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Nanotechnologies — 5-(and 6)-Chloromethyl-2′,7′ Dichloro-dihydrofluorescein diacetate (CM-H2DCF-DA) assay for evaluating nanoparticle-induced intracellular reactive oxygen species (ROS) production in RAW 264.7 macrophage cell line

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National foreword

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Nanotechnologies — 5-(and 6)-Chloromethyl-2',7' Dichloro-dihydrofluorescein diacetate (CM-H2DCF-DA) assay for evaluating nanoparticle-induced intracellular reactive oxygen species (ROS) production in RAW 264.7 macrophage cell line

Nanotechnologies — Essai au diacétate de 5-(et 6)- Chlorométhyle -2',7' Dichloro-dihydro-fluorescéine (CM-H2DCF-DA) pour l'évaluation de la génération intracellulaire d'espèces réactives à l'oxygène induites par les nanoparticules sur la lignée souche 264.7 de macrophages

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Contents

Page

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

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

Introduction

The field of nanotechnology 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 programme 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 interactions of nanoparticles with cells, even more so due to their large surface to volume ratio. Thus, there is a need for reliable fast screening methods to determine the potential toxicity aspects of nanoparticles with characterization of chemical functionalization on nanoparticles.

It is likely that monitoring biological response of cellular model systems to nanoparticle exposure can provide insight into the "modes-of-action" of nanoparticles and which of them may 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 of this, 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 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 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 5-(and-6)-chloromethyl-2',7' Dichloro-dihydro-fluorescein diacetate, acetyl ester (CM-H_2DCF-DA), and propidium iodide assays. The IANH identified a number of factors that increased variability and developed techniques to reduce variability.

Oxidative stress, which leads to DNA damage, is a primary driver leading to the accumulation of mutations which occurs in living organisms, so it is important to assess whether nanoparticles can induce reactive oxygen species in living cells.

This document is a method to assess potential nanoparticle induced radical oxygen species (ROS) generation in cells through *in vitro* measurements. Although multiple techniques are used for determining generation of oxygen radicals in cells, the CM-H_2DCF-DA has been used in round robin testing to evaluate ROS generation in mouse macrophages (RAW 264.7). The CM-DCF-DA assay provides a general measure of oxidative stress rather than detecting specific oxygen radicals or reactive species. [4] While this assay has not been evaluated in a broad range of cells, it does provide insight into the potential for ROS generation in macrophages which may play an important role in scavenging particles from the body.

The CM-DCF-DA assay has a margin of error even when controls are used and a number of factors could produce false negatives.[4] The CM-H_2DCF-DA assay is not optimal for detecting all ROS species, such as the superoxide anion and hydroxyl radical which have short half-lives. In addition, measurement using cytometry should be performed quickly after cells have been exposed in the assay, because DCFH and DCF can leach from cells or the DCFH can be oxidized. Also, the CM-H_2DCF-DA assay is deactivated in serum, so cells should be washed to remove serum and cells could be lost in this process resulting in a potential false negative. Furthermore, some nanoparticles may interact with DCFH and partially quench fluorescence. Thus, negative ROS results with this assay may not be conclusive. ISO/TS 18827 utilizes electron spin resonance (ESR) to detect the presence of ROS species in cells and differentiate between the different reactive oxygen species without interference.

In addition, there are several factors that could produce false-positive results.[5] Some nanoparticles and dead cells can fluoresce. Some nanoparticles can catalytically interact with CM-H_2DCF-DA or the assay components can preferentially adsorb on the surface of the particle.[5] In order to establish true positives, controls should be established to characterize nanoparticles alone under test conditions, as well as distinguish dead cell fluorescence from live cells with ROS.

Furthermore, due to light-induced auto-oxidation, CM-H_2DCF-DA solutions at any concentration should be protected from light and air by storing in the dark in a sealed container filled with nitrogen gas or argon.

Thus, the CM-H_2DCF-DA assay may be applicable to only particular cell lines and nanoparticles and outcomes should be confirmed by additional assays (see Annex A for alternate cell lines). In particular, as a number of factors could lead to false negatives, or positives, other tests should be pursued and a positive result should be confirmed to not be caused by interference.

Controls are needed to determine a baseline of fluorescence of unexposed cells, determine whether cells are affected by non-toxic nanoparticles and also to demonstrate that known ROS generating chemicals and nanoparticles produced ROS which could be determined under assay conditions. Furthermore, it is important to determine whether nanoparticles interfere with the fluorescence of the assay and potentially invalidate assessment of nanoparticle induced ROS generation in cells. Controlled experiments could be performed with cells exposed to Sin-1 with varying concentrations of nanoparticles present to determine whether the nanoparticles quench the fluorescence.

NOTE This assay is considered to be a screening assay that rapidly provides information about a nanoparticle interaction with a cellular system. Although screening type assays are critical for use in evaluating nanoparticle effects on cells, it is important that interpretation of the results be verified with other ROS and related cellular assays.

