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Nanotechnologies — *In vitro* MTS assay for measuring the cytotoxic effect of nanoparticles

Nanotechnologies - Analyse du MTS in vitro pour la mesure de l'effet cytotoxique des nanoparticules

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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).

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

Introduction

The field of nanotechnologies continues to advance rapidly through the development of new materials, products and applications. At the same time, many questions have been raised relating to the potential impact on human health and on the environment of some of these materials. Internationally, a large program of research is underway to better understand and quantify potential hazards. Also the chemicals used to coat the surface of nanoparticles in processing or in products can affect the toxicity of nanoparticles, even more so due to their large surface to volume ratio.

Cellular systems are a fundamental element of living biological systems. It is likely that monitoring toxic response of cellular model systems to nanoparticle exposure will provide insight into the "modes-of-action" of nanoparticles and which of them would need to be further investigated for risk assessment.

In 2008, a number of international researchers concluded that some published results of nanomaterial toxicity could not be replicated across laboratories and that accurate and reproducible nanotoxicology tests were needed. As a result, the International Alliance for NanoEHS Harmonization (IANH) was formed with the goal of developing testing protocols that would accurately assess toxicity and biological interactions of nanoparticles in cellular systems and that these results be reproducible in any laboratory. The IANH performed round robin characterization of particle size distributions in liquid suspensions, and *in vitro* interactions of nanomaterials with cells with the several common cytotoxicity assays (Annex A). This group identified a number of factors that increased variability and developed techniques to reduce it. Research funded by the US NIEHS NanoGo further assessed some of these protocols, in particular, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay protocol[1]. A third team extended the IANH protocol and performed experiments that employed a systematic plate layout to achieve improved analysis and consistency of results (Annex B)[2]. Importantly, each of these protocols used interlaboratory testing between multiple laboratories to identify sources of variability and improve the assay protocols.

This document is a method to assess *in vitro* cell viability with the MTS assay.[3] This assay produces a colourmetric change (absorption peak at 490 nm) in a culture well due to generation of a formazan product in the presence of cytoplasmic reductase enzymes. In general, changes in absorption intensity is directly proportional to cell number although assay conditions that alter reductase activity or reagent availability can result in colourmetric changes that are not directly due to changes in cell viability (i.e. cell number). The MTS reagents are directly added to cell culture well which allows rapid evaluation of potential intrinsic toxicity of nanoparticles. Due to the potential interference effects that can occur with nanoparticles and colourmetric assays, it is important control experiments with the nanoparticles and the MTS reagents are performed before the assay results are accepted. Direct microscopic observation of cells after treatment also provides an orthogonal method to validate an MTS assay result. The normalized protocol presented here is limited to adherent cell types, but it could be modified to be used with suspension cells.

This measurement of toxicity in this assay is a first-tier measurement of nanoparticle effects on individual cellular systems. The normalized method presented here is based on the three MTS assay protocols described above. Differences between the experimental systems are described in Table 1.

Table 1 — Summary of the studies used to develop a normalized MTS assay protocol

Study ID	Cell line[a]	Nanoparticle tested[b]	Positive and negative control materials	Centrifuge step
IANH	RAW-264.7	+PS-NP, CeO_2	$CdSO_4$,no-particle treatment	No
NanoGo	BEAS-2B, RLE-6TN and THP-1	ZnO, TiO_2, MWCNT	No-particle treatment	Yes
EMPA-NIST[c]	A549	+PS-NP	$CdCl_2$, no-particle treatment	No

a ATCC Cell Bank Name

b +PS-NP is a positively charged polystyrene nanoparticle, CeO_2 is cerium oxide, ZnO is zinc oxide, TiO_2 is titanium dioxide, and MWCNT is a multiwall carbon nanotube.

c EMPA is the Swiss Federal Laboratories for Material Science and Technology.

As a result of these differences, some parts in the normalized protocol contains optional steps that were presented in three interlaboratory studies.

