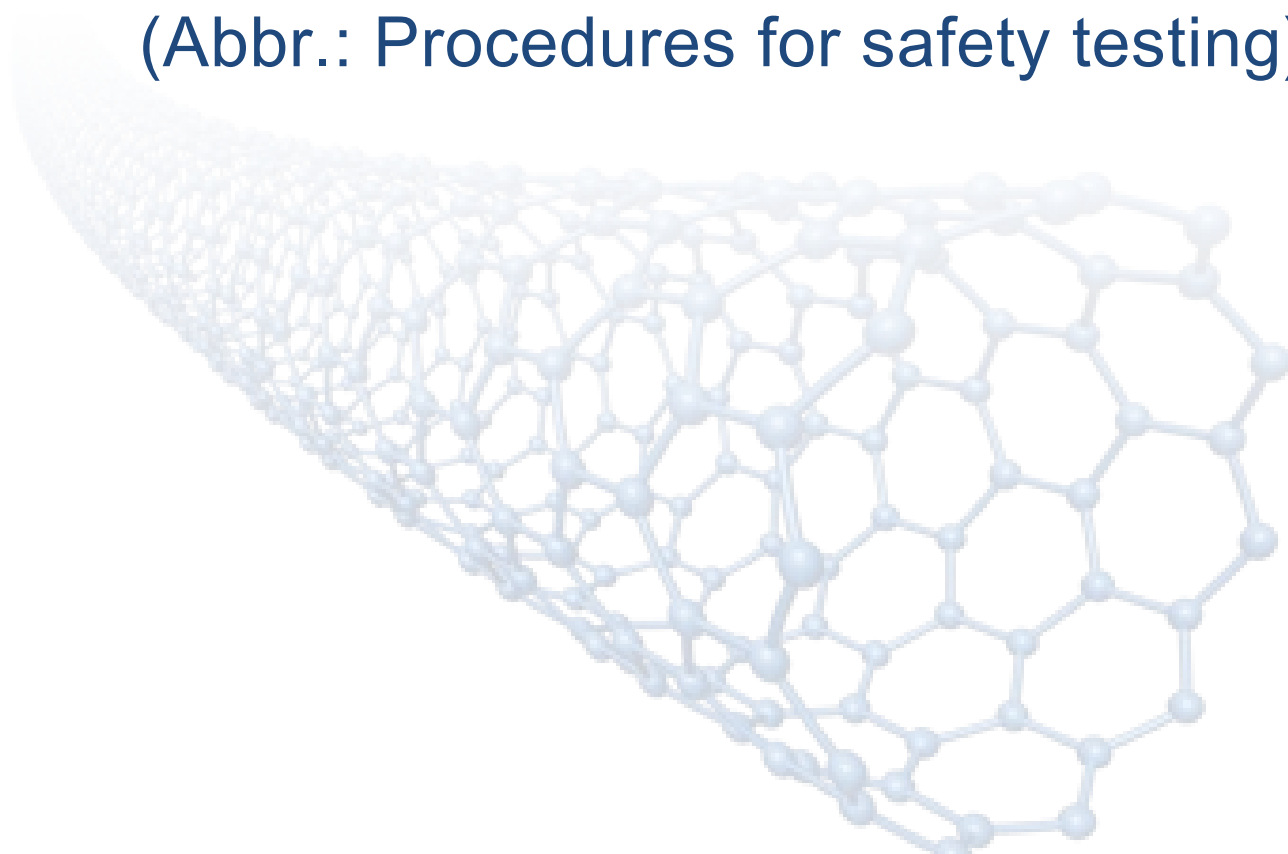

Sample preparation and characterization for safety testing of carbon nanotubes, and *in vitro* cell-based assay

(Abbr.: Procedures for safety testing)

2014



Technology Research Association for Single Wall Carbon Nanotubes (TASC)
Research Institute of Science for Safety and Sustainability (RISS), National Institute of
Advanced Industrial Science and Technology (AIST)

This document is based on results obtained from a project commissioned by the New Energy and Industrial Technology Development Organization (NEDO), “Innovative carbon nanotubes composite materials project toward achieving a low-carbon society (P10024)”.

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PREFACE

In recent years, the development of industrial products using nanomaterials (Manufactured nanomaterials) and their many applications in information-communications, the environment, and energy have been enthusiastically pursued on a worldwide scale. Manufactured nanomaterials are composed of ultrafine particles that are much smaller than previously used conventional chemicals, because of which applications that utilize their new physicochemical properties are very promising; however, the harmful effects on human health and the environment have become a concern.

In August of 2011, “Evaluating risks associated with manufactured nanomaterials” was released, presenting the results of the New Energy and Industrial Technology Development Organization (NEDO) project (P06041). This report covers the assessments of nanomaterial safety and harmful effects, and exposure for three materials: titanium dioxide, fullerene, and carbon nanotubes (CNTs). Along with risk management methods, this report proposed a period-limited occupational exposure limit based on a work period of around 15 years at exposure of five days a week, 8 h per day, and reviewed within about 10 years using new test results obtained in the meantime.

Here at Technology Research Association for Single Wall Carbon Nanotubes (TASC), we are proceeding with a Grant-in-Aid Scientific Research aimed at the implementation of single-walled CNTs (SWCNTs) and graphene and the development of various applications. As a follow-up to the abovementioned NEDO project, we are also developing a simple, autonomous safety management technology for nanomaterials (“Innovative carbon nanotubes composite materials project toward achieving a low-carbon society” (P10024)). Currently, with early commercialization expected for nanomaterials and nanomaterial complexes produced and developed at TASC, we are supplying companies and organizations with nanomaterial samples free of charge. Centering mainly on Research Institute of Science for Safety and Sustainability (RISS) and National Institute of Advanced Industrial Science and Technology (AIST), a safety assessment is in progress at TASC subsequent to the development stage.

This document is to be used as an aid in formulating voluntary safety management methods for the safe handling of CNTs by businesses and business operators for research and development. This document summarizes procedures involved in sample preparation and characterization of CNTs for safety testing, and *in vitro* cell-based assay. As a practical example of SWCNTs developed at TASC, the results and findings of an evaluation based on these procedures are given in an appendix.

As a NEDO project, we at TASC are working on methods for measuring airborne CNTs in a work environment in which CNTs are handled mainly in powder form. The results have been summarized and released as “Guide to measuring airborne carbon nanotubes in workplaces.” The best approach would be to refer to that document in conjunction with the independent safety management plan for each place of business. This document and the “Guide to measuring airborne carbon nanotubes in workplaces” can be downloaded from the AIST website (<http://www.aist-riss.jp/>).

March 2014

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I. Introduction

Purpose

This document provides procedures for simple and rapid safety evaluations of *in vitro* cell-based assay, so that business operators handling CNTs can autonomously implement safety management. The object is to assess the effects on the health—especially regarding the respiratory systems—of workers who might be exposed by inhalation inside facilities for carbon nanotube manufacturing or of those who are involved in their processing. The features of the test method are as follows. (1) Prepare a stable and homogeneous dispersion liquid of the test material, and measure its dispersion conditions and properties in this liquid. (2) Measure dispersion conditions and properties of the test material added to a cell culture medium, as well as changes in medium components. (3) Determine the effects of the test material on the cells—i.e., extract the biomarker (the endpoints) and select a test method for this biomarker that minimizes interference from the test material.

Background

Since nanomaterials are unusual owing to the small size and fibrous shapes, there is increasing concern that apart from their chemical properties, such physical properties may induce nanomaterial-specific biological effects. For efficient research and development of new manufactured nanomaterials, we need to incorporate an investigation into the safety of nanomaterials from the initial stages of all research and development processes.

CNTs have attracted much attention as innovative nanomaterials and may be developed and marketed with various physical adjustments depending on requirements. With usual chemicals, safety and possible harmful effects are evaluated through animal testing for each species. However, owing to high costs and time requirements, it is not feasible to conduct animal testing, especially for nanomaterials whose physico-chemical properties have to be modified according to requirements. Further, using the 3R principles for animal testing (replacement, reduction, and refinement) as a basis, a safety assessment method that uses *in vitro* cell-based assay is needed globally instead of relying on animal testing.

For *in vitro* cell-based assay, it is necessary to add CNTs to the cell culture medium. However, CNTs form aggregates and agglomerations in liquid media (secondary particles) that tend to settle directly onto cells. Often, the way to solve this is to use a dispersant with a surface-active effect. However, many dispersants are toxic themselves. Therefore, to assess the effects on cells that reflect the particular properties of CNTs, it is important to limit the use of dispersants, yet develop preparation methods that disperse CNTs in a stable manner. Furthermore, we must consider how the primary physicochemical properties of CNTs and their secondary properties within the medium affect the cytotoxicity (particle size within the liquid, concentration, ion concentration, and depletion of cell maintenance components that are associated with

adsorption onto CNTs of protein components and mineral salts, and so on, that are contained in the medium and additives). Because it is extremely difficult to measure these within the medium, to the use of *in vitro* cell-based assays is important after establishing an appropriate index to identify the toxicity on cells.

Many aspects of mechanisms for the onset of carbon-nanotube toxicity on organisms with their specific physicochemical properties are unknown. Therefore, beyond the existing method for determining the surviving cells and their capacity for differentiation, it is also necessary to select a new evaluation item (endpoint) that accurately reflects the effects of nanomaterials and to make measurements on the basis of such effects. The development of analytical methods is also important for studying the biological effects of nanomaterials and for comparing cell species of the same nanomaterial. Furthermore, the use of safety testing results obtained by *in vitro* cell-based assay—using either cells or tissue derived from humans and animals—to predict the effects on organisms must be validated using animal testing.

Outline of the Procedure

An outline of the procedure for sample preparation, measurement, and *in vitro* cell-based assay, which is required for safety testing of CNTs, is given in Fig. I.1

1. Sample preparation and measurement for safety testing of CNTs

To conduct *in vitro* cell-based assay for CNTs, a stable suspension must be prepared in a cell culture medium using high-precision measurement technology. Following are the steps required after acquiring data for the appropriate physicochemical component composition for the CNT powder as required for sample preparation and measurement used in safety testing of CNTs. (1) To disperse CNTs uniformly and stably in the cell culture medium and to minimize effects on the cells, the stock suspension should be prepared with minimum amount of dispersant. Then, a working solution is prepared by diluting the stock suspension with the cell culture medium. (2) A stable suspension can be verified by measuring secondary properties such as CNT particle size and concentration, ion concentrations, and the adsorption of such components as protein and mineral salts in the medium of the CNT stock suspension or the working solution. These test results can be used to decide whether to conduct *in vitro* cell-based assay to test for the safety of CNTs.

2. *In vitro* cell-based assay to test the safety of CNTs

Here, we assume that the major health effects are due to inhalation exposure on the respiratory system. Using the medium solution prepared as described in step (1), the procedure continues as follows. (3) While determining the existence of surviving cells owing to various associated cultured cells and their capacity for differentiation, measurements are performed by selecting specific end points, such as an inflammatory marker, oxidant stress, and apoptosis, so that *in vitro* testing can be conducted to assess the safety of CNTs for the onset of different

biological effects mechanisms.

Note that results obtained by adherence to this procedure are for an assessment of the effects of CNTs on the respiratory system. This procedure cannot guarantee the safety of all of them.

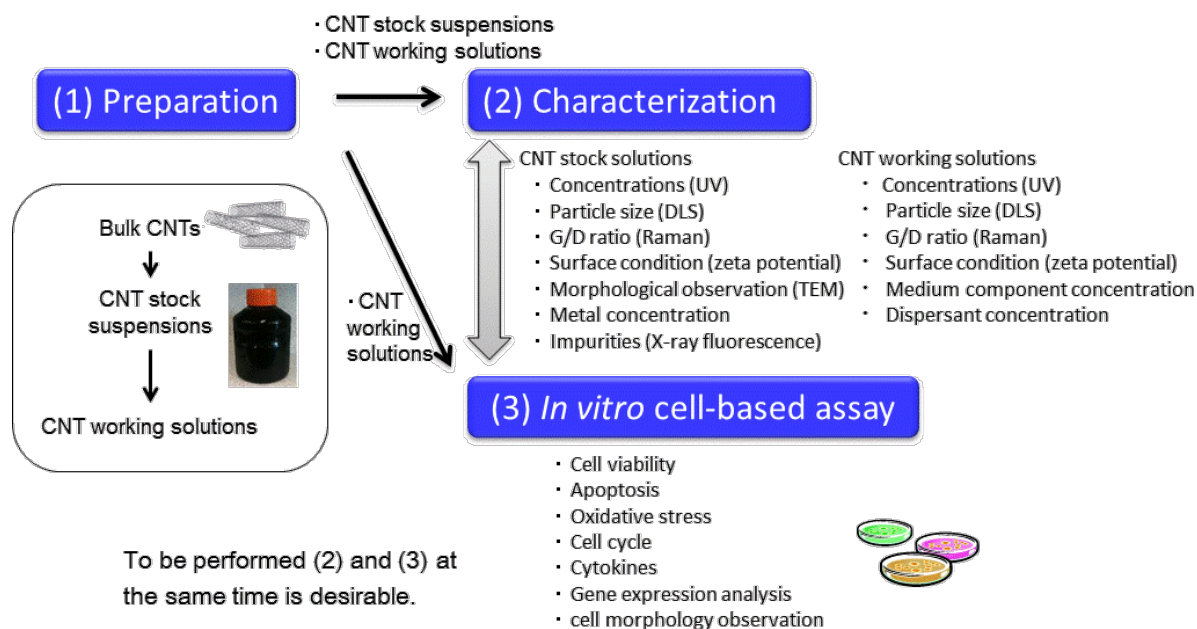


Figure I. 1 Outline of sample preparation and measurement, and the *in vitro* cell-based assay, which are used in the safety testing of CNTs

II. Control of contamination and exposure

1. Sterilization operations

For *in vitro* cell-based assay, sterilization is absolutely essential to prevent contamination by bacteria. Equipment such as stirring bars, beads, and vessels for the bulk powder, dispersion medium, and dispersed samples should all be thoroughly sterilized in an autoclave. Equipment that cannot be autoclave sterilized should be cleaned with ethanol and irradiated with ultraviolet light for over 8 h to kill any existing bacteria. To sterilize the dispersed liquid, suction filtration is used through a filter with a pore diameter of less than 0.45 μm .

Reference standard JIS T 7322 High-pressure steam sterilization equipment for medical use.

2. Countermeasure against endotoxins

Endotoxins, which exist in normal environment, can cause the activation of white blood cells and react with the organism and the cultured cell line. If the dispersion liquid used in the tests is contaminated with endotoxins, it can lead to incorrect evaluations of harmful effects. Therefore, measures must be taken to prevent the introduction of endotoxins when samples are prepared. This is particularly important with *in vitro* cell-based assay in which cells react directly. For example, the water used in the dispersion medium is deionized water treated with a filter to remove endotoxins (ultrapure water). Furthermore, if deionized water is stored for a long time, it can easily be contaminated. Therefore, it is best to prepare only the amount of water needed at the time of use.

In preparing the dispersion liquid for CNTs, measures can be taken to minimize contamination by testing for the presence of endotoxins. A suitable test method is the gelification method (Gel-clot method) for the Limulus amoebocyte lysate (LAL, lysate reagent) test adopted as ISO 29701. This method uses the gelification of the lysate from the action of an endotoxin as an index.

Reference standard ISO 29701 Nanotechnologies—Endotoxin test on nanomaterial samples for *in vitro* systems—Limulus amoebocyte lysate (LAL) test

3. Countermeasure against exposure to CNTs

The effects of CNTs exposure on human health are still unclear, but in accordance with the principle of precaution, CNTs should be handled keeping any exposure to a minimum. For instance, when CNTs are handled in the dry state for weighing and pretreatment as a powder, exposure is possible owing to scattering. To avoid this, work is performed inside a sealed apparatus such as a glove box or under proper local ventilation such as a fume hood with a high-performance filter. However, operating an exhaust fan during weighing may also cause scattering, and hence the fan should be stopped when performing certain work tasks: it should be operated only when removing containers and cleaning. Protective clothing including a mask,

gloves, glasses, and a white coat should be worn.

Owing to the possibility that the dispersion liquid may be scattered during tasks involving wet dispersions, work should be conducted inside a clean bench or using local ventilation equipment to prevent material scattering into the lab. Protective clothing should be worn in this case too.

The ventilation method for glove boxes and local ventilation equipment, methods for selecting protective clothing, and treatment methods for waste material should be chosen after consulting notifications and guidelines from relevant institutions since these are effective measures for preventing contamination by bacteria and endotoxins.

Reference standard “Preventative measures to avoid exposure to nanomaterials” based on the Ministry of Health, Labour and Welfare, Japan. Publication No. 0331013 (March 31, 2009).

III. Sample preparation of carbon nanotubes for *in vitro* cell-based assay

Preface

This section describes the procedure for preparing samples of CNT-dispersed solutions used for *in vitro* testing of CNTs using cells. Here, SWCNTs are dispersed in a solution by using bio-derived materials without any surfactants or polymer electrolytes. Through safety assessment testing over three days from the time the suspension is added to the cells, the method for preparing samples to be used for *in vitro* cell-based assay in which SWCNTs are stably suspended is described. We also present methods for evaluating the CNT concentration, the length of the dispersed SWCNTs, and the presence of endotoxins in the prepared test sample.

1. Scope of application

A method is presented for preparing a sample of suspension for *in vitro* cell-based assay in which SWCNTs are dispersed. Dispersion is accomplished by using ultrasonic irradiation to aggregates/agglomerates of SWCNTs in an aqueous solution of bovine serum albumin (BSA).

2. Reference standards and regulations

This procedure refers to the following standards and regulations.

ISO 22412: 2008, Particle size analysis—Dynamic light scattering

Testing methodology for endotoxins (gelification method using lysate reagent) in the 16th revision of the Japanese Pharmacopoeia

Based on the Ministry of Health, Labour and Welfare, Japan publication No. 0331013, “Preventative measures to avoid exposure to nanomaterials” (March 21, 2009).

3. Terminology

3.1 Stock suspension

A suspension is prepared by dispersing CNTs in BSA aqueous solution.

3.2 Working solution

The SWCNT suspension to be used for *in vitro* cell-based assay is prepared by using the cell culture medium used in the testing in order to dilute the stock suspension by a prescribed scale factor.

3.3 Centrifugal strength

The ratio of centrifugal acceleration to the acceleration due to gravity (g) gives the operating conditions for centrifuge separation. For a centrifugal strength a , this is expressed as ag .

3.4 Bovine serum albumin (BSA)

Purified protein from the serum of cattle is used as an alternative to a surfactant for dispersing SWCNTs in water.

3.5 Endotoxins

Endotoxins are toxins released from microorganisms.

4. Equipment

4.1 Dispersing equipment

An ultrasonic homogenizer is used to irradiate ultrasonic waves from a horn (a probe).

4.2 Containers

A 500-mL glass beaker

4.3 Centrifuge separator

The centrifuge separator should be capable of attaining a centrifugal strength of up to 22,000*g*.

4.4 Centrifuge tube (centrifuging tube)

The centrifuging tube is a sterile 50-mL tube.