Nanotechnologies — 5-(and 6)-Chloromethyl-2',7' Dichloro-dihydrofluorescein diacetate (CM-H2DCF-DA) assay for evaluating nanoparticle-induced intracellular reactive oxygen species (ROS) production in RAW 264.7 macrophage cell line

1 Scope

This document describes how to test and evaluate results obtained from *in vitro* ROS generation in RAW 264.7 macrophage cells exposed to nano-objects, nanoparticles, their aggregates and agglomerates using the CM-H_2DCFDA assay.

The protocol in this document is limited to use of a 24 well plate so if other plates were to be used, volumes would need to be adjusted and the protocol steps validated to ensure confidence in the test results.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 80004-2, ISO 10993-5 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
agglomerate
collection of weakly bound particles or *aggregates* (3.2) 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]

3.2
aggregate
particle comprising strongly bonded or fused particles where the resulting external surface area may be 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]

3.3
culture vessels
vessels appropriate for cell culture including glass Petri dishes, plastic culture flasks or plastic multiwall plates and microtitre plates

Note 1 to entry: These 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.

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

3.4
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 suspoemulsion consists of both solid and liquid phases dispersed in a continuous liquid phase.

[SOURCE: ISO/TR 13097:2013, 2.5]

3.5
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]

3.6
nano-object
discrete piece of material with one, two or three external dimensions in the *nanoscale* (3.8)

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

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

3.7
nanoparticle
nano-object (3.6) with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the *nano-object* (3.6) do not differ significantly

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

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

3.8
nanoscale
length range approximately from 1 nm to 100 nm

Note 1 to entry: Physiochemical particle properties that are not extrapolations from a larger size are predominantly exhibited in this size range.

[SOURCE: ISO/TS 80004-1:2015, 2.1, modified]

3.9
negative control material
material or chemical which does not produce a cytotoxic response

Note 1 to entry: The purpose of the negative control is to demonstrate background response of the cells.

Note 2 to entry: The material or chemical is tested according to ISO 10993-5.

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

3.10
particle
minute piece of matter with defined physical boundaries

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to *nano-objects* (3.6).

[SOURCE: ISO/TS 80004-2:2015, 3.1]

3.11
positive control material
material or chemical which 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, an organotin-stabilized polyurethane has been used as positive control for solid materials and extracts. Dilutions of phenol, for example, have been used as a positive control for extracts. In addition to a material, pure chemicals can also be used to demonstrate the performance of the test system.

Note 2 to entry: The material or chemical is tested according to ISO 10993-5.

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

3.12
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, modified]

3.13
test sample
material that is subjected to biological or chemical testing or evaluation

[SOURCE: ISO 10993-5:2015, 3.5]

4 Symbols and abbreviated terms

CM-H_2DCF-DA	5-(and-6)-chloromethyl-2', 7' Dichloro-dihydro-fluorescein diacetate, acetyl ester
ROS	reactive oxygen species

5 Materials

5.1 Cell line

RAW 264.7, mouse macrophage; Abelson murine leukaemia virus transformed. Verified with DNA identification.

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.

It is important to check cells regularly (e.g. morphology, doubling time, modal chromosome number) because sensitivity in tests can vary with passage number.

Follow the basic principles of cell culture techniques that describing expanding a frozen stock of cells so that the MTS assay for nanocytotoxicity can be performed.

NOTE 1 See Reference [6] for good practices on thawing and culturing cell lines if this information is required.

NOTE 2 See Annex D for example experimental data from RAW 264.7.

5.2 Assay

5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA) is a reagent that becomes fluorescent upon oxidation by ROS. This reagent passively diffuses across the cell membrane and is retained in the cytoplasm. This compound is used as the ROS detection reagent in this document.

NOTE This document is limited to CM-H_2DCF-DA; however, other variants of this assay are listed in Annex B, but their use might require modification of the procedures in this protocol.

5.3 Chemicals, media and sera

All reagents except 5.3.11 and 5.3.12 should be certified as cell culture grade from the manufacturer. The other reagents should be of the highest quality possible.

5.3.1 Dulbecco's Modified Eagle Medium (DMEM).

5.3.2 FBS (Fetal Bovine Serum).

5.3.3 Penicillin.

5.3.4 Streptomycin.

5.3.5 Amphotericin B (antifungal agent).

5.3.6 L-glutamine.

5.3.7 5-(and-6)-chloromethyl-2', 7' Dichloro-dihydro-fluorescein diacetate (CM-H_2DCF-DA).

5.3.8 Phosphate Buffered Saline (PBS) [Ca and Mg Free].

5.3.9 DMEM Phenol red free.

5.3.10 Dimethyl Sulfoxide (DMSO).

5.3.11 3-morpholinosydnonimine (Sin-1) (Positive chemical control).

