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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

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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[1][2][3][4].

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[5][6][7][8].

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

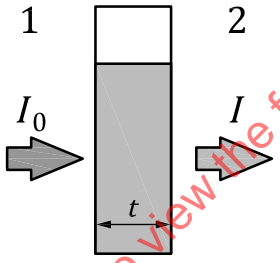
CNH	carbon nanohorn
CNM	carbon nanomaterial
CNT	carbon nanotube

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 $g^{-1} \cdot cm^{-1}$;

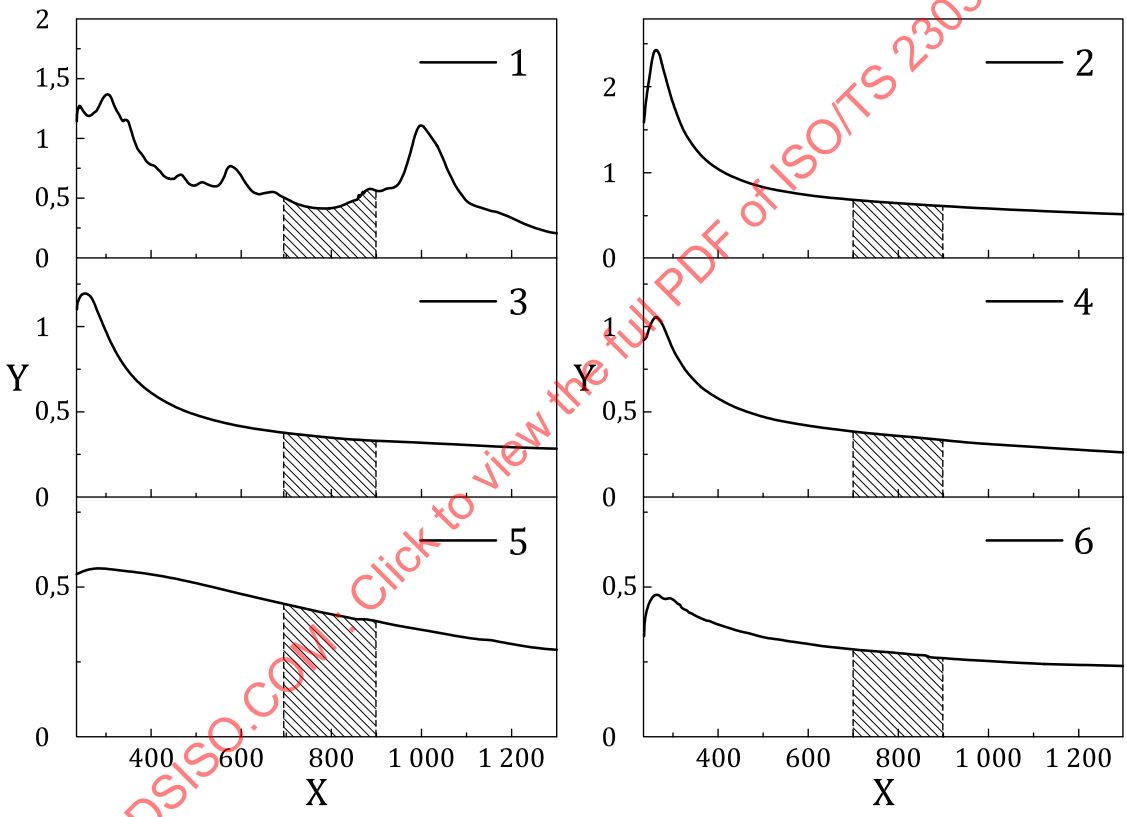
t is the thickness of the solution sample;

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).[9] This π-plasmon absorption peak can be also observed in most graphitic compounds.[10] 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

