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First edition

Nanotechnologies — Method to estimate cellular uptake of carbon nanomaterials using optical absorption

Nanotechnologies — Méthode d'estimation de la captation cellulaire des nanomatériaux carbonés par mesure d'absorption optique

PROOF/ÉPREUVE

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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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

Owing to their unusual physical and chemical properties, carbon nanomaterials (CNMs), such as carbon nanotubes, carbon black, graphene, and carbon nanohorns, have been considered for various applications such as in the fields of electronics, energy, nanotechnology, and biology. With the increase of CNM-based products on the market, the public concern regarding possible toxicities has also increased. Estimation of the amount of CNM associated with the targeted cells is useful for an initial toxicological screening of CNMs and for developing applications in medicine[3][4][5][6].

Fluorescent dyes and/or radioactive isotopes have been routinely used to measure cellular uptake. Because CNMs absorb light in near infrared (NIR) region, where the bio-components such as protein and water in cells or tissues have relatively low light absorption, the cellular uptake of CNMs can be estimated from the absorbance of cell-lysate[7][8][9][10].

Nanotechnologies — Method to estimate cellular uptake of carbon nanomaterials using optical absorption

1 Scope

This document describes a near-infrared optical absorption method to estimate the in vitro cellular uptake of carbon nanomaterials including both internalized and/or tightly adhered to the cell membrane from liquid dispersions. This is a simple method to screen carbon nanomaterials uptake; additional analysis using a different technique can be required if quantification is desired.

2 Normative references

The following document is referenced 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 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO/TS 80004-3, *Nanotechnologies — Vocabulary — Part 3: Carbon nano-objects*

3 Terms, definitions and abbreviated terms

For the purposes of this document, the following terms, definitions and abbreviations as well as the terms and definitions given in ISO/TS 80004-3 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
cellular uptake
internalization or association of a substance by a living cell

3.1.2
cell lysis
destruction or dissolution of cells with release of contents

3.1.3
absorbance
measure of the capacity of a substance to absorb light at a specified wavelength

3.2 Abbreviated terms

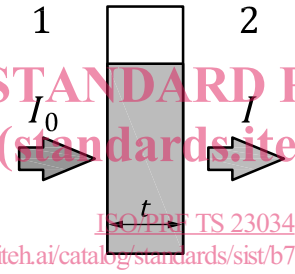
CNH	carbon nanohorn
CNM	carbon nanomaterial
CNT	carbon nanotube

SWCNT	single-wall carbon nanotube
MWCNT	multiwall carbon nanotube
PBS	phosphate-buffered saline
SDBS	sodium dodecylbenzene sulfonate
NIR	near infrared
UV	ultraviolet
Vis	visible

4 Method overview

4.1 General

When an optical beam goes through a solution sample, some of the beam is attenuated by the solution, and the rest is transmitted (see Figure 1). The amount of optical beam attenuated by the solution is related to the property of solution itself and the thickness of the solution sample.



Key

1 incident light
2 transmitted light

Figure 1 — Optical attenuation by a solution sample

The optical absorbance is directly proportional to the concentration of the dissolved substance in a solution. When the concentration of solution is expressed as $g{\cdot}L^{-1}$, the relationship between absorbance and concentration can be written as follows.

$$A = \log_{10}(I_0/I) = k_m t c \quad (1)$$

where

A is the optical absorbance of the solution sample;

I_0 is the radiant fluxes of incident;

I is the radiant fluxes of transmitted beams;

k_m is the wavelength-dependent mass absorptivity coefficient with units of $g^{-1}{\cdot}cm^{-1}$;

