procedures should take into account the reactivity of the nanomaterial.

— Nanomaterials used in medical devices should be analyzed for the presence of contaminants and impurities. The presence of low-level contaminants or impurities can significantly impact the physicochemical and toxicological properties of nanomaterial preparations and can confound analytical and experimental determinations. Examples of contaminants found in nanomaterials include bacterial endotoxin, surfactants, residual solvents, metals and catalysts. Contamination can occur during manufacture, handling and dispersion of nanomaterials; therefore, nanomaterial preparations might need to be analyzed for contamination at more than one stage during the sample preparation process.

— Stability of nanomaterial preparations should be evaluated to determine whether:

  — the nanomaterial dissolves, degrades, or transforms over time (see Clause 7); and

  — the particle size distribution or surface charge changes over time. Stock solutions should be re-made and re-characterized as necessary.

6.5 Description of chemical composition of stock and dosing dispersions

Dispersions of nano-objects used in biological evaluation studies should be compatible with physiologic conditions. For example, dosing solutions for *in vitro* mammalian cell assays should be isotonic, adjusted to a pH of 7,4 and useable in the presence of divalent ions and protein mixtures[71]. The chemical composition used to create physiologic conditions can have unintended effects on the physicochemical characteristics of nanomaterials including the degree of nano-object agglomeration and/or aggregation. Also, the nano-objects themselves can have an effect on the culture media used. It is important to characterize and document the composition of test media and dosing solutions to

ensure reproducibility of experimental conditions. The following parameters constitute a starting list for characterization of media and dosing solutions used for *in vitro* and *in vivo* testing[68]:

— ionic strength;

— calcium and magnesium concentration and anion used as source (e.g. $MgSO_4$ or $MgCl_2$);

— pH and composition of pH buffering system;

— organic additives (e.g. serum, bovine serum albumin, antibiotics);

— identity and concentration of dispersing agents.

6.6 Characterization of stock dispersions

The methods used to create stock dispersions should be documented in detail to enable reproducibility. The following parameters constitute a starting list for characterization of stock dispersions[68]:

— information from the manufacturer;

— analytical data for physicochemical properties as outlined in Clause 5;

— measured mass concentration in the stock dispersion; (also to be expressed as surface area, and number concentration)

— for metal-based nanomaterials, measured concentration of dissolved metal ion(s);

— identity and (where feasible) measured concentrations of impurities and contaminants;

— details of preparation including shape, volume, and material type of the vessel used to create the stock dispersion;

— stability (shelf life) of the stock dispersions.

Characterization of other parameters may be needed on a case-by-case basis.

Steps taken to prevent contamination (e.g. use of ultrapure water and reagents) should be described. The possibility of contamination occurring from deterioration of the probe tip or container surface should be considered when preparations are prepared by ultrasonication of suspensions. Also, contamination from other sources should be considered.

Guidance on the preparation and characterization of nanomaterials for inhalation toxicity testing is provided in ISO 10801 and ISO 10808.

6.7 Characterization of dosing solutions prepared from stock dispersions

The methods used to create dosing dispersions should be documented in detail to enable reproducibility and to aid in the interpretation of study results. The following parameters constitute a starting list for characterization of dosing solutions prior to administration to test animals or *in vitro* systems[68]:

— recommended parameters for characterization of stock dispersions as described in 6.5 and 6.6;

— description of medium used to prepare dosing/sample solution and volume prepared;

— pH of dosing/sample solution and description of pH buffering system;

— description of dispersion procedure(s) applied, including details such as sonication/vortexing time and/or energy input;

— details of dose/sample administration, including volume administered, mixing procedure (for *in vitro* tests), and elapsed time since sonication or mixing of the sample dispersion;

— details of any re-analysis of subsamples from stock or sample/dosing dispersions to verify properties at the conclusion of dosing or after modifications to the dosing solution.

Steps taken to improve dispersion and/or dispensability of the dosing solution should be described in detail and justified.

Detailed recommendations for preparation of nano-object dosing dispersions for oral, inhalation, and dermal toxicology studies are provided in ISO/TR 16196 and Reference [68].

6.8 Dose metrics

Dose levels for toxicology studies are conventionally expressed on a mass concentration basis. However, there are multiple attributes of nanomaterials that can influence their toxicological properties. It is generally accepted that in addition to mass concentration, other parameters including surface area and particle number concentration should be used to fully characterize the dose of nanomaterials. Adequate characterization details should be provided to enable the end user to interconvert various dose metrics, including mass concentration, number of particles, and surface area[72]. However, it is not always possible to accurately measure surface area and/or number concentrations especially when agglomeration occurs. In addition, surface area measurements are currently limited to determinations of nanomaterials in powder form while such measurements for liquid nanodispersions are still under development. Unless the particle number concentration or surface area concentration can be directly measured, they are likely to have higher uncertainties than the mass concentration[73].

There can be situations where mass concentration is appropriate for describing the dose of a nanomaterial. For example, when toxicity is mediated by ions released by the nano-objects, the most appropriate dose metric might be the mass of soluble ions. Also, for inhalation exposure (aerosolization) other dose metrics might apply.

The possibility of fractional deposition should be considered when determining the toxicologically relevant dose in *in vitro* studies of nanomaterials[72] [74] [75]. The contact of small nano-objects (e.g. hydrodynamic diameter <40 nm) with cultured cell layers is primarily determined by diffusion and convection forces. Larger nano-objects and aggregates of nano-objects formed in the cell culture medium settle more rapidly because of the additional influence of sedimentation forces. These factors, as well as interaction with proteins and other constituents of the culture medium, can influence the number of nano-objects that directly contact cultured cells.

Parameters that influence the deposition mechanisms [such as density and the bivariate (length and width) size distribution], the concentration that reaches the cell layer surface (deposited dose), and the amount of NMs taken up by the cells (cellular dose) can give additional information to interpret the observed biological responses[72] [76] [118]. It has to be noted that in practice it can be difficult to measure deposition and cellular uptake.

6.9 Additional considerations

6.9.1 Endotoxin

Bacterial endotoxin, or lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, is by far the predominant type of pyrogen. Endotoxin is omnipresent in the environment and has been identified as a common contaminant of nanomaterials[77].

The presence of endotoxin as a contaminant in nanomaterials can confound biological evaluation tests leading to incorrect conclusions about biocompatibility[77] [78] [79]. Therefore, it is important to evaluate the endotoxin contamination which is usually done at the stock solution and/or source material stage.

Traditional quantification assays for endotoxin might not work reliably for nanomaterials because their properties can interfere with the reagents and/or detection methods used in these assays[80] [81]. In the commonly used Limulus amebocyte lysate (LAL) assay, for example, nano-objects can interfere with the reactivity of endotoxin, the LAL reaction, or detection of the reaction products[81]. Such interference can result in either overestimation or underestimation of endotoxin in the sample. Additionally, the

presence and composition of phospholipids in the test solution can be determined to exclude the presence of endotoxin.

The choice of an appropriate method to assess the endotoxin contamination in medical devices should be made on case-by-case basis. Adapted LAL assay protocols for use with nanomaterials are available. A decision tree to aid in selection of an appropriate LAL format for a particular nanomaterial has been proposed and is useful for understanding the potential issues associated with testing different types of nanomaterials[83]. Alternative methods and their advantages and disadvantages compared to the LAL assay are reviewed in detail in References [82]and [83].

ISO 29701 describes the application of a test using LAL reagent for the evaluation of nanomaterials intended for cell-based *in vitro* biological test systems. The test is suitable for use with nano-object samples dispersed in aqueous media, e.g. water, serum or reaction medium, and to such media incubated with nano-objects for an appropriate duration at 37 °C. ISO 29701 is restricted to test samples for *in vitro* systems, but the methods can also be adapted to nano-objects to be administered to animals by parenteral routes.

Another method is the monocyte activation test (MAT) which is a validated quantitative method that exploits the natural human fever mechanism and can be used to test for endotoxins associated with nanomaterials, as well as other biological pyrogens (e.g. yeast, parasitic and viral pyrogens)[84] [85] [86].

While methods exist to remove endotoxin from nanomaterials, the procedures can result in agglomeration or other undesirable changes. A recommended approach to management of endotoxin contamination is development of practices that prevent contamination during manufacture of nanomaterials[77].

It is important to note that endotoxin is not inactivated by most of the sterilization methods[82]. However, it can be inactivated by at least 30 min of dry heat at ≥250 °C (European Pharmacopeia 8.0, section 2.6.14). Also, European Pharmacopeia 8.0, section 5.1.2, indicates that dry heat at temperatures higher than 220 °C is frequently used for sterilization and depyrogenation of glassware. In this case, demonstration of ≥3-log reduction in heat-resistant bacterial endotoxin can be used as a replacement for biological indicators. The methods for endotoxin inactivation mentioned above can potentially modify or degrade nanomaterials.

6.9.2 Sterilization

Sterilization is an important consideration for the pre-clinical biological evaluation of medical devices and use of the finished product.

— In testing, addition of non-sterile nano-object dispersions to media containing proteins and nutrients can result in growth of contaminating microorganisms such as bacteria, fungi, and viruses. Sterilization of nano-object preparations is required to prevent microbial contamination that can interfere with test systems and confound test results.

— Sterilization of the finished product is often necessary to eliminate potential microbial contamination.

Available methods for sterilization of nanomaterials are reviewed in References [87] and [88] which include:

— autoclaving;

— sterile filtration;

— gamma irradiation;

— ethylene oxide.

High hydrostatic pressure has been proposed as an additional method of sterilization[87], but improvements are needed to increase the ability of this method to eliminate bacterial spores.

The commonly used methods for sterilization listed above have the ability to modify or degrade nanomaterials. Potential modifications resulting from sterilization are material-specific and can include:

— decomposition;

— alterations in size and shape;

— aggregation state;

— type and concentration of impurities;

— degradation profile;

— stability;

— biocompatibility profile of the material;

— and/or functional behaviour.

For example, heat sterilization by autoclaving can alter the mechanical properties of polymers having a glass transition and/or melting point below 120 °C[87]. Sterilization with gamma irradiation or ethylene oxide, methods that can be applied to heat-sensitive materials, can generate free radicals that can produce toxic degradants[88]. Characterization of nanomaterials or nanomaterial-containing products should be performed after sterilization to account for any changes caused by the sterilization process.

Sterile filtration, an alternative method for heat- and chemical-sensitive nanomaterials[87], involves filtration through membrane filters with pore diameters of 0,22 µm (220 nm). As such, it is not applicable to nanomaterials containing fractions with a particle diameter of approximately 220 nm or greater. In general, sterile filtration cannot be used for sterilization of highly viscous suspensions. Also, nano-objects can absorb or adhere to the fibres of the filter, thus reducing the concentration of the nano-objects.