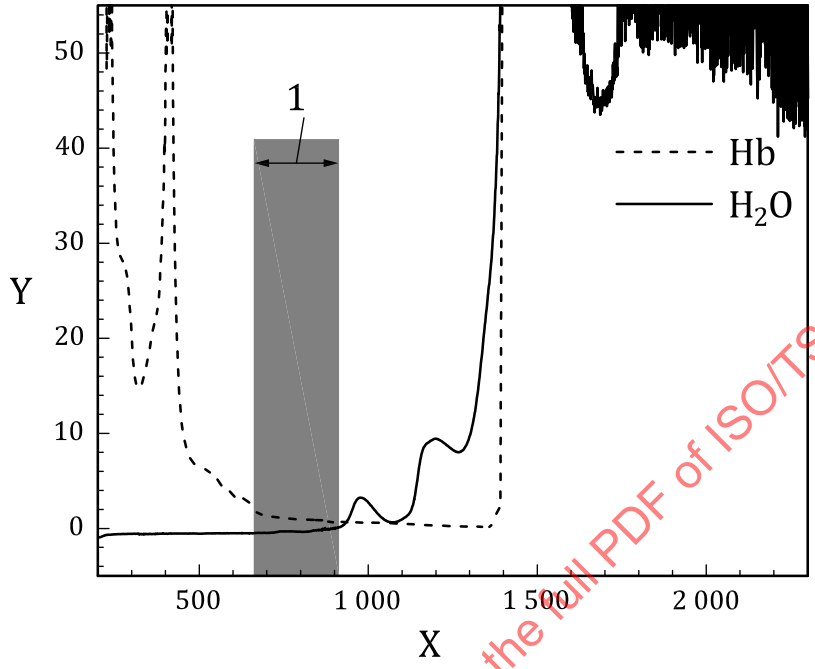
X	wavelength (nm)	3	MWCNTs
Y	absorbance (arbitrary units)	4	CNHs
1	individual SWCNTs	5	carbon black
2	SWCNTs bundles	6	graphene

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) (see 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[11].



Key

X wavelength (nm)

Y absorbance (arbitrary units)

1 low absorption region

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 that 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 SWCNTs, CNHs and 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 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, 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[12][13] 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 µg/ml, 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.

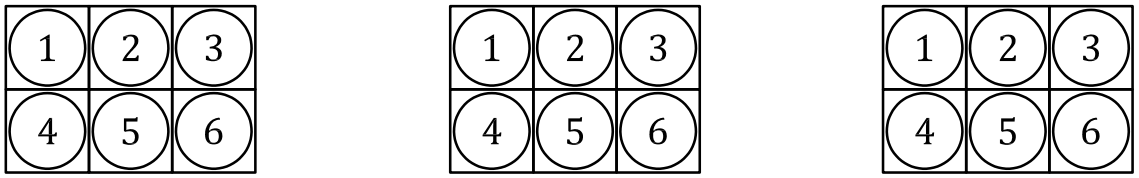
NOTE 1 The calibration curve of the CNM-concentration to absorbance is dependent on the characteristics of CNMs and their dispersion.

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).



a) Control for cell counting **b) Control (n = 6)** **c) CNM testing (n = 6)**

NOTE Each plate corresponds to either a control for cell counting, or control for cell lysis, or for CNM-testing.

Figure 4 — Schematic plates for cell culture

6.5 Treatment of cells with testing suspension

Directly add CNM testing suspension or PBS (control) into cell medium and make sure the concentration of CNM in cell medium is below the one which is toxic to cells (the cell viability is lower than 95 % after treatment with testing suspension). Gently transfer the plates to incubator and incubate cells for 24 h to 48 h. Here, the concentration of CNMs is suggested to be in the range of 10 µg/ml to 100 µg/ml.[14] However this concentration should be adjusted specific to each cell type and carbon nanomaterial and to make sure that the cell viability is larger than 95 % of control. The cell viability can be estimated by using cell proliferation assay reagents such as WST-1 or MTT assay according to the protocol provided by the manufacturer (the MTS assay in ISO 19007:2018 can be referenced).

6.6 Cell counting

Cell numbers in each well can be verified with a cell counter or haemacytometer.[12][13] The detail procedure is described in ISO 20391-1:2018.

6.7 Washing cells and preparation of the cell lysate

6.7.1 General

The washing procedures below are described in detail because they are a significant step in this protocol. However, the processes described below is recommended when the 6-well dishes are used. It can be modified according to the cell lines and the types of culture dishes.

6.7.2 For adherent cells

a) After above incubation (see 6.5), gently remove the culture medium with or without CNMs.

b) For washing, gently pipette 2-ml PBS into each well by careful adding solution along the side of the well to minimize disruption to adherent cells. Gently remove the 2-ml PBS via pipette.

c) Repeat b) three times.

NOTE The steps b) and c) can be modified if necessary for certain cell types by harvesting the cells with detachment agent such as trypsin and then washing cells by centrifuge with PBS twice.

d) Add 0,5 ml solution of cell lysis reagent into each well.

e) Keep the plates in a laminar flow cabinet at room temperature for 30 min.

f) Mix the solution in the well by gently pipetting several times. Transfer the above solution from each well one by one to 1,5 ml micro-tubes.

g) Add 0,5 ml SDBS solution into each well and pipetting several times to disperse any materials on the side or bottom of well into solution.

h) Remove the solution from each well and add it to each respective 1,5 ml micro-tubes.

i) Set the 1,5 ml micro-tube in ice water and treat the solution with a homogenizer one by one or multiply for that homogenizer with one or multi-tips (see 5.2.6).