5.3.12 Positively charged polystyrene nanoparticles, approximately 60 nm diameter (see Reference [20] for description of cationic polystyrene nanoparticles and their mechanism of action).

5.3.13 Distilled water or any purified water suitable for cell culture.

6 Technical equipment

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

6.2 24 multi-well culture plates.

6.3 Flow cytometer.

6.4 Centrifuge.

6.5 Multichannel pipette (at least six channel) with 500 μL volume/pipette.

6.6 Laminar flow cabinet, standard biological hazard.

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

6.8 Inverted phase contrast microscope.

6.9 Stereomicroscope.

6.10 Laboratory balance.

6.11 Cell counter or hemocytometer.

6.12 Pipette.

6.13 Vortex mixer.

7 Nanoparticle sample preparation

The basic principle of sample preparation is that nanoparticles should be dispersed in a biologically compatible fluid with a reproducible procedure. Several procedures have been published that identify methods to reproducibly disperse nanoparticles,[7][8][9]characterize nanosuspensions and their stability. Alternately, nanoparticles can be dispersed with biologically compatible chemical stabilizers,[10] coatings, such as albumin,[11] or directly in culture medium[12] using the appropriate serum. See Annex C for further information on suspension preparation and characterization.

For nanosuspension stability evaluation, two factors should be evaluated:

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

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

Nanosuspensions should be tested for the presence of endotoxins using ISO 29701.

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 A 1 mg/1 ml stock nanoparticle suspension would produce a water content of ~10 % in cell culture media for a 100 μg/mL exposure.

8 Preparations

8.1 General

All solutions (except culture medium), glassware, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). See Figure 1 for ROS experimental characterization flow for nanoparticles.

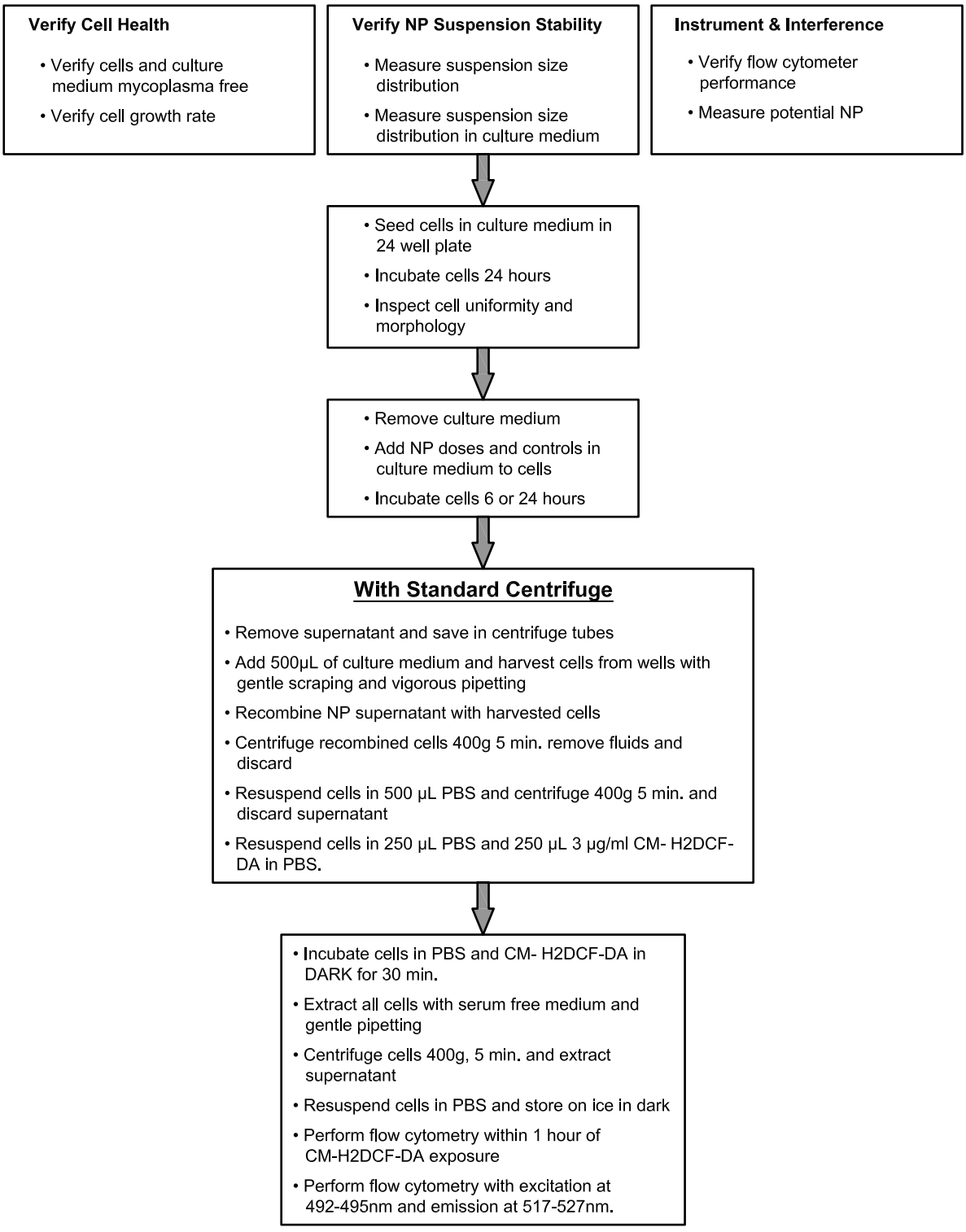


Figure 1 — ROS experimental characterization flow for nanoparticles (NP)