Because the effects of sterilization are likely to be material-specific, impacts of sterilization on nanomaterial properties need to be evaluated on a case-by-case basis. Testing of several methods might be needed to select the optimal mode of sterilization for a particular nanomaterial.

The changes induced by sterilization of a nanomaterial can have prolonged effects. For example, free radicals formed during sterilization using gamma irradiation or ethylene oxide can be transient, but can also initiate cascading reactions that lead to formation of degradants and/or alteration in product function. However, addition of free radical scavengers (e.g. histidine or phenylalanine) can protect against biomaterial degradation as was demonstrated for alginate based biomaterials[89]. A stability program suitable for the detection of changes during the shelf life of the sterilized nanomaterial and/or finished product should be established to determine whether such changes occur.

In some cases, it can be difficult to identify a compatible sterilization method for nanomaterials. Manufacture under sterile conditions can be a solution in such cases.

7 Release of nano-objects from medical devices

7.1 General considerations

A comprehensive identification and characterization of potentially released nano-objects might be necessary under physiological conditions similar to the intended conditions of use. The release kinetics, quantity, migration and bioaccumulation of the nano-objects in biological environments should be evaluated.

7.2 Degradation products

ISO 10993-9 provides a framework and starting point to address the identification and quantification of potential degradation products from medical devices. According to ISO 10993-9:2009, Annex A, degradation studies should be considered if

a) the device is designed to be absorbable,

b) the device is intended to be implanted for longer than 30 days, or

c) an informed consideration of the material(s) system indicates that toxic substances can be released during body contact.

ISO 10993-13, ISO 10993-14 and ISO 10993-15 cover general aspects of degradation of polymers, ceramics, and metals and alloys, respectively.

In conjunction with the release of ions, the occurrence of corrosion can also result in the release of particles at the nanoscale. For certain nanomaterials, it is known that new nano-objects can be formed from the released ions (e.g. nanosilver[90]).

7.3 Release of nano-objects by wear

With many medical devices, there is potential for the device to wear over time during intended use and release nano-objects (e.g. particles, fibres, flakes, fragments) into its environment. This is of particular concern given the known bio-persistence of certain nano-objects which can bind biological elements with great efficiency due to their nanoscale size, large specific surface area and high reactivity[91]. Therefore, release of nano-objects by wear should be properly addressed if the following conditions apply:

a) the device is a nanomaterial;

b) the device is coated with a nanomaterial;

c) the device, under its normal condition of use, generates friction with biological tissue or is subjected to friction between its components or with cement or composites; or

d) if the manufacturing residues can include incidental nanomaterials.

Methods for sampling wear particles generated by joint replacement implants in humans and in joint simulators are described in ISO 17853. It specifies the apparatus, reagents and test methods to isolate and characterize both polymer and metal wear particles from samples of tissues excised from around the joint replacement implant, obtained at revision surgery or post mortem, and from samples of joint simulator test fluids. Some of these procedures could certainly be adapted for isolation and characterization of particles from human biological fluids (e.g. synovial fluid).

In the context of this document, it should be noted that medical devices (e.g. implants, dental fillings) can generate nano-objects due to wear even when nanomaterials are not used in the manufacturing of these medical devices.

7.4 *In situ* processing

Mechanical treatment (e.g. polishing, grinding) of certain medical devices *in situ*, such as performed in dentistry, has the potential to generate materials at the nanoscale, irrespective of whether the original medical device contains nanomaterials[92]. This should be addressed in the risk assessment.

8 Toxicokinetics

8.1 General considerations

The major risk associated with nanomaterials is considered to be related to the presence or release of free nano-objects, ions, or components that comprise the individual nanomaterials. In medical devices,

nanomaterials can be present as free, fixed or embedded materials each with their potential for release of the nano-objects in the body.

The kinetic properties of nano-objects can be described by absorption, distribution, metabolism and excretion/elimination (ADME) as potentially sequential processes. Toxicokinetic studies need to be considered as part of the toxicological risk assessment of medical devices containing nanomaterials. A toxicokinetic study is only required if the nanomaterial has the potential to be released from a medical device and become absorbed, distributed, metabolized, and/or excreted. ISO 10993-16 provides a framework on how to carry out toxicokinetic studies. Overall, the standard is applicable to nanomaterials but some adjustments might have to be made such as labelling techniques, animal model, study duration, dosing strategy and analysing techniques.

Factors such as route of administration, size of the nano-object or its aggregates/agglomerates, surface properties (chemistry and charge), animal species, dose and dosing methods have all been reported to influence the toxicokinetics in animal models[69] [70] [93] [94] [95]. When nanomaterials are used in test systems, one has to be aware that some of the properties that need to be determined can be affected and are largely dependent on the surrounding environment (e.g. tissue culture media, blood/serum, protein presence). Such interactions with the environment can result in a temporal evolution of the nanomaterials themselves e.g. by obtaining/shedding a protein coating, the formation of nano-object agglomerates/aggregates and other changes in the nanomaterials. Such changes can affect the nanomaterial characteristics, which can impact the toxicological profile of a nanomaterial.

Therefore, if the risk assessment concludes that a toxicokinetic study is required; factors that can influence the study design, the performance of the study and interpretation of the results should be addressed.

8.2 Factors influencing the toxicokinetics

8.2.1 Physicochemical properties

Several studies have shown that properties such as size and size distribution, shape, charge, agglomeration and aggregation, hydrophilicity, and surface structure affect the ADME behaviour. For example, while smaller nanoparticles (10 nm to 15 nm) have been reported to have a widespread distribution, larger nanoparticles tend to accumulate in organs of the mononuclear phagocyte system (MPS) notably liver and spleen[69] [95] organs known to play an important role in filtering aged or damaged red blood cells (RBCs) and other particles from the blood[97]. It has been shown that particles larger than 200 nm are more likely to accumulate in the spleen and not in the liver due to the size of the inter-endothelial cell slits (approximately 200 nm in width). In a study conducted on gold nanoparticles, with a size ranging from 1,4 nm to 200 nm, a similar finding has been reported in the liver where accumulation increased with increasing particle size[95]. At the cellular level, it has been reported that nanoparticles larger than 100 nm can be taken up by cells via different endocytic pathways, such as clathrin- or caveolae-mediated endocytosis[98]. Larger nanoparticles are less likely to have skin penetration while their smaller counterparts may be able to access the deeper epidermis, dermis, and lower cell layers[96].

The physicochemical properties of nanomaterial surfaces play a crucial role in determining the interaction with biomolecules[99] and biological systems. Surface properties such as hydrophilicity and hydrophobicity and charge are known to influence protein adsorption, the stability and strength of their adsorption, as well as the nature of adsorbed biomolecules (see 8.2.2). So called capping agents can be used to stabilize nano-objects by binding to the nano-object surface via covalent bonds or by non-covalent chemical interactions. These capping agents are meant to prevent nano-object aggregation. Examples of capping agents include small organic molecules, water-soluble polymers, polysaccharides, lipids, proteins, and surfactants. The capping agents can affect the binding stability and/or toxicity.

Some physicochemical properties were found to affect toxicokinetics. Nanoparticles with sizes at or below 10 nm have a faster excretion through urine[100] and a more widespread distribution[93]. Hydrophilic coating [e.g. polyethylene glycol (PEG)] increases the blood circulation time[69] [70]. Also, for 20 nm silica nanoparticles, the amount in liver and spleen was found to be higher compared to 80 nm silica nanoparticles[101]. Both the 20 nm silica nanoparticles and 80 nm particles were excreted

via the urine with the excretory rate for 80 nm being higher than for the 20 nm silica nanoparticles[102]. It has been reported that objects less than 12 nm in diameter may cross the blood–brain barrier[103]. In addition, the surface charge controls the corona formation (see 8.2.2) as well as the interaction with the cell membranes which also may affect the toxicokinetics. So far, however, it is an open question which parameter is most significant in influencing the ADME.

8.2.2 Biomolecular adsorption

Nanomaterials in biological environments are subject to rapid protein adsorption on their surfaces, forming what is known as the protein "corona". Coronas have been reported to be dual-layered systems composed of an inner core of strongly bound proteins and an outer layer of rapidly exchanging molecules[104]. It should be noted that the protein corona is not static and can change depending on the direct environment of the nano-object. In addition, other biomolecules, such as lipids, can also adhere to the nanomaterial surface. The formation of a serum protein "corona" enhances recognition and uptake by cells of the mononuclear phagocyte system[99] [104] [105]. This generally results in a rather rapid clearance of the nano-objects from the blood, typically into liver and spleen which are key organs of the mononuclear phagocyte system[70] [93] [94] [105] [106] [107] [108] [109] [110].

The structure and composition of the protein corona depends on the synthetic identity of the nanomaterial (i.e. material-intrinsic properties such as size, shape, composition, charge and hydrophobicity[111]), the nature of the physiological environment (e.g. blood, interstitial fluid, cell cytoplasm), and the duration of exposure[112].

The protein corona alters the size and interfacial composition of a nanomaterial, giving it a biological identity that is distinct from its synthetic identity. As a result, physiological responses including signalling, kinetics, transport, accumulation and toxicity are influenced by the acquired biological identity[104] [105] [113]. It is important to note that depending on tissues and cellular compartments, significant differences exist in biochemical composition, ionic strength and acidity of biological fluids. The nature and quantity of proteins adsorbed on nano-objects, and the surrounding biological compartment might contribute to the nature and extent of cellular responses including nano-object cell recognition, internalization (e.g. by macrophages) and subsequent outcomes.

Plasma and serum media are useful for identifying which proteins absorb onto specific nanomaterials. The use of simulant fluids, relevant to the biological compartment, could help assess the fate of nano-objects under physiological conditions. Simulant fluids can be used to determine the biomolecules that could adsorb on the nanomaterial surface and evaluate the life cycle transformations of nanomaterials[114] [115] [116] [117]. Information on the use of simulant fluids has also been included in the OECD dossiers from the sponsorship programme. For example, Osmond-McLeod et al.[117] used simulated biological fluid (Gamble's solution) to assess the durability of different types of carbon nanotubes.

As the nano-object interacts with its environment, a vehicle (e.g. suspending solution) can influence the toxicokinetics of the nano-objects. Some vehicles affect nano-object agglomeration altering the size distribution whereas other vehicles affect the binding of substances to the surface. Preferably, the vehicle should be the same as used for other toxicity tests. In short, it is crucial to carry out appropriate material characterization both of the pure nanomaterial as well as the nanomaterial used in the test system.