6.7.3 For floating cells

a) After the incubation (see 6.5), transfer the cells and culture medium with or without CNMs to a 15-ml centrifuge tube.

b) Wash each well with 1 ml fresh culture medium and transfer into corresponding 15 ml centrifuge tube.

c) Centrifuge cell suspension at 150 g for 5 min at 4 °C.

d) Remove the supernatant.

e) Add 3-ml PBS into each tube and re-disperse cells by pipetting via a pipette with 1-ml tip.

f) Repeat above process of c) to e) for three times to remove CNMs in outside of cells.

g) Add 0,5 ml solution of cell lysis reagent into each obtained cell-pellet.

h) Disperse the cells by pipetting several times and then keep the tubes in a laminar flow cabinet at room temperature for 30 min.

i) Transfer the above solution from each tube to 1,5 ml micro-tubes one by one after pipetting several times.

j) Wash each 15-ml centrifuge tube with 0,5 ml SDBS solution and then transfer them to above 1,5 ml micro-tubes respectively.

k) Set the 1,5 ml micro-tube in ice water and treat the solution with a homogenizer (see 5.2.6).

6.8 Absorbance measurement of the cell lysate

Measurement wavelength can be set at a wavelength in 700 nm to 900 nm (e.g. 750 nm). The reference control blank should be measured by using a mixture solution of SDBS and cell lysis reagent (1:1).

Transfer cell lysate [see 6.7.2, i) or 6.7.3, k)] into a spectrophotometer (see 5.2.2) and measure the absorbance of each sample individually at a wavelength in 700 nm to 900 nm (e.g. 750 nm).

7 Sources of variability

Sources of variability in the results for screening cellular uptake of CNMs by the method proposed in this document could be traced to, for example:

a) The cellular uptake quantity of CNMs estimated by the method provided in this document contain CNMs as well as its impurities of non-CNM impurities.

b) If the cell lysates are not dispersed homogeneously, the measurement result might be incorrect due to the light scatting attenuated light intensity.

c) The number of cells in the CNM testing group might be different from the control group (non-CNMs addition). It is recommended that the concentration of the test CNM dispersion is below the concentration that induces cell death.

d) Cell growth conditions might influence the uptake results of CNMs.

e) The result might be influenced by the change of aggregate size of CNMs in test dispersion during long term storage.

f) Some CNMs may remain adhered to the cell membrane after cell washing and will be included in the uptake measurement.

8 Data output

8.1 General

Measurement and evaluation results obtained according to this document are suggested to be reported in the following format.

8.2 Data analysis and reporting

a) Record the chosen wavelength.

b) Record the absorbance values at the chosen wavelength (e.g. 750 nm) of cell lysates obtained in 6.8.

c) Calculate the concentration of CNMs in a cell lysis solution based on the calibration obtained in 6.3.

d) Calculate the quantity of CNMs in each cell using the results obtained in b) divided by the cell numbers obtained in 6.6. These data can be omitted if it is not required.

e) The result report contains the data as shown in Table 1.

8.3 Data sheet format

The following data sheet format is to be used for reporting.

Table 1 — Example template for CNMs cellular uptake results

		1	2	3	4	5	6	Average (Ac)	SD
Control	**Ac**								
CNMs testing	**Atest**								
	Atest-Ac								
	CNMs µg/well								
	CNMs pg/cell								

SD: Standard deviation.

Ac: Absorbance at 750 nm for cell-lysate obtained from control groups.

Atest: Absorbance of cell-lysate at 750 nm obtained from CNM tests.

CNMs (µg/well): Estimated amount of CNMs in each well based on the calibration line (see 6.3) and absorbance.

CNMs (pg/cell): The value of CNMs (µg/well) divided by the cell number obtained in 6.6.

Annex A
(informative)

Case study with SWCNTs

A.1 Materials

A.1.1 SWCNTs, produced by a super-growth method (Cat. HT0209, AIST, Japan).

A.1.2 CNTs dispersion, SWCNTs dispersed homogenously in BSA solution at concentration of 1 mg/ml[17][18].

A.1.3 Cell line, Raw 264.7, mouse monocyte macrophage; adherent; European Collection of Authenticated Cell Cultures (ECACC), UK[1)].

