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Nanotechnologies — Assessment of peroxidase-like activity of metal and metal oxide nanoparticles

Nanotechnologies — Evaluation de l'activité de type peroxidase des nanoparticules métalliques et d'oxydes métalliques

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Foreword

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

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Enzymes are the biological catalysts that control biochemical reactions. The enzyme peroxidase is a metalloenzyme with many isoforms. It catalyses the oxidation of various organic substrates by hydrogen peroxide, which is used extensively in biochemistry applications. Metal and metal oxide nanoparticles have a wide range of applications in biomedicine, environment protection, and some other fields, such as magnetic separation, detection, anti-bacterial, degradation of contaminants, medical imaging and tumour therapy. In recent years, an intrinsic peroxidase-like activity was observed in some metal and metal oxide nanoparticles, which means that these metal and metal oxide nanoparticles can catalyse the oxidation of substrates of natural peroxidase by hydrogen peroxide under mild reaction conditions in comparable efficiency and kinetics. Iron oxide (Fe_3O_4) nanoparticles are one representative material, and cobalt oxide (Co_3O_4) nanoparticles, copper oxide (CuO) nanoparticles, manganese oxide (MnO_2) nanoparticles, vanadium oxide (V_2O_5) nanoparticles, gold (Au) nanoparticles and platinum (Pt) nanoparticles have been reported to have the peroxidase-like activity as well. These findings extend enzyme mimics from organic compounds to inorganic nanomaterials.

Certain metal and metal oxide nanoparticles can catalyse the transfer of electrons from H_2O_2 to colorimetric indicator under physiological condition. This phenomenon is like the colorimetric reaction mediated by peroxidase and thus is called as peroxidase-like catalysis. Such catalytic property can be used to produce colorimetric, chemiluminescent or electrochemical signals which have great potential applications in biosensors, electrochemical sensors and immunoassays. The nanoparticles with peroxidase-like activity may have anti-tumour, antibacterial or antioxidant functions in biological system. In addition, the nanoparticles with such activity can have potential impacts on health, safety and the environment. Therefore, it is important to assess the peroxidase-like activity of a nanoparticle in practical applications.

The peroxidase-like activity of nanoparticles strongly depends on multiple factors including the composition, size, surface chemistry and crystal structure of the nanoparticles, as well as the measurement conditions. Therefore, it is important to establish a standard method for assessing the peroxidase-like activity of metal and metal oxide nanoparticles.

This document provides a specification for the assessment of peroxidase-like activity of metal and metal oxide nanoparticles. This protocol is useful to enterprises, research laboratories or institutions and metrological organizations that are working on nanomaterials used in biomedical applications and environment protection.

Nanotechnologies — Assessment of peroxidase-like activity of metal and metal oxide nanoparticles

1 Scope

This document specifies a method for assessing the peroxidase-like activity of metal and metal oxide nanoparticles by spectrophotometry. This document can serve as a reference for the measurements of peroxidase-like activities in other types of nanoparticles.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 18153:2003, *In vitro diagnostic medical devices — Measurement of quantities in biological samples — Metrological traceability of values for catalytic concentration of enzymes assigned calibrators and control materials*

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

3 Terms, definitions and abbreviated terms

For the purposes of this document, the terms and definitions given in ISO/TS 80004-2 and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

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

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

3.1 Terms and definitions

3.1.1
nanoparticle
nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

[SOURCE: ISO/TS 80004-2:2015, 4.4, modified — Note 1 to entry has been removed.]

3.1.2
catalytic activity
property of a component corresponding to the catalysed substance rate of conversion of a specified chemical reaction, in a specified measurement system

Note 1 to entry: In this document, the "component" is one kind of metal or metal oxide nanoparticles.

Note 2 to entry: In this document, the catalytic activity is the peroxidase-like activity of metal and metal oxide nanoparticles.

Note 3 to entry: The coherent derived SI unit is "katal" (kat), equal to "mole per second" ($mol \cdot s^{-1}$).

[SOURCE: ISO 18153:2003, 3.2, modified — Notes 1, 2 and 3 to entry have been added.]

3.1.3
specific catalytic activity
catalytic activity per unit mass of metal or metal oxide in nanoparticles

Note 1 to entry: Specific catalytic activity is expressed as $kat \cdot kg^{-1}$.

