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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 1000 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.

7.5 Preparation of spin trapping agent

7.5.1 General

Spin trapping agents contain paramagnetic impurities that cause high backgrounds and noise signals. Use spin trapping agents with ultra-high purity and perform control experiment in which all spin trapping agents are excluded nanomaterial to produce ESR signal. Spin trapping agents with noise signals require pre-purification process such as activated charcoal.

7.5.2 DMPO stock solution

Dissolve the DMPO in deionised water to make a concentration of 500 mM. Exactly 100 µℓ is required for each sample. Use a DMPO with ultra-high purity (≥99 %). Store at –20 °C, protect from light and moisture.

7.5.3 BMPO stock solution

Dissolve the BMPO in deionised water to make a concentration of 500 mM. Exactly 100 µℓ is required for each sample. Use a BMPO with ultra-high purity (≥99 %). Store at –20 °C, protect from light and moisture.

7.5.4 TPC stock solution

Dissolve the TPC in deionised water to make a concentration of 400 mM. Exactly 20 µℓ is required for each sample. Use a TPC with ultra-high purity (≥99 %).

7.6 Reaction of test sample and spin trapping agent

7.6.1 General

Mix by vortexing or pipetting. Do not sonicate because radicals can be generated by sonication[18]. Refer to 8.1. Protect from light for sampling. Radicals can be generated by light[23]. Perform a zero baseline reaction with all the spin trap reagents. Apply the final concentration of reagent as shown in Table 1.

Table 1 — Final concentration of reagent

	Final concentration
DMPO	50,0 mM
$FeSO_4$	1,0 µM
H_2O_2	10,0 µM
BMPO	50,0 mM
Hypoxanthine	62,5 µM
Xanthine oxidase	6,25 µU/mℓ
TPC	10 mM
Rose bengal	25 µM
TEMPOL	1,0 mM × 10^{-3} mM

7.6.2 DMPO reaction

Add 50 µℓ of 500 mM DMPO stock solution to 450 µℓ of metal oxide nanomaterial solution and mix well by vortexing or pipetting.

7.6.3 BMPO reaction

Add 50 µℓ of 500 mM BMPO stock solution to 450 µℓ of metal oxide nanomaterial solution and mix well by vortexing or pipetting.

7.6.4 TPC reaction

Add 50 µℓ of 40 mM TPC stock solution to 450 µℓ of metal oxide nanomaterial solution and mix well by vortexing or pipetting.

7.7 Reaction of positive control and spin trapping agent

7.7.1 DMPO radical adduct form (DMPO/OH)

7.7.1.1 Add 140 µℓ of deionised water to an eppendorf tube.

7.7.1.2 Add 20 µℓ of 500 mM DMPO stock solution and 20 µℓ of 0,01 mM $FeSO_4$.

7.7.1.3 Initiate the reaction with 20 µℓ of 0,1 mM H_2O_2.

7.7.2 BMPO radical adduct form (BMPO/OOH)

7.7.2.1 Add 70 µℓ of 100 mM phosphate buffer (pH 7,4) containing 25 µM DTPA to an Eppendorf tube.

7.7.2.2 Add 20 µℓ of 500 mM BMPO stock solution and 100 µℓ of the 0,125 mM hypoxanthine stock solution.

7.7.2.3 Initiate the reaction with 10 µℓ xanthine oxidase 0 125 U/mℓ.

7.7.3 TPC radical adduct form (TPC/$^1O_2$)

7.7.3.1 Add 100 µℓ deionised water to a 96-well culture plates.

7.7.3.2 Add 50 µℓ of 40 mM TPC stock solution and 50 µℓ of 100 µM rose bengal.

An irradiation time of 10 min has proven to be adequate in many cases. However, the irradiation time will depend on the light source and distance between the light source and sample. Therefore, the optimal irradiation time should be experimentally determined.