Surface nanostructures can have an effect on nanomaterial-protein interaction/adsorption, their circulation/distribution and ultimately on their toxicokinetic profile. The surface chemistry and structure of nanomaterials are particularly important as they determine the nature and extent of primary interaction with biological systems, and subsequent cascade of events. Surface molecules can be inherent to the native structure of nanomaterials or intentionally added moieties intended to alter surface properties of nanomaterials to provide them with specific functionalities. For example, surface coatings can be utilized to alter surface properties of nanoparticles to prevent aggregation or agglomeration, promote preferential protein adsorption or enhance specific tissue or cell targeting[118].

8.2.3 Exposure route

The route of entry is important as it can change the surface of the nanomaterials/nanoparticles and the biodistribution of nano-objects in the body[94] [104] [105] [119] [120] [121]. Depending on the site of application, further kinetics of a released nano-object can be affected by adherence of molecules to the surface of a nanomaterial. In this respect, the formation of a serum protein corona is suggested to enhance recognition and uptake by cells of the mononuclear phagocyte system (MPS)[99] [104] [105]. This generally results in a rather rapid clearance of the nanoparticles from the blood, typically into liver and spleen which are key organs of the MPS[70] [93] [94] [106] [107] [108] [109] [110]. Surface treatment, for example PEGylation of nanomaterials, has been found to delay the blood clearance of IV-administerered nanomaterials [69] [70]. For most exposure routes, other than intravenous, the majority of nano-objects remain at the entry point in nearby tissues, or the local draining lymph node. After local release, nano-objects can enter the systemic circulation directly or indirectly via the lymph drainage.

Translocated Au nanoparticles (1,4 nm) after intratracheal administration showed an organ distribution pattern with a higher uptake in the kidney compared to the liver whereas the liver is the predominant target organ after intravenous administration[122]. Accordingly, nano-objects released from a medical device can be covered by different biomolecules depending on exposure route (e.g. intravenous blood catheters versus intratracheal intubation). Current data in the literature suggest that systemic uptake of nano-objects through skin is limited[123] [124] [125]. Dermal uptake depends on nanoparticle properties, on the anatomical site (skin thickness) and the skin barrier integrity[125]. Also, mechanical movements and chemicals can enhance absorption. If the lung is exposed to nano-objects, the distribution in the lung will depend on the properties of the nano-objects, where nano-object size is an important factor. When the nasal cavity in rodents was exposed to nano-objects, transport of nano-objects to brain via sensory nerves were observed[126] [127] [128].

Nanomaterials have been found in almost all types of organs and tissues, which makes prediction of target organs difficult. The level of nanomaterials found in different organs is often low making identification and quantification of nanomaterials challenging.

8.2.4 Dose

If the doses administered exceed the clearance capacity, accumulation in the body occurs. Nano-objects deposited in the alveoli are primarily phagocytized by alveolar macrophages which are ultimately transported to the mucociliary escalator and cleared mechanically via the airways. If the dose exceeds clearance, overload/saturation of clearance occurs and the kinetics are changed[129]. In blood, nano-objects are mainly cleared via uptake by phagocytic cells in organs of the MPS. However, high or repeated nano-object doses injected into the bloodstream can, for example, overwhelm the phagocytic cells in the liver and spleen, in which case the particles would be redistributed to other organs[130] [131]. Also, it has been reported that toxicokinetics of nano-objects in the bloodstream is affected by the time interval between doses[132].

8.2.5 Species and gender

The physiology and anatomy differ between species and genders, and therefore, it cannot be excluded that this results in modification of the toxicokinetics for nano-objects. Results from toxicokinetic studies on gender differences have shown that female rats are more susceptible to accumulation of silver nano-objects and their derivatives in kidneys than are male rats[133] [134]. Also, toxicokinetic differences between species have been observed. When mice, rats and hamsters were exposed to titanium dioxide nano-objects via inhalation, a disturbance in clearance of nano-objects from lung was observed in mice and rats. As a result, the burden of nano-objects in the lung of mice and rats was higher than for hamsters[135]. When Brown et al.[136] compared clearance of particulate matter in the lungs between rats and humans, they found a faster clearance rate for rats than for humans. Even if the target organ for a nano-object is the same, the localization within the organ can be different[137]. The species and gender in which the toxicokinetics is studied support the systemic toxicity studies, so alignment of toxicokinetic and systemic toxicity studies should be considered when selecting species and gender. In view of the limited information on gender aspects, this issue should be further studied.

8.2.6 Measurement techniques

In order to measure nanomaterials in tissue and organs, the nanomaterial or its elemental components have to be detectable from the biological background. To aid in this, the nano-object can either be labelled with radioactive or fluorescent labels or alternatively determined by its elemental composition, using analytical methods such as inductively coupled plasma-mass spectrometry (ICP-MS). Analysis that relies on the elemental composition has the disadvantage that it does not inform the user of the nature of the nanomaterial (i.e. whether it is present as a nano-object or degraded into elemental form). This limitation could be overcome by using single particle ICP-MS (spICP-MS)[138]. spICP-MS is a relatively new technique that is extremely useful for characterizing and identifying nanoparticles because it is able to provide insight and information into their elemental chemical composition, size, size distribution and number concentration. Furthermore, spICP-MS has the ability to provide critical information to assess the risk level of devices which contain or leach nano-objects by discriminating between elemental ions and nanoparticles which can present different toxicities.

Labelling techniques also have disadvantages. For example, a label can be released from a nano-object and/or it can alter the interaction of the nano-object with its environment, which in turn will affect toxicokinetics. When selecting a labelling technique, the stability of the bond between the label and the nano-object should be considered along with its detection limit. Methods with low detection limits might not be able to detect very low levels of nano-objects in tissues and organs without reprocessing. For some analytical techniques (e.g. ICP-MS), additional processing of the test samples can be necessary in order to increase the sensitivity. If the dose is increased or if repeated dosing is used to assure detectability of nano-objects in tissue/organs, the MPS system can become saturated and hence, the toxicokinetic behaviour modified (as discussed in 8.2.4).

9 Toxicological evaluation

9.1 General considerations

Since it is known that nanomaterials display different physicochemical properties (e.g. mechanical, chemical, magnetic, optical or electric) from that of their bulk forms, it would be logical to expect nanosized materials to affect the biological behaviour responsible for different effects occurring at the cellular, subcellular and biomolecular levels (e.g. genes and proteins), including cellular uptake. As a result, a different toxicological profile from that induced by conventional materials can be expected after exposure to nanomaterials.

Nano-objects have the potential to translocate throughout the body and be taken up by tissues that are downstream from the site of administration (see Clause 8 on toxicokinetics). They have the potential to cross not only the cellular membrane but also the intracellular structural membranes of the nucleus and mitochondria to interact or interrupt DNA synthesis and other cellular functions[139].

When in contact with the biological environment, nano-objects interact with proteins at quantitative and qualitative levels that are dictated by the nature of the physiological environment (e.g. blood, plasma, cytoplasm, etc.) and nanomaterial characteristics. Similarly, when exposed to testing media, nano-objects are expected to interact and/or interfere with the environment depending on their inherent nature and the conditions of exposure; they can then display different behaviour from corresponding bulk materials. It is therefore necessary to specifically validate any testing method designed for biological device evaluation. Similar to medical devices, the choice of endpoints would be dependent on time and site of contact. However, the choice of the actual testing method can also depend on the nanomaterial characteristics as these can interfere with various testing methods.

There are several known pitfalls in toxicity testing of nanomaterials that should be avoided (see 9.2 to 9.8 for specific tests). Also, in the future, more testing pitfalls might come to light as the knowledge of the toxicity and fate of nanomaterials improve. Therefore, it is important to stay up to date with regard to the state of the art on testing of nanomaterials.

Potential biological interaction is not directly dependent on the concentration or number of molecules, but on the nanomaterial/nano-object itself. The dose-response relationship in nanotoxicology might not

be the traditional unit of mass or concentration but rather nano-object numbers or nanomaterial/nano-object total surface area.

In addition to characterization, detailed description of experimental conditions should be documented, including the following.

— for the dose description of the nano-object used in the test, at least three parameters should be recorded: mass, number of particles and surface area, allowing switching between or calculating various dose metrics (ISO/TR 13121);

— suspension/reconstitution medium;

— testing medium (e.g. % serum);

— characteristics of the medium (pH, salt, surfactant, etc.);

— any additives to the medium and their effects on the nano-objects such as dispersion, aggregation, etc. should be listed;

— ion release in a specific medium should be characterized;

— suspension status should be documented in the reconstitution/suspension medium and testing medium;

— container material.

It should be realized that in several areas, alternative testing methods are being developed to replace, reduce and refine *in vivo* animal assays. Whenever possible, such assays, when validated, should also be considered for the evaluation of nanomaterials used in or released from medical devices.

ISO/TR 16197 describes toxicological screening methods for manufactured nanomaterials.

9.2 *In vitro* cytotoxicity testing

9.2.1 General considerations

In general, cytotoxicity is regarded as the impairment of cellular functions including but not limited to the disruption of plasma membrane integrity, interference with organelle function and disruption of the cytoskeleton. The damage depends on the concentration of the agents. Evaluation of cytotoxicity related to nanomaterials used in medical devices involves incubation of cultured cells with a device and/or extracts of a device either directly (direct contact) or through diffusion (indirect contact). The *in vitro* tests are designed to determine the biological response of mammalian cells and potential cytotoxic effects of medical devices and materials in their compositions. Cytotoxicity of nano-objects can be selective depending on cell sensitivity to the toxicant, presence of specific receptors, or uptake mechanisms. Therefore, when assessing nanomaterial cytotoxicity, special attention should be given to the *in vitro* tests chosen. In addition to choosing the relevant cell-culture model, parameters specific to nano-objects should be taken into account.

ISO 10993-5 describes accepted and standardized test methods for assessing the *in vitro* cytotoxicity of medical devices. These include assays such as neutral red uptake, colony formation, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromid] and XTT {2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl] -2H-tetrazolium hydroxide}, that assess the following as indicators of cytotoxicity:

— cell uptake;

— cell lysis;

— cell growth inhibition;

— cell colony formation;

— metabolic activity; and

— other effects on the cells (e.g. morphology, membrane lesions, etc.).

Although these methods have only been accepted for devices with conventional materials, these can be and have been applied to nanomaterials given that their physico-chemical properties are considered. Testing strategies have been proposed for both the *in vitro* testing[140] including evaluation of existing data for their relevance to the expected exposure scenario[141].

