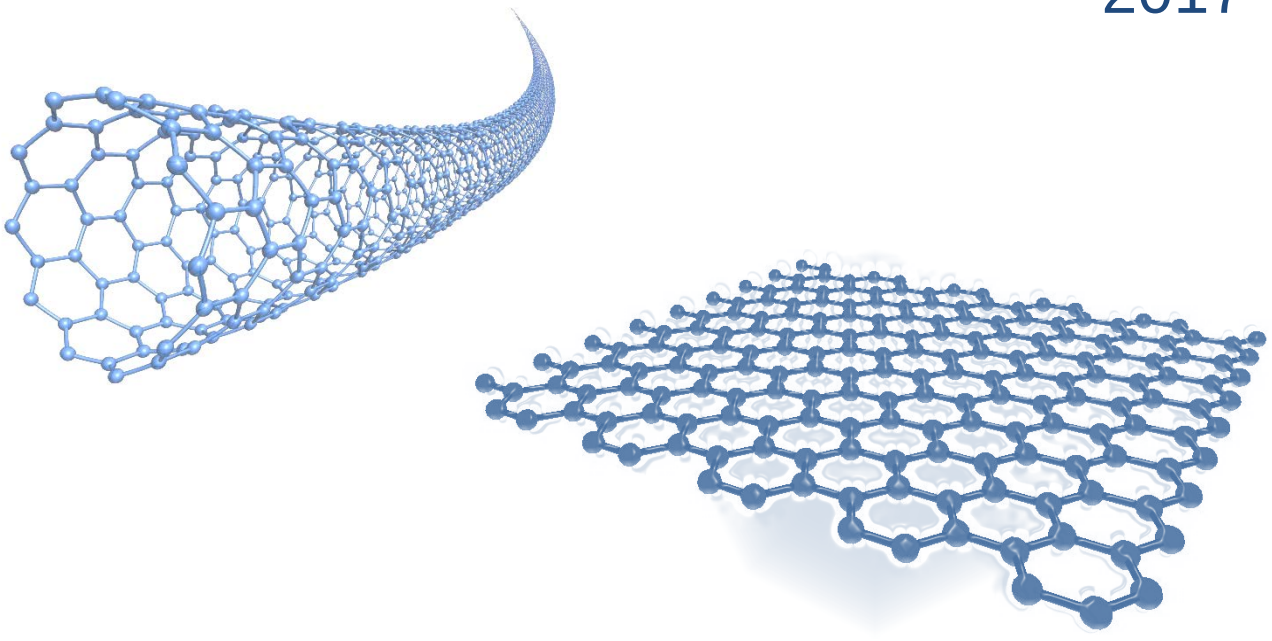
General Procedures for Safety Tests on Carbon Nanomaterials

Procedures for Sample Preparation, Characterization, In Vitro Cell-Based Assays, and Animal Tests on Carbon Nanomaterials

2017



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 the results obtained as part of 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

The development of industrial products that are prepared using carbon nanomaterials such as carbon nanotubes (CNTs) and graphene, as well as their application to information technology and communications, the environment, energy, and the like are being pursued on a global scale. The Technology Research Association for Single Wall Nanotubes (TASC), and National Institute of Advanced Industrial Science and Technology (AIST), have been engaged in basic research as well as application and utilization development aimed at the practical implementation of single-wall nanotubes (SWCNTs) and graphene, through projects established by the New Energy and Industrial Technology Development Organization (NEDO), a national research institute.

On the other hand, there are increasing concerns regarding possible harmful effects that were not an issue in the past, these being a result of physical characteristics such as the ultrafine sizes and shapes of the carbon nanomaterials. It is imperative that business operators implement voluntary safety controls based on preventive concepts, given the current conditions in which rapid technical innovations are appearing and while safety-related knowledge is still being pursued. Assistance provided by public research institutions for voluntary safety control to be implemented by business operators is considered necessary, since the voluntary verification of safety is not easy. TASC and AIST have developed a simple and rapid safety evaluation method to support voluntary safety control by business operators and testing laboratories that handle CNTs as part of a project established by NEDO, that is, the "Development of Innovative Carbon Nanotube Composite Materials for a Low Carbon Emission Society" (2010 to 2014). Subsequently, the "Sample Preparation and Characterization for Safety Testing of Carbon Nanotubes, and in vitro Cell-based Assay" (hereinafter referred to as the "Procedures for Safety Testing") was prepared and published in 2013. This document summarizes those procedures related to sample preparation and characterization of CNTs for safety testing, as well as in vitro testing, with the goal of aiding the implementation of voluntary safety controls by business operators that handle CNTs as part of their business or for research and development. The evaluation results and knowledge derived from these procedures, as performed by TASC and AIST on SWCNTs, are listed as implementation examples.

Furthermore, previously accumulated knowledge was utilized and the subject materials were extended to include carbon nanomaterials, while minimal required safety evaluation standards were established with the aim of building voluntary safety assessment procedures that include data supplementation through animal testing in the project established by NEDO, named the "Innovative Carbon Nanotubes Composite Materials Project toward Achieving a Low-Carbon Society" (2014 to 2016). This "General Procedures for Safety Tests on Carbon nanomaterials" provides a summary of the procedures for not only the sample preparation and characterization of carbon nanomaterials, as well as in vitro cell-based assay, but also animal testing that complements in vitro cell-based assays. Practical applications of sample preparation and characterization, as well as in vitro cell-based assays and animal testing on subjects other than the SWCNTs developed by TASC and AIST, such as multi-wall carbon nanotubes (MWCNTs) and semiconducting/metallic SWCNTs and graphenes, were collected to configure this document in such a way that it would be easy for business operators and testing laboratories to make use of the content.

TASC has also been engaged in the development of accumulation of measurement methods for airborne carbon

nanomaterials, for locations such as work environments. Their case examples and findings were summarized and published as the "Guide to Evaluating Emission and Exposure of Carbon Nanomaterials (carbon nanotubes and graphene)". Furthermore, a document containing existing information on the safety of super-growth SWCNTs, eDIPS SWCNTs, and exfoliated graphenes, information provided by AIST, as well as the evaluation results obtained from activities conducted independently by TASC, was prepared and published as the "Case Study Report." It is hoped that this document, that is, the "General Procedures for Safety Tests on Carbon nanomaterials" will be used as a reference for the voluntary application of safety controls for carbon nanomaterials, together with the "Procedures for Safety Testing," "Guide to Evaluating Emission and Exposure of Carbon Nanomaterials (carbon nanotubes and graphene)," "Guidebook for Evaluation of Carbon Nanomaterial Emissions and Exposures," and the "Case Study Report." Furthermore, these documents are available free of charge for downloading at the website of the Research Institute of Science for Safety and Sustainability at AIST (http://www.aist-riss.jp).