7.7.3.3 After irradiation, the plates should be kept in the dark at room temperature until measurement, but there should be a very short time between irradiation and obtaining the ESR spectrum.

7.8 Preparation of the standard sample for spin calculation

Dissolve the TEMPOL in benzene solution to make a concentration of 1,0 mM × 10^{-3} mM. Exactly 500 µℓ is required.

NOTE 1 The TEMPOL has one stable nitroxide radical per molecule. Therefore, TEMPOL radical can be used as a good spin probe or standard sample.

NOTE 2 The radical number is 3,0 sp × 10^{14} sp in 1,0 mM × 10^{-3} mM TEMPOL[24].

8 Interferences

8.1 Sampling

If nanomaterials are conductive, a loss of microwave power will result. To prevent this, the first option is measuring at the extremely low temperature (4 K) to reduce the conductivity of the sample. If using this method, please check the possible minimum temperature of sample cell for aqueous solutions. The second option is using a filtration or centrifugation method to remove the nanomaterials after reaction with spin trapping agent. The pore size of the filter and conditions of centrifuge should be determined by size and dispersibility of nanomaterials. The third option is the subtraction method to subtract out the uneven ESR signal of samples by a mathematical operation[25].

To check for ROS generated from nanomaterials itself, do not sonicate to disperse nanomaterials because radicals can be generated by sonication[18]. In many cytotoxicity tests, the sonication method is widely used to disperse nanomaterials. Therefore, it is necessary to check ESR signal intensities of both before and after ultrasonic dispersion of nanomaterials. Protect from light while sampling. Radicals can be generated by light[23][26][27].

NOTE 1 Filtration is the physical operation which is used for the separation of nanomaterials from liquids by interposing a membrane through which only the fluid can pass. Use this filtrate after filtration for ESR sampling.

NOTE 2 Centrifuges are used for isolating or separating suspensions of nanomaterials. Use this supernatant after centrifugation for ESR sampling.

NOTE 3 Subtraction is a mathematical operation that represents the operation of removing ESR signal of the samples from ESR signal of sample reacted with spin trapping agent.

8.2 Sampling time

Generation of ROS is a dynamic process wherein both the amount and types of ROS formed are generally time dependent. Half-life of spin adduct is variable. Therefore, it is necessary to measure immediately and multiple times after sampling. The optimal time for beginning ESR measurements after sampling should be experimentally determined.

9 Procedure

9.1 General

The sensitivity is linked to a variety of instrument factors like the cavity, sample cell holder, parameter, etc. and different instruments can have different settings. Table 2 provides examples of settings of ESR parameters for measurements.

Those steps relating to operation of the ESR spectrometer are best addressed by referring to the operating instructions for the ESR spectrometer being used. Figure 1 provides flow chart for measurements.

Table 2 — Settings of ESR spectrometer parameters for measurement of hydroxyl radical, superoxide anion radical and singlet oxygen

Magnetic field parameters	Microwave parameters	Signal channel parameters
Centre field	**Frequency**	**Modulation frequency**
320 mT to 340 mT	9 GHz to 10 GHz	100 kHz
Sweep width	**Power**	**Modulation width**
±5 mT to ±10 mT	1 mW to 40 mW	0,04 mT to 0,25 mT
Sweep time	—	**Amplitude**
1 min to 5 min	—	50 to 300

Table 2 *(continued)*

Magnetic field parameters	Microwave parameters	Signal channel parameters
—	—	**Time constant**
—	—	0,03 s to 0,5 s
—	—	**R.mode**
—	—	First (1st)
—	—	**Phase**
—	—	0 °