Nano-objects are generally taken up by the cells in *in vitro* studies. Once inside the cells, nano-objects can interact with the biological components and disturb the cellular functions. The intracellular localization of the nano-objects inside the cells would depend on their physicochemical properties, size and dose. As *in vivo*, nano-objects tend to end up mainly in cells of the MPS, *in vitro* cytotoxicity testing in both phagocytic (e.g. RAW264,7 murine macrophages, THP-1 human cells as differentiated macrophages) and non-phagocytic cell lines (e.g. 3T3 murine fibroblast, L929 murine fibroblasts, HaCaT human keratinocytes) can be considered (ISO 10993-5, ISO 19007:—, Annex A).

One mechanism reported to be involved in cell toxicity is oxidative stress[142] [143] that triggers activation of signalling pathways that are sensitive to redox potential, which would ultimately culminate in production of cytokines and chemokines involved in pro-inflammatory responses. Especially when nanomaterials are inducing cytotoxicity via reactive oxygen species (ROS), the nano-objects do not necessarily have to enter the cells. A similar situation can exist for nanomaterials that are inducing cytotoxicity via ion release such as ZnO and Ag nano-objects.

Some parameters are considered critical while conducting *in vitro* cytotoxicity assays. Examples are indicated below.

9.2.2 Consideration of nanomaterial interference with the assays

There are several known pitfalls that should be considered when evaluating the toxicity of nanomaterials. A wide variety of interferences is possible when evaluating cytotoxicity of nanomaterials *in vitro*[150]. Due to electric charges and optical properties, nanomaterials can potentially interfere with test protocols relying on colorimetric assays and/or fluorescent agents. Interaction with nutrients, e.g. adsorption, might be another cause of false positive outcomes of a cytotoxicity assay.

Specifically, nano-objects have been shown to interact with dyes used in assays such as MTT, XTT, lactate dehydrogenase (LDH) and dichlorofluorescin (DCF)[151] [152] [153] [154]. Moreover, some nano-objects can themselves disperse/absorb light and therefore interfere with the measurements in colorimetric assays. These aspects need to be considered when using colorimetric methods. In addition to direct interference, it should be noted that nano-objects can directly bind/adsorb to biological mediators leading to altered measurements)[150] [152] [153] [157] [158] [159]. Incorporation of appropriate controls and removal of nano-objects via centrifugation before reading the assay can reduce the variations in data generated for the same nano-objects)[155] [156]. Corroboration of several test results from different methodologies might be required for a scientifically sound interpretation.

9.2.3 Consideration of relevant dose and dose metrics

When using *in vitro* methods, it is important that the doses of nano-objects to be tested include a broad range for evaluation of the cytotoxicity (ISO 19007). Additionally, it is important to realize the existence of different dose metrics and to use a relevant dose metric to express any dose response relationship. Defining the appropriate dose metric of nano-objects represents a challenging issue for cytotoxicity evaluation in cell culture models. Mass, surface area, and number of nanoparticles have all been proposed as important dose metrics and are considered at the OECD level[72] [144] [145] [146].

9.2.4 Consideration of nano-object kinetics

In *in vitro* cell culture models, dissolved compounds reach the cells by diffusion, while nano-objects can get into contact with cells by sedimentation [aggregated nanoparticles (NPs)] and by diffusion (single particles). It has been reported that these processes are predominantly size-dependent[147].

Aggregation is also influenced by the culture media and conditions, as well as the physicochemical properties of nano-objects that dictate their interaction with their environment[148].

The effective size and density of particles in cell culture systems are a function of their agglomeration/aggregation state, packing density and shape. These properties can have profound impact on the rate of delivery to cells in culture by diffusion and gravitational settling[148]. On the other hand, the agglomeration state has been reported to affect the oxidative stress-mediated dose–response profiles *in vitro*.

Nano-objects characterized by their small mass can remain in suspension due to relatively low sedimentation forces[72]. This limits their contact with cells and the dose of delivered nano-objects in culture models where the cells adhere to the bottom surface of culture dishes. Therefore, consideration should be given to the unique kinetics of nano-objects in solution which are subject to media density and viscosity, particle size, shape, charge and density, etc.

Depending on these factors, the nano-objects can then diffuse, settle, or agglomerate, influencing their level of contact and transport in the cells[72]. So, depending on the system/model utilized, the nominal and effective dosimetry (i.e. mass or number or surface area dose of particles that affect the cells) of nano-objects can have great differences, leading to differences between the assessed effect and the real effect of nano-objects[149]. Such issues can be addressed by applying *in silico* modelling, such as *in vitro* sedimentation, diffusion and dosimetry (ISDD) and multiple-path particle dosimetry (MPPD) models, to generate dose profiles for the deposited NMs.

9.3 Genotoxicity, carcinogenicity and reproductive toxicity

9.3.1 General considerations

ISO 10993-3 specifies strategies for hazard identification and tests on medical devices for the following biological aspects: genotoxicity, carcinogenicity, and reproductive and developmental toxicity. In general, ISO 10993-3 is applicable for evaluation of a medical device or its components whose potential for genotoxicity, carcinogenicity or reproductive toxicity has been identified or is unknown. The possibility exists that a nanomaterial can have different genotoxicity, carcinogenicity or reproductive toxicity profiles as compared to the corresponding bulk material. Specific considerations for nanomaterials are discussed in the paragraphs below. There are two major types of genotoxic damage; mutagenic and clastogenic, both of which should be assessed.

Specific strategies for the genotoxicity testing for nanomaterials have been described[160]. Conflicting results regarding the genotoxicity of nanomaterials have been reported. Therefore, possible nanoparticle genotoxic effects should be assessed on a case-by-case basis. However, when some studies show a positive genotoxic outcome whereas other studies do not, for the risk assessment, the nanoparticle should be considered as potentially genotoxic. Some of the negative studies were not relevant as DNA exposure was not demonstrated (e.g. negative Ames test).

Certain nano-objects can cross the cell membrane, enter the nucleus and interact with nuclear DNA and proteins. In addition, direct contact between nano-objects and DNA can also occur during cell division when the nuclear envelope disappears. Metallic nano-objects appear to be of high concern for genotoxicity and carcinogenicity due to their reactive behaviour. Genotoxicity was demonstrated for several metallic nanoparticles, e.g. Ag-NP[157] [161] [162] [163], Au-NP[164] [165], and nickel nanoparticles[139] [166] [167] [168]. However, negative genotoxicity results were also reported[167] [168] [169] [182]. Experimental studies in rats by Hansen et al.[170] showed the development of rhabdomyosarcoma following intramuscular implantation of metallic nickel particles that were of nano-size as well as of fine-size (micrometer scale).

Nano-objects can generate free radicals following interaction with cell constituents of the same scale and can induce DNA lesions or affect chromosome segregation during mitosis, resulting in perturbation of cell division and disorganization of cell trafficking. In contrast, it also has been reported that SWCNT can mitigate ultrasonication-induced DNA damage[172].

The genotoxic effect of nanomaterials can also be the result from indirect mechanisms, involving pro-oxidative effects or DNA repair inhibition.

It is thought that oxidative stress followed by an inflammatory response and abnormal cellular apoptosis are some of the non-genotoxic events that can be elicited by nano-objects[139]. These events can predispose cells to a carcinogenic outcome. Evaluations of these cellular responses in *in vitro* and *in vivo* models can be performed as indicators for the potential to induce indirect DNA damage[139]. Differentiated responses of two types of TiO_2 (anatase and rutile) were observed that were related to the production of reactive oxygen species (ROS)[173]. The most comparative outcome was the production of ROS in human epithelial and lung fibroblast cells. The results in Reference [174] suggest that there is a difference in reactivity of the different TiO_2 particles based on the composition of the crystalline structure (anatase or rutile) versus non-crystalline TiO_2, and sizes larger than 10 microns [174].

In a study by Park et al.[171], researchers showed that nanoparticles composed of zinc, silver and aluminium showed similar toxicity in human alveolar cells. In addition, human epidemiology studies suggest a relationship between metallic nanoparticle exposure by inhalation and the onset of Hodgkins lymphoma[139]. With the exception of tumor induction by chronic inflammation and the situation in lung overload (see 9.3.4), there is little data on carcinogenesis by nanoparticles.

In general, a negative result in a genotoxicity assay has to be considered carefully. To exclude a direct genotoxic effect, an important issue is the potential exposure of the cellular target compartment (nucleus) for *in vitro* and/or tissue for *in vivo* studies. However, it should be noted that also indirect effects (e.g. due to ROS induction) can be responsible for genotoxicity.

9.3.2 *In vitro* genotoxicity tests

The bacterial reverse mutation test (ISO 10993-3) used in genotoxicity testing might not be appropriate for mutagenicity testing of nano-objects due to the uncertainty of nano-object uptake and thus DNA exposure[57] [58] [175]. However, several nano-objects were shown to enter *Salmonella typhimurium* used in the Ames bacterial reverse mutation assay[176]. Weak gene mutations in Salmonella strains TA102, TA104, and YG3003 have been caused by C60 in polyvinylpyrrolidone irradiated by visible light[177]. The problem in the bacterial mutation assays is the interpretation (and conclusions) when a negative result is obtained.

For the genotoxicity testing of nano-objects, mammalian cell systems are recommended because of their potential to take up the nano-objects. The following tests are commonly used to assess *in vitro* genotoxicity of nanomaterials.

— *In vitro* gene mutation test in mammalian cells (the mouse lymphoma *tk* assay, MLA); or the hypoxanthine-guanine phosphoribosyltransferase (HPRT) mutation assay. The MLA has the capability for detection of both gene mutations and chromosomal damage (formation of large and small colonies).

— *In vitro* micronucleus assay for detecting cytogenetic damage in conjunction with a gene mutation assay (e.g. HPRT) or the mouse lymphoma assay detecting small scale sequence alterations (point mutations).

— Comet (single cell gel electrophoresis assay) assay for detecting various types of DNA damage (e.g. single and double DNA strand breaks, oxidized bases and DNA crosslinks) depending on the conditions of the assay, e.g. double strand breaks are neutral comet; single strand breaks are alkaline comet; oxidative damage depends on the addition of enzymes.

For the *in vitro* genotoxicity testing, similar pitfalls might be present as described in 9.2. In addition, there are some specific issues to consider.

The value of adding liver S9 metabolic fractions is dependent on the base material, coating and other additives present in the nanomaterial. While inorganic particles may not be subject to metabolic activation, other materials may be. For testing of some inorganic nano-objects (e.g. metal oxide nano-objects), there might not be value in adding liver S9 metabolic fractions to the *in vitro* assays because the chemical components of the nano-object may not be metabolized. However, for nanomaterials which

contain organic components or chemical entities (e.g. polymer carbon-based nanomaterials), metabolic transformations can occur. In addition, the presence of S9 or other proteins, e.g. in serum, can cover the surface of nano-objects thereby influencing their uptake and reactivity. Thus, the inclusion of liver S9 metabolic fraction should be considered.

