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Nanotechnologies — Testing the photocatalytic activity of nanoparticles for NADH oxidation

Nanotechnologies — Test de l'activité photocatalytique des nanoparticules pour l'oxydation du NADH

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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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

Photocatalytic activity (PCA) is the measure of capacity of a material to promote a specific photochemical reaction under defined conditions (as defined in ISO 20507:2014, 2.3.31). With the expanding use of nanomaterials in various industries, the possible impacts on human health and the environment due to the enhancement of detrimental chemical reactions in the presence of light (both natural and artificial) is an ongoing concern. The absorption of a photon with sufficient energy generates an electron-hole pair that can migrate to the nanoparticle (NP) surface and react with water and oxygen, thus forming extremely reactive radicals and reactive oxygen species (ROS). Generation of the ROS by some wide-bandgap materials, such as TiO_2, ZnO, WO_3, CeO_2, carbon nanotubes, quantum dots and some metal NPs when illuminated by UV-VIS light, can cause oxidative stress, resulting in toxic effects in living organisms[5]. Therefore, measuring the nanomaterial PCA under physiological conditions allows for an assessment of its photo-toxicity potency.

Existing standard test methods for particle and surface PCA measurement (see ISO 10676 and ISO 10678) are not directly applicable to determine nanomaterial PCA leading to photo-toxicity, as they require a large test volume and/or long measurement duration, while utilizing organic dyes as indicators that are not biocompatible.

The in vitro NP PCA test for NADH oxidation is intended to evaluate the nanomaterial photo-toxicity potency when exposed to an ultraviolet (UV) light.

Nanotechnologies — Testing the photocatalytic activity of nanoparticles for NADH oxidation

1 Scope

This document specifies a method for the measurement of the photocatalytic activity (PCA) of nanoparticles (NPs), suspended in an aqueous environment in physiologically relevant conditions, by measuring the ultraviolet (UV)-induced nicotine adenine dinucleotide hydrate (NADH) oxidation.

The measurement is intended to assess the potential for the photo-toxicity of nanomaterials. The method is also applicable to NP aggregates and agglomerates.

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/TS 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core terms*

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

3 Terms, definitions, symbols and abbreviated terms

For the purposes of this document, the terms and definitions given in ISO/TS 80004-1, ISO/TS 80004-2 and the following apply.

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

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

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

3.1 Terms and definitions

3.1.1
actinometry
method to measure the number of photons integrally or per unit of time

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

[SOURCE: ISO 18153:2003, 3.2, modified — The notes have been deleted.]

3.1.3
oxidation
chemical reaction accompanying a gain of oxygen, loss of hydrogen of an organic substrate or loss of one or more electrons from a molecular entity

3.1.4
NADH equivalent specific PCA
PCA measured as the NADH *photo-oxidation* (3.1.5) rate per unit weight of nanoparticles

3.1.5
photo-oxidation
oxidation reactions induced by light

3.2 Symbols and abbreviated terms

DIW	deionized water with ≥ 18 MΩ·cm resistivity
NADH	nicotine adenine dinucleotide hydrate
NaOH	sodium hydroxide
2NB	2-nitrobenzaldehyde
NP	nanoparticle
PB	phosphate buffer
PCA	photocatalytic activity
ROS	reactive oxygen species
TI	trans-illuminator
TiO_2	titanium dioxide
UV	ultraviolet
UV-Vis	ultraviolet and visible
$A_c(i,j)$	phenolphthalein absorbance before exposure to trans-illuminator UV irradiation in each well (i = B, C, D, E, F, G; j = 2, 3, 4, ..., 10, 11)
$A_e(i,j)$	phenolphthalein absorbance after exposure to trans-illuminator UV irradiation in each well (i = B, C, D, E, F, G; j = 2, 3, 4, ..., 10, 11)
$\Delta A(i,j)$	change in phenolphthalein absorbance after exposure to trans-illuminator UV irradiation in each well (i = B, C, D, E, F, G; j = 2, 3, 4, ..., 10, 11)
ΔA_a	average change of phenolphthalein absorbance over all wells before and after UV irradiation by using a UV trans-illuminator
C_0	starting concentration of the NP suspension for a dilution series of test solutions; the suspension absorbance at 310 nm or 365 nm (depending on the used UV trans-illuminator) is $1{,}4 < A < 1{,}6$
$C(i,j)$	light intensity correction factor of each well, which accounts for the UV irradiation intensity variation of the UV trans-illuminator at the location of each well (i = B, C, D, E, F, G; j = 2, 3, 4, ..., 8, 9)
$I_{F,0}(i,j)$	NADH fluorescence intensity measured before UV irradiation in each well (i = B, C, D, E, F, G; j = 2, 3, 4, ..., 8, 9)
$I_{F,t}(i,j)$	NADH fluorescence intensity measured following the UV irradiation of t duration by using a UV trans-illuminator in each well (i = B, C, D, E, F, G; j = 2, 3, 4, ..., 8, 9)
$k_{app}(i,j)$	apparent NADH photo-oxidation rate in each well, expressed in µmol/min (i = B, C, D, E, F, G; j = 2, 3, 4, ..., 8, 9)
λ_{exc}	excitation wavelength used to record fluorescence in multiple well plate readers
λ_{ems}	emission wavelength used to record fluorescence in multiple well plate readers
$A\,\lambda(\max)$	maximum absorbance of NP suspension in a wavelength range from 300 nm to 800 nm
$\lambda(\max,TI)$	wavelength at which a UV trans-illuminator provides the maximum intensity of light
$S(i,j)$	slope of the NADH fluorescence intensity versus the UV irradiation time in each well (i = B, C, D, E, F, G; j = 2, 3, 4, ..., 8, 9)
$S_c(i,j)$	$S(i,j)$ corrected for the trans-illuminator light intensity variation at each well
b	slope of k_{app} versus NP concentration in the linear range, expressed in units of mmol/min·g