Several methods can be used for determining cell viability, including MTS,[3] 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT[4]), (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (XTT[5]), lactate dehydrogenase (LDH[6]), trypan blue exclusion[7] and neutral red assay[8], The MTS assay was used in a multi-group round robin characterization. The MTS assay is an improved version of the MTT assay and provides a simple high throughput characterization for cell viability[1][9]. The optical density of the MTS assay solution increases upon its reduction by the functioning cell enzymes in live cells.

Control experiments are required to determine a baseline optical density of cell viability for untreated cells, and to verify that cells have an expected response to known non-toxic nanoparticles, toxic chemicals and toxic nanoparticles as measured with the assay [10]. Furthermore, it is important to determine whether nanoparticles interfere with the optical readout of the assay and potentially invalidate assessment of the nanoparticle cytotoxicity response. [11]

It is important to note that the MTS assay described here is one of many commercially assays available to assess the cytotoxicity of nanomaterials. Although assays such as the LDH assay which assesses plasma membrane integrity, the ATP assay which evaluates energy metabolism and the BrdU assay for DNA synthesis are not discussed here, the results from these assays in addition to the MTS assay allow for a more comprehensive evaluation of the overall impact of nanoparticles on cells.

Nanotechnologies — *In vitro* MTS assay for measuring the cytotoxic effect of nanoparticles

1 Scope

This document specifies a method for evaluating the effects of nano-objects and their aggregates and agglomerates (NOAA) on cellular viability using the MTS assay. The assay design includes performance requirements and control experiments to identify and manage variability in the assay results.

This document is applicable to the use of a 96-well plate.

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 terminological databases for use in standardization at the following addresses:

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

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

3.1
culture vessel
example assay vessel described in this document based a 96-well tissue culture-grade plate format

Note 1 to entry: Other tissue culture grade vessels (i.e. 384 well plates, 24 well plates, 6 well plates) can be used interchangeably in these methods provided that they meet the requirements of tissue culture grade and are suitable for use with mammalian cells.

Note 2 to entry: Adjustments to the protocol such as cell seeding volumes, cell rinsing volumes, and cell dosing volumes may be required if other tissue culture grade vessels are used during this procedure.

[SOURCE: ISO 10993-5:2009, 3.1]

3.2
dispersion
microscopic multi-phase system in which discontinuities of any state (solid, liquid or gas: discontinuous phase) are dispersed in a continuous phase of a different composition or state

Note 1 to entry: If solid particles are dispersed in a liquid, the dispersion is referred to as a suspension. If the dispersion consists of two or more liquid phases, it is termed an emulsion. A superemulsion consists of both solid and liquid phases dispersed in a continuous liquid phase.

3.3
endotoxin
part of the outer membrane of the cell envelope of Gram-negative bacteria

Note 1 to entry: The main active ingredient is lipopolysaccharides (LPS).

[SOURCE: ISO 29701:2010, 2.3]

3.4
negative control material
material or chemical which, when tested in accordance with this document, does not produce a cytotoxic response

Note 1 to entry: The purpose of the negative control is to demonstrate the basal level response of the cells. This control is often composed of the vehicle solvent used to store the nanomaterial in stock concentrations.

[SOURCE: ISO 10993-5:2009, 3.4]

3.5
positive control material
material or chemical which, when tested in accordance with ISO 10993-5, provides a reproducible cytotoxic response

Note 1 to entry: The purpose of the positive control is to demonstrate an appropriate test system response. For example, a nanomaterial positive control would be positively charged polystyrene.

[SOURCE: ISO 10993-5:2009, 3.2, modified]

3.6
sedimentation
settling (separation) of the dispersed phase due to the higher density of the dispersed particles compared to the continuous phase

Note 1 to entry: The accumulation of the dispersed phase at the bottom of the container is evidence that sedimentation has taken place.