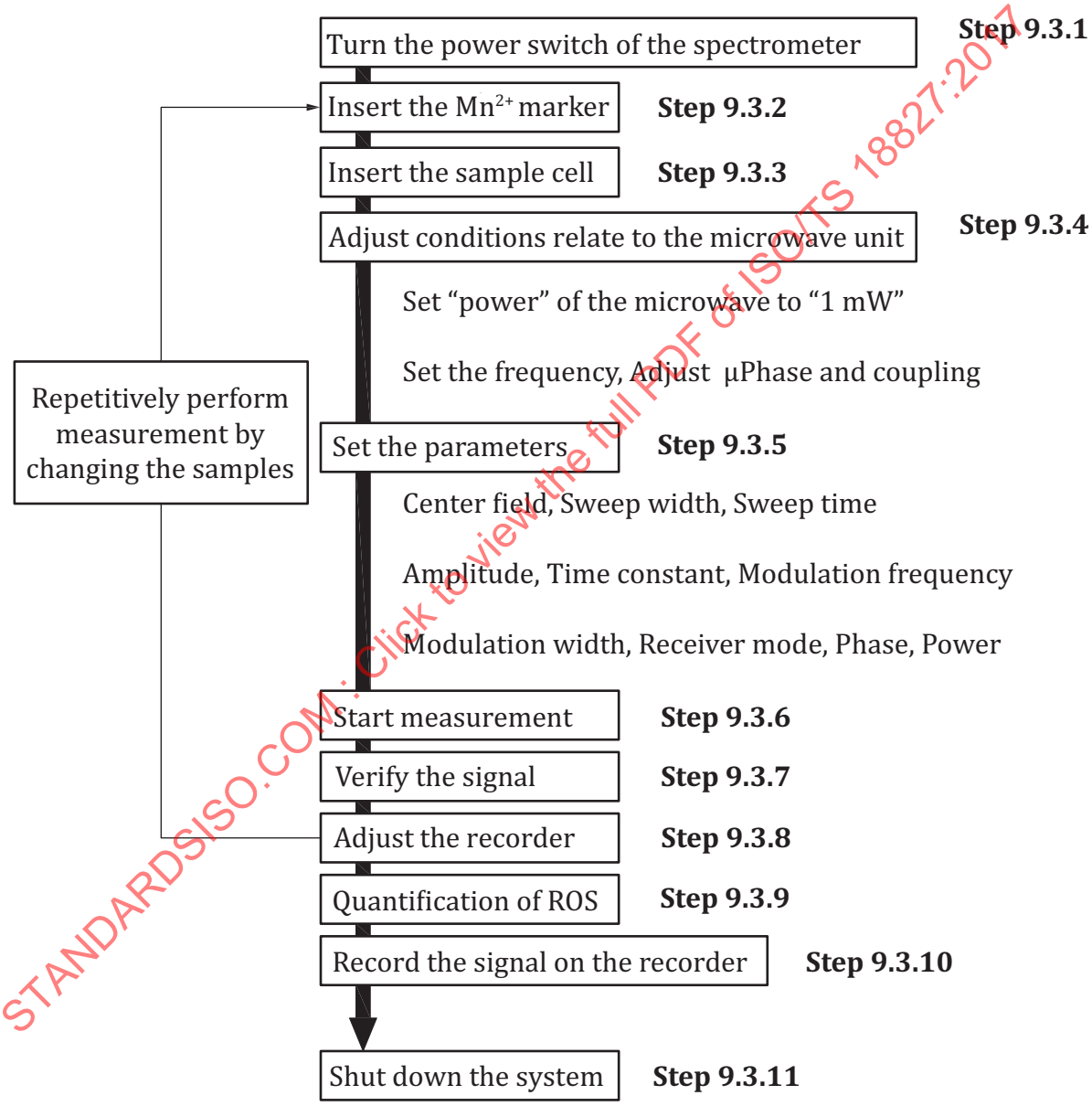


Figure 1 — Example of steps required for acquiring the ESR spectrum

9.2 Injection of sample

Draw the sample into sample cell by utilizing capillary action or drive it in with an injector.

Avoid introducing bubbles during the sample injection process.