4.5 Filtering apparatus

A cell strainer is used to remove coarse aggregates/agglomerates of SWCNTs.

4.6 Measuring apparatus for CNT concentrations in a stock suspension

Concentrations of CNT are obtained from absorbance in the ultraviolet-visible range (UV-Vis).

4.7 Apparatus for measuring secondary particle sizes of suspended CNTs

An apparatus based on dynamic light scattering (DLS) is used to measure particle sizes.

4.8 Observation apparatus of morphology of dispersed CNTs

Transmission electron microscopy (TEM) is used to observe dispersing state and to measure the length of CNTs via an image analysis method.

5. Sample

5.1 Dispersion medium and its preparation

5.1.1 Dispersant

BSA is used as the dispersant.

5.1.2 Dispersion medium

An aqueous solution is prepared by dissolving 10 mg/mL of BSA in pure water that is first prepared by purification in an ultrapure water production system, followed by filtering to remove endotoxins. Next, the BSA aqueous solution is passed through a 0.22- μ m sterilizing filter, and the sterilized solution is used as a dispersion medium to disperse the SWCNTs.

5.2 SWCNTs

The dry state is ideal, and no particular heat treatment is required.

6. Pretreatment

6.1 Sterilization of equipment

Beakers are sterilized in an autoclave at 121°C for 20 min. The horn of the ultrasonic homogenizer, after being disinfected with a 70% aqueous solution of ethanol, is then sterilized by UV irradiation for 3 min. Other equipment is disinfected with a 70% aqueous solution of ethanol.

6.2 Pretreatment and sterilization of SWCNTs

The required amount of SWCNTs is weighed for preparing the CNT suspension sample with a predetermined concentration.

The SWCNT raw material in sheet form is cut into small pieces. Depending on the form of the raw material, tweezers and/or scissors can be used for cutting.

The predetermined quantity of SWCNTs is put into a 50-mL beaker and irradiated for 3 min with UV light.

Warning: In weighing the SWCNTs and their pretreatment, avoid exposure to the SWCNTs by working inside local ventilation equipment. (See “Preventative measures to avoid exposure to nanomaterials,” based on the Ministry of Health, Labour and Welfare publication No. 0331013.) Perform tasks using individual protective clothing, including masks and gloves.

7. Preparation procedure for the stock suspension

7.1 Stock suspension of SWCNTs

The concentration of CNTs in the stock suspension is prepared up to a maximum of 1 mg/mL.

To the beaker containing the collected and sterilized SWCNTs according to Section 6.2, 50 mL of the dispersion medium, as prepared in Section 5.1.2, is added.

The solution is stirred for 20 min to blend it thoroughly.

Warning: To avoid contamination from air, agitation with a stirrer is done inside a clean bench. In addition, tasks are performed using individual protective clothing with masks and gloves.

7.2 Dispersion

Before dispersion, the sonication horn of an ultrasonic homogenizer is inserted directly into a

beaker containing the SWCNTs and the BSA aqueous solution. The position of the horn is adjusted to visually ensure that the circulation of liquid inside the beaker appears to be optimum.

The SWCNTs are dispersed by continuously irradiating with ultrasonic waves for 2 h.

To suppress any rise in temperature of the suspension during ultrasonication, the beaker is cooled with ice intermittently as required.

Note: The ultrasonic output control should be checked in advance to consider, any possible breakage or wear of the container and/or horn and corruption of the SWCNTs themselves, along with the dispersion effect on the SWCNTs.

Warning: During the ultrasonic irradiation treatment, to avoid any exposure due to contamination from the air or ejection of the suspension, the treatment is done inside a clean bench. These tasks are performed using individual protective clothing with masks and gloves.

7.3 Centrifugation

The suspension obtained above is isolated in a 50-mL centrifuge tube.

The supernatant is recovered after centrifugation with centrifuge strength of 6,000*g* for 15 min.

7.4 Filtration

The recovered supernatant is filtered through a cell strainer of pore size 40- μ m in order to obtain the stock suspension as the filtrate.

7.5 Preparing of a stock suspension with an adjusted CNTs state

In addition to the above procedure, the following procedure can be followed to prepare a stock suspension depending on the intended state of the suspended carbon nanotubes (carbon nanotube length and secondary particle size).

- (a) Adjust the sonication
- (b) Recover the supernatant after centrifugation at adequate centrifuge strength over an adequate duration
- (c) Filter with a cell strainer of appropriate mesh size
- (d) If necessary, continue centrifugation to obtain the sediment
- (e) Add the BSA aqueous solution prepared in Section 5.1.2 to the sediment and redisperse in an ultrasonic bath over a short period
- (f) Filter with a cell strainer of appropriate mesh size
- (g) Use filtrate obtained from either (c) or (f) as the stock suspension

8 Preparation of working solution

A working solution is prepared with a predetermined concentration of carbon nanotubes by diluting the stock suspension with the cell culture medium for *in vitro* cell-based assay. For the

dilution, a cell culture medium in which the concentration of BSA is adjusted to be 10 mg/mL of the BSA concentration in obtained working solution is used.

9 Assessment of the suspension

9.1 Measuring the morphology of CNTs

The dispersed state of the SWCNTs is observed using TEM to make an assessment.

The secondary particle size of the suspended SWCNTs is assessed from average particle size using dynamic light scattering (DLS) according to ISO22412.

CNT lengths are evaluated using image measurements taken from TEM micrographs.

9.2 Assessing the CNT concentration in the stock suspension

CNT concentration is assessed on the basis of absorbance in the ultraviolet-visible (UV-Vis) range. Hence, the relationship (the calibration curve) between the SWCNT mass concentration and absorbance of the suspension is found in advance using a standard suspension of sufficiently dispersed SWCNTs and an absorbance of 600–800 nm.

9.3 Test for endotoxins

Following the gelification method for endotoxin testing in the 16th revision of the Japanese Pharmacopoeia, a detection test for endotoxins in the stock suspension is performed. In the limit test based on the lysate reagent coagulation reaction, endotoxin concentration is verified to be less than reagent detection limit.

The lysate used is Pyrotell® (Associates of CAPE COD Inc.) with a detection limit of 0.03 EU/mL.

Appendix A

Working example: preparation of SG-SWCNTs for *in vitro* testing

A.1 Outline

We give an example of CNT suspension to be used for *in vitro* cell-based assay. The sample was prepared according to the above procedure, which is similar to that for SWCNTs manufactured with the super-growth method (SG-SWCNTs). According to the procedure, SG-SWCNTs were dispersed at 1 mg/mL in a 10 mg/mL BSA aqueous solution, and suspensions were prepared containing carbon nanotubes with two different lengths (stock suspensions: samples SG1 and SG2). The properties of the stock suspension were also assessed.

A.2 Preparation of the dispersing medium

Nacalai Tesque (Co., Ltd.) 08587-42 was used as the BSA dispersant.

A 10-mg/mL BSA aqueous solution was prepared using pure water that purified with the Milli-Q ultrapure water production system (Millipore) and passed through the BioPak endotoxin removal filter (Millipore).

The dispersing medium used was what remained after passing the above BSA aqueous solution through a 0.22- μ m sterilizing filter (GPO.22 μ m; Merck).

A.3 Preparation of a suspension of SG-SWCNTs

A.3.1 Treatment with ultrasonication

A 50-mg mass of the SG-SWCNTs was weighed and collected in a 50-mL beaker and then sterilized.

A Branson SONIFIER 250 was used for the ultrasonic homogenizer (Fig. A.1(a)).

The ultrasonic homogenizer was inserted into a suspension prepared according to the procedure in Section 7.1. The out-put control of the homogenizer was set to four and the suspension was treated with continuous ultrasonication for 2 h (sample SG1) and 0.5 h (sample SG2) (Fig. A.1(b)).

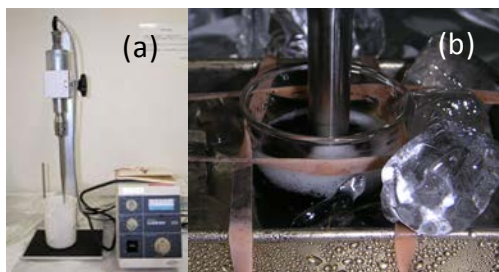


Figure A.1 (a) Ultrasonic homogenizer and (b) dispersion operations

A.3.2 Centrifugation and filtering

For sample SG1, following the procedure in Section 7, the supernatant, which is separated with

a centrifuge at a centrifugal strength of 6,000*g* for 15 min, is filtered with a 40- μ m cell strainer (BD Falcon™), and the filtrate is used as the stock suspension.

On the other hand, sample SG2, according to Section 7.5, was prepared to make a suspension of relatively long CNTs. The supernatant was obtained through centrifugation with a centrifugal strength of 3,000*g* for 15 min. This supernatant was filtered with a 70- μ m cell strainer (BD Falcon™) to remove coarse agglomerates/aggregates. Then, after separating at centrifugal strength of 22,000*g* for 10 min, 10 mg/mL BSA aqueous solution was added to this sediment; after redispersing for approximately 5 min, the suspension was filtered with a 100- μ m cell strainer, and the filtrate was used as the stock suspension (Fig. A.2).



Figure A.2 Prepared suspension of SG-SWCNTs (stock suspension)

A.4 Evaluating the properties of the stock suspension

A.4.1 Assessment of the concentration of SG-SWCNTs

Figure A.3(a) shows the UV-Vis absorption spectrum of the SG-SWCNTs suspension prepared with a 10-mg/mL BSA aqueous solution (measured with the UV-2550, Shimadzu Corporation). From the average absorbance between 600 and 800 nm in wavelength, the relationship (calibration curve) between the mass concentration of CNTs and the absorbance of the suspension was obtained (Fig. A.3(b)).

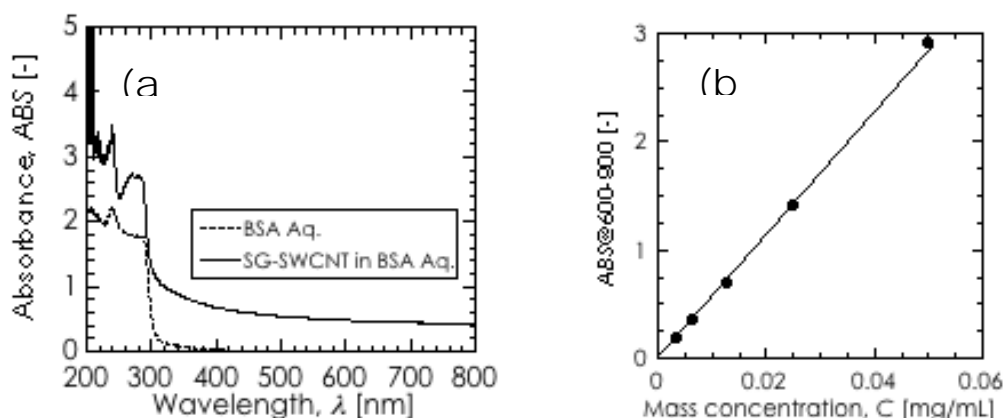


Figure A.3 (a) UV-Vis spectra for a suspension of SG-SWCNTs and an aqueous BSA solution. (b) Relationship between average absorbance of CNT suspension at 600–800 nm and mass concentration.

The SG-SWCNTs concentrations found from this calibration curve are shown in Table A.1. The concentration in SG1 was 0.81–0.88 mg/mL, and in SG2 was 0.78–1.00 mg/mL.

Table A.1 Properties of the dispersed samples SG1 and SG2.

Sample ID	CNT concentration	Cumulant average size	Geometric mean length	Geometric standard deviation
	[mg/mL]	x_{DLS} [nm]	l_g [nm]	s_g [-]
SG1-1	0.82	297	317	2.26
SG1-2	0.88	288	306	2.31
SG1-3	0.81	293	347	2.08
SG2-1	0.78	832	867	2.28
SG2-2	1.00	819	857	2.75

A.4.2 Secondary CNT particle size

The cumulant average particle sizes x_{DLS} measured by DLS using a Zetasizer Nano (Malvern) are shown in Table A.1 (see ISO 22412). For samples SG1 and SG2, which were prepared under different conditions, cumulant average particle size x_{DLS} also differs; on the other hand, x_{DLS} is roughly the same for samples with the same dispersion conditions.

A.4.3 Lengths of SG-SWCNTs

TEM micrographs (JEM-1010, JEOL) of the dispersed SG-SWCNTs are shown in Fig. A.4.

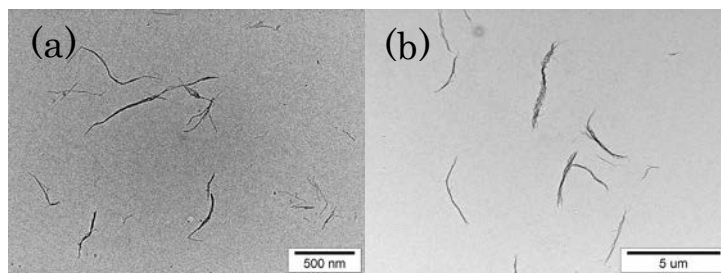


Figure A.4 TEM micrographs of the suspended SG-SWCNTs for (a) sample SG1 and (b) sample SG2.

From the TEM micrographs, the length distribution of approximately 1000 SG-SWCNTs was measured. Figure A.5 shows the cumulative distribution $Q_0(l)$ of lengths on the basis of their numbers. Table A.1 shows the geometric mean l_g and the geometric standard deviation s_g .

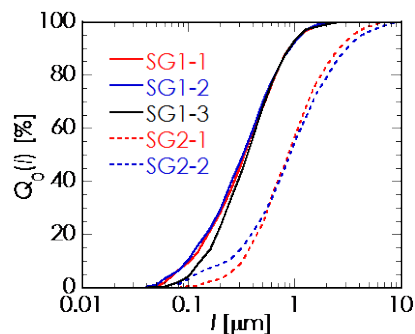


Figure A.5 Length distributions of dispersed CNTs.

A.4.4 Confirming endotoxins

A detection test for endotoxins in the stock suspension was performed using the limiting test for the gelification method in the 16th revision of the Japanese Pharmacopoeia.

After adding 0.2 mL of stock suspension to a Pyrotell™ Single Test vial with the detection limit of 0.03 EU/mL and mixing well, we placed the vial in a thermostatic bath (water bath) and left it to stand at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 60 ± 2 min.

After the vial was removed from the thermostatic bath, it was inverted to observe any gel formation occurrence. By this means, we confirmed that the endotoxin concentration was less than the Pyrotell™ detection limit.

A.5 Preparation of working solution

The stock suspension was diluted with a prescribed cell culture medium to make the working solution for the *in vitro* cell-based assay.

The person in charge of writing Chapter III.

Shigehisa Endoh, Ph.D.; Junko Maru.

IV. Characterization of CNT working solution for *in vitro* cell-based assay

Preface

In this section, we describe the procedure for evaluating the properties of CNT suspensions, especially those suspended in the culture media used for *in vitro* cell-based assay. The properties evaluated are stability of suspension, surface potential, intensity ratio of the G band and D band for Raman scattering (GD ratio), and amount of Bovine Serum Albumin (BSA). Measurement methods corresponding to each property in this procedure are dynamic light scattering (DLS), electrophoretic light scattering (ELS), Raman spectroscopy, and field flow fractionation (FFF).

1. Scope of application

The objects of the measurements are CNT suspension for *in vitro* cell-based assay.

2. Normative reference

The following referenced documents are indispensable for application in this document:

JIS Z8819-1:1999 Representation of particle size measurement results—Part 1: Pictorial method

JIS Z8819-2:2001 Representation of particle size measurement results—Part 2: Calculation of average particle size/diameter and the moment using the particle size distribution

JIS Z8826: 2005 Particle size analysis—photon correlation method

ISO 13321:1996, Particle size analysis—Photon correlation spectroscopy

ISO 22412: 2008, Particle size analysis—Dynamic light scattering (DLS)

JIS Z 8803: 1991, Liquid viscosity—measurement method

JIS K 2283: 2000, Crude oil and petroleum products—kinetic viscosity test method and viscosity index calculation method

ISO 13099-2:2012, Colloidal systems—Methods for zeta-potential determination—Part 2: Optical methods

3. Terms, definitions, and symbols

3.1 Terms and definitions (measurement methods)

3.1.1 Dynamic light scattering (DLS)

DLS is widely used to determine the size of Brownian nanoparticles in colloidal suspensions in the nano and submicron ranges. When particles are suspended in a liquid they are in constant random motion, i.e., Brownian motion, in which a given particle undergoes random position changes in time. Assuming the distribution for the displacement of a particle undergoing Brownian motion in aqueous solution is a Gaussian function, the average rms displacement in two dimensions can be calculated. Namely, the Stokes–Einstein equation can be used to

compute the mean particle size from the diffusion coefficient.

3.1.2 Electrophoretic light scattering (ELS)

ELS is based on DLS in which an electric field with a fixed direction is applied to particles in suspension. The frequency shift or phase shift of an incident laser beam depends on the suspended particles mobility. Thus, the electrophoretic mobility, which depends on the surface potential of individual particles, can be determined from the Doppler shift. This electrophoretic mobility is then used to calculate the zeta potential using the Smoluchowski equation.

3.1.3 Raman spectroscopy

Raman spectroscopy is a measurement method in which nanomaterials or polymers are irradiated with light of a specific wavelength. The modulation of the characteristic wavelength, caused by vibrational modes in the molecules or crystals, is observed as Raman scattered light shift. A method that combines Raman spectroscopy and an optical microscope in a Raman microscope enables analysis of structures and components of local structure.

3.1.4 Field flow fractionation (FFF)

This method is an elution technique wherein nanoparticles, microparticles, and macromolecules are separated by their physicochemical properties. Various FFF methods are attractive techniques for separating materials in colloidal suspensions by means of flow, centrifugal, magnetic, and thermal field control.

3.1.5 Dispersing medium

The dispersing medium is the liquid phase that disperses CNTs. An ordinary culture medium, such as DMEM (Dulbecco's modified Eagle's medium), is used for assessing *in vitro* cell-based assay.

3.1.6 Dispersant

Dispersants are used to suspend CNTs stably in the liquid phase. This procedure mainly uses BSA as the dispersant.

3.1.7 Suspension sample and particle sample

The original CNT bulk powder is called the particle sample, or alternatively, the nanoparticle sample. The liquid containing the particle sample that has been suspended in an appropriate suspension medium is called the suspension sample.

3.1.8 Particle diameter d

Particle diameter is the diameter of a particle assuming its shape to be spherical. If the particle

is a true sphere, then particle diameter equals true diameter.

3.1.9 Particle size distribution and average particle size

The light-scattering intensity averaged diameter, \bar{d}_{DLS} , is obtained from the following formula:

$$\bar{d}_{\text{DLS}} = \frac{\sum_{i=1}^N \Delta Q_{\text{int},i}}{\sum_{i=1}^N \frac{\Delta Q_{\text{int},i}}{d_i}}$$

Here, N is number of segments, d_i is average particle size for segment i , and $\Delta Q_{\text{int},i}$ is scattered light intensity. Depending on the apparatus, the volume-based (or weight) average particle diameter d_w or the number-based (or number) average particle diameter d_n may be available. It is recommended that the definitions of these average particle sizes be checked in operating manuals.

