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Nanotechnologies — Characteristics of working suspensions of nano-objects for in vitro assays to evaluate inherent nano-object toxicity

Nanotechnologies — Caractéristiques des suspensions de nano-objets utilisées pour les tests in vitro évaluant la toxicité inhérente aux nano-objets

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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 document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of 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 www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies.*

This second edition cancels and replaces the first edition (ISO/TS 19337:2016) which has been technically revised.

The main changes are as follows:

— "the flow of measurements" has been improved as shown in Figure A.1;

— the status of Annex A has been changed from informative to normative;

— "5.2 Endotoxin" has been replaced by "5.5 Contamination".

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Before nano-objects enter onto the market, their possible impact on human health and the environment should be carefully evaluated.

In vitro toxicity assays using cultured cells are frequently used as a tool in screening materials for possible hazardous properties. The testing provides essential information for understanding the mechanisms of biological effects induced by the materials. However, nano-objects require specific considerations with respect to the in vitro toxicity assays, because of their unique behaviour in cell culture medium. For example, immediately after the introduction of nano-object samples into the culture medium, the nano-objects can undergo changes in

a) ionic dissolution,

b) corona formation, or

c) aggregation/agglomeration

leading to alteration in particles size and sedimentation. Therefore, it is critical to consider the above-mentioned phenomena in clarifying if the observed effects are related to the tested nano-object itself or from uncontrolled sources and to avoid false interpretation of assay results. For example, the corona formation, metal ion release from the nano-objects and impurities (residual from the nano-object synthesis process) can interfere with some in vitro assays[1], producing inaccurate results. Additionally, the formation of agglomerates and aggregates can alter the toxicity of a suspension. It is important to carefully assess and describe the characteristics of the suspension of nano-objects being tested.

Therefore, the rigorous characterization of the working suspension prior and during in vitro toxicity assays on these characteristics is essential to exclude the in vitro experimental artefacts. In this document, the essential characteristics related to these three phenomena and applicable measurement methods were summarized. On the other hands, this document does not include a strategy to select the appropriate techniques from the applicable measurement methods because the working suspensions that contain nano-object samples for in vitro toxicity assays has the different materials components, concentration and sizes; thus, the appropriate selection is depending on the investigators. While the related informative annexes and the list of references in the Bibliography are included in this document to assist with appropriate method selection by investigators to make allowance for the characterization method selection, optional methods are also given in this document. In Clause 6, the details of the characterization methods/procedures are described; therefore, the appropriateness of the characterization can be judged.

The intention of this document is for reliable test results on nano-object toxicity to be shared and communicated among stakeholders of nano-objects, such as regulators, general public, manufacturers and end users.

Nanotechnologies — Characteristics of working suspensions of nano-objects for in vitro assays to evaluate inherent nano-object toxicity

1 Scope

This document describes the characteristics of working suspensions of nano-objects to be considered when conducting in vitro assays to evaluate inherent nano-object toxicity. In addition, the document identifies applicable measurement methods for these characteristics.

This document is applicable to nano-objects, and their aggregates and agglomerates greater than 100 nm.

This document intends to help clarify whether observed toxic effects come from tested nano-objects themselves or from uncontrolled sources.

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/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 80004-2 and the following apply.

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

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

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

3.1
culture medium
aqueous solution of nutrients required for cell growth

3.2
secondary particle
agglomerate/aggregate of primary particle(s), proteins and other medium components

3.3
stability
properties to remain unchanged over a given time under stated or reasonably expected conditions of storage and use for an in vitro toxicity assay

3.4
working suspension
suspension prepared for an in vitro assay that includes *culture medium* (3.1) and nano-object sample

3.5
contamination
trace amounts of extrinsic substances present in the nano-object samples that affect cellular growth

4 Abbreviated terms

AAS	atomic absorption spectrometry
BCA	bicinchoninate acid
BSA	Bovine serum albumin
C-U/F	ultrafiltration assisted by centrifugation
DLS	dynamic light scattering
AF4	asymmetrical-flow field-flow fractionation
ICP-AES	inductively coupled plasma-atomic emission spectrometry
ICP-MS	inductively coupled plasma mass spectrometry
LAL	limulus amebocyte lysate
LD	laser diffraction
LPS	liposaccharides
MAT	monocyte activation test
PCR	polymerase chain reaction
ppt	parts per trillion
SLS	static light scattering
TFF	tangential flow filtration
TOC	total organic carbon
U/F	ultrafiltration
UV-Vis	ultraviolet-visible