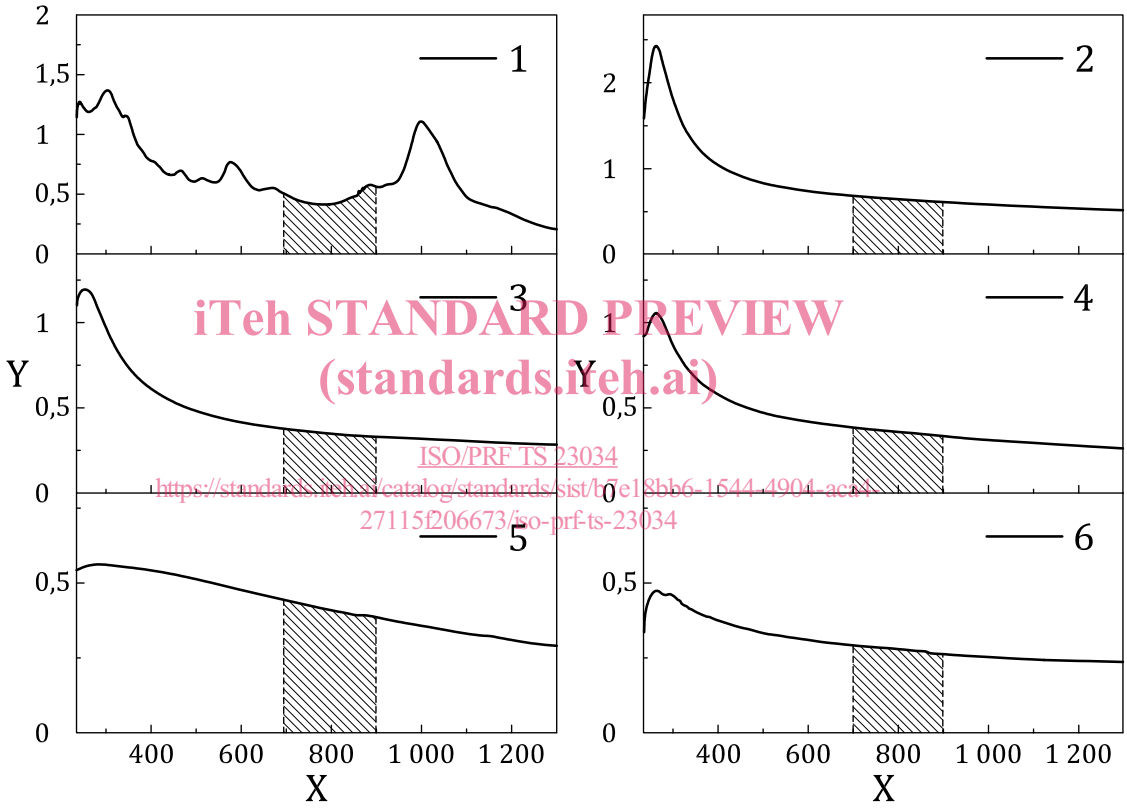
t is the thickness of the solution sample;

c is the concentration of a substance dissolved in the solution sample expressed in units of $g{\cdot}l^{-1}$.

If A, k_m and t are known, then the concentration of a substance dissolved in the solution sample can be obtained. A is obtained by measurement, k_m is obtained by calibration using the known amount of the substance dissolved in the solution sample, and t is a fixed value determined by a cuvette optical pathlength for absorbance measurement.

4.2 Optical absorption of carbon nanomaterials

The optical absorption spectra of carbon nanomaterials in dispersion show a strong absorption peak in 300 nm to 200 nm (4 eV to 6 eV) that is attributed to the collective excitations of π electron systems (π-plasmons).[1] This π-plasmon absorption peak can be also observed in most graphitic compounds.[11] The peak is superimposed on the featureless background extending to the vis-NIR and IR regions. The typical spectra of SWCNTs, MWCNTs, CNHs, carbon blacks and graphene are shown in Figure 2. The broad non-resonant absorbance of MWCNTs, CNHs, carbon black, graphene and/or SWCNTs in vis-NIR region (e.g. 700 nm to 900 nm) is typical for most carbonaceous nanomaterials in samples.