After completion of the measurement, empty any remaining from the cell by turning the screw on the degassing adapter and wash the cell with deionised water. Repeat degassing and exhausting several times. If some water remains in the middle of the cell, hold the cell upright and remove water inside by applying blotting paper to the sample cell mouth or shake the water out. Then, suck in a new sample.

After positive control reactions and sample reactions, if some sample remains including metal ions, nanomaterials in the cell, thoroughly wash with acid water (a 1 % to 30 % solution of hydrochloric or nitric acid) and deionised water in the order named.

9.3 ESR measurement

9.3.1 Turn on the power switch of the spectrometer.

Let the cooling water flow.

Do not place metal objects around the machine.

9.3.2 Insert the Mn^{2+} marker.

The Mn^{2+} marker is positioned in the cavity.

NOTE 1 The ESR marker ensures that measurement results can be related to reference values. Additionally, they are often used for the determination of measurement uncertainties and for calibration.

NOTE 2 The ESR spectrum of Mn^{2+} consists of six lines and the interval between the third (g-factor = 2,034) and fourth (g-factor = 1,981) lines is 8,69 mT ± 0,01 mT[28]. Intervals between signals, other than these two, cannot be used for calibration of the horizontal axis (sweep width) because they change according to the frequency used.

9.3.3 Insert the sample cell.

9.3.3.1 Wipe off dust, moisture and other contaminants from the sample cell wall.

9.3.3.2 Set power of the microwave to 0 mW.

9.3.3.3 Insert the sample cell straight in an exact direction into the cavity resonator. The sample cell should be placed at the same position within the cavity each time. The type of cavity can make a large difference in sensitivity. Cavities are characterized by their quality factor (Q factor), which indicates how efficiently the cavity stores microwave energy. As Q factor increases, the sensitivity of the spectrometer increases. Liquid water strongly absorbs the microwave radiation used to measure the ESR spectrum. If aqueous solutions are tested, the sample should be placed in a quartz flat cell with the thin part of the cell position parallel to the direction of the electric field in the cavity (normally parallel to the direction of the magnetic field).

Make sure that the sample is properly positioned in the cavity. The sample should be at the centre of the cavity.

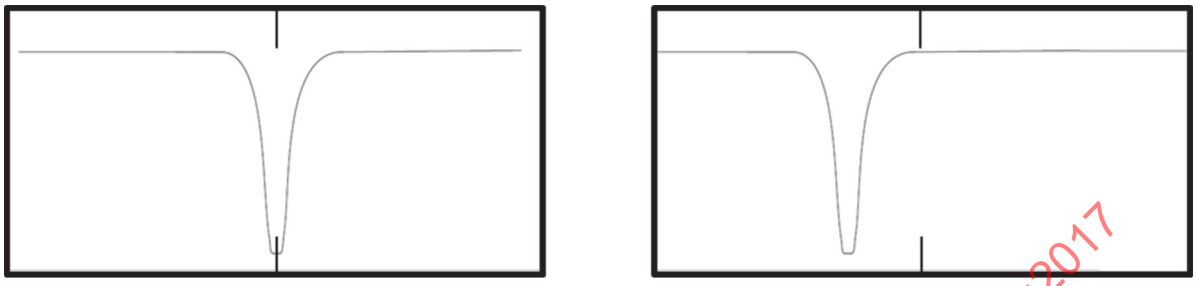
Make sure that the power of microwave is set at 0 mW whenever inserting or removing samples.

9.3.4 Adjust conditions related to the microwave unit.

9.3.4.1 Set power of the microwave to 1 mW.

9.3.4.2 Set the frequency.

Frequency indicates the oscillation frequency of the microwave. Adjust so that the resonance dip comes to the centre. See Figure 2.



(O) (X)

Figure 2 — Adjusting the frequency

9.3.4.3 Adjust μPhase

μPhase indicates the phase of the microwave. The phase value of the microwave has no unit.

If the resonance dip is deformed as shown in Figure 3, adjust "μPhase" so that the dip shape becomes as that marked with Figure 2 (O).