A.1.4 Cell lysis reagent, CelLytic M (Sigma, C2978)[2)].

A.1.5 SDBS solution, SDBS powder dissolved in deionized pure water at concentration of 50 mg/ml.

A.1.6 Other reagents for cell culture are as described in this document.

A.2 Procedure

A.2.1 Preparation of the calibration line

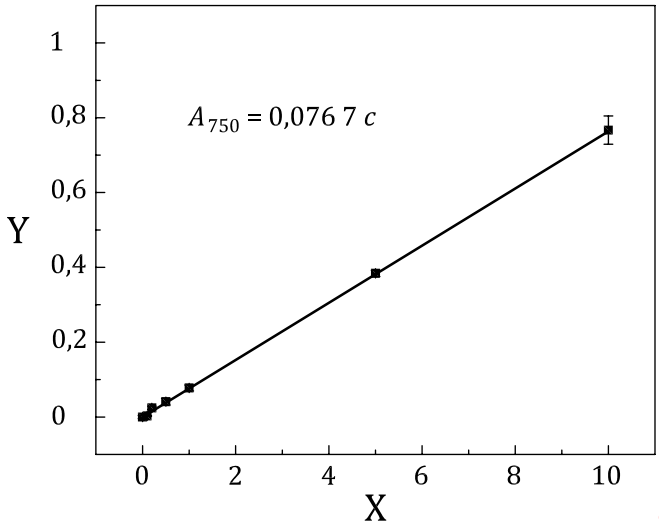
SWCNTs dispersions with concentrations of 0 µg/ml, 0,1 µg/ml, 0,2 µg/ml, 0,5 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml were prepared by dilution of the testing suspension with a mixture solution of SDBS and cell lysis reagent (1:1). Three parallel samples of diluted SWCNTs dispersions for each concentration are prepared. The absorbance values of SWCNTs dispersions in cuvette at 750 nm are measured by using UV-Vis-NIR spectrometer and recorded in the Table A.1. The calibration line is prepared by plotting the average value of absorbance versus concentration as shown below (see Figure A.1).

Table A.1 — Absorbance of testing CNT dispersion at 750 nm for various concentrations

	SWCNTs µg/ml						
	0	**0,1**	**0,2**	**0,5**	**1**	**5**	**10**
Absorbance at 750 nm	−0,004 7	0,003 2	0,028	0,036 0	0,068 5	0,374 5	0,768 1
	0,002 1	0,001 4	0,019	0,035 5	0,082 0	0,391 1	0,766 2
	0,002 6	0,003 8	0,025	0,051 2	0,082 5	0,386 3	0,766 5
Mean	0	0,002 834	0,024	0,040 9	0,077 7	0,383 9	0,766 9
SD	0,004 136	0,001 270	0,004 6	0,008 9	0,007 9	0,008 5	0,001 02

1) Raw 264.7 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

2) CelLytic M is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.



Key

X concentration (µg/ml)
Y absorbance
A_{750} absorbance at 750 nm
c concentration

Figure A.1 — Calibration curve of the absorbance at 750 nm to concentration of SWCNT dispersion

A.2.2 Testing procedure

A.2.2.1 Control test for checking the applicability of the measurement method

The process follows Clause 6 with several modifications as shown below.

a) Raw 264.7 cells with a number of $3{,}3 \times 10^5$ cells/well are seeded on a 6-well plate and incubated for 48 h.

b) After incubation, gently remove the culture medium.

c) Gently add 2-ml PBS into each well, and then remove it. This process is repeated three times.

d) Add 0,5 ml solution of CelLytic M into each well.

e) Keep the plates in a laminar flow cabinet at room temperature for 30 min.

f) Transfer the above solution from each well one by one to 1,5 ml micro-tubes after pipetting several times.

g) Add 0,5 ml SDBS solution into each well and pipetting several times.

h) Remove solution from each well and add it to each 1,5 ml micro-tubes (step f) respectively.