8.2 Flow cytometry calibration

Flow cytometry is a very sophisticated technique that requires calibration and establishment of fluorescence thresholds to separate ROS induced fluorescence from background fluorescence. The instrument should be qualified as described by the manufacturer. The first step is to establish a relative intensity calibration curve by measuring the fluorescence of reference beads.[13][14] Reference beads with assigned molecular equivalents of soluble fluorophore (MESF) are available from several commercial vendors.

8.3 Experimental culture medium

8.3.1 10 % Fetal Bovine Serum (FBS).

8.3.2 90 % DMEM.

8.3.3 100 µg/ml streptomycin.

8.3.4 60 mg/ml penicillin (100 IU/ml).

8.3.5 292 mg/ml L-glutamine.

8.3.6 2,5 mg/ml Amphotericin B.

Due to potential degradation of the L-glutamine, the culture medium should not be stored for more than three weeks.

8.4 Reagent preparation

8.4.1 Shortly before performing the flow-cytometry analysis, dissolve the ROS indicator CM-H_2DCFH-DA in DMSO to make a 1 mg/mL concentrated stock solution.

NOTE Do not expose the CM-H_2DCFH-DA to fluorescent light, because the molecule can be deactivated by the illumination.

8.4.2 Place in a storage container that is then filled with nitrogen gas or argon and seal the container.

8.4.3 Keep the container tightly sealed and stored in the dark until ready to use.

8.5 Preparation of cell stock culture

Using the RAW 264.7 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.

Good cell culture practices should be used.[6]

8.6 Preparing culture for experiments

Suspend the cells in experimental culture medium (10 % FBS) without phenol red to a density of 2×10^5 c/mL.

8.7 Verification of healthy cell growth

Prior to performing experiments on nanoparticles, each laboratory should demonstrate and characterize growth of healthy cells. Cell growth needs to be characterized and monitored in each laboratory, namely viability and doubling rates. Cell viability should remain >95 % (by trypan blue exclusion) for incubation times up to 48 h.

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

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

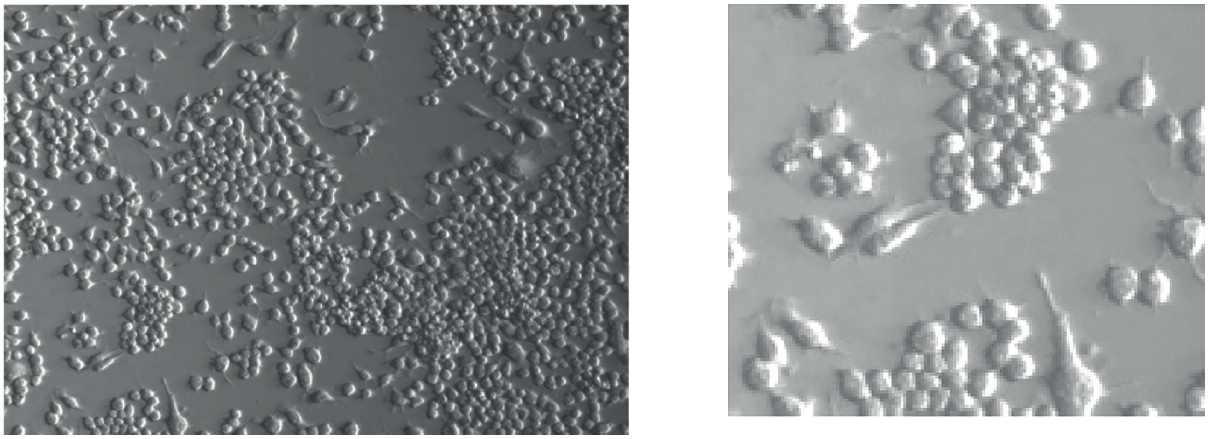
   2) use one plate for each time period (24 h and 48 h);

   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 as illustrated in Figure 2.



NOTE 1 The right panel is a blown up section of the left panel.

NOTE 2 Each round cell is approximately 18 µm in diameter.

Figure 2 — Healthy RAW 264.7 cells (round) a few elongated (activated or dividing) cells

b) Assess cell number and viability with trypan blue:

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

   2) add 500 μL of complete culture medium and harvest the cells;

   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 per cent viability (live/total) by counting the cells in the hemacytometer with a stereomicroscope;[15]

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.