Key

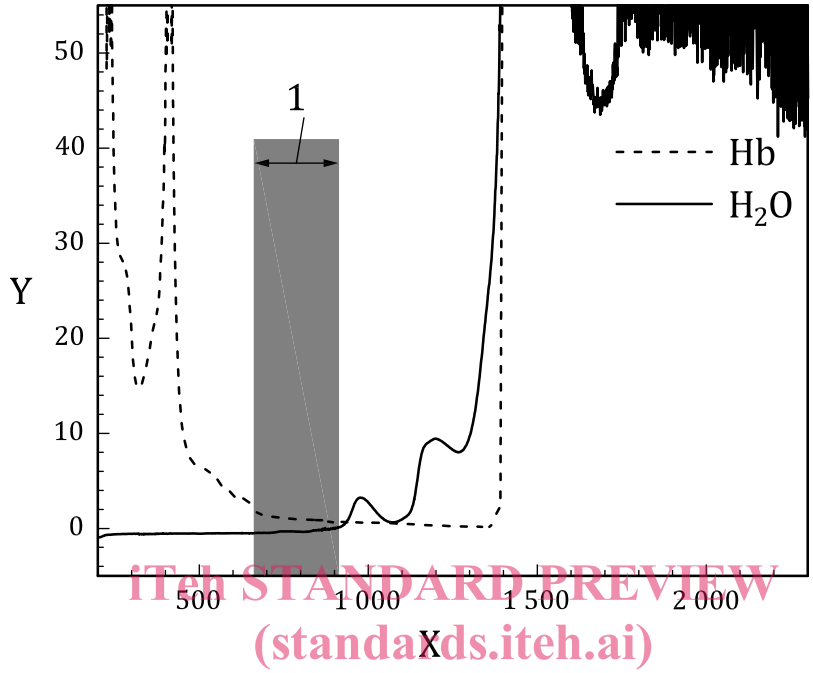
X	wavelength (nm)	3	MWCNTs
Y	absorbance (arbitrary units)	4	CNHs
1	individual SWCNTs	5	carbon black
2	SWCNTs bundles	6	graphene

NOTE The oblique regions show the absorbance of each carbon nanomaterial in 700 nm to 900 nm.

Figure 2 — Typical absorption spectra of individual SWCNTs, SWCNT-bundles, MWCNTs, CNHs, carbon black and graphene nanoplates in dispersions

4.3 Optical absorption of biomolecules

Cell lysate may contain the bio-components, such as proteins, amino acids, fatty acids and DNA, which can absorb light at wavelengths ranged from 200 nm to 600 nm. Water has absorption in IR (> 1 000 nm) (Figure 3). Because the absorption of the bio-components in the region of 650 nm to 900 nm is low, this region is always used for diagnosis and also called as therapeutic window[12].



Key

X wavelength (nm)

Y absorbance (arbitrary units)

1 no absorption

Figure 3 — Absorption spectrum of haemoglobin (Hb) and water

4.4 Determination of the concentration of CNMs in dispersion by absorbance

As shown above, when the light wavelength and the path length of light are fixed, the amount of absorbed light by a certain CNM dispersion would be directly proportional to the concentration of the dispersed CNMs. Then based on a calibration curve obtained by known concentration and its corresponding absorbance, the unknown concentration of CNMs dispersion can be determined from its absorbance. Since most types of CNMs have absorbance in 700 nm to 900 nm where the components in cell lysis do not, any wavelength in this region (e.g. 750 nm) can be chosen to determine the concentration of CNMs dispersion.

NOTE This technique is limited to carbon nanomaterials that absorb strongly in the NIR region of 700 nm to 900 nm, but would not be suitable for some types of carbon nanomaterials such as nanodiamond and nanographene oxide, which have low absorbance in this region.

4.5 Case studies

Case studies with single-wall carbon nanotubes SWCNTs, CNHs and multiwall carbon nanotubes (MWCNTs) are presented in Annexes A, B and C respectively.

5 Materials and apparatus

5.1 Materials

5.1.1 Chemicals

5.1.1.1 Water, deionized and sterilized pure water, grade 1, in accordance with ISO 3696:1987.

5.1.1.2 Culture medium, with or without serum that meets the growth requirements of the selected cell line.

5.1.1.3 Phosphate-Buffered Saline, (PBS; pH = 7,4).

5.1.1.4 Cell lysis reagent, a colourless buffer solution that contains detergents for mammalian cell lysis/extraction.

5.1.1.5 SDBS solution, Sodium dodecylbenzene sulfonate (SDBS) powder dissolved in deionized pure water with concentration of 50 mg/ml.