3.2 Abbreviated terms

ABTS	2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt)
DMSO	dimethyl sulfoxide
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
IONPs	iron oxide nanoparticles
NPs	nanoparticles
OPD	o-phenylenediamine
TMB	3,3',5,5'-tetramethylbenzidine

4 Principle

The HRP reaction can be expressed by Formula (1):

$$\text{Substrate}_{\text{reduced}} + H_2O_2 \xrightarrow{\text{Horseradish Peroxidase}} \text{Substrate}_{\text{ox}} + H_2O \quad (1)$$

To evaluate the activity of HRP, chromogenic substrates are often employed, such as TMB, OPD, ABTS, among these, TMB can be the most widely used one.

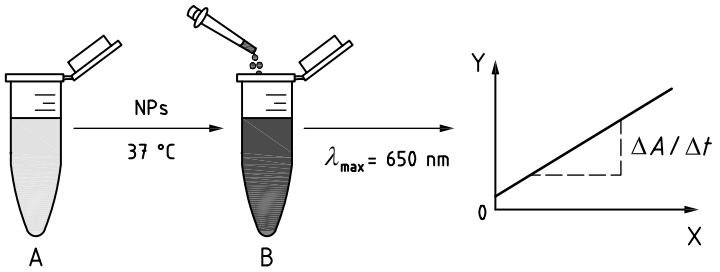
Some metal and metal oxide NPs can also catalyse the substrates of horseradish peroxidase in the presence of H_2O_2, which is referred as peroxidase-like activity in this specification. For the substrate TMB, the chemical reaction can be expressed by Formula (2):

$$\text{TMB} + H_2O_2 \xrightarrow{\text{metal and metal oxide NPs}} \text{TMB}_{\text{ox}} + H_2O \quad (2)$$

The reaction of Formula (2) generates a blue colour oxidized product that has a characteristic absorption peak at a wavelength of 650 nm. The absorbance is measured as a function of time at (37 ± 1) °C (see Figure 1). The measurement time can be determined based on the linear range of the progressing curve of the peroxidase reaction. During the initial phase of the reaction within the first few percent progression towards total completion, there is a linear phase of the reaction. It is recommended to record the absorbance within the linear phase of the reaction. To determine the enzymatic activity, it is sufficient to calculate from the change in absorbance per unit of time during this linear phase.

Usually, metal or metal oxide NPs show peroxidase-like activity under acidic conditions and the activity is weak or even vanishes under neutral or base conditions. The relevance of determining the peroxidase-like activity under acidic environment is considered as the low pH exists ubiquitously in biological systems including lysosomes, tumours, wounds, stomach and in environmental systems including polluted water. At acidic conditions, the dissolution of ions is possible and therefore inclusion of an additional control is needed (see 7.5).

The solubility of TMB is significantly decreased in solutions of pH ≥ 5,5. This document is applicable for suspensions with a pH value in the range of 3,5 to 5,5.



Key

X	time, in s	A	TMB + H_2O_2
Y	absorbance	B	TMB_{ox} + H_2O

Figure 1 — Schematic of peroxidase-like activity measurement for metal or metal oxide NPs

The number of peroxidase-like activity units of metal or metal oxide NPs is calculated according to the Lambert-Beer law:

The initial change rate of absorbance (min^{-1}) is obtained from the slope of the early, linear phase, of the experiment, as shown in Figure 1. After deducting the reagent blank rate, the number of peroxidase-like activity units of metal or metal oxide NPs is calculated according to Formula (3).

$$b_{\text{nano}} = \frac{V}{\varepsilon \times l} \times \frac{\Delta A}{\Delta t} \tag{3}$$

where

b_{nano} is the number of enzyme activity units of metal or metal oxide NPs, in kat;

V is the total volume of the reaction solution, in l;

ε is the molar attenuation coefficient of the TMB derivative, which is 39 000 $mol^{-1} \cdot l \cdot cm^{-1}$;

l is the optical path length of the cuvette, in cm;

$\Delta A/\Delta t$ is the initial change rate of absorbance of the reaction solution after correcting with a reagent blank rate, in s^{-1}.

The specific catalytic activity of NPs, a_{nano}, is calculated by dividing b_{nano} by the mass of the tested NPs.

The peroxidase-like activity of metal or metal oxide NPs is calculated according to Formula (4).

$$a_{\text{nano}} = \frac{b_{\text{nano}}}{m_{\text{M}}} \tag{4}$$

where

a_{nano} is the peroxidase-like activity of metal or metal oxide NPs, in $kat \cdot mg^{-1}$;

m_{M} is the mass of metal or metal oxide NPs, in mg.

NOTE See Annex A for the example from measurement and calculation of the mass.