8.8 Evaluation of nanoparticle interference

NOTE The presence of nanoparticles might interfere with the assay through optical fluorescence, catalytic interactions with the assay or fluorescence quenching.[5] Thus, it is important to determine whether interference is being produced by the nanoparticles.

8.8.1 Transfer 500 μL of nanoparticle suspension diluted in culture medium to 1 μg/mL, 10 μg/mL, 25 μg/mL, 50 μg/mL and 100 μg/mL in the first column of a plate.

8.8.2 Transfer 500 μL of nanoparticle suspension diluted in 1,5 μg/ml CM-H_2DCF-DA/PBS to 1, 10, 25, 50 and 100 μg/mL in a second column of the plate.

8.8.3 Transfer 500 μL of 1,5 μg/ml CM-H_2DCF-DA in PBS in a third column of the plate.

8.8.4 Incubate the plate in the dark for 30 min at 37° C in a humidified, 5 % CO_2 atmosphere.

8.8.5 Using a flow cytometer, characterize the fluorescence of all samples with excitation at 492 nm to 495 nm and emission at 517 nm to 527 nm.

8.8.6 Determine whether the nanoparticle fluorescence or nanoparticle interaction with the CM-H_2DCF-DA may interfere with the assay.

8.8.7 Perform controlled experiments with cells exposed to 5 μM Sin-1 with the full range of nanoparticle concentrations to determine whether the nanoparticles change the assay's fluorescence.

8.8.8 If the nanoparticle suspension interferes with the CM-H_2DCF-DA assay, do not use it to characterize ROS generation in cells caused by the nanoparticles.

NOTE The evaluation of nanoparticle interference uses a different plate layout than is used to characterize potential cell ROS (see Figure 3).

8.9 Control preparation

8.9.1 General

In addition to the controls listed below, nanoparticles shall be evaluated in culture medium without cells to identify potential fluorescent interference.

8.9.2 Control description

8.9.2.1 Sin-1 control is to produce the maximum concentration of ROS as it induces cell death.[16][18] This is a reference of the highest potential concentration of ROS and thus highest fluorescence from the DCF.

8.9.2.2 Neutral and negatively charged polystyrene might not exhibit cytotoxicity[19] in cells or induce ROS in RAW 264.7 cells. So the negatively charged polystyrene is to be used as a negative control.

8.9.2.3 A second negative control is exposing the cells to nanoparticle free dispersant in cell culture medium.

8.9.2.4 Three wells shall receive no treatment and no CM-H_2DCF-DA to determine a baseline of fluorescence from the cells.

8.9.2.5 Two wells shall receive no treatment and shall receive CM-H_2DCF-DA assay treatment to assess the oxidative stress levels of the cells with this number of passages.

8.9.2.6 Separate experiments shall be performed to determine whether the nanoparticles have fluorescence that could interfere with the assay in flow cytometry.

8.9.3 Sin-1 stock solution preparation (1 mM)

8.9.3.1 Dissolve Sin-1 in PBS to achieve a concentration of 1 mM.

8.9.3.2 Store the 1 mM Sin-1 stock solution frozen in aliquots at −20° C for no longer than three months.

8.9.3.3 Adjust positively and negatively charged polystyrene suspension concentrations to 10 mg/mL.

9 Evaluation of nanoparticle impact on ROS generation in cells

9.1 Prepare cells in the 24 well plates

9.1.1 The cells should be collected, counted and then resuspended in culture medium without phenol red at ~2 × 105 c/mL.

NOTE Approximately 3,0 × 10^6 cells are needed for a single plate (~30 % excess cells).

9.1.2 Transfer 1 × 105 cells in phenol red free culture medium with a total volume of 500 μL into each well of a 24-well plate.

9.1.3 Incubate cells at 37° C, with humidity and 5 % CO_2 for 24 h.

9.1.4 Inspect cells for uniformity of distribution in each well, ~70 % confluence and morphology with a stereomicroscope.

9.1.5 Remove supernatant from each well with a pipette positioned at the bottom edge of the well or with gentle aspiration.

9.2 Dose the cells with nanoparticles and controls

9.2.1 Prepare two plates with nanoparticle doses in culture medium to concentrations as shown in Figure 3.

9.2.2 Prepare control doses to concentrations as shown in Figure 3.

9.2.3 Using a pipette, transfer 500 µL total volume of nanoparticle suspension, control, and culture medium into the 24 well plate as shown in Figure 3.

9.2.4 Incubate cells with nanoparticle and controls at 37° C with one plate for 6 h and the second plate 24 h.

9.2.5 While the cells are incubating with the nanoparticles and controls, dilute the CM-H_2DCF-DA/DMSO to 3 µg/ml in PBS.

NOTE 1 Do not expose the CM-H_2DCF-DA to fluorescent light because the molecule can be deactivated by the illumination.