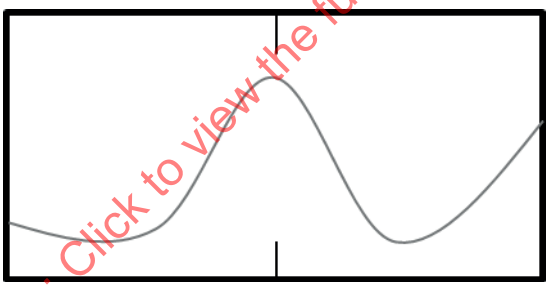
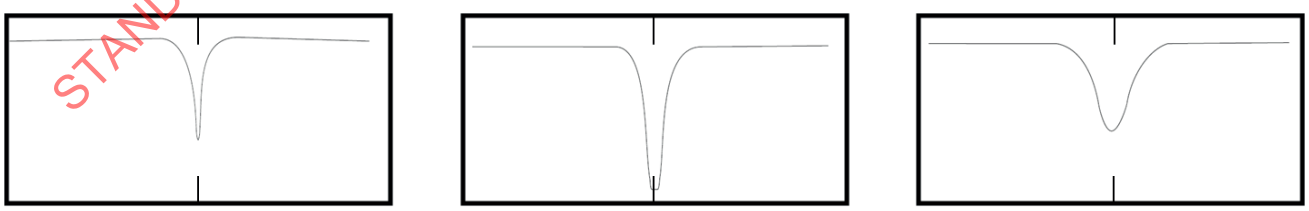


Figure 3 — Deform of the resonance dip

9.3.4.4 Adjust coupling.

This is the most important step in acquiring quality EPR spectra. In most instances, "Auto-tune" will work fine, but for some instances, the cavity shall be tuned manually. See Figure 4.



(x) **(O)** **(x)**

Undercoupled cavity **Matched cavity** **Overcoupled cavity**

Figure 4 — Adjusting the degree of coupling (no unit)

NOTE An overcoupled cavity will have a lower Q factor, resulting in lower sensitivity. These effects can happen when using aqueous solutions or conducting samples. If impedances of cavity and waveguide are not matched with each other, the microwave power transmitted through the waveguide is partially reflected. Not all the power enters into the cavity. Impedance of the cavity is adjusted to that of the waveguide by using the coupler.

9.3.5 Set the parameters.

See Table 2.

9.3.5.1 Centre field: 320 mT to 340,00 mT

Centre field is the abbreviation of the centre of the magnetic field.

NOTE A guideline in the observation magnetic field range is 5 to 10 times the line width of the signal although it varies according to the line shape (Lorentzian, Gaussian).

9.3.5.2 Sweep width (±): 5 mT to 10 mT

Sweep width indicates the magnetic field sweep width. Variable in the range between 0,001 and 750, limited by the centre magnetic field and sweep width though.

9.3.5.3 Sweep time: 1 min to 5 min

A variable has a range between 0,1 s and 120 min. When the sweep time is between 20 s to 120 min, the recorder moves, synchronizing width the sweep and the ESR waveform is drawn on the chart paper. When the sweep is faster than 10 s or less, the recorder bar is fixed at the centre.

9.3.5.4 Amplitude: 50 to 300

Amplitude indicates the gain of the amplifier. A variable has a range from 1 to 10 000.

The best signal-to-noise ratio(S/N) is obtained with an optimal setting. Too low a setting will result in poor S/N and an excessive setting will result in a spectrum that is clipped off at the top and bottom.

9.3.5.5 Time constant: 0,03 s to 0,5 s

Time constant indicates the response time of the lock-in amplifier. The longer the response time, the smaller the noise become, but the worse the response.

The setting for time constant differs according to how finely separate the splitting of the signal is and should be carefully selected. When hyperfine splitting is observed, measurement time should be set sufficiently slow, otherwise the signal does not respond well and the waveform will be deformed. Also, if a large value is set for time constant, which has a function for eliminating noise, the signal response becomes slow and a longer time constant becomes necessary. Since, as indicated above, the sweep time to be set depends on the complexity of the signal and the set time constant, it should be selected and set for each measurement.