The micronucleus test can be used for nanomaterial testing. When the protocol requires using cytochalasin B (cyt-B) to identify cells that have divided, one should determine whether endocytosis and/or exocytosis of nanomaterials is not affected by cyt-B, or use a sequential version of the assay where cyt-B is added after the exposure period and potential uptake. It is known that cyt-B can affect the cellular uptake of nano-objects[178] [179].

9.3.3 *In vivo* genotoxicity tests

When *in vivo* testing is indicated, appropriate methods should be used to establish that the tested nano-object reaches the target organ. If this cannot be demonstrated, a second *in vivo* test in a different target organ, for which uptake can be demonstrated, might be needed to confirm the absence of genotoxicity *in vivo*. *In vivo* subacute and/or subchronic toxicity studies can be used to determine the distribution of the nano-objects. This information on the tissue distribution can guide the selection of the *in vivo* genotoxicity test to ensure the tested organ is relevant to the distribution and accumulation of the nano-objects. A pitfall of the *in vivo* genotoxicity assay can be that the target organ is not exposed to the nano-objects investigated.

Proposals for combining genotoxicity testing with other *in vivo* tests have been described[180] [181].

For each study protocol, the route of exposure in the intended human population should be considered and exposure of the target organs should be demonstrated. Results from the toxicokinetics studies can be used to demonstrate organ exposure.

The following tests are commonly used to assess *in vivo* genotoxicity:

— micronucleus test in rodent erythrocytes or bone marrow;

— chromosomal analysis in rodent bone marrow

— DNA strand break analysis (*in vivo* Comet assay).

The choice of the appropriate test system should be justified and documented.

When other *in vivo* test systems are used to obtain additional information on genotoxicity, the decision should be justified and documented using evidence-based rationale.

For any *in vivo* genotoxicity assay such as the single cell gel assay (Comet assay), it is recommended to identify target organs based on toxicokinetic studies and/or subchronic *in vivo* studies.

Several transgenic animal models are available for determination of DNA damage after *in vivo* exposure (OECD TG 488, 2013). One of those models is the Lac-Z model transgenic mouse model, which has been used to evaluate the genotoxicity of TiO_2 nano-objects *in vivo*[182]. The model consists of C57Bl/6 mice harboring the pUR288 plasmid (containing the LacZ reporter gene) inserted in head-to-tail sequences homozygously on both chromosomes 3 and 4[182] [183]. This mouse model allows assessment of mutagenicity in several organs, making it a valuable *in vivo* model for investigating genotoxic effects and repairing mechanisms after exposure to chemical agents.

Other transgenic models mentioned in the OECD TG 488 for which sufficient data are available to support their use in this TG are: lacZ bacteriophage mouse (Muta™Mouse[2)]); lacZ plasmid mouse; gpt

2) Muta™Mouse is a trademark of HRP Inc. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

delta (gpt and Spi–) mouse and rat; lacI mouse and rat (Big Blue®[3]). The cII positive selection assay can be used for evaluating mutations in the Big Blue® and Muta™Mouse models. Mutagenesis in the transgenic rodent models is normally assessed as mutant frequency; if required, however, molecular analysis of the mutations can provide additional information (OECD 488).

9.3.4 Carcinogenicity

The human genome is continuously exposed to DNA-damaging agents such as reactive oxygen species, ultraviolet light, and genotoxic chemicals[184]. Numerous *in vitro* and *in vivo* studies have found that nanomaterials induce DNA damage and mutations. The link between genotoxicity and cancer is well recognized, so the results of these studies are useful for predicting the carcinogenicity of nanomaterials[185]. Chronic inflammation as suggested for rod-like nano-objects[192] [193] might play a key role because it could lead to elevated oxidant levels. Although a considerable amount of information concerning genotoxicity is available (see 9.3.1), including contradicting results for many nanomaterials, the level of understanding of nanomaterial carcinogenicity is limited.

It is well known that genotoxicity and chronic inflammation can lead to carcinogenicity. It has also been reported that biopersistence and induction of chronic inflammation has been known to induce tumors in the lung[186] [187]. These effects may be due to what is designated as an "overload" dosing[188] [189] [190] [191]. Biopersistence is mainly determined by the chemical composition of the nano-objects' size and shape (e.g. fibers). Some nanomaterials such as nano-TiO2 and carbon nanotubes (CNTs) have been indicated to be involved in tumor development in animal models[193]. Possible mechanisms involve DNA lesions and ROS production during inflammation.

An evaluation of the carcinogenic risk should be considered if human exposure is high or chronic. The most common *in vivo* tests to assess the carcinogenic potential of chemicals are the carcinogenicity test as described in EC B.32 and OECD 451 and the combined chronic toxicity/carcinogenicity test as described in EC B.33 and OECD 453. For medical devices, carcinogenicity testing is described in ISO 10993-3. In addition, transgenic animals like the murine rasH2 model may be used as a short-term alternative test compared to the two-year carcinogenicity study[194] [195].

It should be noted however that the use of such tests should be evaluated on a case-by-case basis, as their suitability has not been demonstrated for nano-objects.

9.3.5 Reproductive toxicity

While data on reproductive toxicity of nanomaterials remain scarce, there is an increasing interest in exploring their potential effects on the reproductive apparatus, cells of the germ line, embryonic development and offspring. Because nanoparticles are able to penetrate through biological barriers, there is a possibility of crossing the reproductive system barriers (e.g. blood-testis barrier and placental barrier) with a potential impact on sperm vitality and function as well as embryo development. Some nano-objects have been shown to cross the biological barrier of the reproductive tissues such as the blood-testis barrier and the placental barrier depending on their type and experimental conditions/models[196]. In a recent review, it was concluded that it is plausible that NP may translocate from the respiratory tract to the placenta and fetus, but also that adverse effects can occur secondarily to maternal inflammatory responses[197]. Damage to genetic material through interaction with DNA molecules can also lead to mutations and affect reproduction and development of the next generations.

Determination of whether reproductive toxicity testing is required can be made based on the exposure and use of a medical device as included in a risk assessment. If required based on a risk assessment of a medical device, assessment of reproductive toxicity of the nanomaterials used should be considered in compliance with the requirement of ISO 10993-3.

3) Big Blue® is a trademark of Stratagene. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

When sufficient and adequate evidence demonstrates that a nanomaterial or its metabolites do not reach the reproductive system organs, no reproductive toxicity testing is necessary. This evidence can be based on data from absorption, distribution, metabolism and excretion (ADME) studies.

When no evidence is available to rule out contact with the reproductive system organs, testing should be considered, especially in the following cases:

a) prolonged or permanent-contact medical devices likely to come into direct contact with reproductive tissues, embryos or foetus;

b) energy-depositing medical devices;

c) absorbable or containing leachable nanomaterials/nanoparticles. for which complete elimination has not been demonstrated.

It should be noted that the use of some exposure methods are not recommended as they can interfere with prenatal development. For example, intraperitoneal administration can cause the tested nano-objects to be directly injected in the uterus itself or pass through the wall of the uterus and directly affect the developing embryos/foetuses. Also, "nose only" inhalation exposure might not be appropriate for pregnant females due to the fact that the animals are kept under stressful conditions and have no access to food and water[59].

In addition to ISO 10993-3, OECD 421 can be used to gather initial information on possible reproductive toxicity effects of a nanomaterial. The results of the test can also be used for initial hazard assessment and can be helpful in the decision-making process as to whether additional tests are required or not. If additional tests are considered necessary, in view of the outcome of the screening test, they should be performed in accordance with OECD 414, OECD 415, OECD 416 or OECD 422, as appropriate.

9.4 Immunotoxicity, irritation and sensitization

9.4.1 General considerations

ISO/TS 10993-20 presents an overview of immunotoxicology with particular reference to the potential immunotoxicity of medical devices.

ISO 10993-10 describes the procedure for the assessment of medical devices and their constituent materials with regard to their potential to produce irritation and skin sensitization. This document includes

— pretest considerations for irritation, including *in silico* and *in vitro* methods for dermal exposure,

— details of *in vivo* (irritation and sensitization) test procedures, and

— key factors for the interpretation of the results.

Inflammation and allergic/autoimmune reactions can result from exposure of the immune system to nano-objects. The extent and type of reactions depend on the antigenic characteristics of nano-objects, their adjuvant potency, inflammatory effect and their ability to activate the complement system. Immune response can then either be stimulated or suppressed.

9.4.2 Immunotoxicity

Most nanomaterials studied to date are nano-objects which upon entering the systemic circulation end up in the MPS cells (e.g. macrophages, dendritic cells, or Langerhans cells) which play a central role in the immune system. Therefore, the potential immunotoxicity of nanomaterials needs specific consideration. In general, immunotoxicity is evaluated during repeat dose toxicity testing (e.g. 28 d or 90 d) during which the first indications for immunosuppression and/or immunostimulation can be detected. General considerations for immunotoxicity testing of medical devices are described in ISO/TS 10993-20. For medical devices, immunotoxicity investigations typically involve prolonged implantation studies (ISO 10993-6 and ISO 10993-11). For nano-objects, alternative routes of exposure

need to be considered like intravenous administration for systemic exposure, e.g. as it has been reported for nanosilver in Reference [198].

Because of the complexity of immune system, *in vitro* models provide a reliable and preferred method of studying the immune cell function. The impact of nanomaterials on immune cell function can be studied by evaluating signaling pathways, such as the nuclear factor kappa B pathway, in specific immune cell lines[199] [200]. A number of *in vitro* assays have been published by the National Cancer Institute's Nanotechnology Characterization Laboratory to specifically suit nanoparticle studies and their effect on imunne cells (http://ncl.cancer.gov/working_assay-cascade.asp). These assays are intended for assessing parameters such as phagocytosis, chemotaxis, and nitric oxide production by macrophages in addition to many other endpoints.

Several reviews are available on effects of nanomaterials on the immune system[82] [207] [208] [209]. Nano-objects have been used as haptens or hapten carriers, which indicates that they are capable of exerting an adjuvant activity affecting the immune system[201] [202] [203] [204]. For silver nanoparticles, the effects on the immune system were found to be the most sensitive parameter of systemic toxicity after intravenous administration for 28 d[198] [205].