5.1.1.6 Trypsin-EDTA (0,25 %).

5.1.2 Cell line

Established cell lines are preferred and shall be obtained from recognized repositories. They can be either adherent cells or floating cells.

5.2 Apparatus

5.2.1 UV-Vis-NIR spectrometer

A spectrophotometer covering a broad ultraviolet to NIR wavelength range shall be used. Equipment should be installed in a clean environment, while avoiding any electrical noise, mechanical vibrations, direct sunlight, etc.

The spectrophotometer should be turned on 30 min prior to the measurement to allow the baseline to stabilize. The spectrophotometer should be calibrated in the absorbance scale, where an absorptive filter of neutral optical density with a value close to zero may be used. An absorption spectrum of CNMs aqueous dispersion should be obtained against a reference of the solution that used for dissolution of cells [e.g. a mixture solution of SDBS and cell lysis reagent (1:1)].

5.2.2 Cuvette for optical absorption measurement

Quartz cuvette.

5.2.3 Incubator, 37 °C, humidified, 5 % CO_2/air.

5.2.4 Culture dishes, single-well or multi-well plates can be used. 6 multi-well plates with flat bottom are recommended.

5.2.5 Centrifuge.

Centrifuge tubes: Sterilized, 13-ml centrifuge tubes and 1,5 ml centrifuge micro-tubes.

Centrifuge: Refrigerated centrifuge equipped with rotors of 15-ml centrifuge tubes and 1 ml to 2 ml micro-tubes. The centrifuge speed in gravitation force is recommended to be 150 g.

5.2.6 Homogenizer.

An ultra-sonicator with a horn tip with a minimum output power of 200 W.

For convenience, an ultra-sonicator with multi-tips is recommended.

5.2.7 Cell counter.

Automated cell counter, or hemacytometer.

6 Cell-uptake testing protocol

6.1 General

Follow the basic principle of cell culture techniques[13][14] regarding expanding a frozen stock of cells so that the cell uptake for CNM can be performed. The processes described below are recommended when the 6-well plates are used for cell culture. It can be modified according to the cell-lines and the types of cell culture dishes.

This method is applicable to all cell types.

6.2 Sample preparation

CNM suspensions for cellular uptake testing should be homogeneous, and stable in aqueous solution. It can contain dispersant such as bovine serum albumin or polyethylene glycol etc. The concentrations of CNMs should be known before use. It is suggested to use freshly prepared CNM dispersions.

6.3 Preparation of calibration curve of CNM dispersions

Prepare CNM dispersions with various concentrations such as 0, 0,1 µg/ml, 0,5 µg/ml, 1 µg/ml, 2 µg/ml, 5 µg/ml and 10 µg/ml by dilution of the testing suspension (see 5.1.1) with a solution that used for dissolving cells [e.g. a mixture solution of SDBS and cell lysis reagent (1:1)]. Collect the absorbance value for each concentration of CNM dispersion in the cuvette at a consistent wavelength in the region of 700 nm to 900 nm, for example 750 nm, using the UV-Vis-NIR spectrometer. Prepare the calibration curve by plotting absorbance at the chosen wavelength against known CNM concentration.

NOTE 1 The calibration curve of the CNM-concentration to absorbance is dependent on the characteristics of CNMs and their dispersion.

The calibration curve should be prepared for each CNM type used for cellular uptake experiment.

NOTE 2 The absorption of CNMs in 700 nm to 900 nm is the cumulative absorbance of the total of the carbonaceous material, including impurities such as amorphous carbon, graphite, etc. It is preferable to use purified CNM samples.

6.4 Cell-seeding

The cells in culture medium at $2,0 \times 10^5$ to $8,0 \times 10^5$ in 3 ml culture medium shall be seeded in each well of 6-well plates and incubated in a humidified incubator at 37 °C with 5 % CO_2 for 24 h. For cellular uptake measurement, 3-plates of cells, called groups, should be prepared. One plate each for cell counting, control of cell lysis, and CNM-testing (see Figure 4).