3.1.10 Zeta potential

The potential of the “slip face” at which a liquid flow starts to occur inside the electrical double layer formed on the hydrodynamic surface of a particle is called the zeta potential (expressed in mV). Although, this is in a strict sense different from the potential on the particle surface, it is used as the surface potential.

3.1.11 GD ratio

The intensity ratio of the G-band to the D-band (the GD ratio) is used as an index to measure crystal defects in a surface. In this procedure, GD ratio is calculated as the ratio of the intensity of the peak at 1600 cm⁻¹, which is derived from the graphite structure, to the intensity of the peak at 1350 cm⁻¹, which is derived from defects.

3.2 Abbreviated terms and units

\bar{d}_{DLS}	Scattered intensity average particle size	nm
\bar{d}_w	Volume-based (or weight) average particle size	nm
\bar{d}_n	Number-based (or number) average particle size	nm
I_s	Scattered light intensity	
c	Particle concentration in suspension sample (number of particles or mass per unit volume)	
η	viscosity	cP (=0.01 g s ⁻¹ cm ⁻¹)
ξ	zeta potential	mV

4. Principles

4.1 DLS

Nanoparticles or their aggregate/agglomerate suspended in a liquid phase exhibit a jiggling motion termed as “Brownian motion,” which is attributed to collisions with the surrounding liquid’s molecules. When illuminated with light, temporal fluctuations in the intensity of scattered light caused by Brownian motion can be observed, which are used to trace the diffusion coefficient D for the particles. If temperature T and liquid viscosity η are known, then particle size d can be determined from the Stokes–Einstein equation,

$$d = \frac{k_B T}{3\pi\eta D}$$

Here, k_B is the Boltzmann constant. Since there is a distribution of particle sizes in the sample, the particle size given by this equation is a type of average particle size.

4.2 ELS

When nanoparticles or their aggregate/agglomerate are distributed in a liquid, their surface contains either a positive or negative charge. When an electrical field is applied in a constant direction, movement associated with the value of the surface potential can be observed. Using the Smoluchowski equation, based on the mobility, the zeta potential can be evaluated from the equation

$$\xi \propto \frac{\eta U}{\epsilon}$$

Here, η is the solvent viscosity, U is the mobility, and ϵ is the dielectric constant of the solvent. In this procedure, we tentatively use the dielectric constant for water. Because there is a distribution of surface potentials, the zeta potential given by the above equation is a type of average value.

4.3 Raman spectroscopy

When nanoparticles or particles in suspension are irradiated with light of a specific wavelength, inelastic scattered light can be observed. This scattered light has basically the same wavelength as the irradiating light; however, there is inelastic scattering of the characteristic wavelength. This inelastic scattering depends on the vibrational mode of the molecules or crystals and is observed as Raman scattered light. By measuring the width of the frequency shift, the vibrational frequency of the molecule can be determined. In a Raman microscope (basically a distributed Raman spectrometer), the Raman scattered light is suspended using a diffraction grating, and using an attached microscope, structural and component analyses of a local structure on the targeted area can be performed at spatial resolutions of less than one micron.

4.4 FFF

For CNTs suspended in suspension, the speed of self-diffusion is small for large particles and large for small particles. To exploit this phenomenon, a distribution of particle speeds is created

lengthwise in an FFF separation cell. By applying an external field opposite to the direction of particle diffusion and using the unevenness of the flow in the laminar flow field, particles can be classified by size from the observed differences in efflux time, which depend on where the particles are located. For example, with Flow-FFF, the external field is a cross flow.

$$t_r = \frac{\pi \eta d w^2}{2kT} \bullet \frac{V_{cross}}{V_{channel}}$$

Here d is the particle diameter in the sample, w is the channel spacer thickness, η is the solvent viscosity, k is the Boltzmann constant, T is the temperature, V_{cross} is the cross-flow flow rate, and $V_{channel}$ is the channel flow rate. If temperature is not constant, by holding crossflow flow rate and channel flow rate constant, the elution time of the sample is proportional to the particle size, making separation by particle size possible.

5. Equipment

5.1 DLS

5.1.1 Apparatus

This procedure assumes the use of commercially DLS equipment.

5.1.2 Validation

Varidation can be verified in advance using standard substances. For example, by measuring a polystyrene latex standard, it can be assessed whether or not the certified value and the measured value agree to within the range of uncertainty. As an example, we recommend that the validity of the equipment be assessed using a monosuspended polystyrene latex standard with an average particle size of 100 nm. The deviation of the measured value from the certified value should be within $\pm 2\%$, and the reproducibility for each repeated measurement should have a relative experimental standard deviation of less than 2% (see ISO 22412).

5.1.3 Temperature control

The DLS equipment must have the capability to ensure that the measurement cell temperature falls within the required temperature range. The recommended accuracy for the temperature shall be within $\pm 0.3^\circ\text{C}$ (see ISO 22412). The temperature may be controlled as needed, using an external recirculating thermostatic water tank.

5.1.4 Test report

The equipment must be capable of calculating the scattered intensity average particle size, the volume-based average particle size, number-based average particle size, particle size

distribution, polydispersity, and reporting results from these calculations. It is desirable to display and output the autocorrelation function, or alternatively, the power spectrum as needed.

5.2 ELS

5.2.1 Apparatus

This procedure assumes the use of commercially available ELS equipment.

5.2.2 Validation

The validity of the equipment can be verified in advance using standard substances. For example, by measuring a polystyrene latex standard substance, one can assess whether the certified value and the measured value agree to within the range of uncertainty. The validity of the equipment should be assessed using a monosuspended polystyrene latex standard substance with a zeta potential value of 60 mV. Furthermore, the deviation of the measured value from the certified value should be within $\pm 10\%$, and the reproducibility for each repeated measurement should have a relative experimental standard deviation of less than 10%. In particular, for ELS in which the electrode is immersed directly in the suspension solution, the electrode can easily become contaminated and affect measured values. Therefore, it is recommended that this operation be performed frequently.

5.2.3 Temperature control

The ELS equipment must have the capability to ensure that the measurement cell temperature falls within the required temperature range. The recommended accuracy for the temperature should be within $\pm 1.0^\circ\text{C}$ (see ISO 22412). The temperature may be controlled using Peltier cooling as needed.

5.2.4 Test report

The equipment must be capable of calculating the electrical mobility, the conductivity of the suspension solution, and the zeta potential. It is desirable that the equipment display and output the power spectrum.

5.3 Raman spectra

5.3.1 Apparatus

This procedure assumes the use of a commercially available Raman spectrometer.

5.3.2 Validation

The validity of the equipment should be verified in advance using standard substances. For example, by measuring a polystyrene standard substance or a neon lamp, one can assess whether the certified and measured values agree to within the range of uncertainty.

Furthermore, the deviation of the measured value from the certified value should be within ± 3 cm^{-1} , and the reproducibility for each repeated measurement should have a relative experimental standard deviation of less than 3%. The alignment (i.e., the focus of the microscope and adjustment of the optical system) is performed using an alignment tool that depends on the measurement apparatus itself. Therefore, it is best to check the alignment every time measurements are started.

5.3.3 Test report

The equipment must be capable of displaying the Raman spectrum and outputting the result.

5.4 FFF

5.4.1 Apparatus

This procedure assumes the use of commercially available FFF equipment. To output a fractogram, a UV detector must be connected as an online concentration detector. To calculate particle size for each fraction, it is recommended to connect a light-scattering detector, such as multiple-angle light-scattering detector.

5.4.2 Validation

The validity of the equipment can be verified in advance using standard substances. For example, by measuring a protein standard substance, it can be assessed whether or not a separation can be reasonably expected. Furthermore, the validity of the equipment can be assessed using a commercially available mixed sample of Cytochrome C and Myoglobin equine, or a standard substance with a distribution of particle sizes made from polystyrene latex. For the former, to confirm that the peaks corresponding to each protein are separated from each other and for the latter that deviations of measured values from certified values of particle size distribution are within 10%. Reproducibility for each repeated measurement should have a relative experimental standard deviation of less than 10%. In particular, because of the inside of the CNT suspension solution system becomes contaminated, it is recommended that the operation be performed with moderate frequency.

5.4.3 Temperature control

The online concentration detector must have the capability to ensure that the temperature of the measurement cell falls within the required temperature range. The recommended accuracy for the temperature should be within $\pm 1.0^\circ\text{C}$.

5.4.4 Test report

The equipment must be capable of displaying FFF fractograms and outputting the results.

6. Reagents and equipment

6.1 Reagents

The solvent used for cleaning the measurement cell and for diluting, must be a pure substance that does not contain any fine particles, especially fine particles larger than the particles in the suspended sample. For example, if water is used as the solvent for suspension, filtered ultrapure water should be used.

Note: The “ultrapure water” in this procedure is defined here as “pure water that has been purified with an ion-exchange filter or a filter smaller than 0.2 μm , has an electrical resistivity higher than 18 $\text{M}\Omega\cdot\text{cm}$, and does not contain fine particles of total organic carbon concentration less than 5 ppb.

6.2 Standard substances

Characteristic values for standard substances should be such that they provide clearly stated traceability. Approximately 100-nm polystyrene latex suspended in water can be used as a standard substance for particle size. When freezing standard substances, care should be taken to ensure that no chemical association or precipitation occurs.

6.3 Equipment

Common containers and dispensing burettes may be used; however, vessels or equipment that can change the physicochemical properties of the contents (such as pH) are not to be used. For example, ordinary soda glassware can change the pH of the contents, so use of quartz glassware is preferred. In contrast, measurements using a Raman microscope have no effect on pH, and thus commercially available glass slides can be used as substrates without any problem.

6.4 Digital camera

Use of a digital camera is recommended for recording the state of suspended samples.

6.5 Viscosity meter

A viscosity meter is used to determine the viscosity of suspended samples. This viscosity meter is selected as one appropriate for the kinematic viscosity of the suspended sample. For a dilute aqueous solution, the kinematic viscosity is 0.894 cP mL g^{-1} (25°C), which means that an Ubbelohde viscosity meter, a Cannon–Fenske viscosity meter, or something similar can be used. Note: Please note that the absolute viscosity should be used for the Stokes–Einstein equation, not the kinematic viscosity.

7. Operations

7.1 DLS

7.1.1 Installing equipment

Equipment should be installed in a clean environment, while avoiding factors such as electrical noise, mechanical vibrations, and direct sunlight (see ISO 22412 Section 8.1).

Warning: The DLS equipment uses a laser as light source, which can damage human eyes. Do not look directly at incident laser light, and do not allow any reflected laser light to enter the eyes. Safety codes established for the facility must be obeyed.

7.1.2 Preparation of the suspended sample

To avoid decomposition of the culture medium, which acts as the dispersing medium for nanoparticles, the suspended samples should be stored in a refrigerator at low temperature but must also not be allowed to freeze.

When reproducibility of data is within experimental error, the measured sample should be taken from a fixed location in the suspension solution that has been left to stand. For example, it should always be collected at the same depth from the surface. This is because we can assume that, even for a stable suspension, a concentration gradient gradually forms as a result of gravity. Thus, even for suspensions prepared in the same way, the concentrations may be significantly different depending on the location at which samples are taken from the solutions. The suspension sample can be measured at room temperature without any pretreatment, such as agitation using an ultrasonic bath. To avoid any scattering of light by dust, all samples related to the measurement should be prepared in a clean environment.

7.1.3 Visual observation of the suspension sample

When the suspension sample is acquired, and again before and after DLS measurements, the presence or absence of precipitates should be visually checked and the degree of precipitation (if any) should be recorded. If possible, precipitates should be recorded using a digital camera.

7.1.4 Measurement of viscosity

Any reliable values available in the literature may be used. However, when such values are not available, the viscosity of the suspended sample should be measured.

Note: When the dispersing medium is a cell culture, an Ubbelohde viscosity meter can be used by immersing the viscosity meter in a thermostatic bath while controlling the temperature to within $\pm 0.1^\circ\text{C}$.

Note: Owing to the presence of CNTs, the viscosity of the suspended sample may rise significantly. In this case, the proportionality of the viscosity of the dispersing medium to the concentration of protein in the medium can be used; measuring the protein concentration with an appropriate method (for example, the flow-FFF separation method or gel electrophoresis) and then computing the viscosity of the suspension medium is recommended.

7.1.5 Cleaning equipment used in DLS measurements

Sample containers and experimental equipment that come into contact with the suspended sample must be kept away from particles. In addition, contamination from previous particle samples must be avoided. For example, measurement cells and syringes that are used repeatedly should ideally be “optically cleaned.” Ordinary disposable Pasteur pipettes can often be used as they are but should be optically cleaned if there are any concerns. If optical cleaning cannot be conducted, the equipment can be cleaned with a filtered/distilled dust-free solvent. Contaminated measurement cells, vessels, and solvents should not be used.

Note: “Optical cleaning” utilizes the reflux of boiled acetone vapor in order to clean repeatedly used equipment. Using a cooling tube containing a flow of cold water, the equipment is washed for around 30 min with acetone droplets that condense out, following which the equipment is dried. It resembles any ordinary reflux apparatus but requires some ingenuity to set up.

7.1.6 Blank measurements

A blank measurement is conducted in advance to verify that there are no contaminating particles. Usually, scattering intensity is measured from the water that is used to clean the measurement cell and dilute the sample. Any contamination results in strong scattering observed.

Note: If the cell cannot be removed from the apparatus, the cell should either be cleaned repeatedly with cleaning solvent or prewashed with the sample. The presence of contamination should be examined by observation for the scattered light.

7.1.7 Measurements

7.1.7.1 Skills

The DLS equipment operator must have sufficient skill acquired through extensive training. Furthermore, they must also have sufficient expertise in measurement techniques; for example, whether they can report values that agree with certified values for particle size standard substances.

7.1.7.2 Measurement environment

The apparatus should be installed in a clean environment. Furthermore, the environment in which the equipment is handled, the environment in which samples are prepared, and lab coats for persons making the measurements should all be clean. In particular, dust can easily be incorporated into water, interfering with the measurements.

7.1.7.3 Preparation of the DLS equipment

Before performing measurements, the apparatus must be warmed up so that stable data can be acquired.

Before starting the measurements, the measurement cell must first reach the temperature set

in advance. The temperature during the measurement must also fall within the prescribed limits, i.e., within $\pm 0.3^{\circ}\text{C}$.

Note: Stability of the laser varies greatly depending on the type of laser, and between 15 and 30 min is required to stabilize laser intensity and measuring cell temperature.

Note: Operators should assess in advance whether the measuring cell has actually attained the set temperature.

If the liquid volume of the measured sample is large (for example, an inner diameter of 9 mm and a volume of 2 mL), it may require more than 45 min to reach the set temperature (from 22°C to $25.0^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$).

Note: CNT samples generally have a strong absorption coefficient over a broad range of wavelengths; thus, except for ultradilute samples, using transmission-type DLS may be problematic for performing particle-size measurements. If scattered light intensities from a CNT sample drop unusually, special care is needed because it is possible that highly reliable particle-size measurements cannot be made using DLS.

7.1.7.4 Measurement and analysis

Measurements and analyses are conducted according to the procedures for each apparatus. A minimum of three measurements should be conducted to assess measurement accuracy.

7.1.7.5 Recording

The following experimental conditions are recorded: information about the suspended sample, time of the measurement, time taken for the measurement, temperature of the suspended sample, refractive index of the suspended sample, viscosity of the suspended sample, concentration of the suspended sample, wavelength of the laser, and scattered intensities of the blank and the suspended sample.

The following results are recorded: scattered intensity average particle size, scattered intensity, and polydispersity. If necessary, the following should also be recorded: volume-based average particle size, number-based average particle size, particle-size distribution, and outer appearance of the suspended sample.

7.2 ELS

7.2.1 Installation of the equipment

The equipment should be installed in a clean environment, while avoiding any electrical noise, mechanical vibrations, direct light, etc. (see ISO 22412 Section 8.1).

Warning: The ELS equipment uses a laser as light source, which can damage human eyes. Do not look directly at incident laser light, and do not allow any reflected laser light to enter the eyes. Safety codes established for the facility must be obeyed.

7.2.2 Preparation of the suspended sample

To avoid decomposition of the culture medium, which acts as the dispersing medium, suspended samples should be stored in a refrigerator at low temperature but must also not be allowed to freeze.

When reproducibility of data is a priority, the measured sample should ideally be taken from a fixed location in the suspension that has been left to stand. For example, it should always be collected at the same depth from the surface because we can assume that a concentration gradient gradually forms due to gravity even for stable suspensions. Thus, for suspension solutions prepared in the same way, the concentrations may be significantly different depending on the location at which samples are taken from the solutions.

The suspension sample can be measured at room temperature without any pretreatment, such as agitation using an ultrasonic bath. To avoid any scattering of light and dust, all samples related to the measurement should be prepared in a clean environment.

7.2.3 Visual observation of the suspended sample

When the suspension sample is acquired before and after ELS measurements, the presence or absence of precipitates should be visually checked and the degree of precipitation (if any) recorded. If possible, precipitates should be recorded using a digital camera.

7.2.4 Measurement of viscosity

Any reliable values available in the literature may be used. However, when such values are not available, the viscosity should be measured.

Note: When the dispersing medium is a cell culture, an Ubbelohde viscosity meter can be used by immersing the viscosity meter in a thermostatic bath while controlling the temperature to within $\pm 0.1^{\circ}\text{C}$.

Note: Due to the presence of CNTs, the viscosity of the suspended sample may rise significantly. In such a case, the proportionality of the viscosity of the dispersing medium to the concentration of protein in the medium may be used; we recommend measuring the protein concentration with an appropriate method (for example, the flow-FFF separation method or gel electrophoresis) and then computing the viscosity of the dispersing medium.

7.2.5 Cleaning equipment used in ELS measurements

Sample containers and experimental equipment that come into contact with the suspended sample must be kept away from fine particles, and contamination from previous samples must be avoided. For example, often-used measurement cells and syringes should ideally be “optically cleaned.” Ordinary disposable Pasteur pipettes, and the like, can often be used as they are, but if there are any concerns, optical cleaning should be performed. If, for some reason, optical cleaning cannot be conducted, the equipment can be cleaned with a filtered/distilled dust-free

solvent. Measurement cells, vessels, and solvents that have been confirmed to be contaminated should not be used.

Note: “Optical cleaning” is a cleaning method that utilizes the reflux of boiled acetone vapor in order to clean equipment that is used repeatedly. Using a cooling tube containing a flow of cold water, the equipment is washed for around 30 min with acetone droplets that condense out; then it is dried. It resembles any ordinary reflux apparatus, but some ingenuity is required to set it up.

7.2.6 Blank measurements

A blank measurement is conducted in advance to verify that there are no contaminating particles. Usually, scattering intensity is measured from water used to clean the measurement cell and dilute the sample. Any contamination results in strong scattering observed.

Note: If the cell cannot be removed from the apparatus, the cell should either be cleaned repeatedly with cleaning solvent or prewashed with the sample. The presence of contamination should be determined by measuring scattered light.