October 10, 2017

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Chapter I: Introduction

Purpose

This document describes the procedures for in vitro cell-based assays and those animal tests that supplement in vitro cell-based assays as simple and rapid safety evaluation methods for voluntary safety controls, to be implemented by business operators that handle carbon nanomaterials. The test procedures described in this document can be applied to the health and safety management of workers in manufacturing- or processing-related facilities handling carbon nanomaterials, regarding their effect on human health through respiratory routes, since workers involved with the manufacturing of carbon nanomaterials are assumed to be at the highest potential risk of exposure.

Background

Carbon nanomaterials are expected to promote the development of application materials that are ultralight, very strong, and highly functional due to their nanoscale structures. On the other hand, there are concerns regarding their effect on the health of humans. While there have been no clear examples that prove that carbon nanomaterials have any serious impact, it is important for business operators to voluntarily implement safety controls to alleviate any possible anxieties arising from the nanosizes of these materials, and to prevent rumors from arising. The concept of such "voluntary safety control" is effective for carbon nanomaterials, given that technical innovations are proceeding rapidly, since business operators would lose out to international competition if they were forced to wait for the establishment of laws and regulations pertaining to safety. Considering this in a positive light, the voluntary implementation of safety evaluations to demonstrate the safety of products can constitute a selling point for these materials. If business operators dealing with carbon nanomaterials were to indicate their hazard evaluation information, it would reflect positively on the social responsibility of such businesses (corporate social responsibility (CSR)) in response to the demands of individuals within such businesses, as well as interested parties and the public at large.

How should business operators implement safety evaluations for carbon nanomaterials as part of their "voluntary safety control"? The determination of the safety of substances and the related hazards is made based on animal tests in the case of ordinary chemical substances; however, animal tests incur substantial cost and are time consuming. Conducting high-cost animal tests over long periods of time for each of many carbon nanomaterials with different physical and chemical characteristics according to their applications is not realistic. Furthermore, there is a demand for safety evaluations through simple in vitro cell-based assays that do not rely on animal tests as far as possible, based on the principle of 3R pertaining to animal testing (replacement, reduction, and refinement).

Carbon nanomaterials shall be added to cultures in which cells are cultured during in vitro cell-based assays. The properties of carbon nanomaterials are such that they coagulate and form coagulates in a liquid culture medium (secondary particles) and settle directly onto the cells. The use of dispersing agents that primarily act as

surfactants is often implemented as a means of resolving such issues, but such dispersing agents themselves are often cytotoxic. This means that the use of dispersing agents shall be restricted, while it is extremely important to develop a means of preparing samples by stably dispersing carbon nanomaterials, to evaluate the impact on cells of the characteristics that are unique to carbon nanomaterials. In addition, the physiochemical characterization of carbon nanomaterials (particle sizes, concentration, ion concentration in liquid, protein compositions contained in cultures and additives, as well as the depletion of cell-sustaining components associated with the absorption of inorganic salts by carbon nanomaterials) shall be conducted using appropriate evaluation methods to not only verify whether the materials are stably dispersed but also to gain an understanding of which parameters have an impact on the cultured cells. There are many unknowns regarding the mechanism of the toxicity development of carbon nanomaterials in living organisms and the available information continues to be insufficient. It is therefore important to not only identify the biological impact on cells by using conventional methods but also to conduct the selection of new evaluation items (endpoints) that correctly reflect the impact of carbon nanomaterials and to conduct measurements accordingly. Furthermore, the development of analysis methods for conducting comparative examinations of the biological impact of carbon nanomaterials, as well as on different cell types for a given carbon nanomaterial, along with the implementation of verification by animal testing to determine the validity of the predicted impact on living organisms, based on safety tests derived by such in vitro cell-based assays, are considered important.

Outline

The characteristics of this testing method are (1) the stable and uniform dispersion of carbon nanomaterials in liquids, followed by the measuring of the dispersion conditions and properties of the liquid; (2) the evaluation of the dispersion conditions and the properties of carbon nanomaterials added to a cell culture medium for the in vitro cell-based assays of liquids administered in animal tests; (3) the selection of the impact of carbon nanomaterials on cells and animal tissues, that is, biological indices (endpoints) to select test methods that eliminate, as far as possible, impediments arising from carbon nanomaterials on such findings; (4) the quantitative analysis of carbon nanomaterials found in biological tissues after intratracheal instillation into animals. The preparation of samples for safety tests on carbon nanomaterials and their characterization, as well as cultured cells and animal testing, are summarized in Figure I-1.

1. Preparation of Carbon Nanomaterial Samples and Characterizations of Suspension Samples

This document describes a fabrication technology to enable the stable dispersion of samples in a cell culture medium or instillation liquid using highly accurate measurement technology to conduct in vitro cell-based assays and animal testing of carbon nanomaterials. The physiochemical properties and the component composition data for the powder of the carbon nanomaterials shall first be secured and then, when preparing the stock suspension, the carbon nanomaterials shall be dispersed uniformly and stably in the cell culture medium while minimizing the use of dispersant to minimize the impact on animal tissues as far as possible (Figure I-1, ①). Furthermore, the prepared concentrate suspension shall be diluted in the cell culture medium or a medium to derive the liquid to be administered to animals. The secondary characteristics of carbon nanomaterials in prepared stock suspensions, as well as the cell culture medium or animal instillation (particle size of carbon nanomaterials, as

well as concentration, ion concentration, absorption of proteins and inorganic salts in the culture) shall be measured to verify their stable dispersion (Figure I-1, ②). The decision to conduct in vitro cell-based assays and animal tests as safety tests for carbon nanomaterials shall be made based on the results of such tests.

2. In Vitro Cell-based Assays for Safety Testing of Carbon Nanomaterials

A cell culture medium, prepared and characterized as described in Section 1, above, is used to conduct in vitro cell-based assays to evaluate the hazards related to carbon nanomaterials by identifying the presence and differentiation potency of cells from relevant cultured cells by assuming the health impact on the respiratory system from exposure through inhalation, while endpoints such as inflammation markers, oxidation stress, and apoptosis are selected to conduct measurements (Figure I-1, ③).

