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Nanotechnologies — Electron spin resonance (ESR) as a method for measuring reactive oxygen species (ROS) generated by metal oxide nanomaterials

Nanotechnologies — Résonance paramagnétique électronique (RPE) pour la mesure des espèces réactives de l'oxygène (ROS) générées par des nanomatériaux sous forme d'oxyde métallique

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Foreword

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

Introduction

Recently, the use of metal or metal oxide-based nanomaterials has dramatically increased in biomedical and industrial applications. However, the scientific basis for the cytotoxicity and genotoxicity of most manufactured nanomaterials are not fully understood. An important mechanism of nanotoxicity is the generation of reactive oxygen species (ROS). The study on the hazardous effects of metal oxide nanomaterials is still in its initial stage. The ability to generate ROS is one main source of toxicity of metal oxide nanomaterials. Overproduction of ROS can induce oxidative stress, resulting in cells failing to maintain normal physiological redox-regulated functions. This in turn may lead to DNA damage, unregulated cell signalling, change in cell motility, cytotoxicity, apoptosis and cancer initiation. There are critical determinants that can affect the generation of ROS. The critical determinants include size, shape, particle surface, surface positive charges, surface-containing groups, particle dissolution, metal ion release from nanometals and nanometal oxides, UV light activation, aggregation, mode of interaction with cells, inflammation and pH of the medium[1]. Thus, to detect and quantify ROS formation on the surface of metal oxide nanomaterials, this document suggests the electron-spin-resonance (ESR) method.

Amongst ROS, the most biologically relevant and widely studied are hydroxyl radical (OH), superoxide anion radical (O_2^-), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2).

However, direct detection of some free radicals (e.g. superoxide anion and hydroxyl radical) is very difficult or impossible[2] in solution at room temperature. ESR spin trapping is a valuable tool in the study of transient free radicals[3]. Spin trapping is a technique, developed in the late 1960s, where a nitrone or nitroso compound (a spin trap) reacts with a target free radical to form a stable and distinguishable free radical (spin adducts) to be detected by ESR spectroscopy.

Spin adducts can be observed directly by ESR spectroscopy. The ESR spectra of these spin adducts are unique and provide a fingerprint for the presence of ROS.

This document specifies methods of detection by ESR of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) hydroxyl adduct, 5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide (BMPO) superoxide adduct and 2,2,5,5-tetramethyl-3-pyrroline-3-carboxamide (TPC) singlet oxygen adduct formation from metal oxide nanomaterials. This document provides a method to assess ROS generation on the metal oxide nanomaterials in a cell free condition. This method may provide valuable information for the prediction of ROS-mediated cytotoxicity without cytotoxicity assay at physico-chemical evaluation phase.

Nanotechnologies — Electron spin resonance (ESR) as a method for measuring reactive oxygen species (ROS) generated by metal oxide nanomaterials

1 Scope

This document provides a procedure for the detection of ROS (OH, O_2^-, $^1O_2$) generated by metal oxide nanomaterials in aqueous solution with a reactive oxygen species-specific spin trapping agent using ESR, but excludes ESR procedures that do not use a spin trapping agent.

2 Normative references

There are no normative references in this document.

3 Terms, definitions and abbreviations

For the purposes of this document, the following terms and definitions 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 http://www.iso.org/obp

3.1 Terms and definitions

3.1.1
nanomaterial
material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of nano-object and nanostructured material.

Note 2 to entry: See also ISO/TS 80004-1:2015, 2.8 to 2.10.

[SOURCE: ISO/TS 80004-1:2015, 2.4]

3.1.2
test sample
material, device, device portion, component, extract or portion thereof that is subjected to biological or chemical testing or evaluation

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

3.1.3
zero baseline control
equivalent of the positive control where no radicals are detected

Note 1 to entry: For example, zero baseline control for the positive control of fenton reaction will be H_2O_2 and DMPO in the absence of iron; for hypoxanthine–xanthine oxidase (HX-XO) system, it will be hypoxanthine and BMPO in the absence of HX-XO; for rose bengal photosensitization, it will be rose bengal and TPC in the absence of light.