4 Description of the test method

In this document, the PCA of NPs in an aqueous suspension is measured as the photo-oxidation rate of NADH present in the NP suspension. By observing the NADH fluorescence intensity decrease before and after successive irradiation with artificial UV light, the fraction of the oxidized NADH due to the photocatalytic action of NPs can be measured. The photo-oxidation rate of NADH is determined at several NP concentrations with a dilution series and the NP concentration range showing the linear dependence of the photo-oxidation rate of NADH versus NP concentration is determined[6]. The photo-oxidation slope in this linear range provides the NADH photo-oxidation rate per unit of NP concentration in the aqueous suspension. The multiplexed assay utilizes a 96-well plate, UV trans-illuminator and a multiple-plate optical reader leading to a fast and accurate measurement. The 96-well platform allows for a simultaneous measure of a range of NP concentrations and provides an option to compare with reference NPs as positive and negative controls. It accounts for the spontaneous NADH photo-oxidation under UV illumination in the absence of NPs and intrinsic NP fluorescence.

5 Reagents and apparatus

5.1 Reagents

5.1.1 NADH, β-Nicotinamideadenine dinucleotide, reduced disodium salt, CAS Number: 606-68-8.

a) Stock solution of NADH:

1) dissolve approximately 35 mg of NADH in 10 ml of 5 mmol/l phosphate buffer, pH = 8.

b) Working solutions of NADH:

1) dilute a stock solution of NADH by a factor of 20 into 5 mmol/l, pH = 8 phosphate buffer;

2) the resulting concentration of the working NADH solution will be about 250 µmol/l;

3) verify the working NADH concentration [NADH] by measuring the absorbance of the solution at $\lambda = 339$ nm. If necessary, adjust the NADH concentration by diluting with a 5 mmol/l phosphate buffer or by adding a NADH stock solution until absorbance $A_{339} = 1,56 \pm 0,05$.

5.1.2 2-nitrobenzaldehyde (2NB), CAS Number: 552-89-6.

a) Prepare 50 ml 0,1 mol/l solution of 2NB by dissolving 0,756 g of the dry 2NB in 50 ml of 50/50 DIW/ethyl alcohol (by volume).

b) Adjust the 0,1 mol/l 2NB solution to pH = 12 ± 0,2 by adding 0,03 mol/l NaOH.

5.1.3 Phenolphthalein, CAS Number: 77-09-8.

a) Prepare 20 ml of stock solution of phenolphthalein by dissolving 20 mg of dry phenolphthalein in 20 ml of 50/50 DIW/ethyl alcohol.

b) Add 100 µl of phenolphthalein stock [prepared in step a)] to a 50 ml 0,1 mol/l 2NB solution (prepared as per 5.1.2). The solution acquires pink colour.

c) Store the solution in light-protected bottle (amber glass or wrapped in Al foil).

5.1.4 Phosphate buffer, sodium phosphate monobasic/dibasic solution for pH buffer of pH 8 (5 mmol/l PB at pH 8).

EXAMPLE The phosphate buffer is prepared as follows.