3. Animal Tests for Safety Testing of Carbon Nanomaterials

The animal instillation liquid prepared and characterized as described in Section 1 above is used, and the health impact on the respiratory organs of inhalation exposure is assumed, with the selection of appropriate endpoints such as the number of cells in bronchoalveolar lavage fluid (BALF), inflammation markers, pulmonary pathology observations, and genetic expressions of pulmonary tissues, while taking measurements and conducting animal testing to evaluate the hazards presented by carbon nanomaterials. The carbon nanomaterials that are present in pulmonary tissues and the like are quantitatively and qualitatively analyzed to determine the distribution of carbon nanomaterials within the tissues of the animals to which the nanomaterials are administered, as well as their migration outside such tissues and changes over time (Figure I-1, ④).

4. Publication of General Procedures for Safety Tests

The test procedures described above and the examples of their implementations, conducted according to such procedures, have been appended to prepare and publish this document, to aid with the implementation of voluntary safety controls implemented for partnership activities conducted with international organizations, as well as by businesses that handle carbon nanomaterials (Figure I-1, ⑤).

A safety evaluation, as described here, targets presumed inhalation routes for the health and safety control of workers who are involved in the manufacture of carbon nanomaterials and, as such, it does not target oral or transdermal routes. Furthermore, it should be noted that test results derived with compliance to the provisions of this document evaluate the impact on the respiratory system, primarily dealing with acute inflammation and genotoxicity, and do not guarantee safety.

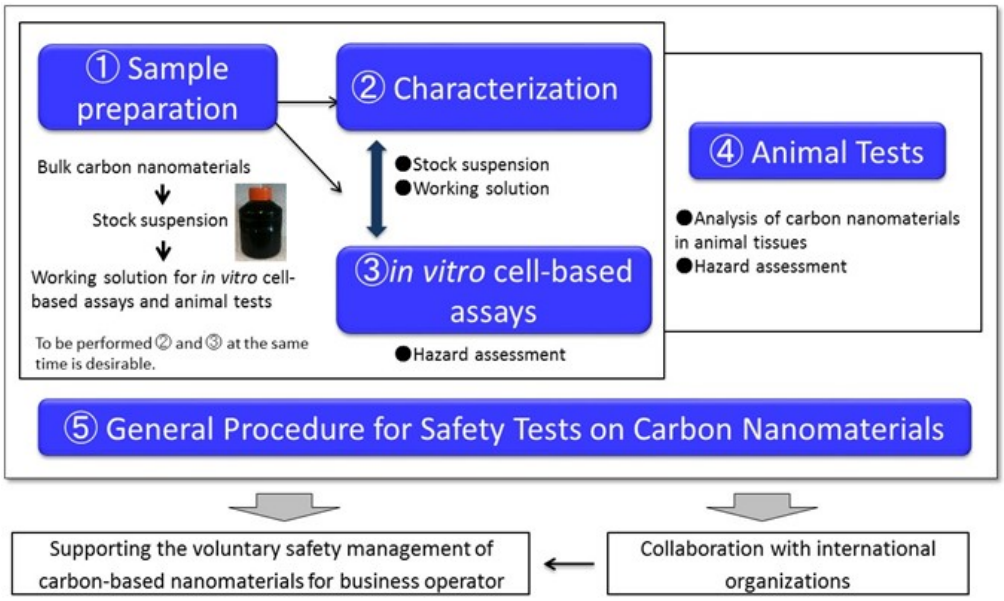

Figure I-1 Overview of sample preparation, characterization, in vitro cell-based assays and animal tests on carbon nanomaterials

Chapter II: Measures for Preventing Contamination and Exposure

1. Sterilization operations

For in vitro cell-based assay, sterilization is essential to the prevention of contamination by bacteria. Equipment such as the stirring bars, beads, and vessels used for the bulk powder, the dispersion medium, and dispersed samples should all (as far as possible) be sterilized in an autoclave. Equipment that cannot be autoclave sterilized should be washed with ethanol and irradiated with ultraviolet light for 3 min to kill any existing bacteria.
Reference standard JIS T 7322 High-Pressure Steam Sterilizers for Medical Use.

2. Countermeasures against endotoxins

Endotoxins can activate white blood cells and react with living organisms and cultured cell systems. Measures shall be implemented to prevent the contamination of samples by endotoxins during sample preparation, since endotoxin contamination of the dispersion liquid used for testing can lead to an erroneous hazard evaluation. This is particularly important for in vitro cell-based assays, in which cells react directly. For instance, deionized water that has been passed through a filter to remove endotoxins (ultrapure water or sterilized water) is used for the dispersion medium. Endotoxins exist in normal environments and can easily contaminate deionized water when it is stored for an extended period. It would therefore be desirable to prepare deionized water only when it is required and only in the required amount.

The presence of endotoxin contamination can be identified in the preparation process of a dispersion liquid used with carbon nanomaterials to prevent contamination as far as possible. The gel-clot method of the Limulus amoebocyte lysate (LAL) test, adopted in the ISO 29701 standard, for instance, is a suitable test method. This method relies on the gelification of the lysate reagent, as caused by the actions of endotoxins, 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 carbon nanomaterials

The health effects of carbon nanomaterials on humans have not yet been clarified, but care shall be taken when handling carbon nanomaterials to prevent exposure, in accordance with the precautionary principles.

There is the potential for exposure when carbon nanomaterials are handled in the dry state, such as when weighing the powder or performing the pretreatment steps, which would lead to scattering of the powder. Work shall, therefore, be performed inside a sealed unit such as a glove box or in a draft chamber with a high-performance filter with suitable local ventilation to prevent this from occurring. There are instances when fans should be stopped during work operations but turned on when taking out containers or cleaning, since running an exhaust fan during a weighing operation can lead to scattering. Furthermore, operatives should wear protective clothing such as dust masks, protective gloves, protective goggles, and lab coats. Work should be carried out on a clean bench or inside a local ventilation unit to ensure that material scattering does not occur, since the

dispersion liquid can scatter during operations such as wet dispersion. Ideally, operatives should also wear protective clothing in such instances.

Refer to the notifications and guidelines issued by the relevant institutions regarding the ventilation method for glove boxes and local ventilation units, as well as the methods for selecting protective clothing, and the methods used to treat waste materials. Protective clothing is also effective as a preventive measure against bacteria and endotoxin contamination.

Reference standard: "Countermeasures for Prevention of Exposure to Nanomaterials" issued by the Ministry of Health, Labour and Welfare, Japan, Publication No. 0331013 (March 31, 2009).