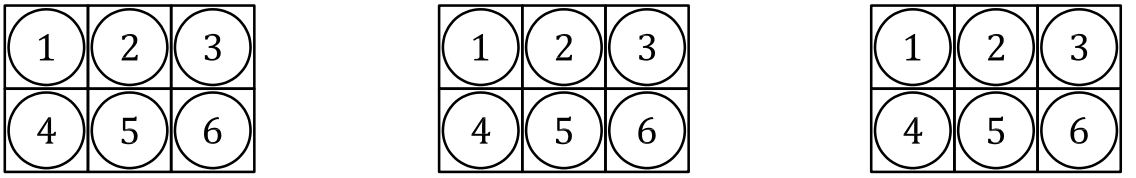
i) Add SWCNTs dispersion (1 mg/ml) into above cell-lysates in 1,5 ml micro-tubes to make sure SWCNTs concentration to be 0 µg/ml, 1 µg/ml, or 5 µg/ml in cell-lysates. The test number is set as $n = 3$.

j) Set the 1,5 ml micro-tube in ice water and treat the solution with a homogenizer (see 5.2.6) for 10 min at 270 W.

k) Measure the absorbance of cell-lysates at 750 nm and calculate the quantity of SWCNTs in cell lysates.

A.2.2.2 Cell uptake test

a) Three 6-well plates are used for three groups (two for control, and one for SWCNTs testing) as shown in Figure A.2. The cell dispersion (about 1,1 × 10^5/ml, 3-ml) is seeded in each well of the 6-well plates and incubated for 24 h.



a) Control (for cell counting) **b) Control (n = 6)** **c) CNT testing (n = 6)**

NOTE Each plate corresponds to either a control for cell counting, or control for cell lysis, or for CNT-testing.

Figure A.2 — Schematic plates for cell culture

b) The culture medium is replaced with test SWCNTs dispersion (0,05 mg/ml in medium) or fresh medium (control)

c) Gently transfer the plates to incubator and incubate the cells for 24 h.

d) Count the number of cells in each well of control (for cell counting) by a cell counter after above incubation (see 6.6).

e) Prepare cell lysates for groups of control and CNT test following the procedure of 6.7.2.

f) Measure absorbance of the cell lysates in each well.

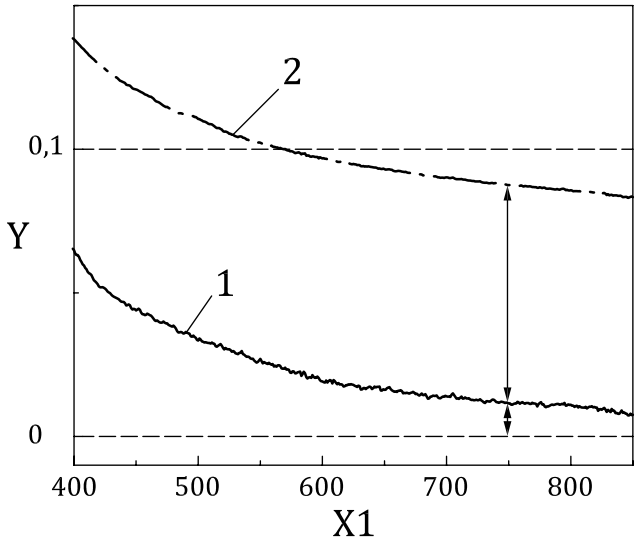
A.3 Test result

A.3.1 Control testing results

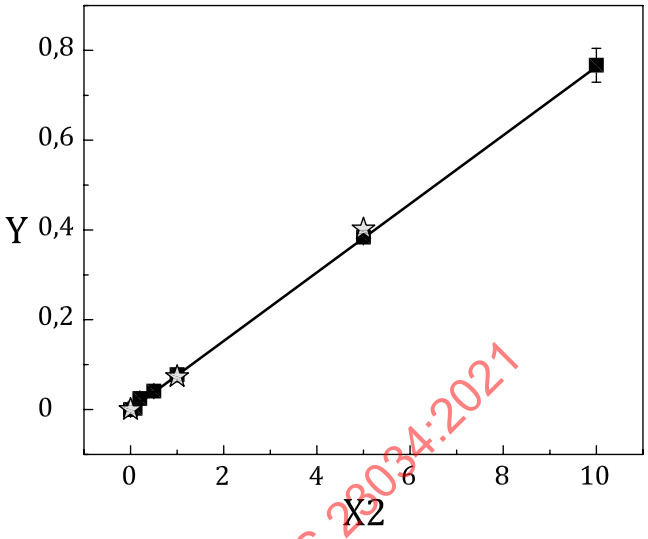
The measurement results are shown in Table A.2, Figure A.3, and Table A.3 as below.