5 Characteristics and measurement methods

5.1 General

To characterize the working suspension for in vitro toxicity assays, it is necessary to identify certain characteristics that can impact the biological system tested. Working suspensions for individual dose shall be divided into two samples, one for toxicity assay and another for characteristics measurements. Clause 5 specifies essential characteristics of the working suspension, listed below, and measurement methods that are applicable to them:

— stability of working suspensions;

— concentration of metal ions;

— concentration of culture medium components;

— contamination.

Measurements of those characteristics shall be made for each dose of working suspensions. The flow of measurements shall be in accordance with Annex A and Figure A.1.

5.2 Stability of working suspensions

5.2.1 General

Stability of working suspension is a key characteristic as it directly impacts the in vitro assay conditions in terms of the dose of the nano-objects to the cells[2],[3],[4]. Aggregation/agglomeration and gravitational settling of the nano-objects are major issues that can affect the stability of the suspended nano-objects. The suspension stability shall be evaluated for the two characteristics, i.e. the relative change of representative size of secondary particles of nano-objects and the relative change of the concentration of nano-objects in the working suspension. The change in size of secondary particles of nano-objects can result from agglomeration of smaller particles in culture media. The relative change of nano-object concentration can result from gravitational settling during an in vitro toxicity assay by considering experimental duration required for the in vitro toxicity assay. Evaluation results of the stability shall be expressed in the unit of per cent over the timescale for an in vitro toxicity assay.

ISO/TR 13097[5] is recommended as comprehensive guidance for stability of working suspension.

5.2.2 Representative size change of secondary particles of nano-objects

An appropriate method shall be selected to directly measure the representative size change of secondary particles of nano-objects from among DLS[3],[6], LD[7] and SLS[8]. Other methods not listed in this document can be used and reported according to the optional methods in 6.6.

See Annex B for measurements.

5.2.3 Concentration change of nano-objects

An appropriate method shall be selected to measure the concentration change of nano-objects suspended in the biological media from among the static light scattering[3],[6],[8], ICP-MS[9],[10],[11], UV-Vis absorption, X-ray transmission[12] and the total organic carbon analysis[13]. Other methods not listed in this document can be used and reported according to the optional methods in 6.6.

See Annex B for measurements.

5.3 Concentration of metal ions

Metal ions, produced as a result of nano-object test sample dissolution, can contribute to observed cell toxicity. The concentration of metal ions in the working suspension shall be measured after separation of particulate matter. Particulate matter can be separated from the ionic fraction by U/F, C-U/F TFF or centrifugation. The measurement shall be made for all metallic elements that are included in the nano-object sample. An appropriate method shall be selected to measure the metal ion concentrations from among ICP-AES, ICP-MS, AAS and the colorimetric method. It shall be noted that many constituents in culture media such as Na and Cl can interfere with metals analysis for some spectrometry techniques, especially ICP-MS[14]-[16]. Other methods not listed in this document can be used and reported according to the optional methods in 6.6. Measurement results of concentrations shall be expressed in the unit of molarity, mass/mass or mass/volume. The measurements can be omitted when a toxic effect is not observed to the cells in the working suspensions. See Annex A for an example of flow of measurements.

See Annex C for measurements.

5.4 Concentration of culture medium components

5.4.1 General

A nano-object sample added to a culture medium to generate a working suspension can adsorb components of the culture medium[1]. This adsorption can result in starvation stress to the test cells. The concentration of protein components and calcium, as surrogates for the nutritional components in the solvent shall be measured after allowing enough time after the addition of nano-object sample to the culture medium. If culture medium components other than protein and calcium that can significantly affect the stability of the working suspension for in vitro toxicity assays are known, the concentration of those components shall be measured as well. The measurements can be omitted when a toxic effect is not observed to the cells in the working suspensions. See Annex A for an example of flow of measurements.