9.3.5.6 Modulation frequency: 100 kHz

Modulation frequency indicates the frequency of the magnetic field modulation.

Magnetic field modulation is a function indispensable for the ESR instrument for observing an ESR spectrum with high sensitivity. The intensity of the magnetic field modulation is related to signal/noise and the signal intensity increases in proportion to an increase of the modulation width, thus enhancing the sensitivity.

9.3.5.7 Modulation width (or modulation amplitude): 0,04 mT to 0,25 mT

Modulation width indicates intensity of the magnetic field modulation. Variable in the range between 0,000 2 and 2, limited by the modulation frequency though.

NOTE If the modulation width is too large (larger than the line width of the EPR signal), the detected EPR signal broadens and becomes distorted. However, low modulation width can result in unacceptable poor signal-to-noise ratio for the signal[29-32].

9.3.5.8 Receiver mode: 1st

The normal first-derivative of the absorption spectrum will be presented. If spectrum has very good signal/noise and has some regions where you would like better resolution, a second-derivative presentation may help.

9.3.5.9 Phase: 0°

Phase indicates the phase (coarse and fine adjustments) of the lock-in amplifier. Normally, this should be set to 0°.

9.3.5.10 Power: 1 mW to 40 mW

Set the microwave power to the measurement value.

The microwave output power necessary for measurement varies according to the sample to be measured and its adjustment is necessary. By observing the ESR signal while varying the microwave output power, the optimum output power can be determined. Although the signal intensity changes in proportion to the root of the microwave output for some samples, saturation of the signal intensity and deformation of the waveform is observed when the microwave output is increased. For the samples which saturate and also for samples which are unknown (whether they saturate or not), always set the microwave output (POWER) starting from the minimum output to the maximum output in several steps while checking the signal intensity and deformation of the waveform. The power setting is determined during the baseline experiment and is kept uniform throughout the experiments.

9.3.6 Start the measurement.

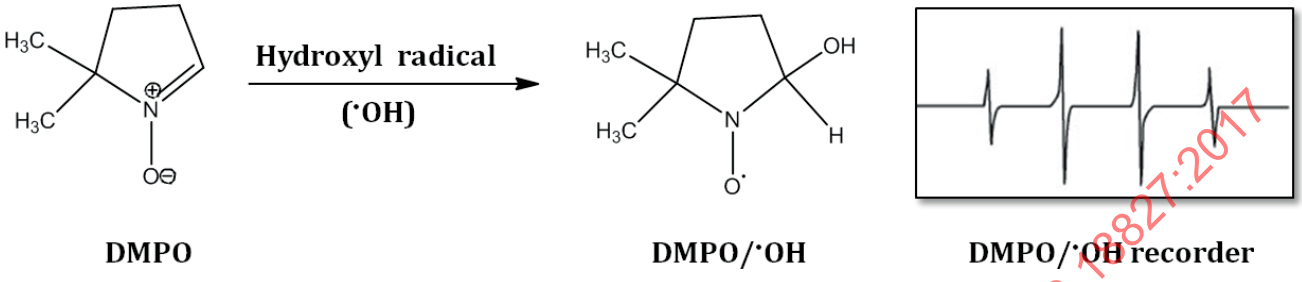
9.3.7 Verify the signal.

If the signal is not at the centre of the screen or not on the screen, adjust it to the centre.

9.3.8 Adjust the recorder.

9.3.8.1 The DMPO/OH formation by the hydroxyl radical[33][34].

See Figure 5.