5 Physicochemical characterization of metal or metal oxide NPs

Prior to the assessment of peroxidase-like activity of metal or metal oxide NPs, the size (distribution), shape, composition, surface chemistry and crystal structure of the nanoparticles should be

characterized according to Reference [1], as the peroxidase-like activity strongly depends on these factors.

6 Apparatus and reagents

6.1 Apparatus and appliances

6.1.1 Spectrophotometer.

A calibrated standard spectrophotometer covering visible wavelength range shall be used.[2] The spectrophotometer shall be turned on 1 h prior to the measurement to allow the baseline to stabilize.

6.1.2 Thermometer, with an accuracy equal to or under ±1,0 °C.

6.1.3 pH meter, with a resolution equal to or under 0,01 and an accuracy of ±0,002.

6.1.4 Thermostatic water bath.

The thermostatic water bath of the spectrophotometer is used to control the tank of cuvette at a constant temperature of (37 ± 1) °C.

6.1.5 Electronic balance, with a precision of 0,01 mg and a repeatability (calibration weight) of ≤0,015 mg (5 g).

6.1.6 Adjustable pipette, of 200 µl (uncertainty, u < 0,3 %) and 1 000 µl (u < 0,3 %).

6.1.7 Volumetric flask, of volume (10 ± 0,04) ml and (100 ± 0,2) ml.

6.1.8 Cuvette, with an optical path length of (10 ± 0,05) mm.

6.2 Reagents

All essential reagents for the assay are listed in Table 1.

Table 1 — Reagents

Classification	Chemical name	Name
Reagents	Anhydrous sodium acetate (AR) [a]	sodium acetate
	Anhydrous acetic acid (AR) [a]	glacial acetic acid
	3,3',5,5'-Tetramethylbenzidine (AR) [a]	TMB
	30 % hydrogen peroxide (AR) [a]	30 % H_2O_2
	Horseradish peroxidase (≥4,167 × 10^{-6} kat/mg solid)	HRP
Solvent	Double distilled water, grade 2, in accordance with Reference [3]	double distilled water
	Dimethyl sulfoxide (AR)	DMSO

[a] AR represents the analytical-reagent grade.

[b] GBW08616, GBW(E)130070 and GBW(E)130071 are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

Table 1 *(continued)*

Classification	Chemical name	Name
Standard substance	Standard solution of metal elements, such as standard solution of iron elements (e.g. GBW08616)[b]	NA
	Potassium biphthalate (e.g. GBW(E)130070)[b]	NA
	Mixed phosphate (e.g. GBW(E)130071)[b]	NA

[a] AR represents the analytical-reagent grade.

[b] GBW08616, GBW(E)130070 and GBW(E)130071 are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

7 Solution preparation

7.1 General requirements

The mass given for each component in solution refers to 100 % content. If the content of the chemical substance is less than 100 % [e.g. y (%)], the factor (F_{content} = 100/y) should be used to calculate the mass of a chemical substance equivalent to the given mass.

The uncertainty should be within ±0,5 % when weighing with an electronic balance.

7.2 TMB solution

Prepare the TMB solution of 4,16 mmol·l^{-1} in DMSO. The TMB should be fully dissolved. The TMB solution is recommended to store in separate packages. Repeated freezing-thawing should be avoided. The solution must not be used if it is observed to be discoloured or if the absorption spectrum has changed. The absorption spectrum of TMB can be found in Reference [4].

7.3 Buffer solution

Prepare the sodium acetate solution of 0,2 mol·l^{-1} and the glacial acetic acid solution of 0,2 mol·l^{-1} with double distilled water.

Mix above two solutions in a certain proportion to prepare the buffer solution with a pH value ranging 3,5 to 5,5 (see Clause 4).

7.4 Nanoparticle dispersion solution

Prepare the stock dispersion solution of metal or metal oxide NPs according to Reference [5]. The stock solution may be diluted to ensure the absorption values fitting within the instrumental detection limit.

7.5 Additional control solution

Metal ions may be released from the surface of nanoparticles in acidic medium and may contribute to the catalytic activity. To subtract the possible contribution, an additional control solution can be prepared (see Reference [6]).

Prepare the additional control solution by mixing the nanoparticle dispersion solution with the buffer solution at (37 ± 1) °C for the same amount of time as the measurement time length. The nanoparticles are then removed from the solution by using ultrafiltration, the ultrafiltration pore size should be smaller than the size of the NPs, or, to remove magnetic nanoparticles from the solution, a magnet can be used. The resulting solution is referred to as the additional control solution.