NOTE 2 Store the CM-H_2DCF-DA in the dark.

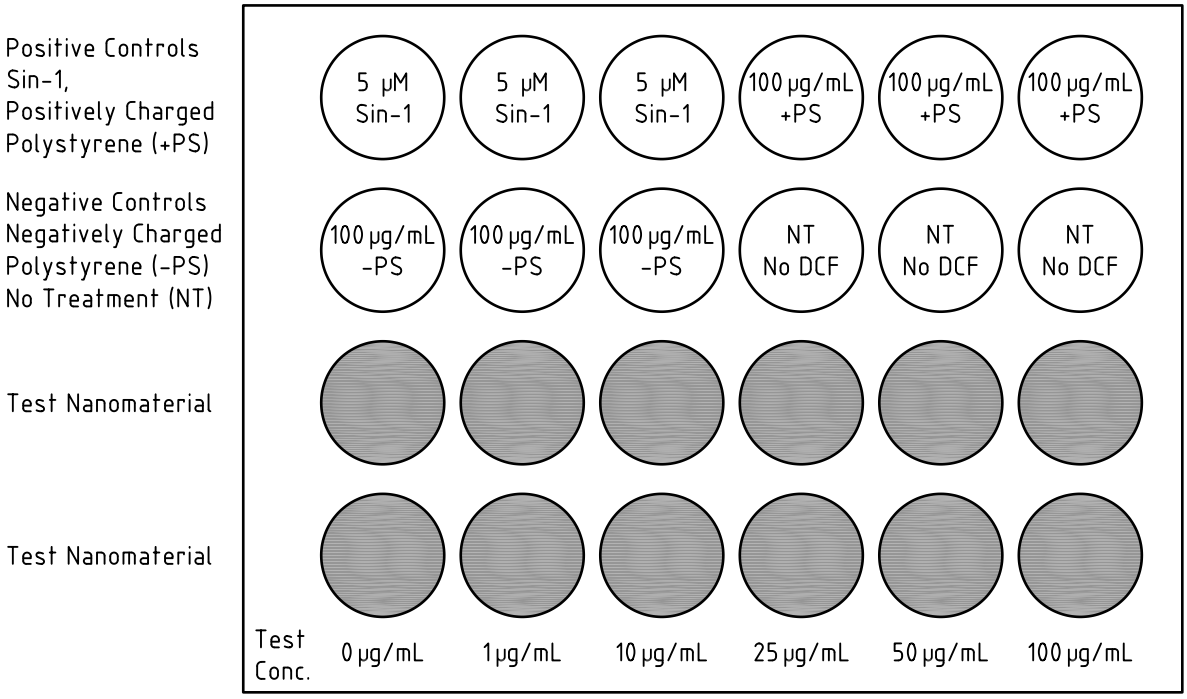


Figure 3 — 24 Well nanoparticle (NP) and control dosing

9.3 Expose the cells to CM-H_2DCF-DA Assay

9.3.1 After incubation, remove the supernatant with a pipette placed at the bottom edge of the wells with gentle aspiration.

9.3.2 Collect the supernatant and non-adherent cells in centrifuge tubes for later recombination with adherent cells.

9.3.3 Add 300 μL of complete medium to each well and harvest the cells with gentle scraping and vigorous pipetting.

9.3.4 Add the cells and supernatant harvested in 9.3.2 to the centrifuge tubes containing supernatant and non-adherent cells harvested in 9.3.1.

9.3.5 Spin the supernatant with the added cells in a centrifuge at 400 g for 5 min to form a pellet and discard the supernatant.

9.3.6 Resuspend the cells in ~500 μL of PBS, then spin in a centrifuge at 400 g for 5 min.

CM-H_2DCF-DA is inactivated by serum so cells should be washed in PBS to remove serum prior to assay exposure.

9.3.7 Extract and discard the supernatant and resuspend the cells with 250 μl of PBS and add 250 μl of the prepared 3 μg/ml CM-H_2DCF-DA in PBS.

9.4 Incubate the cells with CM-H_2DCF-DA

9.4.1 Incubate the cells with the reagent at 37° C with 5 % CO_2 in the dark for 30 min.

9.4.2 Remove the loose cells, PBS, and DCF with pipetting, also remove the adherent cells from the wells with gentle pipetting using serum-free medium (DMEM without phenol red).

NOTE It is important to carefully harvest all cells (both adherent and loose cells).

9.4.3 Spin all of the cells and supernatant in a centrifuge at 400 g for 5 min, discard the supernatant, and resuspend the cells in PBS.

9.4.4 Store the cells on ice in the dark to slow down subsequent dye oxidation while they are awaiting analysis.

9.5 Flow cytometry analysis

9.5.1 Calibrate the flow cytometer with the standards recommended by the instrument manufacturer.

Flow cytometry analyses should be done as soon as possible (within one hour) after loading the cells with CM-H_2 DCF-DA and the study planned in such a way that no more than 20 to 30 samples are analysed at any one time.