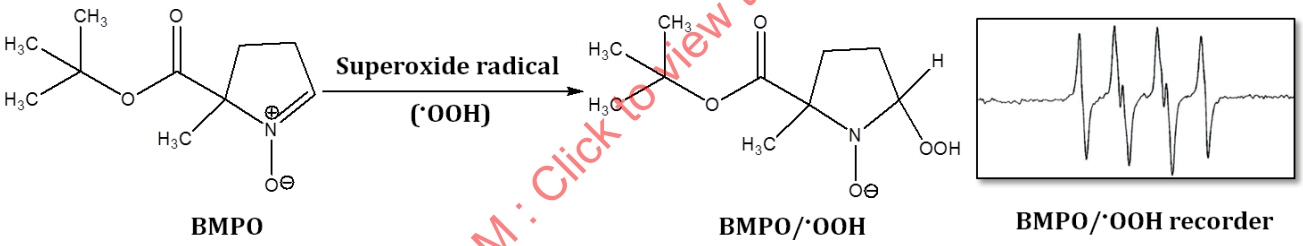
NOTE 1 Quartet with 1:2:2:1 signal intensity.

NOTE 2 $a_N = a_H$ = 14,9 G to 15,0 G in deionised water.

Figure 5 — Formation of the DMPO radical adducts form (DMPO/OH)

9.3.8.2 The BMPO/OOH formation by the superoxide anion radical[9][35].

See Figure 6.

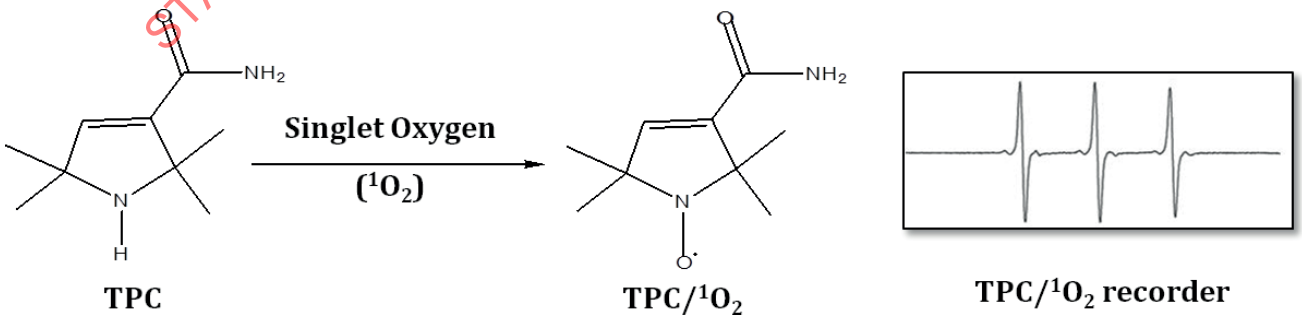


NOTE a_N = 13,4, a_H = 11,8 G in phosphate buffer.

Figure 6 — Formation of the BMPO radical adducts form (BMPO/OOH)

9.3.8.3 The TPC/$^1O_2$ formation by the singlet oxygen[10].

See Figure 7.

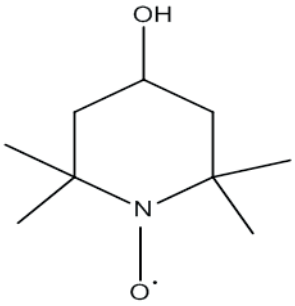


NOTE Triplet with 1:1:1 signal intensity.

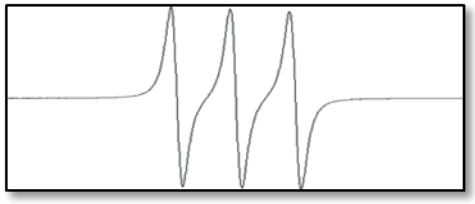
Figure 7 — Formation of the TPC radical adducts form (TPC/$^1O_2$)

9.3.8.4 The TEMPOL standard[36].

See Figure 8.



TEMPOL



TEMPOL recorder

NOTE 1 Triplet with 1:1:1 signal intensity.