Nano-object sample in culture medium shall be incubated with the same conditions of in vitro test. Nano-objects can affect pH, osmolality and other essential characteristics in the culture medium.

5.4.2 Proteins

An appropriate method shall be chosen for the protein concentration measurement from among BCA, Bradford, Lowry, and ultraviolet, refractive index and SLS methods coupled with the AF4[17],[18]. When BCA[19], Bradford[20] or Lowry[21] method are chosen, the protein concentration in the solvent shall be measured after separation of particulate matter from the working suspension. Results of protein concentration measurement shall be expressed in the unit of mass/volume.

See Annex D for measurements.

5.4.3 Calcium

An appropriate measurement method shall be chosen for the calcium concentration measurement from among ICP-AES, ICP-MS, AAS and the colorimetric method. Results of calcium concentration measurement shall be expressed in the unit of molarity, mass/mass or mass/volume.

See Annex D for measurements.

5.5 Contamination

Contamination can be a source of additional toxic action. Endotoxin and mycoplasma shall be determined with appropriate methods.

Endotoxin measurement are available with LAL test (see ISO 29701), the chromogenic-based LAL assays[22], the MAT[23], recombinant Factor C test[24] and high performance liquid chromatography coupled with mass spectrometry methods[25].

Mycoplasma contamination is one of the major problems in vitro assay. Mycoplasma can be detected by PCR based methods[26], culture methods[27] and fluorescence microscopy method[28].

Nano-object sample shall be treated aseptically. It shall be confirmed that there is no history of contamination except that which is described in this subclause.

See Annex E for measurements.

6 Reporting

6.1 General

Measurement and evaluation results obtained according to this document shall be reported describing the source and the constituents of the nano-objects, culture medium and serum, as described in 6.2 to 6.6.

6.2 Name of nano-objects and manufacturing information

Name, catalogue and lot/batch number of nano-objects and manufacturer information including name, address and contact information shall be reported.

6.3 Composition and metallic elements included in the nano-object sample

Define principal and accessory materials, coating materials, catalytic materials and impurities, including their known or estimated quantity.

6.4 Culture medium and serum

Name, manufacturer and lot/batch number of the medium, type and concentration of added serum (volume fraction %), pH values of original medium and pH values during assessment, and type and concentration of other additives, if any, shall be reported.

6.5 Measurement results

The following are required to report for individual doses of working suspensions and measurement time frame. However, the results of the test for contaminants can be reported for the stock of nano-objects instead of individual doses.

Reporting of metal ions, culture medium components and contaminants are not required when toxicity is not observed for the individual doses of working suspension.

— Stability of working suspension

  a) representative size change and concentration change,

  b) date of measurement,

  c) employed measurement methods for representative size change and concentration change,

  d) performing institution and data reliability information,

  e) supporting information on preparation method of working suspension, and

  f) other special supporting information if any;

— metal ions

  a) names of metal ions and their concentrations,

  b) date of measurement,

  c) employed measurement method,

  d) performing institution and data reliability information, and

  e) other special supporting information if any;

— culture medium components

a) protein and calcium concentrations,

b) date of measurement,

c) employed measurement methods for protein and calcium concentrations,

d) performing institution and data reliability information, and

e) other special supporting information if any;

— contamination

a) contamination positive/negative and type of contamination,

b) date of measurement,

c) employed measurement method,

d) performing institution and reliability information, and

e) other special supporting information if any.

6.6 Optional methods

Methods not listed in this document shall be described in the report with the name of the methods employed, its detailed information, reliability and justification.

Annex A
(normative)

Flow of measurements

A schematic showing the steps at which measurements shall be made based on this document is shown in Figure A.1.

Measurements of stability, metal ions and culture medium components are made for each dose of the working suspension prepared by mixing nano-object sample and cell culture medium.

The working suspension is divided into two parts; one is for toxicity assay (assay sample) and the other for measurements of characteristics (characteristics measurement sample). Measurements of metal ions, culture medium components and contaminants are carried out for the characteristic measurement samples when a toxicity effect is observed for the assay sample. Measurements of contaminants can be made for the stock of nano-objects instead of individual doses.