7.2.7 Measurements

7.2.7.1 Skills

The ELS apparatus operator should have sufficient skill acquired through extensive training. They must also have sufficient expertise in measurement techniques; for example, whether they can report values that agree with certified values for particle size standard substances.

7.2.7.2 Measurement environment

The apparatus should be installed in a clean environment. Further, the environment in which the equipment is handled, the environment in which the samples are prepared, and lab coats for the persons making the measurements should all be clean. In particular, care is required because dust and grit can easily be incorporated into water interfering with the measurements

7.2.7.3 Preparation of the ELS equipment

Before performing measurements, the apparatus must be warmed up so that stable data can be acquired.

Before starting the measurements, the measurement cell must first reach the temperature that was set in advance. That temperature during the measurement must also fall within the prescribed limits, i.e., within $\pm 0.3^{\circ}\text{C}$.

Note: Stability of the laser varies greatly depending on the type of laser equipment, and between 15 and 30 min is required to stabilize the laser intensity and the measuring cell temperature.

Note: CNT samples generally have a strong absorption coefficient over a broad range of

wavelengths; so, except for ultradilute samples, using transmission-type ELS to perform particle-size measurements is problematic. If scattered light intensities from a CNT sample drop unusually, special care is needed because it is possible that highly reliable particle-size measurements cannot be made using the ELS equipment.

7.2.7.4 Measurement and analysis

Measurements and analyses are conducted according to the procedures for each apparatus. A minimum of three measurements should be conducted to assess measurement accuracy.

7.2.7.5 Recording

The following experimental conditions are recorded: information about the suspended sample, time of the measurement, time taken for the measurement, temperature of the suspended sample, refractive index of the suspended sample, viscosity of the suspended sample, concentration of the suspended sample, wavelength of the laser, and scattered intensities of the blank and suspended sample.

The following results are recorded: zeta potential, electrical mobility, conductivity, base frequency, and amount of Doppler shift calculated using various equipment.

7.3 Raman spectroscopy

7.3.1 Installing equipment

Equipment should be installed in a clean environment, while avoiding any electrical noise, mechanical vibrations, direct sunlight, etc..

Warning: Raman spectroscopy uses a laser as light source, which can damage human eyes. Do not look directly at incident laser light, and do not allow any reflected laser light to enter the eyes. Safety codes established for the facility must be obeyed.

7.3.2 Preparation of the measurement sample

An adequately CNT bulk powder is placed directly on a microscope slide. To obtain the appropriate measurement sample, a quantity of the suspension is dropped onto the microscope slide and then dried.

Warning: The nanoparticle powder should be prepared under conditions that prevent scattering. When handling CNT powders, nanomaterial safety regulations must be observed, such as wearing protective glasses, gloves, and a mask.

7.3.3 Cleaning of equipment used in the measurements

We recommend the use of ordinary disposable glass slides. Any measuring equipment that has been confirmed to be contaminated should not be used.

7.3.4 Measurements

7.3.4.1 Skills

The Raman spectrometer operator should have sufficient skill acquired through extensive training. They must also have sufficient expertise in measurement techniques; for example, whether they can report values that agree with certified values for spectra.

7.3.4.2 Measurement environment

The spectrometer should be installed in a clean environment. Further, the environment in which the equipment is handled, the environment in which the samples are prepared, and lab coats for the persons making the measurements should all be clean.

7.3.4.3 Preparation of the Raman spectrometer

Before making measurements, the apparatus must be warmed up so that stable data can be acquired. The stability of the laser varies greatly depending on the type of laser equipment, and 15–30 min of warm up are usually required to stabilize the laser intensity. To improve measurement accuracy, the apparatus' alignment tool is used to adjust the optical axis of the spectrometer and laser and to calibrate the instrument in order to obtain accurate spectra. For measurements on minute samples, alignment and calibration must be done the day before the start of measurements.

7.3.4.4 Measurement and analysis

Measurements and analyses are conducted according to the procedures for each apparatus. A minimum of three measurements should be conducted to assess measurement accuracy.

7.3.4.5 Recording

The following experimental conditions are recorded: information about the measurement sample, time of the measurement, time taken for the measurement, and wavelength of the laser. The following results are recorded: measured results for the G-band Raman spectrum and the D-band Raman spectrum.

7.4 FFF

7.4.1 Installation of the equipment

Equipment should be installed in a clean environment, while avoiding any electrical noise, mechanical vibrations, direct sunlight, etc..

Warning: When a multi-angle light scattering detector is used as the online particle detecting device, laser light is used as the light source, and laser light can damage human eyes. Do not look directly at incident laser light, and do not allow any reflected laser light to enter the eyes. Safety codes established for the facility must be obeyed.

7.4.2 Preparation of the suspended sample

To avoid any decomposition of the culture medium, which acts as the dispersing medium, nano particle suspended samples should be stored in a refrigerator at low temperature but must also not be allowed to freeze.

When reproducibility of data is a priority, the measured sample should ideally be taken from a fixed location in the suspension solution left to stand. For example, it should always be collected at the same depth from the surface. This is because we can assume that a concentration gradient gradually forms due to gravity even for a stable suspension solution. Thus, for suspension solutions prepared in the same way, the concentrations may be significantly different depending on the location at which samples are taken from the solutions. The suspension sample can be measured at room temperature without any pretreatment, such as agitation using an ultrasonic bath sonicator. To avoid any scattering of light by grit and dust, all samples related to the measurement should be prepared in a clean environment.

7.4.3 Visual observation of the suspended sample

When the suspension sample is acquired before and after FFF measurements, the presence or absence of precipitates should be checked visually, and the degree of any precipitation recorded. If possible, precipitates should be recorded using a digital camera.

7.4.4 Cleaning of the equipment used in FFF measurements

Sample containers and experimental equipment that comes into contact with the suspended sample must be kept away from fine particles, while avoiding contamination from previous samples. For example, measurement cells and syringes that are used repeatedly should be “optically cleaned.” Ordinary disposable Pasteur pipettes, , can often be used as they are, but if there are any concerns, optical cleaning should be performed. If, for some reason, optical cleaning cannot be conducted, the equipment can be cleaned with a filtered/distilled dust-free solvent. Measurement cells, vessels, and solvents that have been confirmed to be contaminated should not be used.

Note: “Optical cleaning” is a cleaning method that utilizes the reflux of boiled acetone vapor. Using a cooling tube containing a flow of cold water, the equipment is washed for around 30 min with acetone droplets that condense out; then it is dried. It resembles any ordinary reflux apparatus, but some ingenuity is required to set it up.

7.4.5 Blank measurements

A blank measurement is conducted in advance to verify that there are no contaminating particles. Usually, the scattering intensity or UV absorbance is measured for the water used to clean the measurement cell and to dilute the sample. If there is any contamination, there is

strong scattering or specific absorbance.

Note: When the cell cannot be removed from the apparatus, the cell should either be cleaned repeatedly with cleaning solvent or prewashed with the sample. The presence of contamination should be determined by measuring scattered light or UV absorbance.

7.4.6 Measurements

7.4.6.1 Skills

The FFF apparatus operator should have sufficient skill acquired through extensive training. They must also have sufficient expertise in measurement techniques; for example, whether they can report values that agree with certified values for particle size standard substances.

7.4.6.2 Measurement environment

The apparatus should be installed in a clean environment. Further, the environment in which the equipment is handled, the environment in which the samples are prepared, and lab coats for the persons making the measurements should all be clean. In particular, care is required because dust and grit can easily be incorporated into water interfering with the measurements.

7.4.6.3 Preparation of the FFF equipment

Before making measurements, the apparatus, especially the detector, must be warmed up so that stable data can be acquired.

Before starting the measurements, the measuring cell must first attain the temperature that was set in advance. During the measurements, temperatures must stay within the temperature limits, i.e., within $\pm 0.3^{\circ}\text{C}$.

Warning: Stability of the laser in the online light-scattering detector varies greatly depending on the type of laser equipment, but usually it requires 15–30 min to stabilize the laser intensity and the temperature of the measuring cell. This Note also applies to the online UV detector.

7.4.6.4 Measurement and analysis

Measurements and analyses are conducted according to the procedures for each apparatus. A minimum of three measurements should be conducted to assess measurement accuracy.

7.4.6.5 Recording

The following experimental conditions are recorded: information about the suspended sample, time of the measurement, time taken for the measurement, temperature of the suspended sample, refractive index of the suspended sample, viscosity of the suspended sample, concentration of the suspended sample, wavelength of the laser, UV wavelength, and scattered intensity and UV intensity of the blank and suspended sample.

The following results are recorded: FFF fractograms, and values of particle size and light

intensity or UV intensity for each fraction that are displayed with each particular fraction. Also recorded is the calculated decrement (ideally the percentage by weight) for the intended fraction (the amount of BSA in the medium).

8. Data analysis

8.1 Calculation of values

Values reported for average particle size, scattered intensity, and GD ratio, for example, are average values over several repetitions. The method for calculating average values can be chosen, but the details of the calculation must be reported with the averages.

8.2 Evaluation of uncertainties

Uncertainties should be determined for reported values. When this is done, the method used for determining each uncertainty must be clearly specified. This allows readers to judge the validity of the reported uncertainties. For example, since evaluation of detailed uncertainties for DLS measurements poses a great challenge, it is sufficient to use an approximate calculation based on only the main causes of the uncertainty. Sources of uncertainty include the apparatus type, measurement method, analysis method, and fluctuations in repeated measurements.

If the uncertainties due to the difference in DLS instruments cannot be evaluated in advance, they can be estimated from published information. However, the source for the information should be referenced.

9. Report

The following items are reported.

9.1 General

- a) Detailed information that identifies the suspended sample (e.g., information about the particle sample, the suspension sample preparation, information about the dispersing medium, the date the suspended sample was acquired, and the method of transportation).
- b) Measurement date, name of the person doing the measurements, and name of the organization doing the measurements.
- c) Information about standard substances used in verifying the validity of the apparatus (including the manufacturer) and the date on which validation was conducted.
- d) Storage conditions for the suspended sample (e.g., storage temperature, storage facility, and storage time).
- e) Results of visual observations related to precipitation formation (e.g., images from a digital camera).

9.2 Apparatus, and experimental and analysis conditions

- a) Name of the apparatus, model, and name of the manufacturer
- b) Standard substance used in validating particle-size measurements and validation results.
- c) Cleaning conditions for measuring cells, etc.
- d) Information about the reagent if dilution is needed
- e) Concentration of particles in the suspended sample, if possible
- f) Refractive index and viscosity of the suspension (information on the literature if data were obtained from it)
- g) Measurement temperature
- h) Measurement method (time correlation or frequency analysis for DLS, and suspended or FT for the Raman) and analysis method (e.g., the cumulant method for DLS)
- i) Measurement angle
- j) Information on composition of the dispersing medium (qualitative information also acceptable)
- k) Volume of the suspended sample in the cell
- l) Method for calculating reported values (e.g., method used to find averages)
- m) Method for calculating uncertainties and source materials, when used
- n) Number of repeated measurements

9.3 Results

9.3.1 DLS

- a) The scattering-intensity average particle size or its variation fraction with standard deviation over repetitions or the uncertainty
- b) The standard deviation of the volume-based (or weight-based) average particle size or the number-based average particle size including the repeatability of the measurements
- c) The scattering intensity or its variation fraction with standard deviation or the uncertainty

9.3.2 ELS

The zeta potential and its standard deviation over repetitions or the uncertainty

9.3.3 Raman spectroscopy

- a) The GD ratio from the ratio of the G-band and D-band spectra and its standard deviation including the repeatability of the measurements or the uncertainty
- b) For SWCNTs, the RBM spectral values and values of the calculated diameter, their standard deviation including the uncertainty by the repeatability of the measurements

9.3.4 FFF

The free protein content in the medium (amount of BSA) or the percentage by weight of the decrement and its standard deviation over repetitions or the uncertainty

Appendix A

System diagram of the methodology for evaluating individual properties

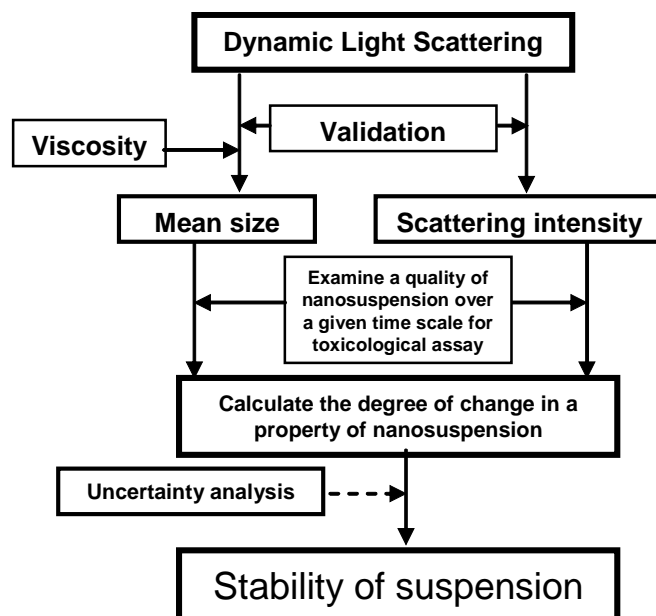


Figure A.1 Flow of methodology for evaluating suspension stability.

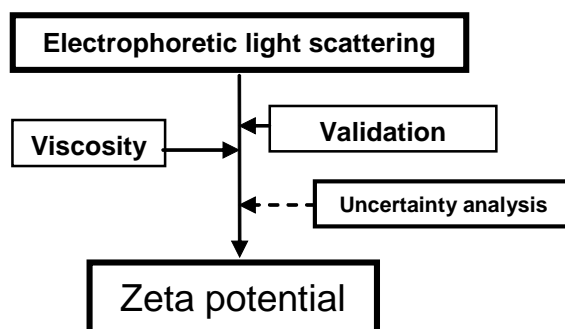


Figure A.2 Flow of methodology for evaluating zeta potential.

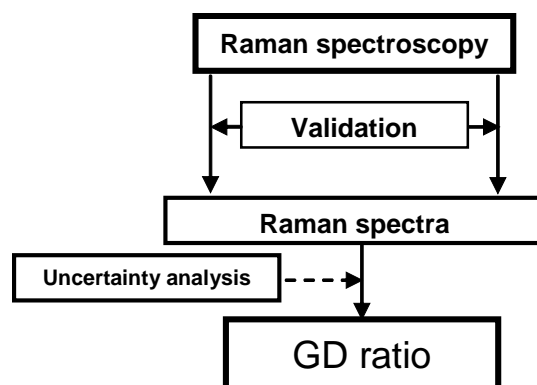


Figure A.3 Flow of methodology for evaluating GD ratio.

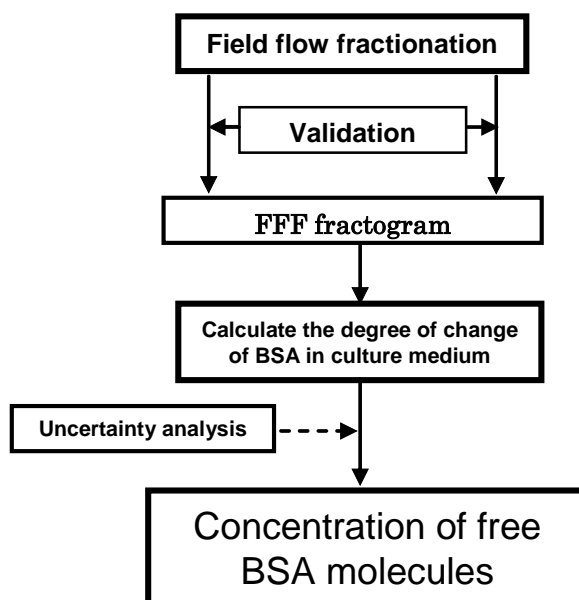


Figure A.4 Flow of methodology for evaluating bovine serum albumin (BSA) amount.

Appendix B

Example: results from a DLS measurement for a SG-SWCNT sample

B.1 Evaluating the stability of suspension for the evaluation period (concentration)

Figure B.1 shows the results of plotting daily variations in light-scattering intensity for each SG-SWCNT suspended sample, which is expressed by

$$I \propto I_0 \frac{c}{2r^2} \left(\frac{2\pi}{\lambda} \right)^4 \left(\frac{d}{2} \right)^6 \left| \frac{m^2 - 1}{m^2 + 2} \right|^2 \quad (\text{B.1})$$

Assuming all particles are the same size, it is possible to predict the number of particles by comparing particles whose differences in relative refractive indices are roughly the same. Here, d is the particle size, I_0 is the incident light intensity, I is the actual measured light intensity, r is the distance, c is the number concentration of particles, and m is the relative refractive index. Figure B.1 shows that unacceptable changes in the scattering intensity were not observed in any of the samples, and all samples were rated as being extremely stable. This result reflects the fact that no significant changes in the values of particle size or apparent gravitational sedimentation occurred because of the DLS measurement.

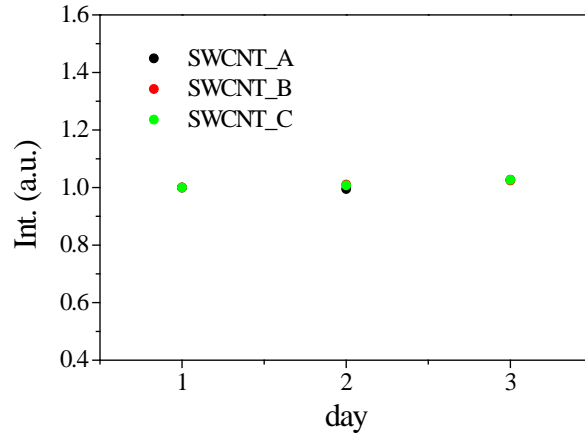


Figure B.1 Time dependence for changes in relative light-scattering intensity for a SG-SWCNT suspension sample. Relative intensity values are plotted relative to that of day one.

B.2 Evaluating the stability and particle size of the suspension for the evaluation period (secondary particle size)

Details for particle size calculation and uncertainty assessment are given below.