Chapter III: Preparation of Suspension Samples of Carbon Nanomaterials

Introduction

The procedure for preparing the suspension samples used in "in vitro cell-based assays using suspension samples of carbon nanomaterials" is described in Chapter V, as are the "animal tests using suspension samples of carbon nanomaterials." A characteristic of both these procedures is that bio-derived materials are used to produce a suspension of carbon nanomaterials without the use of any surfactants or polymer electrolytes. Evaluation Tests on the hazards presented herein are conducted over three days from the time that the suspension is added to the cells as part of the in vitro cell-based assay. It is important for the carbon nanomaterials to be suspended stably during this period. A stable method of preparing samples during the test period is described below. Also described are methods of evaluating the carbon nanomaterial concentrations of samples prepared for testing, as well the presence of endotoxins. These methods may be used to prepare the samples administered in animal tests, such as intratracheal instillation tests and genotoxicity tests.

1. Scope of Application

Suspension samples of the carbon nanomaterials used for in vitro cell-based assay are prepared by ultrasonic irradiation to disperse any aggregates of carbon nanomaterials in an aqueous solution of bovine serum albumin (BSA). Furthermore, the concentration of the carbon nanomaterials in the liquid is evaluated by measuring the absorbance.

This method can be used to prepare samples for animal tests, in which the samples are administered to the animals by endotracheal injection.

2. Referenced Standards and Regulations

This procedure refers to the following standards and regulations:

Testing Methodology for Endotoxins (Gelification Method Using Lysate Reagent) in the 16th revision of the Japanese Pharmacopoeia.

"Countermeasures for Prevention of Exposure to Nanomaterials" (March 21, 2009) standard issued by the Ministry of Health, Labour and Welfare, Japan. Publication No. 0331013 (March 31, 2009).

3. Terminology and Definitions

3.1 Stock Suspension

Suspension prepared by dispersing carbon nanomaterials in aqueous BSA solution.

3.2 Working Solution

Carbon nanomaterial suspension used for in vitro cell-based assay. The solution is prepared by diluting the prepared stock suspension with the cell culture medium used for testing, to a prescribed concentration.

3.3 Centrifugal strength

Ratio of centrifugal acceleration to the acceleration due to gravity (*g*). This figure describes the operating conditions used for the centrifugal separation. This value is expressed as *xg* when the centrifugal strength is *x*.

3.4 Bovine Serum Albumin (BSA)

Protein derived by purifying the serum of cattle. BSA shall be used as an alternative to a surfactant for dispersing carbon nanomaterials in water.

3.5 Endotoxins

Toxins released by microorganisms.

4. Equipment and Instruments

4.1 Dispersing Equipment

Equipment used to disperse carbon nanomaterials in a liquid, using ultrasound to disperse particles in an agglomerate or aggregate state (i.e. clumps). An ultrasonic homogenizer produces ultrasonic irradiation from a horn (probe) inserted directly into the suspension or via an ultrasonic bath, with ultrasonic waves emitted by an ultrasonic emitter irradiating out of the container via the liquid.

4.2 Specimen Containers

Containers are either 50 mL glass beakers or 100 mL sterilized glass vials. These are used as containers for dispersion operations.

4.3 Centrifuge Separator

Equipment used to remove coarse clumps and/or fine particles from a carbon nanomaterial suspension by centrifugal separation. Supernatants or sediments are collected after centrifugal separation.

4.4 Centrifuge Tube (Centrifuging Tube)

A 50 mL sterilized tube.

4.5 Cell Strainer

A cell strainer is used to remove any coarse clumps of carbon nanomaterials that remain in the solution if the suspension has been insufficient.

4.6 Carbon Nanomaterial Concentration Measuring Equipment

Absorbance measurement equipment that takes measurements in the ultraviolet-visible light (UV-Vis) absorption range to evaluate the carbon nanomaterial concentration.

4.7 Suspended Carbon Nanomaterial Secondary Particle Size Measurement Equipment

Particle size measurement equipment based on either the dynamic light scattering (DLS) principle or the differential centrifugal sedimentation (DCS) principle.

4.8 Carbon Nanomaterial Observation Equipment

Equipment used to observe the dispersion state or shape of carbon nanomaterials, or to conduct profilometry based on an image analysis method. A transmission electron microscope (TEM) or scanning electron microscope (SEM), as well as other types of microscopes, can be used to observe individual particles.

5. Samples and Reagents

5.1 Carbon Nanomaterials

The materials shall be in a good, dry condition, with no need to perform heat treatment.

5.2 Dispersion Medium and Its Preparation

5.2.1 Dispersant

Bovine serum albumin (BSA) is used as the dispersant.

5.2.2 Dispersion Medium

A liquid medium in which carbon nanomaterials are suspended. BSA shall be dissolved in distilled water to a concentration of 10 mg mL. This aqueous BSA solution shall be sterilized with a 0.22-μm sterilization filter and used as the dispersion medium.

Note: The water may instead be prepared by first purifying with a purified-water production system and filtering with an endotoxin filter, after which it can be used as purified water.

5.3 Lysate Reagent

Reagent used to detect the endotoxins in a stock suspension, based on a coagulation reaction (gelification method).

6. Pretreatment

5.1 Sterilization of apparatus

Sample containers are sterilized using an autoclave at 121 °C for 20 min

The horn of the ultrasonic homogenizer shall be sterilized by disinfecting with 70% aqueous ethanol solution and then irradiating with ultraviolet light for 3 min.

Other equipment shall be disinfected with 70% aqueous ethanol solution.

6.2 Pretreatment and Sterilization of Carbon Nanomaterials

The amount of nanocarbon material necessary to prepare a suspension sample of the prescribed concentration shall be weighed and sampled.

When the carbon nanomaterial is supplied as a raw material in sheet form, then that carbon nanomaterial shall be diced into small pieces using a pair of tweezers and/or scissors.

The prescribed amount of collected carbon nanomaterial shall be placed in a sample container and irradiated with ultraviolet light for 3 min.

Note: A suspension derived without performing any preparation operations such as centrifugal separation may be used as a prepared solution when using an ultrasonic bath to disperse the solution. Carbon nanomaterials sampled with a dispersion concentration suitable for use in hazard testing shall be placed in a vial.

Warning: Perform the weighing and pretreatment of carbon nanomaterials in a local ventilation unit and avoid exposure to carbon nanomaterials according to the "Countermeasures for Prevention of Exposure to Nanomaterials" standard issued by the Ministry of Health, Labour and Welfare, publication No. 0331013. Furthermore, tasks shall be performed by operatives wearing protective clothing, including masks and gloves.