Table A.2 — Background absorbance of cell lysate

Control	Well number			
	1	2	3	Average (Ac)
A750	0,024 9	0,010 35	0,018 97	0,018 07



a) Typical optical absorption spectra of a cell lysate (1) and following the addition of SWCNTs dispersion (2)



b) Measurement results of absorbance at 750 nm for SWCNTs with known concentrations (0 µg/ml, 1 µg/ml, and 5 µg/ml) in cell lysates, shown as points (star) on the calibration curve of SWCNTs in dispersion

Key

X1	wavelength (nm)	1	cell lysate
X2	concentration (µg/ml)	2	1 µg/ml SWCNT
Y	absorbance		

Figure A.3 — Evaluation for applicability of the measurement method

Table A.3 — Absorbance of test SWCNT dispersion at 750 nm

	Known µg/ml		
	0	**1**	**5**
Atest	0,019 1	0,092 2	0,420 1
Atest-Ac	0,001 0	0,074 13	0,402
Measured µg/ml	0,013 4	0,97	5,24
Error	±1,34 %	±3 %	±4,8 %

This result indicated that the measurement error would be less than 5 % for concentration of SWCNTs in cell lysate >1 µg/ml.

A.3.2 SWCNT uptake test results

A.3.2.1 Cell counting

The number of cells in each well of control group for counting is counted by a cell counter (see Table A.4).

Table A.4 — Cell numbers after incubation

	Well number						
	1	2	3	4	5	6	Average
Cell number/well $\times 10^5$	5,71	6,15	5,84	5,94	6,13	5,96	5,95

A.3.2.2 Preparation of the cell lysate and absorbance measurement

The processes for preparation of cell lysate and absorbance measurement are followed the procedure of 6.7 and 6.8.

Table A.5 — The results of cellular uptake of SWCNTs

		Well number							
		1	2	3	4	5	6	Average (Ac)	SD
Control	**Ac**	0,018 3	0,021 8	0,017 6	0,018 7	0,019 3	0,023 4	0,019 8	0,002 3
CNTs test	**Atest**	0,250 6	0,213 0	0,255 0	0,272 8	0,202 0	0,213 0	0,234 4	0,028 7
	Atest-Ac	0,230 7	0,193 2	0,235 1	0,252 9	0,182 2	0,193 2	0,214 6	0,028 7
	SWCNTs µg/well	3,008	2,519 0	3,065 2	3,297 3	2,375 5	2,518 9	2,797 9	0,374 2
	SWCNTs pg/cell	5,055	4,233 6	5,151 5	5,541 6	3,992 4	4,233 4	4,702 3	0,062 89

SD: Standard deviation.

Ac: Absorbance at 750 nm for cell-lysate obtained from control groups.

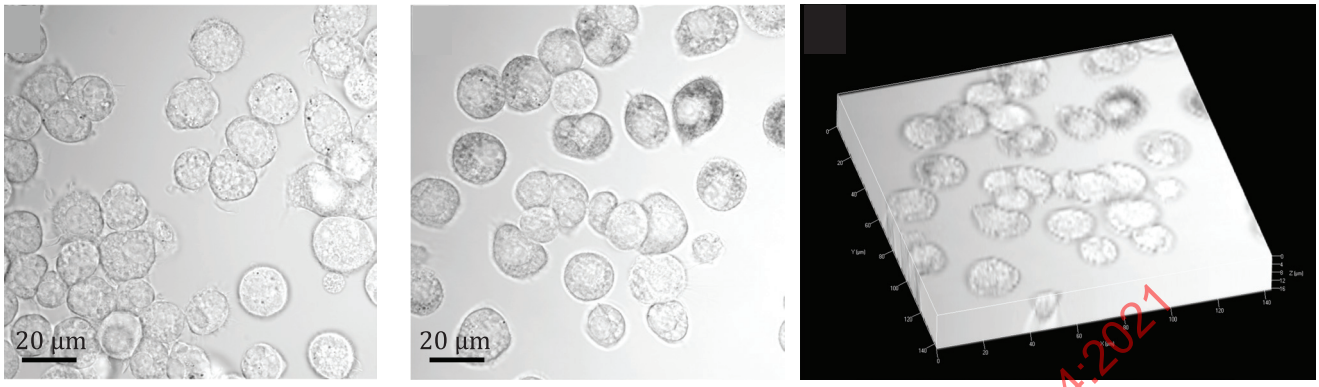
Atest: Absorbance at 750 nm for cell-lysate obtained from test SWCNTs groups.

SWCNTs (µg/well): Estimated amount of SWCNTs in each well based on the calibration line (see Figure A.1) and absorbance.

SWCNTs (pg/cell): The value of SWCNTs (µg/well) divided by the cell number from A.3.2.1.