An issue of Methods in 2007 was specifically dedicated to animal models for the evaluation of immunotoxicity of chemicals[206]. WHO has published three Environmental Health Criteria documents within the International Program on Chemical Safety (ICPS) dealing with immunotoxicity (EHC 180), sensitization (EHC 212) and autoimmunity (EHC 236). An EHC document on immunotoxicity of nanomaterials is in preparation.

9.4.3 Sensitization

Nano-objects and nanomaterials might lead to sensitization, however, their sensitization potential is relatively unknown. Additionally, nano-object interaction with proteins leads to the formation of nano-object/protein complexes, which as a secondary effect can result in sensitization[210]. In addition, possible immune reactions against the protein as part of the nano-object/protein complex might need to be considered. See also 8.2.2 regarding the formation of a protein corona.

So far, the sole type of (hyper)sensitivity response that has been documented for nano-objects is pseudoallergy based on complement activation (CARPA; see 9.5.2).

The currently available literature describes mainly lack of sensitization capacity for nanoparticles[211] [212] [213] [214] [215] [216] [217]. Various assays described in ISO 10993-10 were used including the Buehler test (BT), guinea pig maximization test (GPMT), local lymph node assay (LLNA), human patch test (HPT), and a modified GPMT (GPMT with surface application). Included in these assays were gold nanoshells (150 nm), silver nanoparticles (10 nm), zinc oxide nanoparticles (20 nm), titanium dioxide nanoparticles (20 nm to 100 nm), polystyrene latex beads (50 nm), C60 fullerenes (1 nm), silicon dioxide nanoparticles (7 nm to 10 nm), and carbon nanotubes (1,8 nm to 60 nm).

The BT, LLNA, HPT, and modified GPMT sensitization tests might not be effective for many nanomaterials due to the barrier function of skin[218]. The target cells and organs for sensitization, dendritic cells in the skin and the draining lymph node, might not be reached by the nano-objects. Therefore, a negative outcome cannot be interpreted as the nanomaterials having no sensitizing properties; this negative result can be due to the inability of the nano-objects to penetrate the skin surface. In the modified GPMT, the test substances were not injected concomitantly with the Freunds complete adjuvant (FCA) but topically applied at the (inflammatory) site in which the FCA was injected [58] [219]. More research is required to explore this issue. If confirmed, then new nanomaterial-specific sensitization assays might need to be developed.

There are a number of *in vitro* sensitization assays that were developed in response to the ban in Europe to perform animal testing on cosmetic ingredients and the REACH legislation specifically addressing non animal testing methods. Among those that have been the most extensively tested are the direct

peptide reactivity assay (DPRA), the human cell line activation test (h-CLAT), KeratinoSens[4] and SenCeeTox[5]. At this time, it is unclear if these *in vitro* assays are capable of assessing the sensitization potential of engineered nanomaterials.

9.4.4 Irritation

In compliance with ISO 10993-1, irritation tests (including intracutaneous reactivity) should be considered to estimate the irritation potential of medical devices, materials and/or their extracts, using an appropriate site for application such as skin, eye and mucous membrane in a suitable model. This requirement is also applicable to nanomaterials. The test(s) performed should be appropriate for the route (skin, eye, mucosa) and duration of exposure or contact as per ISO 10993-10.

Various properties of nano-objects can affect their uptake after skin, eye, or mucosa exposure; these properties include (but are not limited to) size, shape, surface area, surface charge, surface energy/activity, solubility, aggregation state, polydispersity and ion dissolution kinetics[218]. Larger nano-objects are less likely to have skin penetration, while their smaller counterparts can be able to access the deeper epidermis, dermis, and lower cell layers[96] [220] [221]. In addition, the effective size (i.e. hydrodynamic radius) of functionalized nano-objects should also be considered, as that will determine their interactions with keratinocytes that may affect penetration after exposure. Shape can also have an effect on skin penetration, with spherical nano-objects showing a higher permeation than elongated nano-objects[222].

Chemical composition of the main nanomaterial can also affect potential irritation caused by nanomaterials. Some research has shown that Au nano-objects penetrated into the deeper skin layers, while Ag[223] and TiO_2 nanoparticles stayed in the stratum corneum[224]. There is some evidence that specific nano-objects (e.g. carbon-based dendrimers) are capable of penetrating the skin to some degree[225]. However, single-walled carbon nanotubes were found to have minimal skin and eye irritation when tested *in vivo* per OECD TG 404 and OECD TG 405, respectively[216]. Surface composition of the nano-object is also important for potential skin penetration. For example, surface charge has been reported to affect quantum dot penetration and cytokine release[226]. In fact, the surface functionalization or derivatization of the nano-object coating can be a cause for toxicity, rather than the nano-object itself[173] [227]; therefore, care should be taken when designing test methods and interpreting test results.

Most published data suggest that intact and even sunburned skin is a good barrier against penetration of nano-objects and that skin penetration beyond the epidermal layers does not occur[228] [229] [230] [231] [232] [233]. However, hair follicles, sweat glands, and compromised surfaces due to mechanical injury (e.g. cuts, abrasions), dermatological conditions (e.g. dermatitis, psoriasis, eczema), pathological conditions (e.g. infection, inflammation), and photodamage (e.g. sunburn) might be more susceptible to nanomaterial uptake through the skin. In addition, the use of certain cosmetic ingredients can increase skin penetration[234].

For materials or devices with patient contact other than intact skin, the intracutaneous reactivity test should be considered to assess the localized reaction of tissue to nanomaterials. This test may be applicable where the determination of irritation by dermal or mucosal tests is inappropriate (e.g. where medical devices are implanted or have blood contact). The intracutaneous introduction of the nano-objects bypasses the stratum corneum, and thus nano-objects may have a higher irritation potential for fibroblasts than dermal exposure[235] [236] [237] [238]. This test might also be useful where nanomaterials are hydrophobic (see ISO 10993-10). For other clinical routes of exposure, irritation might be assessed by topical application of nanomaterials.

4) KeratinoSens is a trademark of Cyprotex. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5) SenCeeTox is a trademark of Cyprotex. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Under the current scheme of ISO 10993-10, mucosa irritation tests are listed under Annex B as special irritation tests. Choice of these test methods need to be rationalized based on the specific use conditions and exposure routes of the materials and devices. Studies on several nano-objects have shown that ocular irritation tests are comparable or more sensitive than dermal irritation tests in detecting potential detrimental effects caused by these materials[216] [239]. Little information is available in published literature on the use of other mucosa irritation methods to test nano-objects.

Various attempts have been made to evaluate and validate *in vitro* skin irritation models as alternative methods to test nanomaterials. However, many considerations have to be made in the selection of models and study design. While some studies showed equivalent results from *in vitro* and dermal irritation models, others reported potential artefacts caused by interference due to nano-object interaction with test reagents or interleukins. There is also a lack of studies to compare the effectiveness of the different types of skin models in assessing nanomaterials[239] [240] [241].

The bovine corneal opacity and permeability assay (BCOP) and EpiOcular™[6)] eye irritation test (EIT) were used to evaluate several NMs for their eye irritating capacity[242]. Some irritating activity was observed only for nanosilver in the EpiOcular™ eye irritation test (EIT) while in the BCOP, variable results were noted. Several dry powder nanomaterials [metal oxides (ZnO, TiO_2, CeO_2), amorphous SiO_2 and MWCNTs], three organic pigments, quartz, and talc were negative in both assays.

9.5 Haemocompatibility

9.5.1 General considerations

Evaluation of haemocompatibility should be performed on medical devices containing nanomaterials in direct or indirect contact with blood. Moreover, even for devices which are not intended to be blood-contacting, if the toxicokinetic study reveals a potential translocation of free nanosized particles originated from the medical device into the systemic blood circulation, then haemocompatibility should also be considered.

ISO 10993-4 provides general requirements for evaluating the interactions of medical devices with blood. It describes:

a) a categorization of medical devices that are intended for use in contact with blood, based on the intended use and duration of contact as defined in ISO 10993-1;

b) the fundamental principles governing the evaluation of the interaction of devices with blood;

c) the rationale for structured selection of tests according to specific categories, together with the principles and scientific basis of these tests.

Blood interactions are classified into several categories based on the primary process or system being measured: haematology, haemolysis, thrombosis, coagulation, platelet activation and complement system activation.

These parameters also apply to haemocompatibility assessment for nanomaterials, together with specific considerations as discussed below. Due to their size, nano-objects might be able to migrate and reach the blood circulation system, where they can induce prothrombotic effects and platelet activation. Haemocompatibility assessment should therefore be considered for nanostructured materials, devices containing nanomaterials, and nano-objects released from devices in direct or indirect contact with circulating blood.

Many of the responses to medical devices that can occur in blood relate to the device surface area coming into contact with the blood. Therefore, the ratio of the device's surface area to the volume of whole blood exposed (cm^2/ml whole blood, WB) is an important factor for assessing hemocompatibility. Other factors influencing interactions with blood include geometry and surface chemistry. Dose response

6) EpiOcular™ is a trademark of MatTek Corporation. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

testing should be considered with nanomaterials using appropriate *in vitro* and *in vivo* models to gauge safety for the appropriate blood interaction categories mentioned above. See also ISO 10993-4.

9.5.2 Complement system activation

The complement system is involved in the innate immune defences against non-self-entities via opsonization of materials to allow recognition and uptake by macrophages. It has been reported that the interaction between nano-objects and the complement system is regulated by several factors including size, morphology and surface[128]. Abnormal increases in complement system activation due to the presence of nanoscale materials in blood can induce significant inflammatory reactions. Therefore, complement activation should be included as part of a biological risk assessment of nanomaterials especially if they are intended to come into direct contact with circulating blood, or if there is a potential migration of free nanosized particles into the systemic blood circulation.

Complement activation can result in an acute hypersensitivity reaction[243]. This specific type of hypersensitivity syndrome is called C-activation related pseudoallergy (CARPA). There has been a number of reports of this type of complement cascade activation being induced by polyethylene glycol (PEG)ylated liposomes and other nanosized substances. Such CARPA inducers include the following: liposomal drugs, micellar solvents, radio contrast agents, carbon nanotubes, liposome- and polymer-based nanomedicines, PEG-coated vesicles, and other lipid-based and phospholipid-methoxyPEG conjugate stabilized preparations[244] [245].

9.5.3 Specific considerations for haemocompatibility testing

Haemocompatibility is a key consideration for blood contacting nanomaterials. This is because proteins from blood are immediately adsorbed on the surface of nano-objects, leading to a cascade of events that would ultimately result in success or failure of a medical device. Depending on their size/surface area and other inherent or acquired surface characteristics, nano-objects can cause variable haemocompatibility outcomes. Indeed, size and surface roughness have been reported to play important roles in the extent of blood serum protein adsorption, platelet adhesion and activation, and whole blood clotting kinetics[246]. See also 8.2.2.