7. Preparation Procedure for Stock Suspensions

7.1 Preparation of Carbon Nanomaterial Suspension

The carbon nanomaterial concentration in the prepared stock suspension shall be no more than 1 mg mL.

Add the prescribed amount of dispersion medium (for instance, 50 mL) prepared by the procedure described in Section 5.1.2, to the sample container (beaker or vial) containing the carbon nanomaterials sampled and sterilized according to the procedure described in Section 6.2.

Thoroughly wet the carbon nanomaterial with the dispersion medium.

Warning: The solution shall be agitated with a stirrer on a clean bench, to avoid contamination from the air. Furthermore, tasks shall be performed by operatives wearing protective clothing, including masks and gloves.

7.2 Dispersion

7.2.1 Outline

The dispersion of carbon nanomaterials in a liquid shall be performed by ultrasonication. An ultrasonic homogenizer or an ultrasonic bath shall be used.

7.2.2 Dispersion with Ultrasonic Homogenizer

The horn of the ultrasonic homogenizer shall be inserted directly into the dispersion medium (aqueous BSA solution) prepared according to the procedure described in Section 7.1. The ultrasonic waves shall be used to blend the carbon nanomaterial with the dispersion medium and the horn shall be positioned in such a way that the circulation of the liquid in the beaker is optimized.

The carbon nanomaterials shall be dispersed by continuously applying ultrasonic irradiation for the prescribed duration.

Cool the beaker with ice if necessary, to suppress a rise in the temperature of the suspension when subjected to ultrasonic irradiation.

Note: The ultrasonic output shall be checked in advance to verify its ability to disperse the carbon nanomaterials relative to the output, as well as to identify any damage or wear to the container and horn, along with damage by the carbon nanomaterials.

Warning: The ultrasonic treatment shall be performed on a clean bench, to avoid exposure to airborne contamination and the scattering of the suspension. Furthermore, tasks shall be performed by operatives wearing protective clothing, including masks and gloves .

7.2.3 Dispersion with Ultrasonic Bath

The vial prepared according to the procedure described in Section 7.1 shall be installed at the prescribed location in the ultrasonic bath. The liquid height in the ultrasonic bath shall be adjusted so that the maximum resonance is derived at the liquid surface of the suspension in the vial.

The carbon nanomaterials shall be dispersed by applying ultrasonic irradiation for the prescribed duration.

Note 1: The vial shall be installed directly above the ultrasonic emitter, to obtain the most intense vibrations in the ultrasonic bath, to effectively perform ultrasonic irradiation.

Note 2: Care shall be taken to prevent any damage or wear to the container.

Note 3: When the suspension is prepared using an ultrasonic bath, the prepared stock suspension may be derived without performing the adjustments described in Sections 7.3 and 7.4.

7.3 Centrifuge Separation

The suspension obtained using the procedure described in Section 7.2 is collected in a 50 mL centrifuge tube. Perform centrifugal separation according to the prescribed conditions (pertaining to centrifugal strength and separation time) and then collect the supernatant.

7.4 Adjustment of Concentration

The suspension collected according to the procedure described in Section 7.3 shall be passed through a cell strainer with a pore diameter of 40 μm, to check for the presence of any coarse clumps. In the event of any clump being observed on the filter, the solution shall be discarded and newly prepared.

The concentration of carbon nanomaterials in the suspension shall be evaluated according to the procedure described in Section 8.1. Any necessary concentration adjustments shall be performed by using the dispersion medium to derive the required stock suspension.

Note: A cell strainer with pores of a size that allows the verification of coarse clump shall be used.

7.5 Preparation of Stock Suspensions with Varying States of Carbon Nanomaterials

Other than the preparation described above, suspension may be prepared according to the procedure described below, based on the required of the intended suspended carbon nanomaterials (such as the secondary particle sizes).

a) The suspension, derived by adjusting the ultrasonic irradiation time, shall be centrifugally separated under appropriate centrifugal separation conditions (pertaining to centrifugal strength and separation time) and the supernatant shall be recovered.
b) The recovered supernatant shall be centrifugally separated for a longer period and at a greater centrifugal acceleration than that of (a), after which the sediments shall be collected.
c) The dispersion medium prepared according to the procedure described in Section 5.1.2 shall be added to the sediments and redispersion shall be performed using a vortex mixer and ultrasonic bath.
d) The suspension shall be filtered according to the procedure described in Section 7.4, after which the filtrate shall be collected.
e) The carbon nanomaterial concentration in the suspension shall be evaluated according to the procedure described in Section 8.1 and any necessary concentration adjustments shall be made by using the dispersion medium to derive the stock suspension.

8. Characterization of Suspension

8.1 Evaluation of Carbon Nanomaterial Concentration in Stock Suspension

Evaluations based on absorbance measurements in the ultraviolet visible light range shall be performed as described in Chapter IV "Characterization of Dispersion Liquid Samples for Carbon Nanomaterials," Section IV-4 "Evaluation of Carbon Nanomaterial Concentration by Absorbance" in this document.

8.2 Measurement of Dispersion State and Morphology of Carbon Nanomaterials

a) The dispersion state of carbon nanomaterials shall be observed using a device such as a TEM.
b) The secondary particle sizes of suspended carbon nanomaterials shall be evaluated based on their average particle sizes and the like, as described in Section IV-2 "Evaluation of Particle Size and Particle Size

Distributions by Dynamic Light Scattering Method" or Section IV-3 "Evaluation of Particle Size and Particle Size Distribution by Differential Centrifugal Sedimentation" of Chapter IV "Characterization of Dispersion Liquid Samples for Carbon Nanomaterials" in this document.

c) Shapes shall be evaluated based on image measurements, such as TEM micrographs.

8.3 Endotoxin Test

The test shall be performed using the gelification method, which is a testing methodology for endotoxins as defined in the 16th revision of the Japanese Pharmacopoeia. The endotoxin detection test shall be performed on the suspension (working solution), prepared by diluting the stock suspension to the maximum carbon nanomaterials concentration for an in vitro cell-based assay.

The concentration of endotoxins shall be verified to ensure that it is equal to or less than the sensitivity level of the reagent based on the limit test method, according to the coagulation reaction of the lysate reagent.

The desirable level of detection sensitivity for the lysate reagent shall be 0.03 EU/mL or less.

9. Preparation of Working Solution

In the case of in vitro cell-based assays, the stock suspension is diluted with the culture medium used for the tests to prepare a working solution of the prescribed carbon nanomaterial concentration. A culture medium, shall be prepared in advance with the working solution having a BSA concentration of 1 mg/mL and shall be used for this dilution process.