B.2.1 Calculation of the measured average value

Each value of average particle size (subscripts on d are n {number average} and I {light intensity average}) is labelled with the analytical method (in parentheses: C {Cumulant method} and M {Marquadt method}) and the measurement date (time), as a subscript of characters to the right {measurement day (the first day as 1, the second day as 2, the third day as 3, and for repeated measurements also adding, 1 for the first time, 2 for the second time)}. Final values were calculated as arithmetic average of all the measured values. For example, when the most reliable analysis method is the Marquadt method, particle sizes based on the Marquadt method were calculated as shown in equation B.2 and B.3.

$$\overline{d_n} = \overline{d_n(M)} = \frac{\left(\sum_{i=1}^n d_n(M)_{1,i} + \sum_{i=1}^m d_n(M)_{2,i} + \sum_{i=1}^p d_n(M)_{3,i} \right)}{n + m + p} \quad (\text{B.2})$$

$$\overline{d_I} = \overline{d_I(M)} = \frac{\left(\sum_{i=1}^n d_I(M)_{1,i} + \sum_{i=1}^m d_I(M)_{2,i} + \sum_{i=1}^p d_I(M)_{3,i} \right)}{n + m + p} \quad (\text{B.3})$$

B.2.2 Uncertainty between analysis methods

For the calculation of the uncertainty between analytical methods (u_{method}), the uncertainty between the Cumulant and Marquadt methods was calculated as Type B using (B.4) and (B.5). For average particle sizes based on the Cumulant method, values calculated on the basis of (B.6) and (B.7) were used.

$$u_{method}(\overline{d_n}) = \frac{|\overline{d_n(M)} - \overline{d_n(C)}|}{\sqrt{3}} \quad (\text{B.4})$$

$$u_{method}(\overline{d_I}) = \frac{|\overline{d_I(M)} - \overline{d_I(C)}|}{\sqrt{3}} \quad (\text{B.5})$$

$$\overline{d_n(C)} = \frac{\left(\sum_{i=1}^n d_n(C)_{1,i} + \sum_{i=1}^m d_n(C)_{2,i} + \sum_{i=1}^p d_n(C)_{3,i} \right)}{n + m + p} \quad (\text{B.6})$$

$$\overline{d_I(C)} = \frac{\left(\sum_{i=1}^n d_I(C)_{1,i} + \sum_{i=1}^m d_I(C)_{2,i} + \sum_{i=1}^p d_I(C)_{3,i} \right)}{n + m + p} \quad (\text{B.7})$$

B.2.3 Uncertainty between equipment

The standard uncertainty between machines (here, equipment A, B, and C) is evaluated as the disparity between average values over the whole period.

$$u_{app}(\overline{d}_n) = \frac{\sqrt{(d_n(A) - \overline{d}_n)^2 + (d_n(B) - \overline{d}_n)^2 + (d_n(C) - \overline{d}_n)^2}}{\sqrt{2}} \quad (\text{B.8})$$

$$u_{app}(\overline{d}_l) = \frac{\sqrt{(d_l(A) - \overline{d}_l)^2 + (d_l(B) - \overline{d}_l)^2 + (d_l(C) - \overline{d}_l)^2}}{\sqrt{2}} \quad (\text{B.9})$$

B.2.4 Uncertainty from day to day and between repetitions

The uncertainty from day to day and between repetitions was evaluated assuming that the weights for the reliability of each measured value for a measurement day or repeated measurements are equal.

$$u_{day}(\overline{d}_n) = \sqrt{\frac{\left(\frac{\sum_{i=1}^n d_n(M)_{1,i}}{n} - \overline{d}_n\right)^2 + \left(\frac{\sum_{i=1}^m d_n(M)_{2,i}}{m} - \overline{d}_n\right)^2 + \left(\frac{\sum_{i=1}^p d_n(M)_{3,i}}{p} - \overline{d}_n\right)^2}{2}} \quad (\text{B.10})$$

$$u_{day}(\overline{d}_l) = \sqrt{\frac{\left(\frac{\sum_{i=1}^n d_l(M)_{1,i}}{n} - \overline{d}_l\right)^2 + \left(\frac{\sum_{i=1}^m d_l(M)_{2,i}}{m} - \overline{d}_l\right)^2 + \left(\frac{\sum_{i=1}^p d_l(M)_{3,i}}{p} - \overline{d}_l\right)^2}{2}} \quad (\text{B.11})$$

B.2.5 Compound standard uncertainty

Compound uncertainty is evaluated from (B.12) and (B.13).

$$u(\overline{d}_n) = \sqrt{u_{method}(\overline{d}_n)^2 + u_{day}(\overline{d}_n)^2 + u_{app}(\overline{d}_n)^2} \quad (\text{B.12})$$

$$u(\overline{d}_l) = \sqrt{u_{method}(\overline{d}_l)^2 + u_{day}(\overline{d}_l)^2 + u_{app}(\overline{d}_l)^2} \quad (\text{B.13})$$

B.2.6 Measured results

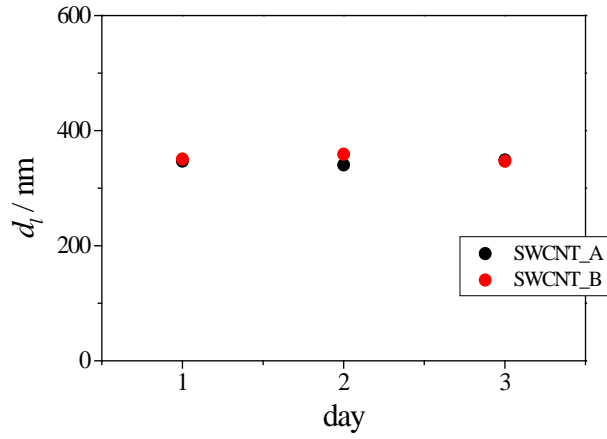


Figure B.2 Time dependence of light-intensity-averaged secondary particle size calculated by the Marquadt method (an improved histogram) for the measured results using a photon correlation spectroscopic method applied to a suspended sample of SG-SWCNT particles. No apparent change in particle sizes was observed with the DLS measurements over three days.

Table B.3 Average particle sizes of SG-SWCNT secondary particles in culture solution and their uncertainties.

<i>sample</i>		(nm)	Combined standard uncertainty (nm)	Standard uncertainties arising from repeated DLS measurements (nm)	Standard uncertainties caused by the differences in DLS instruments (nm)	Standard uncertainties caused by the differences in analytical method (nm)
SG-SWCNT-A	d_l	333.5	21.4	1.4	18.5	10.7
	d_n	114.7	29.2	0.1	27.5	9.9
SG-SWCNT-B	d_l	338.7	37.3	3.8	37.1	0.3
	d_n	110.4	44.9	0.9	42.1	15.7

Appendix C

Example: ELS measurement results for SG-SWCNT suspension

The example of measured results is summarized in Table C.1. The zeta potential takes similar negative values, and the absolute values are not large.

Table C.1 Measured results for the zeta potential for suspended samples of various SG-SWCNT.

<i>sample</i>	zeta potential (mV)	standard uncertainty (mV)
SG-SWCNT-A	-11.6	1.1
SG-SWCNT-B	-10.0	0.7

Appendix D

Example: Raman spectroscopic measurement results for a sample of SG-SWCNTs

The example of measured results is summarized in Table D.1. A significant reduction in the GD ratio was confirmed after ultrasonic treatment.

Table D.1 Measurement results for the GD ratio of a suspended sample of SG-SWCNTs.

<i>sample</i>	GD ratio	standard uncertainty
SG-SWCNT-A	4.2	0.3
SG-SWCNT-B	4.4	0.1

Appendix E

Example: FFF measurement results for a sample of SG-SWCNTs

Figure E.1 and Table E.1 show quantitative results from FFF for the amount of free BSA in a sample of SG-SWCNTs suspended in a culture solution. No decrease in the amount of BSA was observed relative to the control.

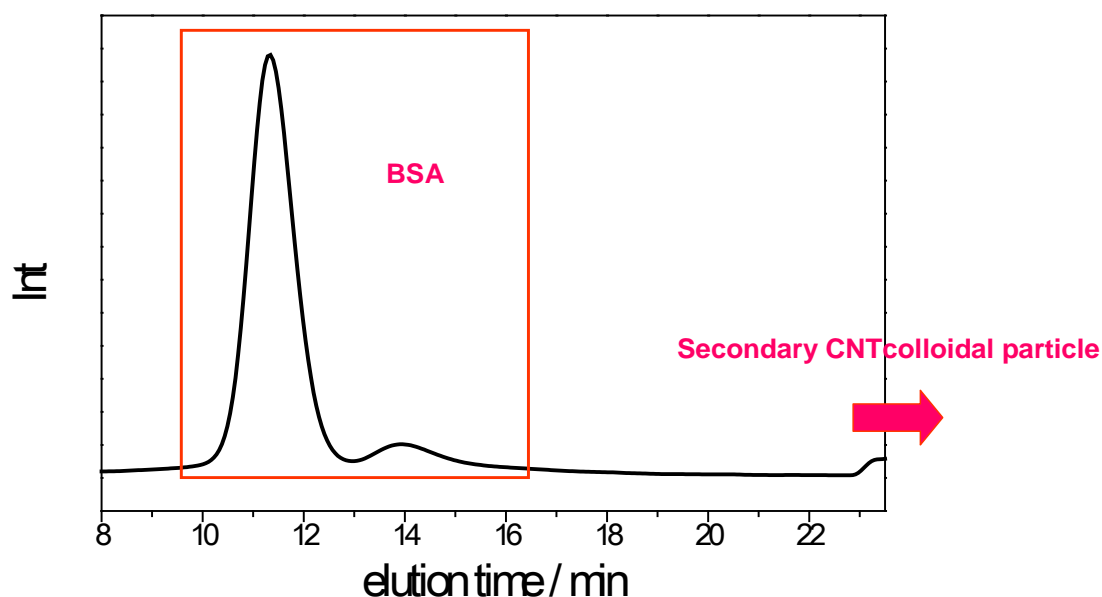


Figure E.1 The UV fractogram at 210 nm for CNT sample separated by the Field Flow Fractionation method. Peaks: BSA, 7–12 min; carbon black colloidal particles: 20–25 min.

Table E.1 Quantitative results for free BSA using the FFF method.

<i>sample</i>	amount of BSA in	amount of BSA in
	culture media	SG-SWCNT
	(mg/mL)	suspension (mg/mL)
SG-SWCNT-A	3.58	3.23
SG-SWCNT-B	3.42	2.96

The person in charge of writing chapter IV.

Haruhisa Kato, Ph.D.; Ayako Nakamura; Shinichi Kinugasa, Ph.D.

V. Concentration of carbon nanotubes suspended in culture medium

Preface

This section defines a procedure for evaluating the concentration of CNT suspensions, especially those suspended in the culture media used for *in vitro* cell-based assay. The procedure is an evaluation method to measure the concentration of an unknown sample by creating a calibration curve using suspension of CNTs of known concentrations.

1. Scope of application

The object of the measurement is CNT suspension for *in vitro* cell-based assay.

2. Normative reference

The following referenced documents are indispensable for the application in this document.

ISO 11358:1997 Plastics—Thermogravimetry (TG) of polymers—General principles

ISO/TS 11308:2011 Nanotechnologies—Characterization of single-wall CNTs using thermogravimetric analysis

3. Terms, definitions, and symbols

3.1 Terms and definitions

3.1.1 Ultraviolet-visible absorption spectroscopy (UV-Vis absorption spectroscopy)

This is a measurement method in which suspended light of different wavelengths is incident on an absorbing medium; the absorbance is calculated using the Beer–Lambert law.

3.1.2 Thermogravimetric analysis (TGA)

This is a method for measuring the change in weight of a sample when air or an inert gas is circulated at a specific flow rate while the sample is heated with an electric furnace in order to adjust the temperature or alternatively to maintain a constant temperature.

3.1.3 Dispersing medium

This is the liquid phase that disperses CNTs. For *in vitro* cell-based assay, an ordinary culture solution is used, for example, DMEM.

3.1.4 Dispersant

This is a substance used to make it easier for CNTs to break up in a dispersing medium. This procedure mainly uses BSA as the dispersant.

3.1.5 Suspended sample and particle sample

The original CNT bulk powder is referred to as the particle sample or the nanoparticle sample.

A suspended sample is the liquid that contains the particle sample suspended in an appropriate dispersing medium.

3.2 Abbreviated terms and units

I_{obs}	actual measured transmitted light intensity	nm
I_0	incident light intensity	nm
ε	molar absorption coefficient	nm
c	particle concentration in the suspended sample (mass per unit volume)	
l	cell length	cm

4. Principles

4.1 UV-Vis absorption spectroscopy

If specific wavelengths are incident on nanotubes or their aggregates, which are suspended in a liquid, specific wavelengths of light are absorbed according to the particular substance because of which the transmitted light has a different wavelength distribution compared to that of the incident light. In this case, the absorbed light intensity is proportional to the concentration of the substance. Thus, measuring the transmitted and incident light intensities determines the concentration c according to the Beer–Lambert law,

$$\log\left(\frac{I_{obs}}{I_0}\right) = -\varepsilon cl$$

4.2 TGA

Air is circulated at a specific flow rate through nanoparticles while the medium is heated with an electric furnace to adjust the temperature or alternatively to maintain a constant temperature, following which the change in weight is measured. Ratios by weight can be found between substances of different apparent combustion temperatures. For example, the CNT and catalyst can be found quasi-quantitatively.

5. Equipment

5.1 UV-Vis absorption spectroscopy

5.1.1 Apparatus

This procedure assumes the use of commercially available UV-Vis absorption spectroscopy equipment.

5.1.2 Validation

The validity of the equipment is verified in advance using standard substances. However, one should determine whether the measured value and the certified value agree to within the range of uncertainty when, for example, measuring a specific wavelength filter whose values are well known. An alignment tool and specific lamp emission line, which depend on the particular measuring equipment, are used for equipment alignment. Ideally, equipment alignment should be conducted every time measurements are started.

5.1.3 Temperature control

The UV-Vis absorption spectroscopy equipment must possess the capability to maintain the temperature of the measuring cell within the required temperature range.

5.1.4 Test report

The equipment must be able to display the UV-Vis spectra and have the capability to output the results.

5.2 TGA

5.2.1 Apparatus

This procedure assumes the use of commercially available TGA equipment.

5.2.2 Validation

The equipment's validity is verified in advance using standard substances. For example, the Curie point of a specific material can be measured for validating an electric furnace by determining whether the measured value agrees with the certified value to within the range of uncertainty. When validating mass measurements, they should ideally be evaluated using a standard weight of specific mass.

5.2.3 Temperature control

The TGA equipment must have the capability to keep the temperature of the measuring cell within the required temperature range.

5.2.4 Test report

The equipment must possess a function that saves thermogravimetric changes at specified intervals and outputs the results.

6. Reagents and equipment

6.1 Reagents

The solvent used for diluting and for cleaning the measuring cell should be pure and not contain any fine particles, especially fine particles larger than the particles in the suspended

sample. For example, when the solvent used for the suspension is water, it is best to use so-called ultrapure water that has been passed through a filter.

Note: The “ultrapure water” in this procedure is defined as cleaning water purified with an ion-exchange filter or a filter smaller than 0.2 μm . It should have an electrical resistivity higher than 18 $\text{M}\Omega \cdot \text{cm}$ and an organic carbon concentration of fine particles of less than 5 ppb.

6.2 Standard substances

Regarding the characteristic properties of standard substances, it is desirable to use those whose traceability is stated clearly. For example, in the case of a calibration filter, a filter with a calibration certificate that has been issued by JCSS is recommended. Furthermore, this filter also requires that it be calibrated near the wavelength to be measured.

6.3 Equipment

Common containers and dispensing burettes may be used; however, vessels or equipment that can change the physicochemical properties of the suspended sample (such as pH) are not to be used. For example, ordinary soda glassware can possibly change the pH of samples and thus quartz glassware is preferred.

7. Operations

7.1 UV-Vis absorption spectroscopy

7.1.1 Installing equipment

Equipment should be installed in a clean environment, while avoiding any electrical noise, mechanical vibrations, direct sunlight, etc.

Warning: The UV-Vis absorption spectroscopy equipment uses lamp light as a light source that can damage human eyes. Do not look directly at the incident light, and do not allow any reflected light to enter the eyes. The safety code established for the facility must be obeyed.

7.1.2 Preparation of the measurement sample

To avoid decomposition of the culture medium, which acts as the dispersing medium, suspended samples (with nanoparticles already suspended within) should be stored in a refrigerator at low temperature but must also not be allowed to freeze.

When data reproducibility is a priority, the measured sample should ideally be taken from a fixed location in the suspension solution that has been left to stand. For example, the sample should always be collected at the same depth from the surface. This is because even for a stable suspension solution, a concentration gradient gradually is supposedly formed due to gravity. Thus, even for suspension solutions prepared in the same way, the concentrations may be significantly different depending on the location at which samples are taken from the solutions. The suspension sample can be measured at room temperature without any pretreatment, such

as agitation using an ultrasonic bath. To avoid any scattering of light by dust, all samples related to the measurement should be prepared in a clean environment.

7.1.3 Preparation of the suspended sample used for calibration

A standard CNT suspension is prepared for which the concentration of the nanoparticles intended for measurement is already known. To ensure reliable results, the concentration of the suspended sample should be evaluated beforehand, i.e., not just the preparation concentration with a mass balance but using a different measurement method, for example, a total organic carbon meter.

7.1.4 Cleaning equipment used in UV-Vis absorption spectroscopy measurements

Sample containers and experimental equipment that come into contact with the suspended sample must be kept away from fine particles, and contamination from previous particle samples must be avoided. For example, measurement cells and syringes that are used repeatedly should ideally be “optically cleaned.” Ordinary disposable Pasteur pipettes can often be used as they are, but if there are any concerns, optical cleaning should be performed. If, for some reason optical cleaning cannot be conducted, the equipment can be cleaned with a filtered/distilled dust-free solvent. Measurement cells, vessels, and solvents that have been confirmed to be contaminated should not be used.

7.1.5 Blank measurements

A blank measurement is conducted in advance to verify that there are no contaminating particles. Usually, scattering intensity is measured from the water used to clean the measurement cell and dilute the sample. Any contamination results in strong scattering observed.

Note: If the cell cannot be removed from the apparatus, the cell should either be cleaned repeatedly with cleaning solvent or prewashed with the sample. The presence of contamination should be determined by measuring scattered light.

7.1.6 Measurements

7.1.6.1 Skills

The UV-Vis apparatus operator should have sufficient skill acquired through extensive training. They must also have sufficient knowledge of measurement techniques; for example, whether they can report values that agree with certified values for a specific filter.

7.1.6.2 Measurement environment

The apparatus should be installed in a clean environment. Further, the environment in which the equipment is handled, the environment in which samples are prepared, and lab coats for the

persons making the measurements should all be clean.

7.1.6.3 Preparation of the UV-Vis absorption spectroscope equipment

Before making measurements, the apparatus must be warmed up so that stable data can be acquired.

When performing UV-Vis absorption spectroscopy with temperature control, the measurement cell must first reach the temperature that was set in advance. That temperature during the measurement must also fall within the prescribed limits, i.e., within $\pm 0.3^{\circ}\text{C}$.

Note: Stability of the lamp varies greatly depending on the type of equipment, and between 15 and 30 min are required to stabilize lamp intensity and measuring cell temperature.

Note: Operators should assess in advance whether the measuring cell has actually attained the set temperature.

If the liquid volume of the measured sample is large, it may require more than an hour to reach the set temperature (from 22°C to $25.0^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$).

Note: CNT samples generally have a strong extinction coefficient over a broad range of wavelengths. If scattered light intensities from a CNT sample drop unusually, special care is needed because of the possibility that this equipment cannot make highly reliable absorption intensity measurements.

7.1.6.4 Measurement and analysis

Measurement and analysis are conducted according to the procedures for each apparatus. A minimum of three measurements should be conducted to assess measurement accuracy. Further, the measurements should be under the same conditions as used for the standard suspended solution during calibration (the bandwidth, for example).

7.1.6.5 Recording

The following experimental conditions are recorded: information about the suspended sample, time of the measurement, time taken for the measurement, and temperature of the suspended sample.

The following results are recorded: UV-Vis absorption spectra calculated by the equipment.