[SOURCE: ISO/TR 13097:2013, 2.13]

3.7
test sample
material that is subjected to biological or chemical testing or evaluation

[SOURCE: ISO 10993-5:2009, 3.5]

4 Symbols and abbreviated terms

cells/mL	cells/mL (cells per millilitre)
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NPS	nanoparticle suspension
PMS	phenazine methosulfate
PS	polystyrene

5 Materials

5.1 Cell line

Established cell lines are preferred and where used shall be obtained from recognized repositories. Follow the basic principles of cell culture techniques regarding expanding a frozen stock of cells so that the MTS assay for nanocytotoxicity can be performed [12].

If a stock culture of a cell line is stored, storage shall be at −80 °C or below in the corresponding culture medium but containing a cryoprotectant, e.g. dimethylsulfoxide or glycerol. Long-term storage (several months up to many years) is only possible at −130 °C or below.

Only cells free from mycoplasma shall be used for the test. Before use, stock cultures should be tested for the absence of mycoplasma.

NOTE 1 It is important to check cells regularly [e.g. morphology, doubling time, modal chromosome number, short tandom repeat (STR) testing] because sensitivity in tests can vary with passage number.

NOTE 2 Nanoparticle can interact with cells through different mechanisms. It is useful to include both a phagocytic cell line (i.e. macrophage) and a non-phagocytic cell line (i.e. epithelial or fibroblast) in these studies. Assay results with the use of these two cell types can provide insight into the mode of action for nanoparticle toxicity.

5.2 Assay

5.2.1 MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]\PMS- phenazine methosulfate [CAS#138169-43-4].

The reagent is reduced in the presence of cellular enzymes and forms a coloured product that is soluble in the culture media. The optical density of the culture media is correlated with cell count in a culture vessel in the absence of artefacts that can occur if the culture conditions affect reductase activity within the cells and if the nanoparticle causes interference effects in the assay readout. The reagent is described in Reference [2] and the reagent materials are available from different vendors.

5.3 Controls

5.3.1 Chemical positive control material, $CdSO_4$, shall be used as positive chemical control.

NOTE 1 Cd^{+2} ions are toxic to animals and cells through an oxidative stress mechanism, see Reference [13].

NOTE 2 Cadmium containing compounds, including water soluble compounds such as $CdCl_2$ and $CdSO_4$ are assigned the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) signal word of Danger.

Cadmium (Cd) is a toxic heavy metal and its disposal and use are regulated in some countries. In the case where Cd cannot be used as a positive chemical control, an alternative chemical control shall be selected. The control compound should be soluble in aqueous media, sufficiently stable for the time course of the experiment and readily available as a purified product from commercial vendors. Non-metallic chemicals such as phenol, DMSO and detergents such as Tween 80 can be used as positive chemical controls, with the protocol undergoing additional validation for the use of these chemicals.

5.3.2 Positively charged polystyrene nanoparticles, (diameter 60 nm, dispersed in water) shall be used as a nanoparticle positive control material. The use of these nanoparticles as positive controls in A549 and Raw 264.7 cells has been validated in interlaboratory studies (see Table 1).

NOTE 1 For dispersion protocol and biological activity of the cationic polystyrene nanoparticles, see Reference [10].

NOTE 2 Positively charged polystyrene (amine terminated) induce toxic oxidative stress in many cells, see Reference [14].

NOTE 3 Nanoparticles of quartz, silica and silver are also cytotoxic to many cell types and could be used as positive controls, see Reference [15].

6 Apparatus

6.1 Incubator, 37 °C ± 1 °C, humidified, 5 % CO_2/air.

6.2 Flat bottom 96 multi-well plates.

6.3 96 multi-well plates with U bottom, for dosing plate use.

6.4 24 multi-well plates with flat bottom, for cell health and growth rate only.

6.5 96 well plate photometer microtitre plate reader.