10. Reports

10.1 Preparation and Characterization Methods

a) Information pertaining to carbon nanomaterials (material name, manufacturer, manufacturing method, composition, etc.)

b) Information for identifying dispersants and dispersion media (reagent name, producer, lot number, etc.)

c) Information for identifying culture media (in cases where a working solution preparation liquid is to be prepared: Reagent name, producer, lot number, etc.)

d) Information pertaining to the dispersion of samples (preparation date and time, person responsible for preparation and affiliated institution name, dispersing equipment name, dispersion conditions, etc.)

e) Information pertaining to centrifugal separation (centrifuge name, centrifuge strength, centrifuging time, etc.)

f) Information pertaining to cell strainers (product name, pore size, manufacturer, etc.)

g) Information pertaining to sample characterization methods (name of concentration measurement equipment and preparation method for concentration calibration curve, measurement principles and name of equipment for measurement of secondary particle sizes, name of image observation equipment for dispersion states, etc.)

h) Endotoxin evaluation methods (lysate reagent name, sensitivity, manufacturer)

10.2. Stock Suspension

a) Stock suspension ID
b) Means and standard deviations of carbon nanomaterial concentration
c) Calculation methods and results obtained for secondary particle sizes (measurement principles and standards, as well as average particle sizes, polydispersity indices, etc.)
d) Images of dispersion states
e) Shapes of dispersed carbon nanomaterials (averages of lengths and widths, as well as standard deviations or distributions)
f) Endotoxin evaluation results

10.3 Working Solution (in cases where cell culture medium preparation liquid is to be prepared)

a) Working Solution ID
b) Dilution ratio

Appendix A

Practical Example: Preparation of Suspension Sample for SWCNTs

A.1 Outline

This example illustrates the preparation, in accordance with the procedure manual, of the CNT suspensions used in animal tests and in vitro cell-based assays of two types of SWCNTs, i.e., metallic and semiconducting, separated and purified from SWCNTs (eDISP-CNT) that were previously prepared according to the eDIPS method. In this instance, an ultrasonic homogenizer was used to prepare the stock suspension with a SWCNT concentration of 1 mg/mL.

A.2 Preparation of Dispersion Media

The dispersant BSA was product 08587-42, manufactured by Nacalai Tesque Co., Ltd.

An aqueous BSA solution with a concentration of 10 mg/mL was prepared by dissolving BSA in distilled water (Gibco®, manufactured by Thermo Fisher Scientific). The dispersion medium was prepared by filtering the aqueous BSA solution described above through a sterilization filter with a pore size of 0.22 μm (GPO.22 μm, manufactured by Merck).

A.3 Preparation of SWCNT Suspension

A.3.1 Ultrasonic Treatment

An ultrasonic homogenizer was used for the ultrasonic dispersion.

Metallic SWCNTs and semiconducting SWCNTs were weighed and collected to ensure that the CNT concentration at the time the dispersion operation was performed is 1.5 mg/mL. These were placed in a 50 mL beaker, and then sterilized.

The dispersion medium prepared according to the procedure described in Appendix A.2 (10 mg/mL aqueous BSA solution) was added to the beaker described above and then the CNTs were blended thoroughly with the dispersion medium.

The ultrasonic dispersion was performed according to the procedure described in Section 7.2. A Branson Sonifier 250 was used as the ultrasonic homogenizer (Figure A.1 (a)).

The probe of the ultrasonic homogenizer was inserted into the dispersion medium suspending CNTs described above, the output control of the homogenizer was set to 4 and ultrasonic treatment was performed continuously for 5 h (Figure A.1 (b)).

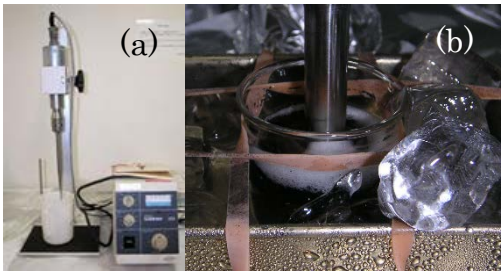

Figure A.1 (a) Ultrasonic homogenizer; (b) Dispersion operation

A.3.2 Centrifugation and Filtering

a) Centrifugal separation was performed for 15 min, with a centrifugal strength of 6000 g. The supernatant was then collected.

b) The supernatant obtained in Step a) was passed through a cell strainer with pore size of 40 μm. The filtrate was verified to ensure that there were no clumps and was then collected. A cell strainer manufactured by BD Falcon™ was used.

A.4 Characterization of Stock Suspension

A.4.1 Evaluation of CNT Concentration

The relationship (calibration curve) between the CNT mass concentration C_{CNT} and the absorbance of suspension A_{av} was derived using the procedure described in Section IV-4 "Evaluation of Carbon Nanomaterial Concentration by Absorbance" of Chapter IV "Characterization of Dispersion Liquid Samples for Carbon Nanomaterials," based on the average absorbance of suspensions of metallic SWCNTs and semiconducting SWSWCNTs with wavelengths of 600 nm to 800 nm.

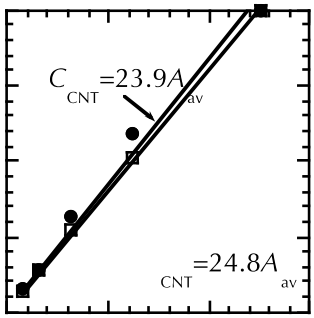

Figure A.2 Relationship between CNT concentration and absorbance of metallic and semiconducting SWCNT suspension

The average absorbance of the raw material for the stock suspension prepared in Step A.3 at wavelengths of 600 nm to 800 nm was derived and the CNT concentration was calculated based on the calibration curve. As shown in Table A. 1, the CNT concentrations in the suspensions of the metallic SWCNTs and semiconducting SWCNTs

were 1.38 mg/mL and 1.24 mg/mL, respectively. The concentration of CNT in the stock suspensions for hazard testing was adjusted to 1 mg/mL using the dispersion media prepared in Step A.2.

A.4.2 Secondary Particle Size of CNTs

The average particle sizes x_{DLS} yielded by the cumulant method, as measured by DLS (Zetasizer Nano manufactured by Malvern), are listed in Table A.1.

Table A.1 Characteristics of prepared suspension

ID	SWCNT	CNT concentration C_{CNT} [mg/mL]	Average particle size x_{DLS} [nm]	CNT length	
				l_g [nm]	s_g [-]
140604-03	Metallic	1.38	786	573	2.32
140603-03	Semiconducting	1.24	656	533	2.49

A.4.3 Length of CNTs

TEM micrographs of the dispersed CNTs are shown in Figure A.3. These images were obtained using a JEM-1010 TEM, manufactured by JEOL.