7.2 TGA

7.2.1 Installation of the equipment

The equipment should be installed in a clean environment, while avoiding any electrical noise, mechanical vibrations, direct sunlight, etc..

Warning: Because the temperature can increase up to 1500°C in TGA equipment, care should be taken when handling it. Safety codes established for the facility must be obeyed.

7.2.2 Preparation of the suspended sample

A sample of bulk powder that has been appropriately suspended is placed directly in a platinum container.

Warning: Since a powder of nanoparticles is being used, preparation should be done under conditions that do not allow any scattering. When handling nanomaterials, nanomaterial safety regulations must be observed, such as wearing protective goggles, gloves, and a mask.

7.2.3 Cleaning the equipment used in TGA measurements

Sample containers and experimental equipment that come into contact with the suspended sample must be kept away from fine particles, and contamination from previous samples must be avoided. For example, measurement cells and syringes that are used repeatedly should be “optically cleaned.” Ordinary disposable Pasteur pipettes can often be used as they are, but if there are any concerns, optical cleaning should be performed. If, for some reason, optical cleaning cannot be conducted, the equipment can be cleaned with a filtered/distilled dust-free solvent. Measurement cells, vessels, and solvents that have been confirmed to be contaminated should not be used.

7.2.4 Blank measurements

A blank measurement is conducted in advance to verify that there are no contaminating particles, and that the equipment is operating properly. If there is any contamination, a thermogravimetric change will be observed.

7.2.5 Measurements

7.2.5.1 Skills

The TGA apparatus operator should have sufficient skill acquired through extensive training. They must also have sufficient expertise in measurement techniques; for example, whether they can report values that agree with combustion temperatures for already known substances.

7.2.5.2 Measurement environment

The apparatus should be installed in a clean environment. Further, the environment in which the equipment is handled, the environment in which samples are prepared, and lab coats for the persons making the measurements should all be clean.

7.2.5.3 Preparation of TGA equipment

Before making measurements, the apparatus must first be warmed up. This is so that any dust in the system will be burned off to provide clean conditions so that stable data can be acquired.

7.2.5.4 Measurement and analysis

Measurements and analyses are conducted according to the procedures for each apparatus. A minimum of three measurements should be conducted to assess measurement accuracy.

7.2.5.5 Recording

The following experimental conditions are recorded: information about the sample, time of the measurement, and time taken for the measurement.

The following results are recorded: the thermogravimetric reduction curve recorded by the apparatus.

8. Data analysis

8.1 Calculation of values

Reported values for the measured sample concentration, for example, are average values over several repetitions. The method for calculating average values can be chosen, but the details of the calculation must be reported with the averages.

8.2 Evaluation of the uncertainty

Uncertainties have to be determined for reported values. When this is done, the method used for determining each uncertainty must be clearly specified. This allows readers to judge the validity of the reported uncertainties. For example, since evaluation of detailed uncertainties of UV-Vis absorption spectroscopy measurements poses a great challenge, it is sufficient to use an approximate calculation based on just the main causes of the uncertainty. Sources of uncertainty include apparatus type, measurement method, analysis method, and fluctuations in repeated measurements.

If the deviations of the apparatus from the other apparatus cannot be evaluated in advance, they can be estimated from published information. However, the source for the information should be referenced.

9. Report

The following items are reported.

9.1 General

- a) Detailed information that identifies the suspended sample (e.g., information about the particle sample and suspension preparation sample, information about the dispersing medium, the date the suspended sample was acquired, and the method of transportation)
- b) Detailed information that identifies the suspended sample used for the calibration (e.g., information about the particle sample, the suspension sample preparation method, information about the dispersing medium, the date the suspended sample was acquired, and the method of transportation)
- c) Measurement date, name of the person doing the measurements, and name of the

organization doing the measurements

d) Information about standard substances used in verifying the validity of the apparatus (including the manufacturer) and the date on which validation was conducted.

e) Storage conditions for the suspended sample (e.g., storage temperature, storage facility, and storage time)

9.2 Apparatus and experimental and analysis conditions

a) Name of the apparatus, model, and name of the manufacturer

b) Particle-size standard substance used in validation, and results of validation

c) Cleaning conditions for the measuring cell, etc.

d) Information about the reagent if dilution was needed

e) Measurement temperature

f) Information on composition of the dispersing medium (qualitative information also acceptable)

g) Volume of the suspended sample in the cell

h) Method for calculating reported values (e.g., method used to find averages)

i) Method for calculating uncertainties and the source for materials when used

j) Number of repeated measurements

9.3 Results

9.3.1 UV-Vis absorption spectroscopy

Calculated concentration and its standard deviation/uncertainty due to the repeatability of the measurements

9.3.2 TGA

Calculated concentration of the residue of catalyst and its standard deviation/uncertainty due to the repeatability of the measurements.

Appendix A

Flow chart for evaluating CNT concentration

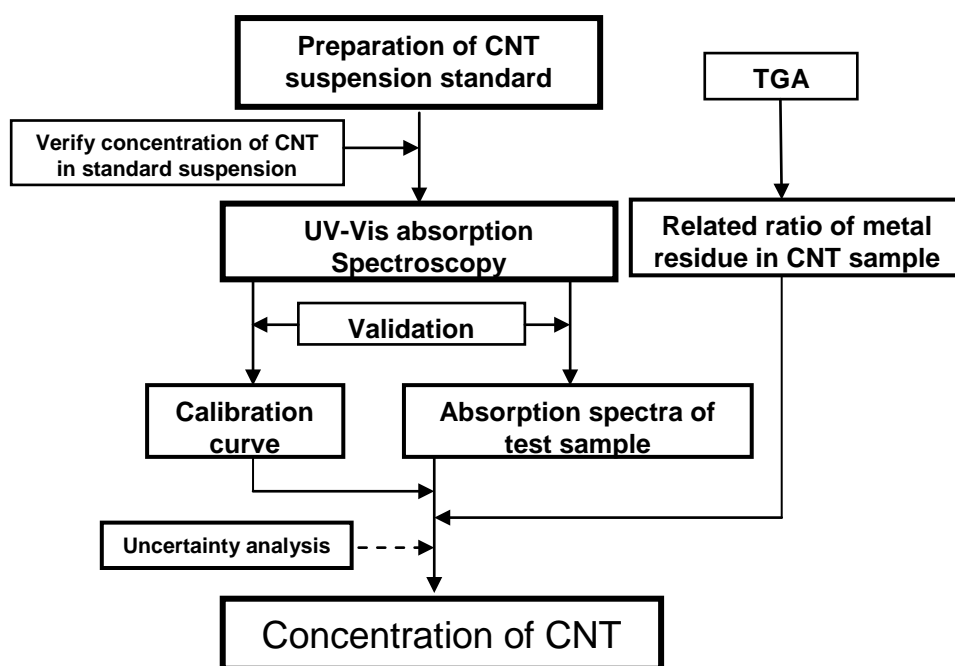


Figure A.1 Flow of methodology for evaluating CNT concentration.

Appendix B

Example: TGA measurement with a sample of SG-SWCNTs

The residue from heating up to 1500°C was assumed to be from impurities in the underlying source, and a purity assessment was conducted for CNT carbon concentration . An example of the measurement results is shown in Fig. B.1(summarized in Table B.1). The standard deviation in the table was evaluated as the standard uncertainty from three repeated measurements.

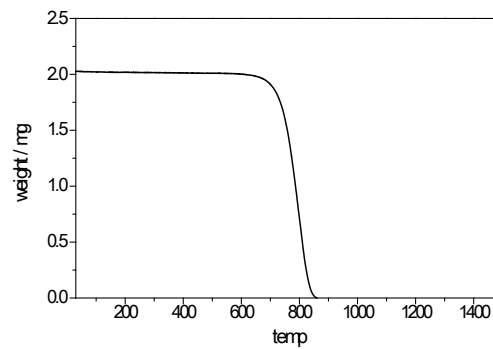


Figure B.1 TGA measurement results for a suspended sample of SG-SWCNTs.

Table B.1. Carbon purity calculated from TGA measurements on a suspended sample of SG-SWCNTs (units are wt%).

<i>sample</i>	<i>percentage of carbon</i>	<i>standard uncertainty</i>
SG-SWCNT	98.2	1.2

Appendix C

Example: UV measurements for a sample of SG-SWCNTs

C.1 Evaluation of the background for UV absorption measurements

For standard suspensions of concentration of SG-SWCNTs prepared at given concentrations, the absorption spectrum is measured, and at wavelengths greater than or equal to 650 nm (650, 700, 750, and 800 nm), the relationship between concentration and absorbance was evaluated using a multipoint calibration curve.

C.2 Calibration curve

Suspended samples are prepared on the basis of this procedure at various concentrations. Absorption spectra of these samples are measured, and the relationship between concentration and absorbance is plotted at multiple points for wavelengths higher than or equal to 650 nm.

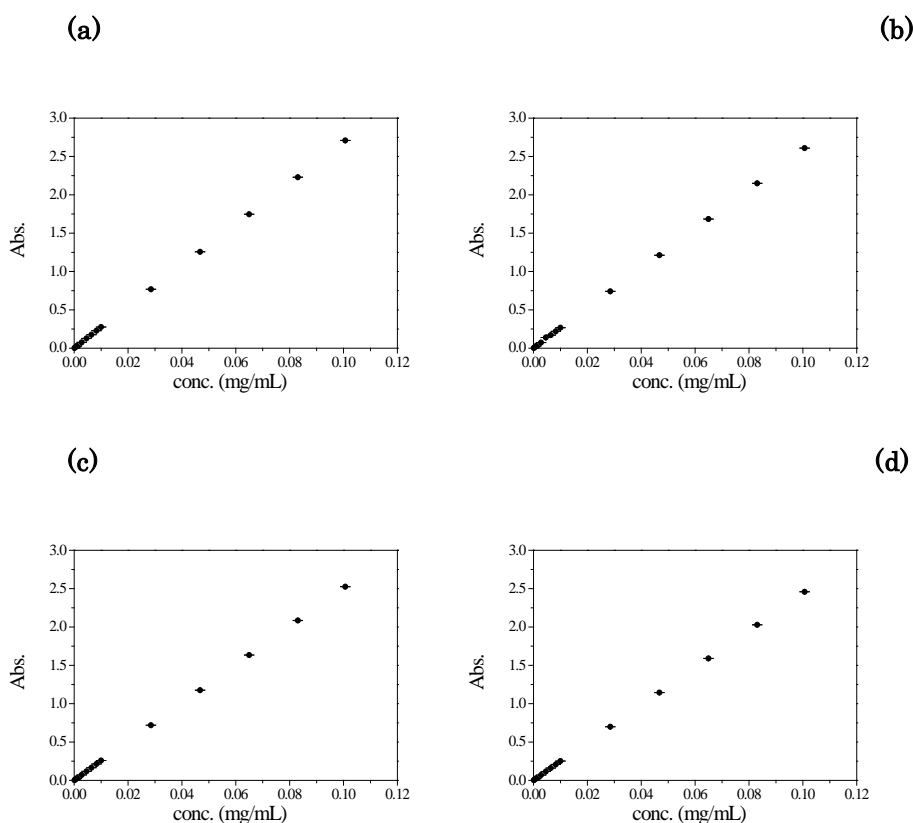


Figure C.1 Example of measurement results for concentration dependence of absorbance for a suspended sample of SG-SWCNTs at selected wavelengths: (a) 650 nm, (b) 700 nm, (c) 750 nm, and (d) 800 nm. Bars in the figure are standard deviations over three repeated measurements at each concentration. Within the given range of wavelengths, the observed absorbance show little change when the dispersing media is varied. Standard deviations over repetitions were not used in evaluating the concentration calculation.

C.3 Example of calculated results for the concentration of SG-SWCNTs

C.3.1 Calculation of the measured average value of SG-SWCNTs

Although it will be noted later, the uncertainties from a regression by a least-squares method at each wavelength were nearly identical. Therefore, all calculated average values were presumed to be equivalent, and the SG-SWCNT concentration was computed using simple averaging,

$$C_{SWCNT} = \frac{C_{cal,650nm} + C_{cal,700nm} + C_{cal,750nm} + C_{cal,800nm}}{n} \quad (C.1)$$

C.3.2 Uncertainty using the straight-line approximation by a least-squares method

The uncertainty due to the straight-line approximation by a least-squares method of the calibration curve was computed by first evaluating this quantity:

$$s_{y/x}(c_{cal}) = \left\{ \frac{\sum_{i=1}^n [A_i - A_{cal,i}]^2}{n-2} \right\}^{0.5} \quad (C.2)$$

Here, A_i is the actual measured absorbance for each concentration, A_{ii} is the absorbance computed using an approximating equation for the calibration curve for each concentration, n is the number of data points when the calibration curve was created, A_{cal} is the absorbance computed with the approximating equation to the calibration curve for the concentrations to be found. Next, the uncertainty is computed from

$$u_{cal}(c_{cal}) = \frac{s_{y/x}(c_{cal})}{b} \left\{ \frac{1}{m} + \frac{1}{n} + \frac{(A_{cal} - \bar{A})^2}{b^2 \sum_i (c_i - \bar{c})^2} \right\}^{0.5} \quad (C.3)$$

where m is the number of repeated measurements, c_i is the SG-SWCNT concentration for each measurement point, b is the slope of the approximating straight line, and the bar indicates the average for each value. For the evaluation of the uncertainty in this report, the uncertainty found for 750 nm was adopted. Ten points were used for n .

C.3.3 Uncertainty from averaging the measured values for each wavelength

The uncertainty from averaging the measured values for each wavelength was computed using

$$u_{av}(c_{SWCNT}) = \sqrt{\frac{(c_{cal,650nm} - c_{SWCNT})^2 + (c_{cal,700nm} - c_{SWCNT})^2 + (c_{cal,750nm} - c_{SWCNT})^2 + (c_{cal,800nm} - c_{SWCNT})^2}{3}} \quad (C.4)$$

C.3.4 Uncertainty in measured values from using different cells

These measurements use disposable cells but the uncertainty due to the difference in the absorbance of the PA-made cell, which should only absorb at short wavelengths, can also have an effect on measured values. Thus, three kinds of cells were extracted at random and evaluated for absorbance measurements at a constant temperature of 25°C. Table C.1 shows the results of evaluating the standard deviation of the measured absorbance that is attributed to using different disposable cells. All wavelengths showed an uncertainty of approximately 1.5×10^{-4} .

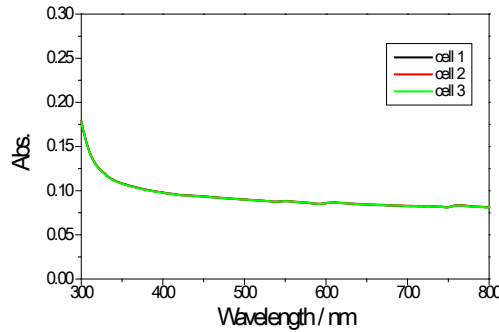


Figure C.2 Measured absorption spectra for different disposable cells extracted at random showed no significant differences.

Table C.1 Standard deviations in measured absorbance from differences between cells.

wavelength (nm)	standard uncertainty
650	1.5×10^{-4}
700	1.8×10^{-4}
750	1.1×10^{-4}
800	1.7×10^{-4}

C.3.5 Uncertainty in measured values associated with instabilities in BSA concentration

Because the BSA concentration is not constant in the method of manufacturing the suspended samples used here, differences in the measured values associated with differences in the BSA concentration in the suspension medium were also investigated. Results are summarized in Fig. C.3 and Table C.2. Realistically, a prepared concentration of 10 mg/mL is not possible; hence, to estimate the uncertainties in our measurements, measurements were performed and assessed

for a range of concentrations. Table C.2 shows that the uncertainties were approximately 3.0×10^{-3} at each wavelength.

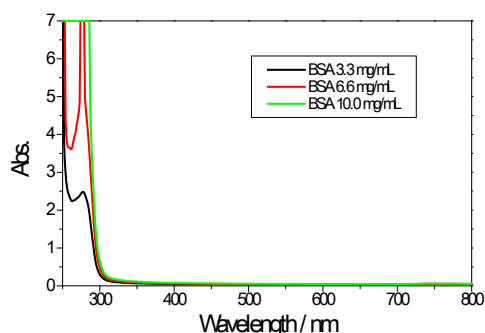


Figure C.3 Measured absorption spectra for different BSA concentrations.

Table C.2 Standard deviations of measured absorbance due to variations in BSA concentration.

wavelength (nm)	standard uncertainty
650	3.7×10^{-3}
700	3.0×10^{-3}
750	2.3×10^{-3}
800	2.3×10^{-3}

C.3.6 Uncertainty in the measured value due to instabilities in pH during measurement

Figure C.4 shows the results for changes in the absorption wavelength with phenol red when the pH is adjusted at a constant temperature of 25°C. The standard deviations for the measured absorbance at multi wavelength (650, 700, 750, and 800 nm) caused by variations in pH are summarized in Table C.3. Each wavelength gives an uncertainty of approximately 3.5×10^{-4} .

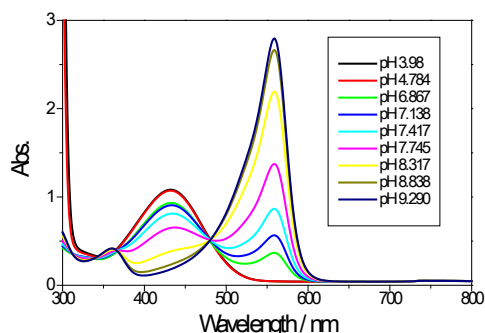


Figure B.4 Effect of pH on absorption spectra at long wavelengths for absorption component phenol red in a culture medium. There is little effect of pH on absorption for wavelengths above 650 nm.

Table C.3 Standard deviations of measured absorbance due to variations in pH.

wavelength (nm)	standard uncertainty
650	3.5×10^{-4}
700	3.5×10^{-4}
750	3.8×10^{-4}
800	3.5×10^{-4}

C.3.7 Uncertainty in evaluation between CNT mass measurement and total organic carbon measurement when the sample is prepared for creating the calibration curve

When a sample is prepared for creating the calibration curve, the uncertainty evaluated between a CNT mass measurement and a total organic carbon measurement had a maximum of approximately 10%. Therefore, the uncertainty is assumed to be Type B, and 5.8% was added to the evaluation of the uncertainties for all measured values; this uncertainty was used in preparing the sample for the calibration curve.

C.3.8 Uncertainty in measurement due to changes in the measurement date for an evaluation

Depending on the size of the sample, some measurements may take longer than a day; this means that the background assessment verified previously may not apply to that measurement. Thus, a suspension analysis was conducted to verify day-to-day measurements and repeated measurements. The measured results at a wavelength of 700 nm for the BSA solution are organized in Table C.4. Since the F_0 value far exceeded the limit level, the table indicates that it was best to avoid measurements that take more than a day. If, hypothetically, measurements that span more than a day are conducted, we would need to consider the uncertainty involved in this evaluation. In other words, in the evaluation of the uncertainty related to the inhomogeneous nature of the sample, the standard deviation derived from its inhomogeneous nature ($= 5.72 \times 10^{-3}$), evaluated using the suspension analysis results in Table C.4, is significantly larger than the uncertainty derived from repeated measurements ($= 7.93 \times 10^{-5}$), so when measurements are conducted over more than a day, it is necessary to evaluate the uncertainty as the former. Here, each evaluation is calculated using (C.5) and (C.6).

$$s_{bb} = \sqrt{\frac{V_A - V_E}{r}} \quad (\text{C.5})$$

$$u_{bb} = \sqrt{\frac{V_E}{r}} \sqrt{\frac{2}{v_{V_E}}} \quad (\text{C.6})$$

Table C.4 Analysis of variance of the results in absorbance associated with absorbance measurements over more than one day.