Step 1: Dissolve 1,261 g of disodium phosphate, heptahydrate (CAS Number: 7782-85-6) in 1 l of DIW.

Step 2: Add 0,041 g of monosodium phosphate, monohydrate (CAS Number: 10049-21-5) to the solution prepared in Step 1.

Step 3: Measure the solution pH, following the complete dissolution of salts.

5.1.5 NP suspension.

a) Prepare 50 ml of NP suspension in a 5 mmol/l phosphate buffer according to the recommended dispersion protocol for the particular nanomaterial (e.g. Reference [7]).

b) Adjust the NP dispersion concentration C_0 so that the highest absorbance reading of the NP dispersion in a range of 300 nm to 800 nm is $1,4 < A < 1,6$. Calculate the stock solution NP concentration C_0 (in mg/l) following the adjustment.

c) From the mass concentration of the prepared stock NP suspension, dilution factors for the preparation of the target concentration of working suspensions can be calculated.

5.1.6 Ethyl alcohol, anhydrous, > 99,5 % pure, less than 0,005 % water. CAS Number: 64-17-5.

5.2 Apparatus

5.2.1 UV-Vis spectrophotometer, wavelength range: 190 nm to 800 nm, absorbance range: 0,1 to 3,0.

5.2.2 Cuvette for UV-Vis absorption measurement, quartz or optical glass, 1 cm optical path length.

5.2.3 96-well plate, [flat bottom surface transparent at λ(max,T): T > 60 %], dark plastic sides preferable.

5.2.4 Microplate absorbance and fluorescence reader, capable of absorbance and fluorescence measurement in a range from 300 nm to 800 nm.

5.2.5 Multi-pipette loader, which has at least six channels with at least 300 µl channel capacity.

5.2.6 300 µl pipette tips, compatible with the multi-pipette loader.

5.2.7 UV trans-illuminator, 365 nm light source with a horizontal illumination area larger or equal to the 96-well plate.

6 Measurement procedure

6.1 Measurement of NP suspension basic properties

6.1.1 UV-Vis absorption spectrum measurement

a) Measure the UV-VIS absorption spectrum of the NP suspension (see 5.1.5) in a range from 300 nm to 800 nm in a 10 mm optical path-length quartz or optical glass spectrophotometer cuvette against 5 mmol/l PB as reference.

NOTE Filling a standard 10 mm cuvette usually requires around 3 ml of sample.

Preferably, use the same cuvette for both the reference and the sample.

b) If the absorbance of suspension at λ(max) [A λ(max)] exceeds 1,6, dilute the suspension with a phosphate buffer (5 mmol/l PB, pH = 8) until $1,4 < A$ λ(max) < 1,6. If it's below 1,4, increase the NP concentration accordingly.

6.1.2 NP suspension stability measurement

NOTE This is required to ensure NPs stay suspended during the measurement duration.

a) Measure the baseline (reference absorbance) using 5 mmol/l PB, pH = 8,0.

b) Measure the UV-Vis absorption spectrum of the NP working suspension, with the concentration adjusted in accordance with 6.1.1.

c) Wait for 20 min while maintaining the cuvette in the spectrophotometer, then re-measure the UV-VIS absorption spectrum of the working suspension.

d) Compare the two spectra and verify that a change in the maximum absorbance is less than 5 % at λ(max). The NP working suspension is not regarded as stable if absorbance at λ(max) decreases more than 5 % over 20 min.

6.2 UV trans-illuminator light intensity calibration based on 2NB actinometry

a) Prepare a 50 ml 0,1 mol/l solution of 2NB, containing phenolphtalein in accordance with 5.1.2 and 5.1.3. Use an amber glass container to store the solution.

b) Fill each well of the 96-well plate with 300 µl of solution, as prepared in a).

c) Place the 96-well plate in the reader, programme it to shake the plate for 5 s, and measure and record the absorbance at 540 nm – $A_c(i,j)$.