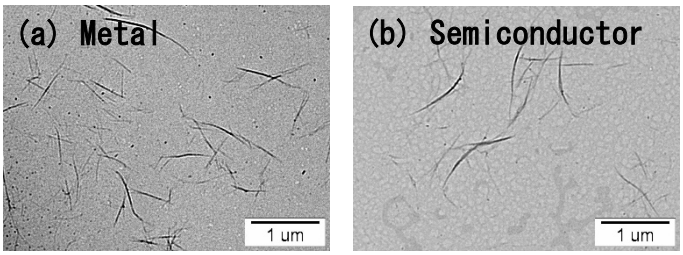

Figure A.3 TEM micrographs of dispersed SWCNTs

The distribution of about 1000 bundles of CNTs with a length l was measured using TEM micrography. The cumulative size distribution $Q_0(l)$ for the length, based on the number of bundles, is shown in Figure A.4. Furthermore, their geometric mean l_g and the geometric standard deviation s_g are also given in Table A.1.

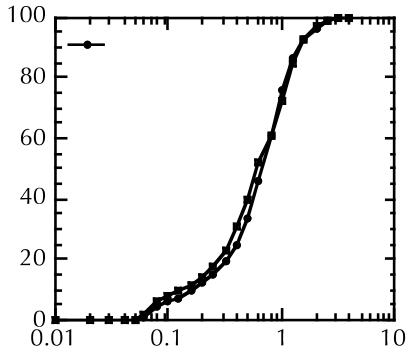

Figure A.4 Length distributions of dispersed SWCNTs

A.4.5 Verification of Endotoxins

A test for detecting endotoxins in the stock suspension was conducted using a limit test based on the gelification method, as described in the 16th revision of the Japanese Pharmacopoeia. We added 0.2 mL of stock suspension, diluted ten times, to a Pyrotell™ single-test vial with a sensitivity of 0.03 EU/mL and then mixed well. Then, the vial was placed in a thermostatic water bath at 37 °C ± 1 °C for 60 ± 2 min. The vial was then taken out of the bath and turned upside down to check for the formation of any gel. No gel formation was observed, thus verifying that the level of the endotoxin concentration was at or under the sensitivity level of the Pyrotell™.

A.5 Preparation of Working Solution

A working solution for in vitro cell-based assays was prepared by diluting the stock suspension using a prescribed cell culture with a BSA concentration of 1 mg/mL.

Chapter IV: Characterization of Dispersion Liquid Samples for Carbon Nanomaterials

IV-1. Evaluation of Zeta Potential

Introduction

The charge on a particle surface is an extremely important characteristic determining the stability of the particles in a suspension used for performing hazard tests on a carbon nanomaterial. The charges on the particles in aqueous solutions are generally evaluated according to their zeta potential. The method of measuring the zeta potential is described below. Refer to Chapter IV "Characterization of Dispersion Sample in Liquid for in vitro Tests Using Cells" of the "Procedures for Safety Testing" for details on those items described in Chapter IV of this document.

1. Scope of Application

The target of the evaluation shall be the suspension containing dispersed carbon nanomaterials. This section describes the method used to measure the migration speed of particles in an electric field, based on light scattering and an evaluation of the zeta potential of suspended particles.

2. Referenced standards and regulations

This procedure refers to the following standards and regulations:

"Countermeasures for Prevention of Exposure to Nanomaterials" (March 21, 2009) standard issued by the Ministry of Health, Labour and Welfare, Japan. Publication No. 0331013 (March 31, 2009).

3. Terminology and Definitions

3.1 Zeta Potential

Index representing the electric charge on the surface over which a liquid flows due to the spatial distribution of ions formed near the surface, as the suspended particles move through the liquid. The zeta potential is given by the difference in electric potential between that at the slip plane and that of bulk liquid.

3.2 Electrophoresis and Electrophoretic Mobility

Movement of charged particles in a liquid caused by application of external electric fields and the velocity of charged particles per unit electric field strength.

3.3 Electrophoretic Light Scattering (ELS)

A measurement method used when calculating the zeta potential, based on the electrophoretic mobility derived from the Doppler shift of light scattering due to the electrophoresis of particles in a liquid.

4. Principles

Particles suspended in a solvent have either a positive or negative charge on their surfaces. Applying an electric

field to a system consisting of suspended charged particles causes those particles to move according to their charge states. This phenomenon is referred to as electrophoresis, while the electrophoretic mobility is a value derived by converting the velocity of movement in this instance per unit electric field strength. By letting the electrophoretic mobility be B_E, the zeta potential ζ can be derived using Smoluchowski's equation, below.

$$\zeta = \frac{\eta B_E}{\varepsilon} \quad (1)$$

where η and ε are the viscosity and dielectric constant of the solvent, respectively. This means that, where there is a distribution of the charged state, then there will also be a distribution of mobility and so the zeta potential would be derived as an average value.

5. ELS Equipment

5.1 Equipment Types and Requirements

The descriptions provided in this document assume the use of commercially available ELS equipment.

Temperature control based on ELS measurements, as well as functions related to data output with regards to electrophoretic mobility, dispersed liquid electric conductivity, and zeta potential shall be as stipulated in Sections **5.2.3** and **5.2.4** of Chapter IV of the "Procedures for Safety Testing."

5.2 Validation

The validity of the equipment shall be verified according to the stipulations given in Section **5.2.2** of Chapter IV of the "Procedures for Safety Testing," using reference materials (RM) and the like. Quantified reference material shall be measured to evaluate whether the certified values and measured values coincide within the range of uncertainty. The use of certified reference material (CRM) with zeta potentials indicated as a certified value or reference value, such as AIST CRM5702, is recommended for evaluating the validity of the equipment. The deviation of measured values from the certified values should be within ± 10% and the reproducibility of the measurements based on the relative experimental standard deviation (coefficient of validation) should be equal or less than 10%. The frequent repetition of this validation is desirable, particularly in the case of ELS where the electrode is immersed directly in the suspension, such that the electrode can be easily contaminated, causing the measured values to be influenced by the contamination.

6. Reagents and Instruments

Refer to Section **5** of Chapter III "Preparation of Dispersion Liquid Samples for Carbon Nanomaterials" of this document, as well as Sections 6.1 and 6.2 of the "Procedures for Safety Testing" for details on the reagents, solvents for cleaning, and reference materials for calibration.