6.6 Centrifuge, capable of at least 2 000 g acceleration.

6.7 Multichannel pipette (at least 8 position), with 200 µL volume/pipette.

6.8 Laminar flow cabinet, standard biological hazard.

6.9 Tissue culture flasks, 25 cm^2 and 75 cm^2.

6.10 Inverted phase contrast microscope.

6.11 Stereomicroscope.

6.12 Laboratory balance.

6.13 Electronic cell counter or hemocytometer

6.14 Micropipette.

6.15 Vortex mixer.

7 Nanoparticle test sample preparation

Following the basic principle of sample preparation, nanoparticles shall be dispersed in a biologically compatible fluid with a reproducible procedure. These can include sonication and mixing by vortexing. Alternatively, nanoparticles can be dispersed with biologically compatible chemical stabilizers, coatings, such as albumin, or directly in culture medium using the appropriate serum. Specific dispersion techniques are not discussed in this document. Details for dispersion can be found in the references cited in the NOTEs and in ISO/TS 19337.

NOTE 1 Several procedures have been published that identify methods to reproducibly disperse nanoparticles[15][16][17] and characterize nanosuspensions and their stability. Dispersion protocols from the NANOGENOTOX Joint Action are publicly available on the internet.

NOTE 2 For biologically compatible chemical stabilizers see Reference [19]. For coatings such as albumin see Reference [20]. For compatible culture medium, see Reference [21].

NOTE 3 Chemical stabilizers such as albumin can introduce high background levels in cell viability assays. It is important to use control experiments (i.e. stabilizer only) to determine the effect of the stabilizer on the assay readout.

With nanoparticles dispersed in a liquid media such as H_2O, the volume fraction of the nanoparticle media in the cell culture media shall be below the fraction that is toxic to the cell culture.

The liquid media supporting the nanoparticle suspension can be toxic to cells and cause a false positive toxicity measurement. Control experiments with liquid media should be performed to determine at what volume fraction is the liquid media toxic to the cells.

NOTE 1 A 1 mg/1 ml suspension would produce a water content of ~10 % in cell culture media for a 100 µg/ml exposure. When using water as a dispersion vehicle, a guideline is to keep the final concentration of water below 10 % of the total volume to reduce significant vehicle effects. If higher concentrations of vehicle are required for nanoparticle dose preparation, it is important to validate the higher concentration of vehicle does not interfere with the assay results.

The type of suspension process used shall be carefully considered in order to rule out false positive cytotoxic effects that are not due to the nanoparticles

For nanosuspension stability evaluation, two factors shall be evaluated:

a) stability against agglomeration (reflected in the mean particle size); and

b) stability of the colloidal suspension (reflected by precipitation and sedimentation).

Nanosuspensions should be tested for the presence of endotoxins in accordance with ISO 29701.

8 Preparations

8.1 General

All solutions (except culture medium), glassware, etc., shall be sterile and all procedures should be performed under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

8.2 Culture medium

The culture medium shall be sterile.

The culture medium with or without serum shall meet the growth requirements of the selected cell line. Antibiotics may be included in the medium provided that they do not adversely affect the assays.

Storage conditions such as refrigerator temperature shall be validated.

NOTE The stability of the culture medium varies with the composition and storage conditions.

8.3 Preparation of cell stock culture

Using the chosen cell line and culture medium, prepare sufficient cells to complete the test. If the cells are to be grown from cultures taken from storage, remove the cryoprotectant, if present. Subculture the cells at least once before use.

When subculturing cells, remove and resuspend the cells by enzymatic and/or mechanical disaggregation using a method appropriate for the cell line. Additional cell line information is in Annex A.

Good cell culture practices should be used. See Reference [12] additional instructions if required.