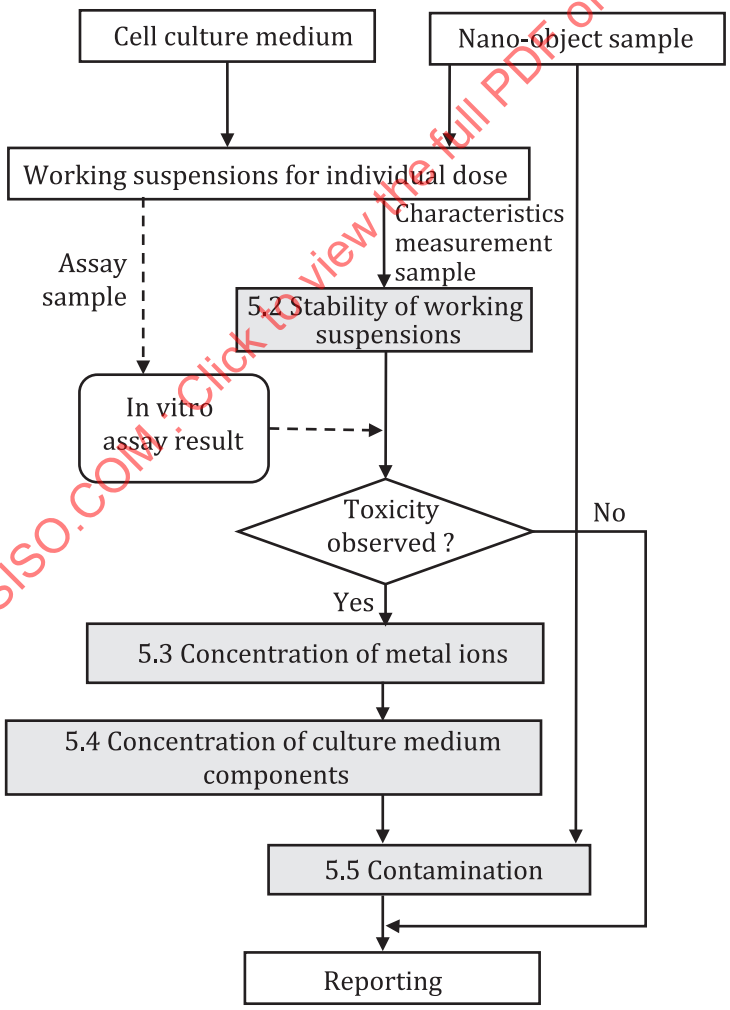

Figure A.1 — Flow of measurements

Annex B
(informative)

Measurement and evaluation of stability

B.1 General

Examples of measurement methods of stability of nano-object samples are provided for the benefit of the user. The user is cautioned that these methods have not necessarily been validated for use in characterizing multiple types of nano-object samples. Due to the diversity of nano-object samples, the user should select an appropriate method to evaluate the stability of working suspension during in vitro toxicity assays.

All measurements should be performed on working suspension or the nano-object suspensions prepared in the same manner as the in vitro toxicity assay. The same sample stocks of the nano-object suspension should be used for the measurements and for the in vitro toxicity assessment experiments. Evaluated results of the change of values should be expressed in the units of percent; namely, dividing the change of values during an in vitro toxicity assay by the initial values. It is recommended to perform multiple measurements (at least three times) and to present the data as an average of the measurements.

B.2 Representative size change of secondary particles of nano-objects

In order to monitor the changes of the representative size of secondary particles of nano-objects, the user should select an appropriate method from DLS, LD and SLS. The method should be selected by observable size range in the respective measurement principles.

B.3 Change of concentration of nano-objects

In order to monitor the changes of the concentration of a majority of the types of nano-objects, light scattering intensity monitoring methods such as DLS or SLS can be applied, as long as the unit size of the nano-object is larger than protein molecules in the working suspensions. TOC method can be applicable to carbon-based nano-object analysis, when combustion temperatures or pyrolysis temperatures of the carbon-based nano-object and the culture media are different. In the case that the background carbon is subtracted accurately, the TOC method can be also applicable. UV-Vis absorption method is applied to all groups of nano-objects when their UV-Vis absorbance can be decoupled from the culture medium background absorbance. X-ray transmission measurement is applicable to nano-object samples with high background in the UV-Vis absorption method, however, it is practically restricted to suspended nano-objects of atomic numbers less than carbon.