NOTE 2 a_N =16,8 G in deionised water.

Figure 8 — TEMPOL standard

9.3.9 Quantify ROS using Formula (1).

$$X = (B / A) \times (D / C) \times (3,0\text{sp} \times 10^{14}\,\text{sp}) \quad (1)$$

where

A is the area of absorption peak with a standard sample (1,0 mM × 10^{-3} mM TEMPOL);

B is the area of absorption peak with a test sample;

C is the area of absorption peak of Mn^{2+} marker with a standard sample;

D is the area of absorption peak of Mn^{2+} marker with a test sample;

X is the radical number of test sample

Sp is the spin.

The radical concentrations of test samples (nanomaterials) are determined by comparing the area of absorption peak with that of a standard sample (TEMPOL). The radical number is 3,0 sp × 10^{14} sp in 1,0 mM × 10^{-3} mM TEMPOL. The standard sample and test sample should be measured under the same settings of ESR spectrometer parameters (see Table 2) respectively. Quantification is calculated with the area of the absorption peak of the Mn^{2+} marker and the signal by double integration of the ESR spectrum[37][38]. The radical number of the test sample can be calculated by comparing the relative area of the standard sample.

NOTE The ESR technique has a detection limit of approximately 1 nmol/L (10^{-9}M)[39][40].

9.3.10 Record the signal on the recorder.

9.3.11 Shut down the system.

9.3.11.1 Set power of the microwave to 0 mW.

9.3.11.2 Select EXIT.

9.3.11.3 Turn off the power switch of the spectrometer.

10 Examples of results

10.1 DMPO/OH

See Figure 9.

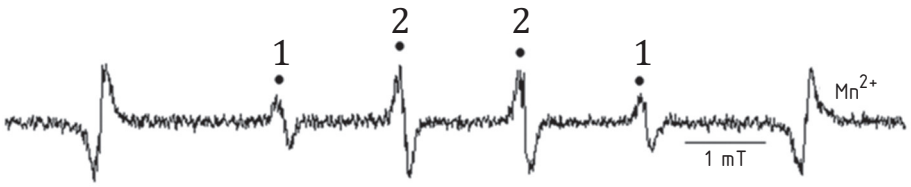


Figure 9 — ESR spectrum of the DMPO radical adducts form (DMPO/OH)

10.2 BMPO/OOH

See Figure 10.

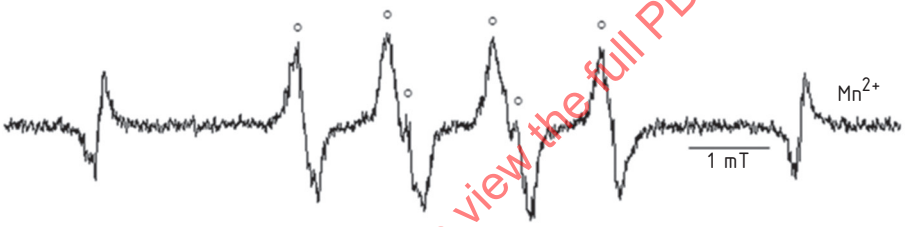


Figure 10 — ESR spectrum of the BMPO radical adducts form (BMPO/OOH)

10.3 TPC/$^1O^2$

See Figure 11.

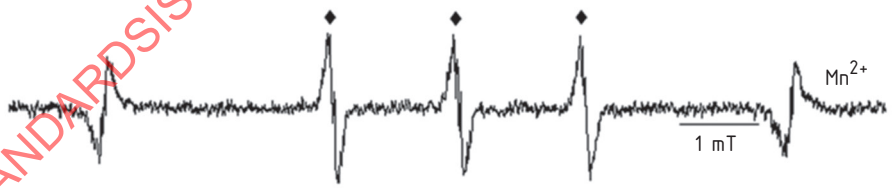


Figure 11 — ESR spectrum of the TPC radical adducts form (TPC/$^1O_2$)