3.1.4
positive control
well-characterized material or substance that, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriately positive or reactive response in the test system

[SOURCE: ISO/TS 10993-12:2012, 3.12]

3.2 Abbreviations

ROS	reactive oxygen species
ESR	electron spin resonance
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
BMPO	5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide
TPC	2,2,5,5-tetramethyl-3-pyrroline-3-carboxamide
DTPA	diethylenetriaminepentaacetic acid
OH	hydroxyl radical
OH-	hydroxide ion
O_2	superoxide anion radical
$^1O_2$	singlet oxygen
TEMPOL	4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl

4 Principle

4.1 General

In most atoms and molecules, electrons are paired. The paired electrons do not give an ESR signal while atoms and molecules with unpaired electrons give an ESR signal. When an atom or molecule with an unpaired electron is placed in a magnetic field, the spin of the unpaired electron can align either in the same direction or in the opposite direction as the field. These two alignments of electron spin have different energies. The application of a magnetic field to an unpaired electron lifts the degeneracy of its spin states. ESR spectroscopy measures the absorption of microwave radiation associated with the transition between these non-degenerate spin states[4].

4.2 Spin trapping method

4.2.1 General

Spin trapping is used in ESR spectroscopy for detection and identification of short-lived free radicals. Ideally, the adduct formed between a spin trapping agent and a free radical has an ESR spectrum characteristic and specific to that free radical. Advanced ESR studies employing spin-trap agents were adopted to distinguish the different types of ROS.

4.2.2 DMPO

DMPO has significant advantages over other nitrone spin traps. It is particularly useful for identifying oxygen-centred radicals, e.g. superoxide anion and hydroxyl radicals. The spin adduct formed between

DMPO and the hydroxyl radical has an ESR signal consisting of a quartet with intensity ratio of 1:2:2:1 and hyperfine splitting of $a_N = a_H$ = 1,49 mT to 1,5 mT, which is consistent with the DMPO-OH adduct[5].

4.2.3 BMPO

BMPO is suitable for the specific in vivo or in vitro detection of short-lived superoxide anions and hydroxyl radicals by forming distinguishable adducts measurable with ESR spectroscopy[6]. Other nitrone spin traps, such as DMPO, do not distinguish superoxide and hydroxyl radical easily because of spontaneous decay of DMPO-superoxide adduct (t1/2 = 0,9 min to 1,3 min) into the DMPO-hydroxyl adduct. BMPO-superoxide adduct does not decay into a hydroxyl adduct and has a much longer half-life (t1/2 = 8,5 min to 15,7 min)[7]. The BMPO-superoxide adduct were fitted with a_N = 1,34, a_H = 1,18 mT[8][9].

4.2.4 TPC

TPC has proper sensitivity and dynamic range for detecting the formation of singlet oxygen[10]. TPC-singlet oxygen adduct spectrum shows a triplet with 1:1:1 signal intensity[11]. The TPC/$^1O_2$ adduct has a hyperfine splitting of a_N = 0,172 mT[12].

NOTE 1 The hyperfine coupling constants of magnetic nuclei (a = the hyperfine splitting of the spectrum, ai where i = type of nucleus, e.g. ^1H, ^{13}C, ^{14}N) and the pattern of an ESR spectrum contains the information about the structure and geometry of such radicals.

NOTE 2 The width of spectral line is characteristic of resonance frequency-energy absorption conditions[13].

4.3 Positive control for generating free radicals

Fenton reaction, hypoxanthine–xanthine oxidase (HX-XO) system and rose bengal photosensitization are well-characterized systems that can generate hydroxyl radical, superoxide anions and singlet oxygen, respectively. These systems demonstrates the suitability of the spin trapping agents to yield a reproducible and ESR signal patterns of the spin adducts such as intensity ratio and hyperfine splitting.

4.3.1 Fenton reaction[14]

Transition metal ions can activate H_2O_2 to form hydroxyl radicals which are strong oxidants. This system is called the fenton reaction. Iron (II) (ferrous ion) is oxidized by hydrogen peroxide to produce iron (III) (ferric ion), a hydroxyl radical and a hydroxyl anion (Reaction 1). The hydroxyl radical produced in the fenton reaction might be then trapped by DMPO to yield the spin adduct, DMPO/OH (Reaction 2).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\cdot}OH + OH^- \qquad \text{Reaction 1}$$

$$OH + DMPO \rightarrow DMPO/OH \qquad \text{Reaction 2}$$

4.3.2 Hypoxanthine–xanthine oxidase system[15]

Hypoxanthine–xanthine oxidase (HX-XO) system is a well-characterized system that can generate superoxide anions (Reaction 3 and Reaction 4). The superoxide anion can then be trapped by BMPO to form the spin adduct, BMPO/OOH (Reaction 5).

