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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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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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Foreword

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

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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.

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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.

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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.

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[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.

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