A.4 Observation of cellular uptake of SWCNTs by Raw 264.7

To make sure that the washing process in 6.7 with PBS is enough to remove the CNTs out of cells, the Raw 264.7 cells after SWCNTs uptake and washing by PBS before cell lysis are observed by optical microscopy. The results show that the SWCNTs are inside of cells in the condition of this testing (see Figure A.4).



a) Raw 264.7 cells as control

b) Image extracted from c) of Raw 264.7 cells after incubation with SWCNTs dispersion with PBS washing

c) Three-dimensional reconstruction of 10 Z-stack images of Raw 264.7 cells after incubation with SWCNTs dispersion and PBS washing

NOTE The black stains in cells of images Figures A.4, b) and c) indicate the uptake of SWCNTs by cells. Scale bars: 20 µm.

Figure A.4 — Confocal microscopy images of Raw 264.7 cells and of Raw 264.7 cells after SWCNTs uptake

Annex B
(informative)

Case study of CNHs

B.1 Materials and equipment

B.1.1 Materials

B.1.1.1 CNHs, as-grown CNHs sample is obtained from NEC Japan. The sample is sterilized in an autoclave at 121 °C for 20 min before use.

B.1.1.2 CNH, test suspension: as-grown CNHs are dispersed homogenously in BSA solution at concentration of 1 mg/ml.

B.1.1.3 Cell lysis reagent, CelLytic M (Sigma, C2978).

B.1.1.4 SDBS solution, SDBS powder dissolved in deionized pure water at concentration of 50 mg/ml.

Other chemistry and reagents for cell culture are the same as described in this document.

B.1.2 Cell line

B.1.2.1 RAW 264.7, mouse monocyte macrophage; European Collection of Authenticated Cell Cultures, UK.

B.1.2.2 Culture properties, adherent.

B.1.3 Equipment

B.1.3.1 Homogenizer, ultrasonic homogenizer with horn-tip, disinfected with a 70 % aqueous solution of ethanol.

B.1.3.2 UV-Vis-NIR spectrometer.

B.1.3.3 Quartz cuvette for absorption measurement, a micro-volume quartz cuvette with path-length of 10 mm and volume of 700 µl.

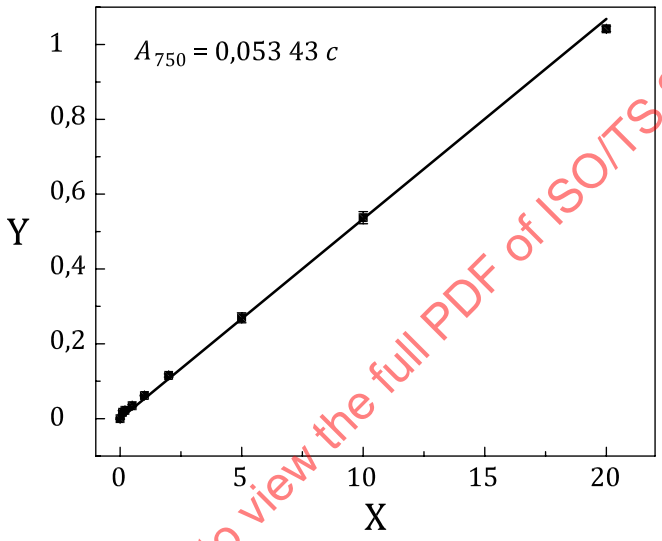
All containers and deionized pure water are sterilized in an autoclave at 121 °C for 20 min before using.

B.2 Calibration line of CNHs in aqueous suspension

CNHs dispersions with various concentrations of 0 µg/ml, 0,1 µg/ml, 0,2 µg/ml, 0,5 µg/ml, 1 µg/ml, 2 µg/ml, 5 µg/ml, 10 µg/ml and 20 µg/ml are prepared by dilution of the test suspension with a mixture solution of SDBS and cell lysis reagent (1:1). Three parallel samples of diluted CNHs dispersions for each concentration are prepared. The absorbance values of CNHs dispersions in cuvette at 750 nm are measured by using UV-Vis-NIR spectrometer and recorded in the Table B.1. The calibration line is prepared by plotting the average value of absorbance versus concentration as shown in Figure B.1.