While materials with nano-structures on their surface area can reasonably be directly evaluated using the conventional methods described in ISO 10993-4, the haemocompatibility evaluation of free nano-objects can be much more challenging. Due to their higher surface/volume ratio than bulk materials, considerably more serum protein may readily adsorb onto free nano-objects distorting the subsequent cascade of reactions occurring in the blood as soon as foreign bodies enter systemic circulation. Also, interactions with platelets, coagulation factors and endothelial cells might be altered due to potential aggregation/agglomeration of free nano-objects once in contact with blood. In light of these potential *in vitro* test interferences, specific attention should be paid to the reproducibility, reliability and sensitivity of the methods used before arriving at any conclusions regarding the haemocompatibility of free nano-objects. Of note, a standard test method for the analysis of haemolytic properties of nanoparticles was published by the American Society for Testing and Materials (ASTM E2524) adapted from the ASTM Standard Practice F756 usually followed to evaluate haemolytic properties of bulk materials.

It should be noted that pro-inflammatory and pro-coagulant factors (e.g. TNF-a, IL-6, IL-8, MCP-1, tissue factor) from endothelial cells can be induced by NPs[247]. Both the pro-coagulant and pro-inflammatory potential of NPs could be detected in an endothelial cells and monocytes co-culture model[248] [249]. So, it is recommended that in addition to the basic hemocompatibility screening, also the activation of the endothelial cells and/or monocytes as well as the interaction of endothelial cells, monocytes with NPs should be determined (e.g. by evaluation of markers for cell adhesion molecules like CD54/CD106/CD62E, pro-inflammatory cytokines like TNF-α, IL-6, IL-8, MCP-1, and pro-coagulant factors).

9.6 Systemic toxicity

The systemic toxicity profile of nanomaterials cannot be predicted by the systemic toxicity profile of the corresponding bulk materials. This is especially due to their ability to translocate in the body and reach organs fairly inaccessible to conventional materials. Nano-objects have been described as

potentially crossing all protective barriers including the nuclear membrane, blood-brain and foeto-placental barriers. Systemic toxicity of nano-objects should therefore be considered.

One key parameter to consider when addressing the systemic toxicity potential of nanomaterials is their solubility profile. Soluble nanomaterials will dissolve when they contact tissues or fluids just like soluble bulk materials in medical devices do. However, with poorly soluble nanomaterials, the body's clearance capacity and defence mechanisms can be quickly overwhelmed, leading to long-term systemic accumulation which can cause adverse effects. The biopersistence of insoluble nanomaterials could lead to changes in lysosomal permeability and enzymatic activity and macrophage apoptosis. Depending on their clinical use and the intrinsic properties of the nanomaterials, the evaluation of their potential systemic toxicity should consider several exposure durations (see ISO 10993-11).

Because nano-objects can potentially be distributed throughout the body, the selection of tissues/organs intended for histopathological analyses (ISO 10993-11) should be considered on a case-by-case basis with special emphasis on the MPS (e.g. notably liver, spleen), kidneys, brain, bone marrow and others, depending also on the route of administration and intended clinical use.

Special attention should be paid to the administered dose; the dose metric of mass or concentration usually applicable to bulk materials may not be suitable for nanomaterials whose systemic toxicity will mainly depend on the particle itself interacting with a biological system. The particle number administered and/or the resulting surface area to which a patient was exposed might be better parameters to describe a dose response relationship.

However, mass is a convenient dose metric in terms of dosing. When all metric parameters are known, the dose in mass can easily be translated to dose per number of particles and/or dose per surface area.

Also, the size of the dose and the dosing frequency can influence the outcome of a systemic toxicity test. Exposure to nano-objects can saturate or stimulate uptake in organs, especially organs of the MPS. Studies on rodents where carbon and poly(lactide-co-glycolide) nano-objects were intravenously injected have shown that phagocytic cells in liver and spleen can be saturated, with redistribution of nano-objects to other organs as a result[130] [131]. When Biozzi et al.[132] injected carbon nano-objects to the blood of rats, they observed that the uptake in the liver was enhanced by repeated dosing. They suggested that the change in behaviour was related to an adaptation of the MPS to the new conditions especially to the level of nano-objects in blood[132]. In other studies where nano-objects as carbon colloids were injected intravenously in a dose that caused blockage of particle uptake to liver, the phagocytic activity of the liver went back to normal after 3 d to 6 d[250] [251].

9.7 Pyrogenicity

Like any material surfaces, nanomaterials can be coated with bacterial endotoxins or lipopolysaccharide (LPS), especially when the nanomaterials are produced under non-sterile conditions or in the presence of water. The presence of endotoxins on nanomaterial surfaces may interfere with the interaction between nanomaterials and the biological systems and affect assessment results (e.g. non-specific inflammation). It should be noted that terminal sterilization (dry heat, moist heat, ethylene oxide, gamma and electron beam radiation) does not inactivate bacterial endotoxin. The US Pharmacopeia recommends treating materials for sufficient time and temperature to achieve a ≥3-log reduction in the activity of endotoxin (typically at least 250°C for at least 30 min) (USP 1995) (See 6.9.1).

ISO 29701 describes the limulus amebocyte lysate (LAL) test for the determination of endotoxin in nanomaterial samples for use in cell based *in vitro* biological systems. Another method is the monocyte activation test (MAT) which is a validated quantitative method that exploits the natural human fever mechanism and can be used to test for endotoxins associated with nanomaterials as well as other biological pyrogens (e.g. yeast, parasitic and viral pyrogens)[84][85][86]. Information relevant for endotoxin testing of nanomaterials is also included in 6.9.1 and ISO 10993-11. Additional resources which may be helpful include USP 85, USP 151, and ANSI/AAMI ST72. In addition to endotoxin-mediated pyrogenicity, nanomaterial-mediated pyrogenicity should be considered as part of the biological evaluation of nanomaterials, per ISO 10993-11.

9.8 Implantation

Implantation tests for medical devices are described in ISO 10993-6. Depending on the type of medical device, various implantation sites can be considered (e.g. subcutaneous, intramuscular, intracranial, etc.). For free nano-objects, direct injection into the appropriate tissue should be considered.

Specific attention should be focused on migration of the nano-objects into the local draining lymph nodes regarding possible release of nano-objects from a medical device.

When an implantation test is used to evaluate potential systemic toxicity, the requirements of both ISO 10993-6 and ISO 10993-11 should be considered.

It should be realized that a control material is commonly used in implantation tests. Some certified reference nanomaterials are available that are standardized for size measurements. In addition, so called "representative" nanomaterials are now available which are widely used commercial nanomaterials; see 5.3.

10 Presentation of characterization and test results

The test report containing data on the characteristics and biological effects of nanomaterials should include, where applicable, but is not limited to, the following information:

a) device and nanomaterial description and details using the appropriate physicochemical characteristics noted herein:

   1) identification of nanomaterials, including chemical composition, and structure;

   2) size;

   3) morphology;

   4) agglomeration and aggregation rate/state;

   5) solubility;

   6) surface charge, surface area and chemistry;

   7) description of intended use, nature and duration of biological contact/interaction;

   8) evidence of purity of the nanomaterial in the final device if applicable, and identification and quantification of any leachable and/or chemical residues;

   9) analytical data to support stability of the nanomaterial (e.g. for nano-objects, this might include aggregation rate/state using stock samples and various dosing solutions used in the various assays);

b) sample preparation (e.g. extracts, direct contact) and testing conditions (nanomaterial concentration and medium, container material) including identification of standard protocols applied;

c) standard analytical methods, if applicable;

d) description of analytical test methods, including quantification limits;

e) assessment of medical device material degradation and potential nano-object release including rationale to support the adequacy of experimental conditions to the device's intended use environment;

f) description of medical device material degradation and potential nano-object release test methods, test conditions, test materials and procedures, including controls;

g) identification and quantification of degradation products (e.g. form and condition of degradation products, their stability and controls used);

h) evidence of test validation, including use of appropriate controls and supportive justifications, where applicable;

i) summary of results;

j) interpretation, discussion of results and conclusions;

k) statement of compliance to appropriate good laboratory practices and/or to quality management systems for test laboratories (e.g. ISO/IEC 17025).

When developing and validating new methods, the testing performed should contain the information in accordance to ISO/TR 13014.

11 Risk assessment

11.1 General considerations

Although the general principles of chemical risk assessment are considered applicable to nanomaterials[55] [59] [252] [253], nanomaterials present some special challenges, including unique physicochemical properties, greater compositional uncertainty, changing properties in biological systems, exposure measurement difficulties, and appropriate dose metric decisions that might require specific guidance. Therefore, transparency is important during the risk assessment process where inclusion and exclusion of data, assumptions, variability and uncertainties should be discussed. In absence of essential data, the risk assessor will be unable to complete the risk assessment. Hence, it should be clear from the assessment on how information has been taken into account when the final risk assessment is determined. It should be noted that the biological risk assessment of nanomaterials comprises the assessment of both the soluble and insoluble fractions of the nanomaterial. It is recognized that critical information needed for risk assessment of nanomaterials can often be lacking, leading to significant uncertainty. Such uncertainties are expected to diminish as relevant new data become available from nanomaterial testing and research. Periodic reassessment of the risk associated with use of a nanomaterial should be considered as new information becomes available.

Potential biopersistence/bioaccumulation of nanomaterials presents a specific challenge to the risk assessment relative to chemical risk assessment. In many cases, the body can be incapable of metabolizing or actively breaking down the nanomaterial which results in a potential indefinite persistence within tissues or cells. The persistence within tissues or cells has been demonstrated for various types of nanomaterials including silver[90] [254], zinc oxide, titanium dioxide[255], gold nanoparticles[237] [238], and carbon nanotubes[239]. Also, for TiO_2 nano-objects, prolonged persistence was reported[259]. The specific biological effects associated with this persistence are not well understood, yet there is some evidence to suggest that this phenomenon can result in a constant state of inflammation that may eventually lead to carcinogenesis[192] [260]. In addition, one should consider the release of nano-objects as a result of wear/age/use of medical devices manufactured without the use of nanomaterials.