Factor	sum of squares	degree of freedom	variance	F0 value	threshold
day	4.1×10^{-6}	4	1.025×10^{-6}	24.3	3.478
repeatability	4.2×10^{-7}	10	4.218×10^{-8}		
sum	4.5×10^{-6}	14			

C.3.9 Combination of uncertainties

The uncertainties for the six items from C.3.2 to C.3.7 are combined as the quantified uncertainty in the concentration of the SG-SWCNTs,

$$u(c_{\text{SWCNT}}) = \sqrt{u_{\text{cal}}(c_{\text{cal}})^2 + u_{\text{av}}(c_{\text{cal}})^2 + u_{\text{cell}}(c_{\text{cal}})^2 + u_{\text{BSA}}(c_{\text{cal}})^2 + u_{\text{pH}}(c_{\text{cal}})^2 + u_{\text{conCA}}(c_{\text{cal}})^2} \quad (\text{C.7})$$

C.4 Evaluation results for the concentration of SG-SWCNTs

Based on the evaluation method above, Table C.5 summarizes the calculation results and the uncertainties for the SG-SWCNT concentration in culture mediums for these samples.

Table C.5 Calculated concentrations (mg/mL) for each SG SWCNT and corresponding uncertainties

<i>sample</i>	Concentration of CNT	Combined standard uncertainty	Standard uncertainties arising from calibration curve	Standard uncertainties arising from averaging	Standard uncertainties arising from difference of cells	Standard uncertainties arising from BSA absorption	Standard uncertainties arising from difference of pH	Standard uncertainties arising from CNT concentration standard
SG-SWCNT-A	9.76×10^{-2}	5.65×10^{-3}	1.3×10^{-4}	2.00×10^{-4}	2.16×10^{-7}	4.52×10^{-6}	7.47×10^{-7}	5.64×10^{-3}
SG-SWCNT-B	9.59×10^{-3}	5.55×10^{-4}	9.64×10^{-6}	2.23×10^{-5}	2.16×10^{-7}	4.52×10^{-6}	7.47×10^{-7}	5.54×10^{-4}

The person in charge of writing Chapter V.

Haruhisa Kato, Ph.D.; Ayako Nakamura; Shinichi Kinugasa, Ph.D.

VI. Metal concentrations of carbon nanotubes suspended in culture medium

Preface

This section presents a procedure for metal analysis in a suspension of SWCNTs for *in vitro* cell-based assay. A method is given for determining metal ions contained in the culture medium, such as sodium and calcium, because the metal ion could decrease owing to adsorption on CNTs. In addition, another method is given for determining the concentrations of impurities, such as catalytic metals that are used in the production of SWCNTs. These methods involve decomposing the sample with acid and then analyzing for metal ions using either inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma atomic emission spectrometry (ICP-MS).

1 Scope of application

A method is presented for metal analysis in a suspension of SWCNTs dispersed in a Bovine Serum Albumin (BSA) aqueous solution and their diluted suspension with culture medium such as DMEM.

2 Reference standards

In this procedure, the following standards are referenced or quoted.

JIS K 0116 General rules for emission spectrometry

JIS K 0133 General rules for high-frequency plasma mass spectrometry

3 Terminology, definitions, and symbols

3.1 Stock suspensions

A suspension of SWCNTs dispersed in BSA aqueous solution

3.2 Working solutions

A suspension of SWCNTs for *in vitro* cell-based assay made by diluting the stock suspension by a certain scaling factor with the culture medium used in the test

3.3 ICP-MS

A method for the qualitative and quantitative analysis, achieved by ionizing the metal with inductively coupled plasma of argon gas and then isolating and counting those ions using a mass spectrometer; quadrupole and double-focusing types have been used as mass spectrometers.

3.4 ICP-AES

A method for the qualitative and quantitative analysis, achieved by atomization and excitation

of the metal with inductively coupled plasma of argon gas and then the determination the spectrum of light emitted when the excited elements return to their ground states

4 Equipment and instrumentation

4.1 Hot plate

Used for the acid treatment of samples at up to 200 °C, this can regulate the temperature to around $\pm 10^{\circ}\text{C}$.

4.2 Container for acid treatment of samples

The container is a lid and 100-mL beaker made from PTFE or quartz, and resistant to both heat and acid.

4.3 Centrifuge

This centrifuge is capable of operating up to a centrifugal force of 8,000*g*.

4.4 Ultrafiltration filter

This filter is capable of ultrafiltration of a 15-mL sample. The molecular weight cutoff is around 50,000.

4.5 Funnel and filter paper

An acid-resistant funnel and filter paper are used for filtration of the acid treated sample to remove the residue.

4.6 Measuring flask

This is a container for diluting the acid treated sample following filtration. Because the dilution factor differs according to concentration, a flask of size 10–100 mL is used. Since the use is temporary, normal glassware can be used.

4.7 Centrifuge tube

An acid-resistant tube of 50 mL volume used for storage

4.8 ICP-MS

This is commercially available ICP-MS equipment for the analysis of metal impurities and metal ions. A pretreated sample solution is sprayed into the plasma, and the ion species of the elements generated in the plasma are isolated and quantified with the mass spectrometer. Because interference from matrix components can occur easily, a double-focusing mass spectrometer is sometimes required for some species of metal.

4.9 ICP-AES

This is commercially available ICP-AES equipment for analyzing species of metal ions. A pretreated sample solution is sprayed into the plasma, and the emission spectrum of the elements is determined when excited metal return to their ground states.

5 Reagents

5.1 Nitric acid

A high-purity analytical grade nitric acid is suitable for use in this method, provided the content of the analytes or interfering substances should be negligible compared to the lowest concentration to be determined, i.e., less than 0.1 mg/mL.

5.2 Hydrogen peroxide

A high-purity analytical grade is suitable. It is suitable for use in this method, provided the content of the analytes or interfering substances should be negligible compared to the lowest concentration to be determined, i.e., less than 0.1 mg/mL.

5.3 Ultrapure water

This water is distilled and passed through an ion-exchanger as specified in JIS K 0557 for all sample preparations and dilutions. The concentration of the analytes or interfering substances in the water should be negligible compared to the lowest concentration to be determined. Ultrapure water for metal analysis is either manufactured using a water purification system for ICP-MS or commercially obtained.

5.4 Standard solution

Both single-element standard stock suspensions and multi-element standard stock suspensions with adequate specification stating the acid used and the preparation technique are commercially available. They are used as internal standard solution, tuning, or standard of analytes. In such cases, the solution should have guaranteed traceability. Many metals used in general metal analysis as internal standard substances are also used as catalysts in the manufacture of SWCNTs. Therefore, it should be verified that the standard solution does not contain any element serving as an internal standard in the sample.

6. Procedure for metal analysis in the suspension

6.1 Cleaning the equipment

Before use, the equipment, such as the beaker, should be cleaned with 5%–10% nitric acid and then rinsed thoroughly with ultrapure water.

6.2 Isolating metal ion components

A 5-mL aliquot of the stock suspension in cell culture medium in ultrafiltration tubes are centrifuged for 2 h at 6,000*g* (N=3). Two mL of ultrapure water is added to the residue in each ultrafiltration tube, and each is centrifuged, for 30 min at 6,000*g* twice.

Comment: Since various types of ultrafiltration tubes can be used and their filtration efficiencies depend on the directions of the filter paper and centrifugal force, care must be taken that force acts in the directions of the filter paper.

6.3 Decomposition of cell-derived organic substances and dissolution of metals

Total volume of filtrated solution containing metal ions described in Section 6.2 or a 5 mL aliquot of the suspension containing both metal ion and metal particle impurities are put into either a PTFE or quartz beaker, a 5% nitric acid solution is added, and then these are heated on a hot plate for approximately 8 h at about 160 °C. Hydrogen peroxide is timely added to promote decomposition of cell-derived organic substances. Heating continues until the liquid becomes transparent.

6.4 Filtration

After 6.3, the acid solution is filtrated into a measuring flask. After the beaker and filter are washed several times with 5% nitric acid, the washed solution and 5% nitric acid is also added into a measuring flask, the size of which depends on the metal concentration in the acid solution.

6.5 Analysis

Metal ions in the culture medium, such as sodium and calcium, are analyzed using either ICP-AES or ICP-MS. Catalytic metal impurities such as iron, nickel, and cobalt are analyzed using ICP-MS. Sample analysis is conducted in triplicate. If variations among triplicate are large, the acid treatment and analysis have to be conducted again.

6.6 Blank analysis

Additionally, for BSA solution without SWCNTs, BSA solution diluted with cell culture medium without SWCNTs, and ultrapure water, acid treatment and analysis are performed as described in Sections 6.2–6.5 as blank analysis. The lower limit of quantitation is defined as 10 times the standard deviation of blank analysis (N = 5).

Appendix A

Example: metal analysis in a CNT suspension

A.1 Outline

An example, performed according to the above procedures, is given for the metal analysis in a suspension of CNTs in a cell culture medium. The suspension contained enhanced direct injection pyrolytic synthesis (eDIPS) method SWCNTs (defined as eDIPS-SWCNTs) intended for *in vitro* cell-based assay. As the stock suspension in cell culture medium, a solution of approximately 1 mg/mL of eDIPS-SWCNTs, dispersed in a 10 mg/mL solution of BSA, was diluted by a factor of 10 using DMEM. Metal ions in the stock suspension in cell culture medium were separated out using ultrafiltration.

A.2 Pretreatment

A.2.1 Preparation of the acid solution

Using ultrapure water for metal analysis (ultrapure water for ultratrace analysis, Wako Pure Chemical Industries, Ltd.), nitric acid (1.42 ultrapur-100 nitric acid, Kanto Kagaku, Ltd.) was diluted to prepare a 5% nitric acid solution as the nitric acid of stipulated dilution for washing equipment, for diluting, and for use in ICP analysis.

A.2.2 Cleaning the equipment

Before use, a 100-mL PTFE beaker (beaker (PTFE), ASONE), PTFE watch glass (watch glass (PTFE), ASONE), filter funnel (polyrot, ASONE), and measuring flask (a Super-grade measuring flask, Shibata Scientific Technology, Ltd.) were all washed with 5% nitric acid. The equipment was then rinsed with ultrapure water for metal analysis (ultrapure water for ultratrace analysis, Wako Pure Chemical Industries, Ltd.).

A.2.3 Ultrafiltration

A 5 mL aliquot of the stock suspension in cell culture medium was put into each of three ultrafiltration tubes (50,000 molecular weight cutoff off, centrifugal concentrator VIVASPIN20, Sartorius Mechatronics, Japan, Ltd.) and ultrafiltration was performed with a high-speed refrigerated centrifuge Suprema21 (TOMY) over 2 h at 6,000*g*. In addition, 2 mL of ultrapure water was added to the residue in the ultrafiltration tubes, and this was centrifuged twice for 30 min each at 6,000*g*.

A.2.4 Analysis of coexisting substances (organic matter) and dissolution of metals (acid treatment)

The total volume of the filtrated solution after ultrafiltration (A2.3) or a 5 mL aliquot of the

stock suspension in cell culture medium was put into a PTFE beaker, to which approximately 70 mL of 5% nitric acid was added; the solution was then heated on a hot plate (Sand bath style hot plate, Tokyo Glass Kikai Ltd.) for about 8 h at around 160°C. Hydrogen peroxide (hydrogen peroxide for atomic absorption analysis, Wako Pure Chemical Industries, Ltd.) was timely added to promote decomposition of organic matter as biogenic components. The heat treatment was continued until the liquid became transparent.

A.2.5 Filtration

The acid solution following acid treatment was transferred to a 50-mL measuring flask through filter paper (quantitative filter paper No.5C, ADVANTEC) using a funnel. After washing and filtering the PTFE beaker and filter paper several times with a 5% nitric acid, the total acid solution was diluted in the measuring flask. The liquid for sodium analysis was further diluted by a factor of 100.

A.2.6 Analysis using ICP-MS

An analysis was performed using a high-resolution ICP mass spectrometer (ICP-MS, Thermo Scientific) with $N = 3$ for a single sample. Calcium (Ca standard solution, Wako Pure Chemical Industries, Ltd.), phosphorus (P standard solution, Wako Pure Chemical Industries, Ltd.), sodium (Na standard solution, Wako Pure Chemical Industries, Ltd.), nickel (Ni standard solution, Wako Pure Chemical Industries, Ltd.), iron (Fe standard solution, Wako Pure Chemical Industries, Ltd.), titanium (Ti standard solution, Wako Pure Chemical Industries, Ltd.), and yttrium (Y standard solution, Wako Pure Chemical Industries, Ltd.) were used as standard solutions for the calibration curves. In addition, scandium (Sc standard solution, Wako Pure Chemical Industries, Ltd.) was used as an internal standard solution. Examples of calibration curves are shown in Fig. A.1. Results were recalculated using the dilution factor (50 or 5000 times) and the amount of the stock suspension in cell culture medium (5 mL).

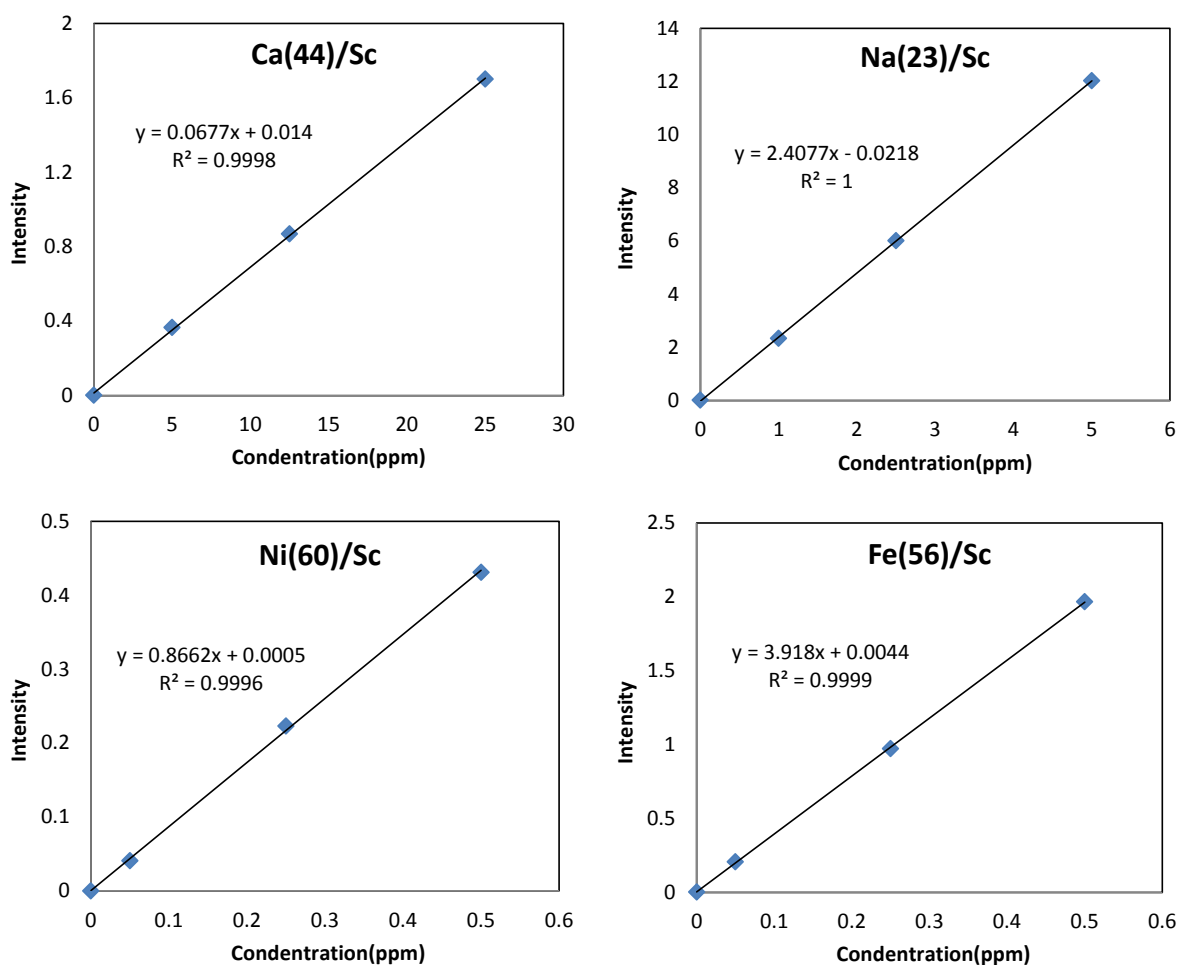


Figure A.1 Example calibration curves.

A.2.7 Results

The measured metal ion and impurity metal concentrations in the stock suspension in cell culture medium are shown in Table A.1. Metal ions in the stock suspension in cell culture medium were the same as the control, and there was no observable reduction through adsorption onto CNTs. Furthermore, 4.4–5.5 $\mu\text{g/mL}$ of iron was found to be present as a metal impurity.

Table A.1 Concentrations of metal ions and metal impurities in the medium preparation solution.

		B033-1	B038-1	Control
Metal ion concentration [μg/mL]	P	33	34	34
	Ca	64	66	65
	Na	2600	2600	2400
	Y	N.D. (<0.04)	N.D. (<0.04)	N.D. (<0.04)
	Ni	N.D. (<0.1)	N.D. (<0.1)	N.D. (<0.1)
	Fe	N.D. (<0.7)	N.D. (<0.7)	N.D. (<0.7)
Metal ion and solid impurity metal concentration [μg/mL]	P	36	32	32
	Ca	80	79	78
	Na	2500	2900	2600
	Y	N.D. (<0.04)	N.D. (<0.04)	N.D. (<0.04)
	Ni	N.D. (<0.1)	N.D. (<0.1)	N.D. (<0.1)
	Fe	5.5	4.4	N.D. (<0.7)

The person in charge of writing Chapter VI.

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VII. *In vitro* cell-based assay for safety assessment

Preface

In this section, a procedure is given for *in vitro* cell-based assay as a simple and rapid safety assessment of CNTs. Here, we choose items for *in vitro* cell-based assay to more properly reflect biomarkers (endpoints) in humans, which center mainly on inflammation of the lungs due to inhalation exposure. We also describe an *in vitro* cell-based assay, with which it is possible to make proper measurements without inhibiting the prepared dispersed CNTs in a cell culture.

1. Scope of application

Using a sample of a stock CNTs suspension, we present a method for *in vitro* cell-based assay to serve as a safety assessment of their effects.