Annex C
(informative)

Measurement of metal ions

C.1 Separation of ions from particulate matter

C-U/F membrane with an optimal fractionation molecular weight is selected depending on the size of nanoparticles. Conditions of C-U/F, namely can be employed in order to separate particulate matter and metal ions. In the U/F, a relevant centrifugal acceleration and duration of centrifugation should be adequately set to recover the filtrate containing ions by checking relationship a plot recovery versus duration of centrifugation or centrifugal acceleration[1],[22],[23].

The filter membrane can adsorb ions and it shall be tested before use[24].

Alternatively, centrifugation can be employed especially for metal nano-objects. Conditions of the centrifugation, namely centrifugal acceleration and duration of centrifugation should also be adequately set to recover the solution containing ions by checking capability of absorption of corresponding ion using concentration standard previously.

The filtrate or supernatant is analysed by the methods described in Clause C.2.

C.2 Measurements

C.2.1 Method choice criteria

ICP-MS is a highly sensitive technique and can detect the level of parts per trillion. On the other hand, AAS is highly reliable.

If a chelating agent for the colorimetric measurement is suitable for detection of the expected metal ion, the colorimetric method can be employed. The chelating agent should have high specificity of substrate, and no cross-reactivity. When there is no suitable chelating agent for expected metal ion, colorimetric method should not be employed.

C.2.2 Inductively coupled plasma-atomic emission spectrometry

Calibration curves for the metal ions of interest are necessary using standard solutions of the metal ion.

Measurement of ICP-AES should be made in accordance with ISO 11885[25].

C.2.3 Inductively coupled plasma mass spectrometry

Pre-treatment of the working suspension and generation of the calibration curves are conducted in accordance with Clause C.1.

The measurements are recommended to follow the relevant standards of ISO 17294-1[26] and ISO 17294-2[27].

C.2.4 Atomic absorption spectrometry

The calibration curves for the ions of interest are necessary using standard solutions of the ions.

C.2.5 Colorimetric method

If metal ions in the working suspension have characteristic absorption band with/without chelator in the visible or ultraviolet region, the colorimetric methods can be used to determine the concentration. Before the quantitative analysis, the absorption spectra are measured and identified for the ions. A characteristic absorption band that does not interfere with that of other ions is selected for the analysis.

C.2.6 Ion selective electrode

Ion selective electrodes can be used to determine the concentration of dissolved ions in the medium[35].

Annex D
(informative)

Measurement of culture medium components

D.1 Proteins

If separation of the particulate matter from the dispersant is achievable by centrifugation/ultra-centrifugation[18], the protein concentration in the medium is determined by the BCA method[19], Bradford method[20] or Lowry method[21] using BSA as the protein standard.

If separation of the particulate matter from dispersant is complicated, the AF4 method can be employed.

When the culture medium does not include any protein, such as in the case of a serum-free medium, evaluation of protein concentration is not required.

D.2 Calcium

Nano-objects are separated from the working suspension by C-U/F. Centrifugation or ultracentrifugation can also be employed if materials can be easily separated from the working suspension. In the U/F, relevant membrane having an optimal fractionation molecular weight is selected to separate them, depending on the size of nano-objects. When C-U/F is employed, conditions of C-U/F, namely centrifugal intensity and duration of centrifugation should be adequately set up to fully recover the solution containing calcium (Ca^{2+}). After the appropriate separation of nano-objects from working suspension, Ca^{2+} content of the culture medium can be determined by ICP-AES, ICP-MS or AAS. If nano-objects are separated from the working suspension by centrifugation or ultracentrifugation, Ca^{2+} concentration in the suspension medium can be determined by the colorimetric method (see Annex C).

D.3 Others

Other culture medium components that are important for healthy growth of the test cells can be reported. In order to measure the concentration of those component(s) in the working suspension, the particulate matter is separated in the same way as for the Ca^{2+} measurement. Those component(s) are then measured by appropriate methods. Details of the measurement methods for those component(s) should be described in the reporting.