8.4 Verify viable cell growth

Prior to performing experiments on nanoparticles, characterize viability and doubling rates of the cells. Cell growth rates: viability and doubling rates shall be characterized and monitored. Cell viability should remain > 95 % by using a trypan blue exclusion assay:

a) Grow the cells in 24 well plates for 24 h and 48 h:

   1) transfer 200 000 cells/ml in 500 µL of culture medium per well with eight replicates per time period;

   2) use one plate for each time period (24 h and 48 h);

   3) gently move the plates into the incubator without agitation to avoid disturbing cell attachment resulting in non-uniform deposition;

   4) verify that incubators have been recently calibrated for: temperature, humidity, CO_2 concentration. Record metrics in a laboratory notebook to establish charting metrics;

   5) at each time point (24 h and 48 h), remove one plate from the incubator;

   6) make note of the apparent health and morphology of the cells with a stereomicroscope.

b) Assess cell number and viability with trypan blue:

   1) remove culture medium from the wells with gentle pipetting;

   2) harvest the cells using the manufacturer's instructions;

   3) collect the culture medium containing cells in a centrifuge tube;

   4) spin the supernatant with the added cells in a centrifuge at 400 g for 5 min to form a pellet;

   5) discard the supernatant;

   6) add 25 µL (0,4 % trypan blue in PBS) to 100 µL culture medium;

   7) resuspend the pellet in trypan blue/culture medium with a pipette;

   8) deposit the cells on a hemacytometer;

   9) record the total number of live and dead (blue) cells and the percent viability (live/total) by counting the cells in the hemacytometer with a stereomicroscope. See Reference[22] for details;

   10) cell doubling times should be consistent with those expected for the cell line and the percentage of viable cell through 48 h should be > 95 % prior to continuing with nanoparticle exposure experiments.

8.5 Verification of plate reader uniformity

Ensure that the instrument is operating properly prior to performing the measurement.

8.6 Control preparation

8.6.1 Control description

Positive control materials shall be $CdSO_4$ and positively charged polystyrene. See Clause 5 for more detail on the selection of these positive controls.

Separate experiments shall be performed to determine whether the nanoparticles and antibiotics can potentially interfere with the assay.

Prepare stock solutions in sterile and endotoxin free ultra pure water (<1,1 µS/cm at 20 °C).

8.6.2 $CdSO_4$ stock solution preparation (10mM)

Cells shall be exposed to $CdSO_4$ at final concentrations of 1 µM, 10 µM, 25 µM, 50 µM, and 100 µM.

a) Dissolve and dilute $CdSO_4$ in ultra pure water to 10mM concentration;

b) Store the 10mM $CdSO_4$ at 4 °C. Sterile filtration is not required.

8.6.3 Nanoparticle control suspension preparation

Adjust positive polystyrene concentration to 10 mg/ml with ultra pure water.

NOTE At this concentration, the maximum dosing concentration in the test plate (100 µg/ml) will result in 1.0 % (w/v) water vehicle in the cell culture media. This stock preparation, which was used in study described in Annex B, is used as an example for all the following procedures. If a more dilute nanomaterial is used as a stock solution, the fraction of vehicle in the cell culture media will be increased. See Clause 7 for preparation of other nanoparticle suspensions.

8.7 Precision pipetting

A calibrated pipette shall be used to dispense reagents into the 96-well plate used for this assay. If possible, a calibrated multichannel pipette that is capable of simultaneously dispensing from at least 6 pipette tips during a single ejection in a 96-well plate should be used to perform this procedure. A previous study indicated that variability in cell seeding between wells using a multichannel pipette is lower than the variability from separate pipetting steps. [21]

NOTE With the small volumes of fluids, cells and nanoparticles used in the 96 well experiments, it is important that the pipette system is carefully calibrated and procedures used to dispense fluids precisely. A more detailed description of procedures vendor. As an example, Cerionix application note AN1-12 12/06 describes "Precision- and Accuracy-Based Validation of Pipette Tips Used on Automated Liquid Handling Platforms Following Multiple Cleanings with 'Cold' Atmospheric Plasma".

9 Characterization of nanoparticle impact on cell viability

9.1 General

Due to potential variability in nanosuspensions, three independent replicate assays should be conducted on different days with new suspensions. The protocol steps for each assay are summarized in the flow chart shown in Figure 1.