WARNING — Observe that it is positioned at the same location and orientation as during the NADH/NP UV exposure. Follow the directions in Annex A for the plate positioning.

d) Turn on the trans-illuminator [lambda = λ(max,TI)] and warm up for 30 min.

e) Position the 96-well plate on the trans-illuminator.

f) Expose the plate to UV light for 10 min.

g) After 10 min, turn off the trans-illuminator, and measure and record the absorbance at 540 nm using the 96-well plate reader – $A_e(i,j)$.

h) Subtract the absorbance values, recorded in step c), from the absorbance values recorded in step g) for each well, as shown by Formula (1):

$$\Delta A(i,j) = A_e(i,j) - A_c(i,j) \quad (1)$$

i) Calculate the average differential absorbance, as shown by Formula (2):

$$\Delta A_a = \sum \Delta A(i,j) \quad \text{all 60 working wells}/60 \quad (2)$$

j) Calculate the light intensity correction factors for light intensity at each well, as shown by Formula (3):

$$C(i,j) = \Delta A_a / \Delta A(i,j) \quad (3)$$

k) The light intensity correction factors for individual wells will be multiplied by the slope of NADH florescence decrease, calculated as shown by Formula (5) in 6.3.2.3.

A sample calibration of UV trans-illuminator light intensity is given in Annex B.

6.3 Measurement of NADH solution fluorescence intensity

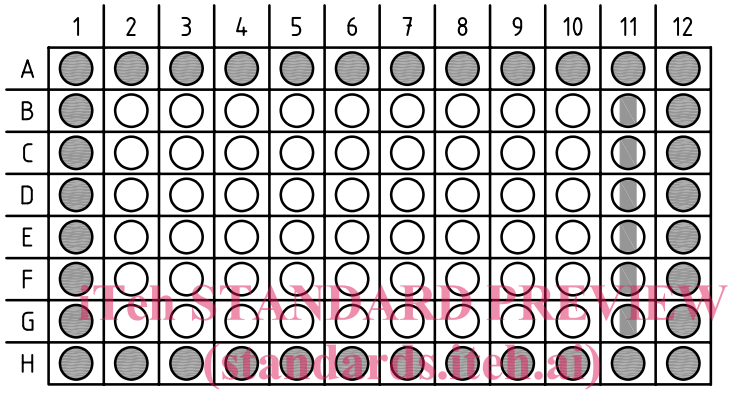
6.3.1 NADH photo-oxidation rate measurement at various NP concentrations

6.3.1.1 Prepare the 250 µmol/l NADH solution in accordance with 5.1.1.

6.3.1.2 Prepare the stock NP suspension (using the appropriate dispersion procedure, see 5.1.5) C_0 = 100 %. The absorbance at λ(max) (in a range from 300 nm to 800 nm) should be 1,4 < A < 1,6.

6.3.1.3 Prepare the dilution (by volume) series of NP suspension (each 2 ml using a phosphate buffer) at concentrations (relative to C_0 = 100 %). See 6.3.1.2: 100 %, 80 %, 60 %, 40 %, 20 %, 10 %, 8 %, 5 %.

6.3.1.4 Fill wells marked in white with 100 µl of 250 µmol/l NADH solution, as shown in Figure 1.



NOTE Wells marked in white contain 100 µl of 250 µmol/l NADH solution and: a buffer for col. 2; 100 µl NP suspension at C_0 for col. 3; 100 µl NP suspension at 80 % C_0 for col. 4; 100 µl NP suspension at 60 % C_0 for col. 5; 100 µl NP suspension at 40 % C_0 for col. 6; 100 µl NP suspension at 20 % C_0 for col. 7; 100 µl NP suspension at 10 % C_0 for col. 8; 100 µl NP suspension at 8 % C_0 for col. 9; 100 µl NP suspension at 5 % C_0 for col. 10; 100 µl NP suspension at C_0+ 100 µl buffer (5 mmol/l PB, pH = 8) solution for col. 11.

Figure 1 — Schematic diagram of a 96-well plate for NADH photo-oxidation rate measurement at various NP concentrations

6.3.1.5 Add 100 µl of the NP suspension dilution series and fill the wells as described below. Mix the solutions in the individual wells by pipetting in and out at least three times.

— A1, B1, ⋯, G1, H1 (column): No use.

— A2, A3, ⋯, A10, A11 (row): No use.

— B2, C2, D2, E2, F2, G2: Blank buffer.

— B3, C3, D3, E3, F3, G3: NP suspension at C_0.

— B4, C4, D4, E4, F4, G4: NP suspension at 80 % of C_0.

— B5, C5, D5, E5, F5, G5: NP suspension at 60 % C_0.

— B6, C6, D6, E6, F6, G6: NP suspension at 40 % C_0.

— B7, C7, D7, E7, F7, G7: NP suspension at 20 % C_0.

— B8, C8, D8, E8, F8, G8: NP suspension at 10 % C_0.