Flow cytometry should use fluorescence excitation at 492 nm to 495 nm and emission at 517 nm to 527 nm.

9.5.2 CM-H_2DCF-DA-stained cells are analysed on a flow cytometer (collect 10 000 events).

a) Characterize the emission data for nanoparticle exposed cells.

b) Characterize emission data for positive and negative controls including cells with no exposure treatment.

NOTE Collect and store all intensity data for further analysis.

9.5.3 Review the fluorescence of cells with no exposure treatment, negative controls and positive controls in order to establish thresholds for positive cell fluorescence.

10 Data analysis and results

If the nanoparticle suspension interferes with the DCF assay, do not use it to characterize ROS generation in cells caused by the nanoparticles as discussed in 8.8.

a) Determine the mean fluorescence intensity (MFI) for all samples characterized including controls.

b) Prepare a spreadsheet where the MFI of nanoparticle exposed cells and controls are divided by the MFI of control cells that were not exposed to nanoparticles.

NOTE The results are expressed as the fold increase in fluorescence of the exposed cells to the unexposed cells. Thus, the mean fluorescence intensity of the unexposed cells is 1,0.

c) Determine from the data collected in 8.8 and 9.5.2 whether the nanoparticles interfere with the CM-H_2DCF-DA Assay.

d) Record relevant experimental parameters such as exposure time, dosing concentrations, control results and NP dispersion properties to aid in reproducing the experimental results.

Annex A
(informative)

Alternate cell lines

While the scope of this document is limited to the RAW 264.7 cell line and the CM-H_2DCF-DA Assay, other cell lines should be applicable, but the protocol would need to be validated with other cell lines.

Other cell lines that should be applicable (based on limited evaluation) include human bronchial epithelial (BEAS-2B), rat alveolar type II epithelial (RLE-6TN), mouse liver epithelial hepatocyte (HEPA-1), human microvascular endothelial (HMEC) and rat aorta, thoracic/medial layer (A10) cell lines. These cell lines are adherent similar to the RAW 264.7 cell line and have been used to evaluate ROS in the presence of nanoparticles in some experiments.

Annex B
(informative)

Alternate fluorescence characterization techniques

This document is limited to use of CM-H_2DCF-DA with flow cytometry to characterize fluorescence. Other techniques such as fluorescence plate readers or epifluorescence microscopes can be used as an alternative to characterize the CM-H_2DCF-DA Assay. When using fluorescent plate readers, the wells in the plates should be black to ensure accurate readings. The protocol would need to be validated with other instruments to characterize fluorescence.

Other compounds including 2',7' –dichlorofluorescin diacetate (DCFDA), 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA), carboxy-H_2DCF-DA or other variants of this assay. The use of these other assays should be experimentally validated and may require modification to the procedures in this protocol.

Annex C
(informative)

Suspension preparation and characterization

Nanoparticles dispersed in aqueous solution need to be dispersed at a concentration that should allow high cell exposure concentrations without requiring high vehicle solvent content for cell exposure (i.e. a 1 mg/1 ml suspension would produce a water content of ~10 % for a 100 μg/mL exposure). Consideration of the suspension process is critical for ruling out false positive cytotoxic effects that are not due to the nanoparticle itself.

NOTE Nanosuspension stability is evaluated by two factors: 1) agglomeration (reflected in the mean particle size) and 2) precipitation of the colloidal suspension.

Annex D
(informative)

Example experimental data from RAW 264.7

D.1 General

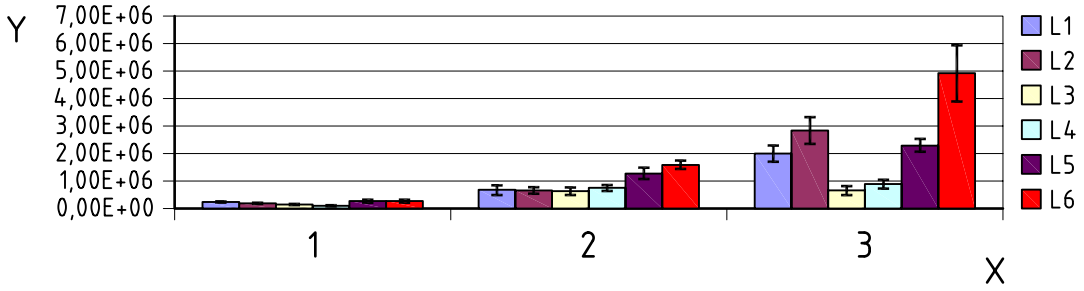
This document was applied to the RAW 264.7 cell line to verify cell growth rate and health and then to assess the reactive oxygen species generated in cells by exposure to positively charged polystyrene and ceria nanoparticles.

