TECHNICAL SPECIFICATION

ISO/TS 19337

First edition
2016-03-15

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

Nanotechnologies — Caracteristiques des suspensions de nano-objets utilisées pour les tests in vitro *évaluant la toxicité inherente aux nano-objets*

Reference number
ISO/TS 19337:2016(E)

ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

Contents

Page

Foreword

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

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

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

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

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

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

Introduction

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

In vitro toxicity assays using cultured cells are frequently used as a tool in screening hazardous materials. This 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 their behaviour is distinct from water soluble chemicals. For example, immediately after the introduction of nano-object samples into the culture medium, the nano-objects undergo changes, such as (a) dissolution, which is the dissolving of nano-objects into their ionic counterparts, (b) corona formation, which is the adsorption of the components of culture medium onto the nano-object surface, or (c) changes in aggregation/agglomeration state, leading to alteration in particles size and sedimentation. Therefore, it is critical to consider the aforementioned phenomena in clarifying if the observed effects are related to the tested nano-object itself or from other uncontrolled sources and to avoid false interpretation of assay results.

The rigorous characterization of the working suspension prior and during *in vitro* toxicity assays is essential to exclude the *in vitro* experimental artefacts. 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. Therefore, it is important to carefully assess and describe the characteristics of the suspension of nano-objects being tested.

This Technical Specification describes the essential characteristics and applicable measurement methods of working suspension containing nano-object samples for *in vitro* toxicity assays. Intention is that reliable test results on nano-object toxicity could be shared and communicated among stakeholders of nano-objects, such as regulators, general public, manufacturers and end users. This Technical Specification does not describe a procedure for validation of working suspension.

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

1 Scope

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

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

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

2 Normative references

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

ISO 29701, *Nanotechnologies — Endotoxin test on nanomaterial samples for in vitro systems — Limulus amebocyte lysate (LAL) test*

3 Terms and definitions

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

3.1
culture medium
aqueous solution of nutrients required for cell growth

3.2
secondary particle
complex 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 and nano-object sample

4 Abbreviated terms

For the purposes of this Technical Specification, the following abbreviated terms apply.

AAS	atomic absorption spectrometry
BCA	bicinchoninic acid
C-U/F	ultrafiltration assisted by centrifugation
DLS	dynamic light scattering
FFFF	flow field-flow fractionation
ICP-AES	inductively coupled plasma-atomic emission spectrometry
ICP-MS	inductively coupled plasma mass spectrometry
LD	laser diffraction
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 determine certain characteristics that might impact the biological system tested. This Clause specifies essential characteristics of the working suspension, listed below, and measurement methods that are applicable to them.

— Presence of endotoxins,

— stability of working suspensions,

— concentration of metal ions, and

— concentration of culture medium components.

Measurements of those characteristics shall be made for each dose of working suspensions. The measurement of endotoxin can be made alternatively for the stock nano-object suspension to be dosed. See Annex A for an example of flow of measurements.

5.2 Endotoxin

Contamination with endotoxins, part of the outer membrane of Gram-negative bacteria may significantly alter the results of the *in vitro* toxicity test. Therefore, it is critical to quantify the concentrations of endotoxins in the working suspension. The concentration of endotoxins in the working suspension shall be measured by Limulus amebocyte lysate (LAL) test in accordance with ISO 29701 and the monocyte activation test (MAT).[2][3]

5.3 Stability of working suspensions

5.3.1 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.[4][5] Aggregation/agglomeration and gravitational settling of the nano-objects are major issues that may affect the stability of the suspended nano-objects. The 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, resulting 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 *in vitro* toxicity assay.

NOTE ISO/TR 13097[6] is recommended as a comprehensive guidance for stability of working suspension.

5.3.2 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 dynamic light scattering (DLS),[4][7] laser diffraction (LD)[8] and static light scattering (SLS).[9] Other methods deviating from this Technical Specification can be used and reported in accordance with 6.6.

See Annex B for measurements.

5.3.3 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 light scattering,[4][7][10] inductively coupled plasma mass spectrometry (ICP-MS),[11][12][13] ultraviolet-visible (UV-Vis) absorption, X-ray transmission[14] and the total organic carbon analysis.[15] Other methods deviating from this Technical Specification can be used and reported in accordance with 6.6.

See Annex B for measurements.

5.4 Concentration of metal ions

Metal ions, produced as a result of nano-object test sample dissolution, can contribute to test 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 ultra-filtration (U/F), ultra filtration assisted by centrifugation (C-U/F) or tangential flow filtration (TFF). 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 inductively coupled plasma-atomic emission spectrometry (ICP-AES), ICP-MS, atomic absorption spectrometry (AAS) and the colourimetric method. Other methods deviating from this Technical Specification can be used and reported in accordance with 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.

5.5 Concentration of culture medium components

5.5.1 General

A nano-object sample added to a culture medium to generate a working suspension may adsorb components of the culture medium.[1] This 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 by setting aside enough time after the addition of nano-object sample to the culture medium. If culture medium components other than protein and calcium that may significantly affect the stability of 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.

NOTE Nano-objects can affect pH, osmolality and other essential characteristics in the culture medium.