Table B.1 — Absorbance of CNH dispersion at 750 nm

	CNHs µg/ml								
	0	**0,1**	**0,2**	**0,5**	**1**	**2**	**5**	**10**	**20**
Absorbance at 750 nm	0,002 2	0,012 1	0,022 2	0,0316 7	0,060 42	0,111 1	0,258 9	0,522 2	1,043 2
	−0,001 7	0,016 4	0,019 0	0,035 8	0,062 3	0,119 3	0,265 0	0,534 4	1,041 5
	−0,000 4	0,021 2	0,024 4	0,036 3	0,062 1	0,115 3	0,284 4	0,554 5	1,040 9
Mean	0	0,016 6	0,021 9	0,034 6	0,061 6	0,115 2	0,269 4	0,537 0	1,041 87
SD	0,002 0	0,004 6	0,002 7	0,002 6	0,001 0	0,004 2	0,013 2	0,016 3	0,001 2



Key

X concentration (µg/ml)
Y absorbance
A_{750} absorbance at 750 nm
c concentration

Figure B.1 — Calibration of CNH dispersion absorbance at 750 nm

B.3 Cell uptake test

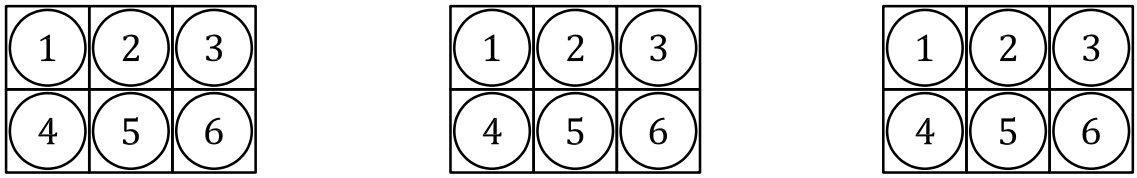
B.3.1 Cell culture

Raw 264.7 cells are cultured in RPMI-1640 medium containing 10 % FBS and streptomycin/penicillin (Gibco)[3)] at 37 °C in a 5 % CO_2 atmosphere.

B.3.2 Cell culture with CNHs dispersion

a) Three 6-well plates are used for three groups (two for control, and one for CNHs test) as shown in Figure B.2. The cells dispersion (about 1,1 × 10^5/ml, 3-ml) is seeded in each well of 6-well plates and incubated for 24 h.

3) RPMI-1640 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.



a) Control for cell counting **b) Control (n = 6)** **c) CNM testing (n = 6)**

NOTE Each plate corresponds to either a control for cell counting, or control for cell lysis, or for CNH-testing.

Figure B.2 — Schematic plates for cell culture

b) The culture medium is replaced with testing CNHs dispersion (0,05 mg/ml in medium) or fresh medium (control).

c) Gently transfer the plates to incubator and incubate the cells for 24 h.

B.3.3 Cell counting

The number of cells in each well of control group after above incubation (see B.3.2) is counted by a cell counter. The results are shown in Table B.2.

Table B.2 — Cell numbers after incubation

	Well number						
	1	**2**	**3**	**4**	**5**	**6**	**Average**
Cell number/well $\times 10^5$	5,71	6,15	5,84	5,94	6,13	5,96	5,95

B.3.4 Preparation of the cell lysates

The process for preparation of cell lysates follows the procedure of 6.7.2.

B.3.5 Absorbance measurement of the cell lysates

The measurement follows the procedure of 6.8.

B.3.6 Test result

The test results are shown in Table B.3.

Table B.3 — The results of CNH cellular uptake

		Well number							
		1	**2**	**3**	**4**	**5**	**6**	**Average (Ac)**	**SD**
Control	**Ac**	0,024 9	0,032 5	0,010 3	0,019 0	0,025 9	0,023 7	0,022 7	0,007 5
CNHs test	**Atest**	0,131 3	0,145 2	0,141 0	0,135 9	0,141 7	0,140 8	0,139 3	0,004 9
	Atest-Ac	0,108 6	0,122 5	0,118 3	0,113 2	0,119 0	0,118 1	0,116 6	0,005 4
	CNHs µg/well	2,032 6	2,292 7	2,214 1	2,118 7	2,227 2	2,210 4	2,182 6	0,092 2
	CNHs pg/cell	3,410 3	3,846 8	3,714 9	3,554 8	3,736 9	3,708 7	3,662 1	0,154 7

Ac: Absorbance at 750 nm for cell-lysate obtained from control groups.

Atest: Absorbance of cell-lysate at 750 nm obtained from CNH tests.

CNHs (µg/well): Estimated amount of CNHs in each well based on the calibration line (see B.2) and absorbance.

CNHs (pg/cell): the value of CNHs (µg/well) divided by the cell number from B.3.3.