2. Reference standards

None in particular

3. Terminology, definitions, and symbols

3.1 *In vitro* cell-based assay

An experimental condition where various experimental conditions are controlled as artificial conditions inside a test tube or in a Petri dish

3.2 Antibacterial and antifungal agents

In a culture of either human or animal cells, a mixture of antibacterial and antifungal agents is added to the culture medium to avoid contamination by bacteria or fungi. A concentrated solution is commercially available that is simply added to the culture medium. In many cases, this solution contains penicillin and streptomycin as antibiotic agents, and amphotericin B as an antifungal agent.

3.3 Cell culture medium

This is a liquid that provides a suitable growing environment for the cultivation of human and animal cells. The amino acids, vitamins, salts, and so on, required for cell growth are all included. The pH is adjusted to be close to the optimum neutral for cell growth. In many cases, it is made by adding fetal bovine serum (FBS) to the above basal medium to about 10%. Various typical formulas have been established including Eagle's basal medium, DMEM, Roswell Park Memorial Institute (RPMI) 1640 culture medium, and so on.

3.4 Prepared stock suspension

This is a suspension of CNTs that has been dispersed with an aqueous solution of BSA.

3.5 Culture medium preparation solution

This is a suspension of CNTs used for *in vitro* cell-based assay. A preparation stock suspension is prepared by diluting it by a prescribed factor with the cell culture that is used for *in vitro* testing.

4. Test method

4.1 Cell line

A cell line is used for the respiratory tract model that is intended for simple and rapid safety assessment of CNTs. For example, to model Type II alveolar epithelial cells, human alveolar epithelial adenocarcinoma cells (A549) are used, and to model macrophages, rat macrophage cells are used (NR8383). Storage and preparation of the cell line is conducted according to the specified methods.

4.2 Cell culture

With prescribed concentration, cells in the logarithmic growth phase are planted in a multi-well plate having an appropriate well number for the number of test items. It is best to perform the test using about four wells for every sample. After incubation overnight under conditions of optimum temperature, humidity, and carbon dioxide, the states of the cells are studied with an inverted microscope to verify that they have grown appropriately. The cells are then withdrawn by suction using an aspirator to either replace the culture medium preparation solution or add to it. The culture medium preparation solution in this case is for the CNT treatment group, apart from which negative and positive control groups are also prepared. Cells are remarkably influenced by the dispersant used for the preparation of CNTs in the stock suspension. Thus, the cytotoxicity of the dispersant is necessary to check in advance (Horie et al, 2014). The negative control group uses cells that have been cultivated by adding a preparation stock suspension containing no CNTs to the cell culture. For the positive control group, some chemical reagent is chosen to serve as an index for each respective end point, cell culture medium is added, following which those cultivated cells used. The results of cell tests for the negative and positive control groups serve as indices for a relative assessment between the reliability and the effect of CNTs on each respective cell test system. Following that, after a prescribed incubation time, the following tests are performed.

4.3 Cell viability test

For a cell viability test, we have the measurement of color changes in formazan dye of tetrazolium salts due to the mitochondrial hydrogenase in living cells (such as the WST-1 method; WST stands for “water soluble tetrazolium salts”); we also have the measurement of cell proliferating ability from its capacity to form colonies. These should be chosen and used as

is thought appropriate. Measurements are made after being cultivated in the culture medium preparation solution for each of the prescribed times. The MTT method (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole), which uses mitochondrial enzyme activity as an end point, is not considered suitable since colorimetry is inhibited by the addition of CNTs (Wörle-Knirsch et al, 2006).

4.4 Measurement of intracellular reactive oxygen species (ROS)

It has been reported that, due to the presence of CNTs, oxidative stress is induced from the reactive oxygen species (ROS) that are generated (Fujita et al, 2013). Therefore, cell permeable fluorescent probes (such as DCFH-DA (2', 7'-Dichlorodihydrofluorescein diacetate)) are used for the ROS, and the fluorescent intensity of the fluorescent probe captured inside the cells (such as DCFH (2', 7'-Dichlorodihydrofluorescein)), which have been cultivated in the culture medium preparation solution for the prescribed times, is then used as a measure of the activity of the reactive oxygen species inside the cell. In animal testing using rodents such as rats, measuring the ROS inside these cells can be considered to be a useful biomarker for verifying the validity of damage assessment methods.

4.5 Cell cycle analysis

Inhibition of specific cell cycles by CNTs has been reported. For that reason, a fluorescent probe that intercalates with the DNA double helix is used (Sargent et al, 2009). These fluorescent probes captured inside cells are measured for each prescribed time the cells were cultivated in the culture medium preparation solution. The measurements reveal the state the cell cycles are in, and thus analyze for any inhibition of cell cycles.

4.6 Measurement of inflammatory cytokines

It has been reported that inflammatory cytokines are produced due to CNTs (Herzog et al, 2009). Since inflammatory cytokines are involved as a causal factor in evoking various inflammatory responses in the organism, they are a useful index. Furthermore, in animal testing using rodents, such as rats, inflammatory cytokines are considered to be a common biomarker for verifying the validity of damage assessment methods. There are several different kinds of inflammatory cytokines (examples: IL-1b, MCP-1, MIP-1a, IL-1b, IL-6, IL-10, IL-12p70, IFN- γ , MCP-1, TNF- α , etc.) depending on the animal species. By selecting the optimum from amongst these, various inflammatory cytokine measurements are possible either simultaneously or independently. Both single and multiple cytokine measurements use the supernatants from cell cultures that have been cultivated over prescribed times in the cell culture medium preparation solution.

4.7 Gene expression analysis

Genes activated or repressed by CNTs are considered a useful biomarker by pinpointing the biomarkers (endpoints) verifying the validity of safety assessment methods that use animal testing with rodents, such as rats. Using cells that have been cultivated over prescribed times in a culture medium preparation solution, the total RNA is extracted, and based on this, with quantitative real-time polymerase chain reaction (qRT-PCR) and DNA microarrays, specific or comprehensive gene expressions can be analyzed.

4.8 Transmission electron microscopy (TEM) analysis

The uptake of CNTs by cells and the characteristic of incorporated CNTs are considered useful data when observing each biomarker (end point). Using cells that have been cultivated over prescribed times in a culture medium preparation solution, the CNTs are washed with a phosphate buffer, prefixed with glutaraldehyde, washed again, and postfixed with osmium tetroxide, following which the cells are dehydrated and embedded in the resin in order to prepare ultrathin-sections that are then stained and reinforced. The cell samples are then observed using an electron microscope.

5. Report

Report on the following items.

5.1 General

- a) Detailed information for identifying the dispersed sample (such as information about the particle sample, dispersion preparation method, information about the dispersing medium, date the dispersed sample was acquired, the method of transportation, characterization of the dispersed sample in solution)
- b) Measurement date, name of person doing the measurements, and name of the organization doing the measurements
- c) Date on which the validity of the apparatus was verified
- d) Storage conditions for the dispersed sample (such as temperature, storage facility, and storage time)
- e) Results of visual observations related to precipitation formation (such as images from a digital camera)

5.2 Apparatus and measuring reagents, experimental and analytical conditions

- a) Name of the apparatus, model, and name of manufacturer
- b) Positive control substance used in the validation and the validation results
- c) Name of measuring reagent and its manufacturer
- d) Name of software used in analysis and its manufacturer

5.3 Results

5.3.1 Cell viability test

Cell survival rate of the CNT treatment group compared with the negative control group and standard deviation over repetitions

5.3.2 Measurement of intracellular reactive oxygen species (ROS)

Absolute value of the intracellular ROS level in all groups (such as the DCF value), or the cell ROS level of the CNT treatment group compared with that of the negative control group and standard deviation over repetitions

5.3.3 Cell cycle analysis

Proportions for each cell cycle (G1 period, S period, G2 period, M period) in all groups and their standard deviations over repetitions

5.3.4 Measurement of inflammatory cytokines

Value of each inflammatory cytokine in all groups and the standard deviation over repetitions

5.3.5 Gene expression analysis

Gene expression variable ratio for the carbon nanotube treatment group compared with that of the negative control group; depending on the case, the significant difference in the expression gene functional group analysis, clustering such as hierarchical clustering, and analysis of the network with the pathway database.

5.3.6 TEM analysis

Observational record of cell samples for all groups

Appendix A

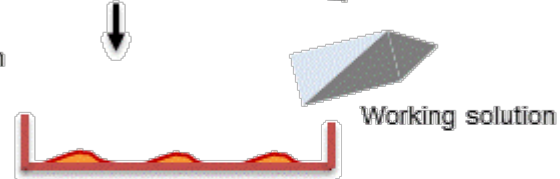
System diagram of *in vitro* cell-based assay for safety assessment

(1) Preculture

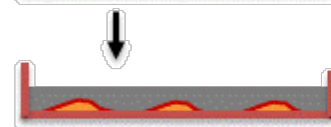
(2) Cells in a logarithmic growth phase



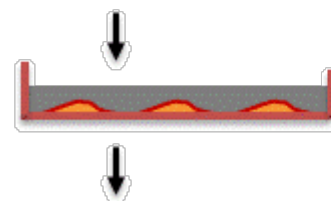
(3) Replacing a stock suspension
to cell culture medium



(4) Culture at time zero



(5) Prescribed cultivation time



in vitro assay

Appendix B

Example: *In vitro* cell-based assay for SG-SWCNTs using A549 cells

B.1 Outline

An actual case example is presented of the method of *in vitro* cell-based assay; the example uses A549 cell line and was conducted according to the above procedures. For the A549 cell line, a culture preparation solution (concentration of approx. 0.1 mg/mL) of SG-SWCNTs was exposed for 48 h, while simultaneously, a test to measure the activity of the intracellular ROS, cell cycle analysis, a comprehensive gene expression analysis, and observations of cell morphology using TEM, were conducted (Fujita et al. 2013).

B.2 Preparation of sample for use and characterization

The culture medium preparation solution was prepared according to the sample preparation method for *in vitro* cell-based assay in the procedure given in Section III: “Sample preparation of carbon nanotubes for in vitro cell-based assay”. Both the stock suspension and the working solution were evaluated according to the procedure given in Section IV: “Characterization of CNT working solution for *in vitro* cell-based assay”, Section V: “Concentration of carbon nanotubes suspended in culture medium” and Section VI: “Metal concentrations of carbon nanotubes suspended in culture medium”.

B.3 Cell and culture conditions

For a cell test using cultivated cells, the A549 cell line (RIKEN) was used. The cells were cultivated in a DMEM with 10% added FBS in 5% CO₂ at 37°C, and the culture preparation solution was diluted in concentrations of ×1, ×10, and ×100. The cells were cultivated for 48 h. The code names for each of the samples are abbreviated below. For details about SG1-1 and SG1-2, please refer to Appendix A of Section III: “Sample preparation of carbon nanotubes for in vitro cell-based assay”

#	Sample	Code	Dilution conditions
1	SG-SWCNT working solution	SG1-1	×1
2	SG-SWCNT working solution	SG1-2	×10
3	SG-SWCNT working solution	SG1-3	×100
4	SG-SWCNT working solution	SG2-1	×1
5	SG-SWCNT working solution	SG2-2	×10
6	SG-SWCNT working solution	SG2-3	×100
7	Negative control medium (BSA included)	Control	-

B.4 Cell viability test

Effects on cell survival due to SG-SWCNTs (for 24 and 48 h exposures) were measured according to WST-1 (Takara Bio, Inc.) and WST-8 (Kishida Chemical Co., Ltd.); these were used

as indicators of mitochondrial dehydrogenation activity in the living cells. Results from measuring the cell growth potential for each group using WST-1 and WST-8 showed no significant differences. In both tests, a positive control group was provided, including 100 μ M Mitomycin C, and WST-1 test system was verified.

B.5 Measurement of intracellular reactive oxygen species (ROS)

Effects on induction of oxidative stress due to SG-SWCNTs (exposed for 24 and 48 h) were observed by measuring the production of level of ROS within cells using flow cytometry (Merck Millipore) with the DCFH (2', 7'-Dichlorodihydrofluorescein diacetate) method. Results showed that compared to the negative control group, the ROS production level increased over time and the ROS production level depended on the SG-SWCNT concentration.

B.6 Cell cycle analysis

After exposure, the cells were fractionated using flow cytometry (Merck Millipore) into M1 (the mitotic phase), G1 (the gap phase), S (the synthesis phase), and M2 (the mitotic 2 phase), and the inhibition of SG-SWCNTs on cell cycles were investigated for an exposure of 24 h. Results showed that the proportions of fractionated cells in each group were similar. The inhibition of SG-SWCNTs on cell cycles can be considered small. We note that 10 μ M of Nocodazole, as a cell cycle progress inhibitor, was provided for the positive control group, and its test system was verified.

B.7 Comprehensive gene expression analysis

Effects of SG-SWCNTs (exposure of 24 and 48 h) on the comprehensive gene expression were analyzed using a DNA microarray. After 24h cultivation, the total cell RNA was extracted with a commercially available kit (Qiagen), and then the quality of decomposition was checked using a Nanodrop 2000 (Thermo) and Bioanalyzer (Agilent). Based on the total RNA, an comprehensive gene expression analysis was conducted using a human DNA microarray (Agilent). With this result, significant expression of the main genes associated with inflammation or apoptosis, oxidative stress, or extracellular degrading enzyme, for example, was not shown. We can therefore consider that the effect of SG-SWCNTs on gene expression is small. Details about these results are included in the gene expression information database, GEO (Gene Expression Omnibus) at the U.S. National Center for Biotechnology Information (NCBI), accession number: GSE41101.

B.8 TEM analysis

Using TEM (H-7000, Hitachi Ltd.), we investigated the shapes inside cells and the uptake of SG-SWCNTs following exposure for 24 and 48 h. Results showed that uptake of SG-SWCNTs s occurred both in the SG-1 and the SG-2 groups for exposures of both 24 and 48 h (Fig. B.1). Fine

bundles thought to be SG-SWCNTs make up several massive forms localized in places thought to be vacuoles. However, these were not observed in the control group for exposures of either 24 or 48 h. For both groups, no abnormal morphology was found in the cells.

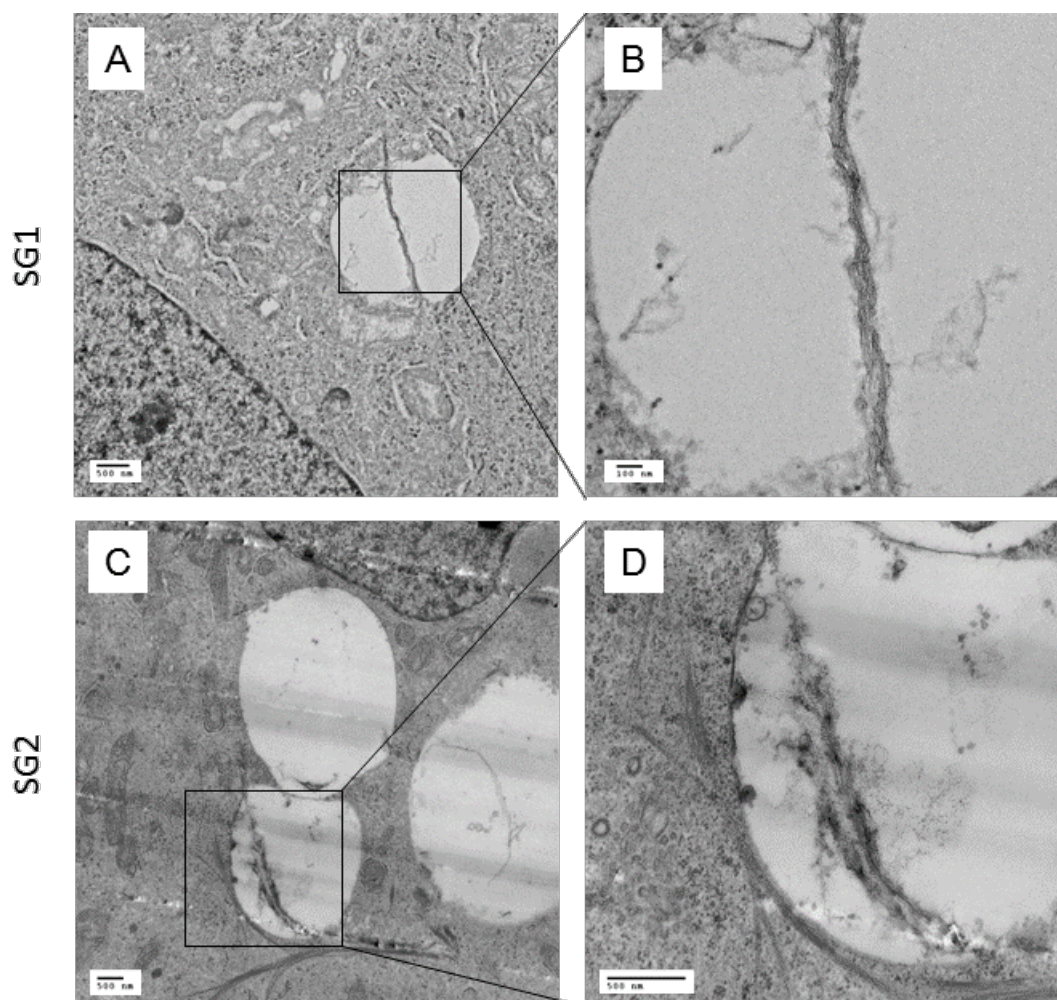


Figure B.1 SG-SWCNTs from A549 after exposure for 24 h viewed using a transmission electron microscope. Panel B is an enlarged view of the cell exposed to CNT-1 (Panel A). Panel D is an enlarged view of the cell exposed to CNT-2 (Panel C).

B.9 References

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Appendix C

Findings: Suitability of SG-SWCNT *in vitro* testing methods using A549 cells

We present here the technical suitability of the SG-SWCNTs *in vitro* testing methods for each of the test items in the example of Appendix B. We also give research results for the number of actual implementations of each item in the existing literature.

End point	Test method	Suitability of the test method	Findings from the examples	Number of implementations (137 reports) of <i>in vitro</i> cell-based assay in the existing literature (October, 2005)
Cell viability	MTT	Unsuitable	Not appropriate due to inhibition of colorimetric values by CNT exposure	45
Cell viability	WST-1	Suitable	Considered a suitable method	9
Cell viability	WST-8	Suitable	Slightly higher sensitivity than WST-1	3
Cell viability	release of lactate dehydrogenase (LDH)	Unsuitable	Not appropriate due to inhibition of colorimetric values by CNT exposure	19
Apoptosis	Caspase	Suitable	Considered a suitable method.	4
Oxidative stress	DCFH	Suitable	Increase in ROS inside the cell	12
Cell cycle	pH3- (Ser10)	Suitable	Possible.	0
Inflammation marker	cytokines	Suitable	Choice of several from 13 kinds of cytokine	23
Gene expression analysis	DNA microarray, q-PCR	Suitable	Marker search and profile using gene expression	4
Observed cell morphology, CNT uptake	TEM	Suitable	Requires effort for a cell prefixing method	6

The person in charge of writing Chapter VII.

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Sample preparation, measurement, and procedures for *in vitro* cell-based assay as a safety test for carbon nanotubes (abbreviation: Procedures for safety testing)

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