The risk management process for biological evaluation of medical devices is described in ISO 10993-1:2009, Annex B. ISO 10993-1 does not define terms such as risk assessment, risk analysis and risk evaluation but follows the wording of ISO 14971 and the guidance document ISO/TR 15499. However, in ISO 10993-1:2009, Annex B, the risk analysis is extended and also comprises risk evaluation and risk control. ISO/TR 15449 provides more detailed advice on carrying out biological risk evaluations within a risk management process than ISO 10993-1. An overview of the risk management process is provided in the flowchart in Figure 1, but the scope of this document is limited to risk assessment, which comprises risk analysis and risk evaluation. The risk is described in ISO 14971 as being the product of two independent factors: the probability of harm and the severity of that harm.

The risk assessment process for nanomaterials is evolving and many tools are under development to assist in the evaluation of nanomaterial risks such as nanoinformatics *in silico* techniques[261],

quantitative nanoparticle activity relationship (QNAR)[262] [263] [264], exposure models[265] [266], physiologically based pharmacokinetic (PBPK) modelling[248] [249], systemic toxicology testing and high throughput screening[269]. See ISO/TR 16197. At present, these tools are used infrequently to support regulatory submissions for nanomaterials. However, their importance may grow in the future. Weight of evidence is another term that is often mentioned in risk assessment documents on nanomaterials and may be usefull[271] [272]. OECD provides a summary of important issues to consider in the risk assessment of nanomaterials that also are applicable for medical devices[252].

Figure 1 provides an overview of the entire risk management process as based upon ISO 10993-1, but the scope of this document is limited to risk assessment that is presented in Figure 1 above the shaded area. The shaded area in Figure 1 indicates risk control as part of the risk management.

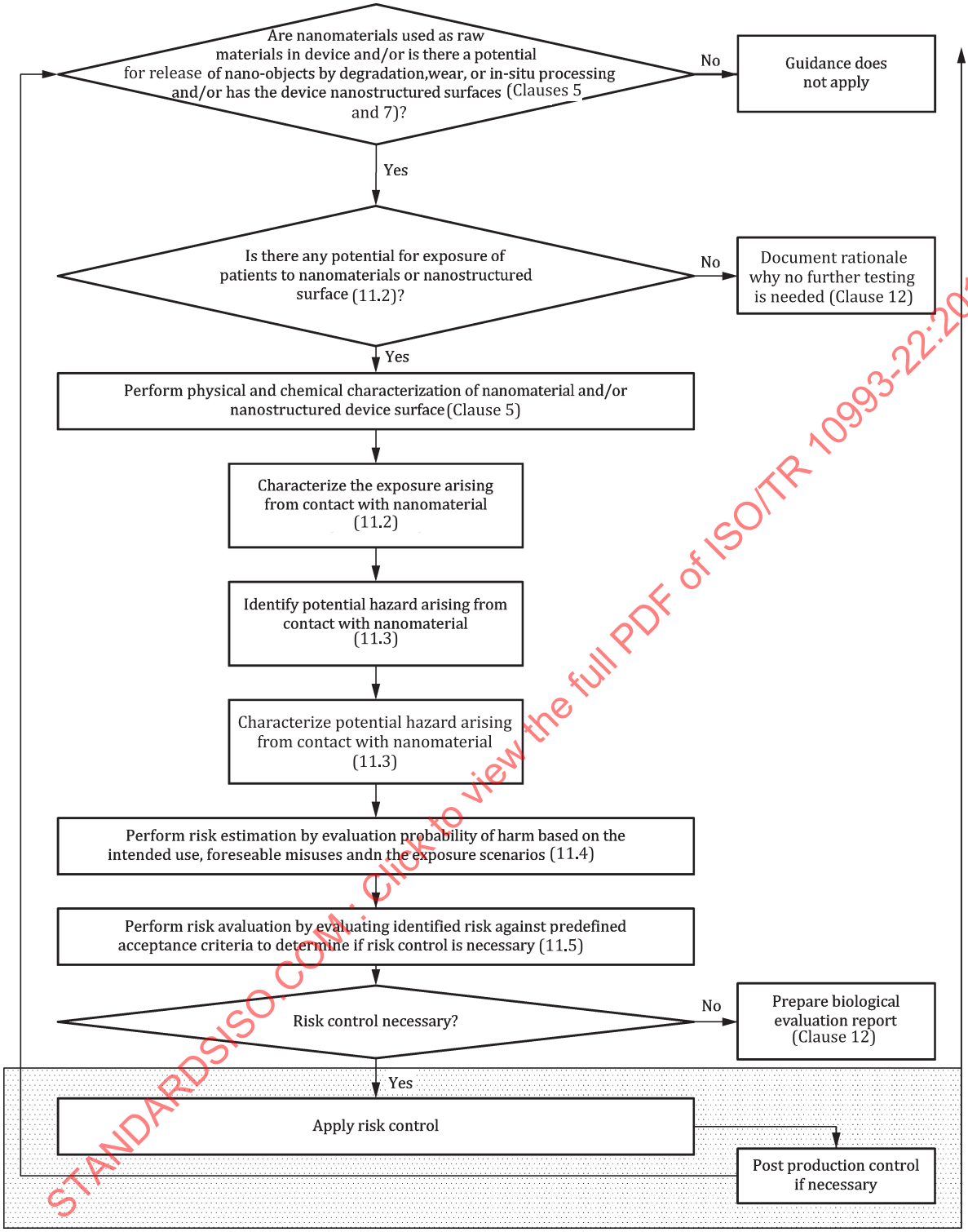

Figure 1 — Overview of the risk management process

11.2 Exposure assessment

The probability of harm is most often characterized by exposure assessment, clinical experience or comparison to similar applications. For nanomaterials used in medical devices, this is associated with the potential release and formation of nano-objects from the device. Exposure assessment aims to establish how much nanomaterial the patient is exposed to, and if the exposure makes the nano-objects available for local tissue responses and/or systemic exposure. Therefore, it becomes important

to describe the exposure scenario and establish release, migration and formation of nano-objects from the medical devices by selecting accurate test methods. Consider for the exposure assessment:

— intensity, frequency and duration of contact;

— route of exposure (e.g. tissue/blood, dermal, oral or respiratory);

— intake or uptake rates;

— bioavailability.

Initially, the focus is on the estimation of the likelihood and extent of absorption/distribution of nano-objects into the body either by simulating the exposure, measuring actual exposure, or making assumptions of exposure. The systemic uptake can, however, vary between different exposure conditions, exposure routes, health conditions of the patient, and bioavailability. Exposures via inhalation, intravenous injection or tissue implantation are often of greater concern than oral or dermal exposure.

A challenge during the exposure assessment is the characterization of the nanomaterial. It can be difficult *in vivo*, especially in tissue and body fluids, due to limited availability of analytical methods but also due to low concentrations. Another limitation is that chemical analysis does not always provide information about the particulate nature of the nanomaterial. The characterization, therefore, might have to confirm if the nanomaterial is in a solid, solubilized, degraded form. It has been shown that some nanomaterials (e.g. nanosilver) can dissolve or shed metal ions, that may precipitate to new nanomaterials with a different chemical composition[90] [272]. The analytical methods should be described and justified. The exposure assessment selected should take into account aspects such as:

— suitability;

— sensitivity;

— robustness;

— accuracy;

— detection limits;

— material form (solid, soluble or degraded);

— sources of variability and uncertainty.

11.3 Biological hazard identification

A hazard is a potential source of harm. All known and reasonably foreseeable hazards associated with the nanomaterial in both normal and reasonable fault conditions should be identified. Nevertheless, to cause harm, a hazardous situation has to occur which often involves a sequence of events. Identification and characterization of these events are important when risks are to be estimated.

When risk assessors are conducting the hazard identification/characterization step, they should consider the following:

— results of literature review;

— results of material characterization;

— samples preparation;

— dose selection and dose description;

— test methods;

— toxicokinetics;

— description of the hazard, including dose response and mode of action.

For some types of biological effects, it is not possible to derive a typical toxicological dose-response relationship. In these cases, other methods should be used to characterize the hazard and classify the severity of harm. Here, special attention should be paid as to how hazardous situations arise and the nature of their outcome. Examples of such biological effects include foreign body reactions to implanted nanomaterials and blood coagulation due to nanomaterial surface structure and design. Appropriate documentation of these other types of biological effects is necessary to assure transparency and traceability.

11.4 Risk estimation

The principles for risk estimation are considered to be applicable for nanomaterials, but might be more complex due to the currently limited understanding of how nanomaterials interact with test systems and the human body. Weight of evidence approaches and principles on how to derive tolerable intake values described in ISO 10993-17 can be useful in this process. ISO 10993-17 provides guidance on how to use uncertainty factors and set a tolerable intake value for leaching substances and may also be useful for nanomaterials that may become available for systemic exposure by release, degradation, wear or by its constitution.

All available and appropriate information from literature reviews, biological evaluation, clinical use, and exposure assessment should be used to estimate the health risk posed by the nanomaterial. Furthermore, the quality, reliability and relevance of information should also be taken into account. Data used for the risk estimation should be evaluated for reliability based on:

— confirmation of the identity and physicochemical properties of the material tested in relation to the material being evaluated;

— whether or not the data have been generated according to an accepted testing or measurement approach;

— whether the applied approach is accepted for use in testing nanomaterials.

Identified and characterized risks are compared to the exposure or the probability of a hazard to cause harm. A risk arises if there is a probability for harm. When it is not possible to determine the exposure (the probability of harm), the risk assessment, after assuming a worst-case exposure scenario, might have to depend on the severity of the hazard alone. Nevertheless, since risk is a function of exposure, and frequency and duration of use are both exposure factors, the probably of harm will rise when either of these two factors increase.

11.5 Risk evaluation

Risk evaluation is a process in which estimated risks are evaluated against predefined acceptance criteria to determine if risk control is necessary. This process is identical for all medical devices whether or not they contain nanomaterials. As with all devices, a qualitative risk evaluation might be necessary when data available to perform a quantitative risk evaluation are insufficient. The risk level depends not only on the potential severity of the hazard and the probability of harmful exposure, but also on the device's intended use.

12 Biological evaluation report

The biological evaluation report should contain the items as described in ISO 10993-1, including more specific information regarding the use of nanomaterials. Expert assessors who have the necessary knowledge and experience should determine and document:

a) the strategy and program content for the biological evaluation of the medical device that contains, generates or is composed of nanomaterials/nano-objects;

b) the criteria for determining the acceptability of the risk related to the nanomaterial(s) for the intended purpose, in line with the risk management plan;

c) the adequacy of the material characterization of the medical device and of the nanomaterials;

d) the rationale for selection and/or waiving of tests and the degree of uncertainty in the interpretation of those tests;

e) the interpretation of relevant existing data and results of testing relevant to the medical device;

f) any additional data collected to complete the biological evaluation;

g) overall biological safety conclusions for the medical device and any degree of uncertainty in coming to these conclusions.

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