D.2 Experimental procedures

The health and growth rate of the RAW 264.7 cells are verified by using procedures in 8.7. The effect of nanoparticles on generation of reactive oxygen spices in RAW 264.7 cell is characterized using procedures in Clause 9 and Clause 10.

D.3 RAW 264.7 Health assessment through growth rate and cell viability

Two important factors to assess in the heath of cells are the growth rate and viability through the time of the experiments. Six groups in the IANH performed these experiments and determined that the cells multiplied and doubled within 24 h and continued to multiply through 48 h as is shown in Figure D.1. Furthermore, most groups found the cells to have viability greater than 90 % through the 48 h experiments is shown in Figure D.2. Increasing the number of cells counted and the number of cells used to assess viability should improve the reliability of the health assessment.

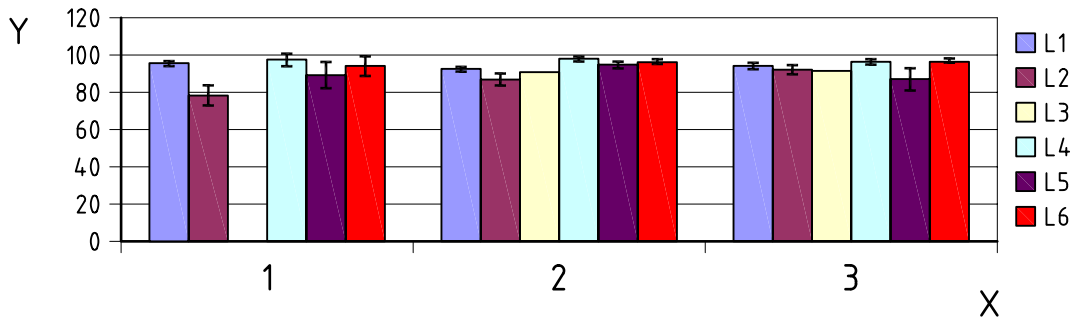


Key

1 24 h cell
2 48 h cell
3 96 h cell
X growth time
Y number of cells

NOTE L1 through L6 represent the six laboratories that participated in the interlaboratory testing.

Figure D.1 — Counts of RAW 264.7 cells for six different laboratories at 24 h and 48 h



Key

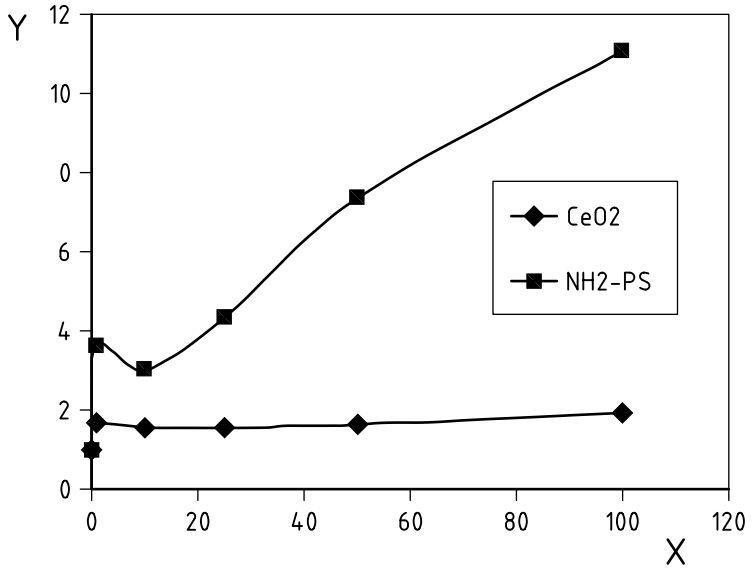
1 24 h viability
2 48 h viability
3 96 h viability
X time
Y cell viability (%)

NOTE Lab 3 did not measure viability at 24 h. Most laboratories reported viabilities of over 90 % for both 24 h and 48 h. Lab 3 did not report cell viability at 24 h. L1 through L6 represent the six laboratories that participated in the interlaboratory testing.

Figure D.2 — RAW 264.7 cell viability measured at 24 h and 48 h

D.4 ROS generation in RAW 264.7 cells with exposure to nanoparticles (Figure D.3)

RAW 264.7 cells were exposed to positively charged polystyrene (60 nm) and ceria (~200 nm) for 24 h and then exposed to CM-H_2DCF-DA. The fluorescence of the cells exposed to positively charged polystyrene increased significantly with exposure dose, while the fluorescence remained flat with increasing exposure to ceria particles.



Key

X dose (µg/ml)

Y fold increase in MFI

Figure D.3 — RAW 264.7 relative mean fluorescence change with exposure to positively charged polystyrene (NH_2-PS np) and ceria (CeO_2) after 24 h exposure

These measurements are reported for one laboratory only as an example. The mean fluorescence increased for both materials relative to unexposed cells, but increased dramatically with exposure to positively charged polystyrene while it remained relatively flat with exposure to ceria.

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