$$\text{Hypoxanthine} + H_2O + O_2 \xrightarrow{\text{Xanthine oxidase}} \text{Xanthine} + H_2O_2 \qquad \text{Reaction 3}$$

$$\text{Xanthine} + H_2O + O_2 \xrightarrow{\text{Xanthine oxidase}} \text{Uric acid} + O_2^- \qquad \text{Reaction 4}$$

$$O_2^- + BMPO \rightarrow BMPO/OOH \qquad \text{Reaction 5}$$

4.3.3 Rose bengal photosensitization[16][17]

Rose bengal is known as a photosensitizer for generation of singlet oxygen. When photoexcited, rose bengal transfers its energy to oxygen producing singlet oxygen (Reaction 6 and Reaction 7). The singlet oxygen can then be trapped by TPC to form the adduct, TPC/$^1O_2$. (Reaction 8).

$$\text{rose bengal} \xrightarrow{\text{Light}\,(\lambda\,\text{max} = \text{around 550 nm})} \text{rose bengal*} \qquad \text{Reaction 6}$$

$$\text{rose bengal*} + O_2 \rightarrow \text{rose bengal} + {}^1O_2 \qquad \text{Reaction 7}$$

$${}^1O_2 + \text{TPC} \rightarrow \text{TPC}/{}^1O_2 \qquad \text{Reaction 8}$$

5 Reagents

Use only reagents of recognized analytical grade and only distilled water or water of equivalent purity.

5.1 Spin-trap agent, for example DMPO, BMPO and TPC.

5.2 Reagents for positive control, for example $FeSO_4$, H_2O_2, phosphate buffer (pH 7,4), diethylenetriaminepentaacetic acid (DTPA) or chelating ion exchange resin, hypoxanthine, xanthine oxidase, rose bengal.

5.3 Deionised water (18,2 MΩ at 25 °C).

5.4 Standard sample for spin calculation, for example TEMPOL.

6 Apparatus

The usual laboratory apparatus are required and, in particular, the following:

6.1 Laboratory balance.

6.2 1,5 mℓ centrifuge tube.

6.3 Pipettes, with 10 µℓ to 1 000 µℓ volume.

6.4 Pipette tips, for 10 µℓ to 1 000 µℓ volume.

6.5 Vortex mixer.

6.6 Centrifuge, for 1,5 mℓ centrifuge tube.

6.7 Sample cell (flat sample cell or fine capillary-like sample tube), for samples of aqueous solutions; consisting of quartz (pure silicon dioxide, SiO_2).

6.8 Light source, λ max = around 550 nm.

6.9 ESR spectrometer.

7 Sampling

7.1 Preparation of test sample (metal oxide nanomaterial suspension)

The metal oxide nanomaterial is freshly prepared in deionised water and agitated on a vortex mixer or pipetting immediately. Approximately 500 μℓ is required for each sample. Mix well by vortexing or pipetting. Do not sonicate because radicals can be generated by sonication[18]. Refer to 8.1 and the OECD document[19].

Types and levels of ROS generated from nanomaterials can vary in accordance with the type of nanomaterials. Sample concentration of nanomaterials should be experimentally determined. Compare the ROS levels generated from same concentration of nanomaterial according to the ROS type. Refer to 9.3.9.

7.2 Preparation of solution for generating the hydroxyl radical

7.2.1 $FeSO_4$ solution

Dissolve the $FeSO_4$ in deionised water to make a concentration of 0,01 mM. Exactly 50 μℓ is required for each sample. Only freshly prepared solutions of $FeSO_4$ should be used.

7.2.2 H_2O_2 solution

Dilute the H_2O_2 in deionised water to make a concentration of 0,1 mM. Exactly 50 μℓ is required for each sample. Freshly prepared solutions of H_2O_2 should be used.

7.3 Preparation of solution for generating the superoxide anion radical

7.3.1 Phosphate buffer

Prepare a solution of phosphate buffer (100 mM, pH7,4) removed transition metal ions in deionised water. Exactly 70 μℓ is required for each sample. To remove transition metal ions, refer to NOTE 1 or NOTE 2.

NOTE 1 DTPA is used to eliminate possible artefactual oxidation by trace amounts of contaminating metal ions[20].

NOTE 2 Chelating ion exchange resins are specific exchangers or chelating. Prepare a solution of phosphate buffer by treating with chelating ion exchange resin[21][22].

7.3.2 Hypoxanthine solution

Dissolve the hypoxanthine in 100 mM phosphate buffer to make a concentration of 0,125 μM. Exactly 100 μℓ is required for each sample.

7.3.3 Xanthine oxidase solution

Dissolve the Xanthine oxidase in 100 mM phosphate buffer to make a concentration of 0,125 unit/mℓ. Exactly 10 μℓ is required for each sample. Freshly prepared solutions of xanthine oxidase should be used. Store at 2 °C to 8 °C.

7.4 Preparation of solution for generating the singlet oxygen

Dissolve the rose bengal in deionised water to make a concentration of 100 μM. Exactly 50 μℓ is required for each sample. Protect from light.