Refer to Sections 6.3 to 6.5 of Chapter IV of the "Procedures for Safety Testing" for details on the sample containers, the digital cameras used to observe the samples and viscometers used to measure the viscosity of the suspension.

7. Operations

7.1 Preparation Prior to ELS Measurement

Refer to Section 7.2 of Chapter IV of the "Procedures for Safety Testing" for details on the installation of the

equipment and the cleaning of the instruments.

Prepare the sample. The amount of sedimentation shall be recorded by visually checking for sedimentation in the prepared suspension.

The viscosity shall be measured by referring to Section 7.2 of Chapter IV of the "Procedures for Safety Testing" for details on the measurement. The viscosity is a physical property necessary to calculate the zeta potential based on the measured electrophoretic mobility as well as blank measurement of the dispersion medium, which shall also be required.

7.2 Measurement

7.2.1 Skills and Measurement Environments

The skills of the measurement equipment operators, as well as the measurement environments, shall comply with the stipulations given in Sections 7.2.7.1 and 7.2.7.2 of Chapter IV of the "Procedures for Safety Testing".

7.2.2 Preparation of ELS Equipment

The equipment shall be allowed to warm up in accordance with the procedure described in Section 7.2.7.3 of Chapter IV of the "Procedures for Safety Testing".

The measurement cells shall be allowed to reach the preset temperature prior to the start of measurement. Furthermore, the cells shall remain within the stipulated temperature range during the measurement operations. The recommended temperature range is ± 0.3 °C.

7.2.3 Measurements and Analyses

Measurements and analyses shall be performed according to the instruction manual provided with the equipment. If possible, measurements should be performed at least three times, to evaluate the repeatability.

7.2.4 Records

The measurement date and time, information related to solid samples and dispersion liquids, information on the ELS equipment and other details of the experiment conditions, as well as results such as zeta potentials and electrophoretic mobility, shall be recorded according to the stipulations provided in Section 7.2.7.4 of Chapter IV of the "Procedures for Safety Testing."

8. Analysis of Data

Calculate the value of the zeta potentials and evaluate the uncertainties according to the stipulations given in Section 8 of Chapter IV of the "Procedures for Safety Testing."

9. Reports

9.1 General Items and Conditions Related to Equipment, Experiments, and Analyses

Information relating to the samples, information relating to the ELS equipment, as well as the ELS measurement conditions and the analysis methods shall be reported according to stipulations provided in Sections 9.1 and 9.2 of Chapter IV of the "Procedures for Safety Testing".

9.2. Results

The zeta potentials, as well as the repetition standard deviations or uncertainties, shall be reported.

Appendix A

Practical Example: Example of Measuring Zeta Potential

A.1 Outline

The zeta potential of the CNTs dispersed in the liquid according to the stipulations provided in Chapter III "Preparation of Dispersion Liquid Samples for Carbon Nanomaterials" of this document was evaluated for SWCNTs fabricated using a super-growth method.

A.2 Preparation of Suspension Samples

The prepared suspension was identical to that prepared according to the stipulations provided in Section IV-2 "Evaluation of Particle Size and Particle Size Distributions by Dynamic Light Scattering Method" (see Appendix B) of Chapter IV "Characterization of Dispersion Liquid Samples for Carbon Nanomaterials", of this document.

A.3 Measurement of Zeta Potentials

An example of the change in the zeta potentials of the CNT suspensions, i.e., sample immediately after preparation according to the procedure described in A.2, and of the same suspension after having been left in a refrigerator for 8 days, is shown in Figure A.1.

The Zetasizer Nano manufactured by Malvern was used for the ELS measurement. The measurement temperature was 25 °C and the measured liquid volume was 1 mL.

The suspension was diluted 20 times using ultrapure water, since the scattered light intensity is high when the CNT concentration is the 1 mg/mL of the working solution. Three measurements were taken and the error bars in the figure represent the relevant standard deviations.

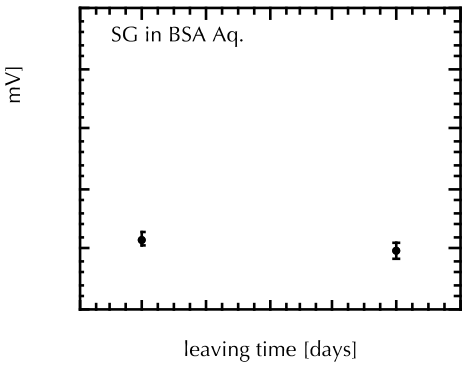

Figure A.1 Example zeta potential measurements

IV-2. Evaluation of Particle Size and Particle Size Distributions by Dynamic Light Scattering Method

Introduction

The primary particle size of solid samples is regarded as being an important physical and chemical characteristic for conducting hazard evaluations for carbon nanomaterials. However, the secondary particle size, which expresses the assemblage state (as nanoparticles generally form agglomerates or aggregates), is also regarded as being an important characteristic. This section describes a method for determining the secondary particle size of solid samples in the suspensions used for hazard tests using a dynamic light-scattering method. Readers should refer to Chapter IV "Characterization of Dispersion Sample in Liquid for *in vitro* Tests Using Cells" of the "Procedures for Safety Testing" for details on the items described in this procedure manual.

1. Scope of Application

The target of the evaluation shall be the suspension, consisting of an aqueous solution containing dispersed carbon nanomaterials. This section describes the method for evaluating the primary and secondary sizes of the particles in a suspension using a dynamic light scattering method.

2. Referenced standards and regulations

This procedure refers to the following standards and regulations:

JIS Z 8819-1: Representation of results of particle size analysis Part 1: Graphical representation

JIS Z 8828: Particle Size Analysis: Dynamic Light Scattering Method

"Countermeasures for Prevention of Exposure to Nanomaterials" (March 21, 2009) standard issued by the Ministry of Health, Labour and Welfare, Japan. Publication No. 0331013 (March 31, 2009).

3. Terminology, Definitions, and Symbols

3.1. Terminology and Definitions

3.1.1 Particle Size

Diameter of a particle, the shape of which is assumed to be spherical. If a particle is a true sphere, the particle size would be equal to the diameter.

3.1.2 Secondary Particle Size

Diameter of assemblages of particles that are either rigidly jointed or weakly bound, in other words, the diameter of a clump.

3.1.3 Stokes-Einstein Equation

Relationship that links the Brownian diffusion and Stokes' resistance of particles due to random thermal motion. It provides the relationship between the particle size and the Brownian diffusion coefficient.

3.1.4 Dynamic Light Scattering (DLS)

Method used to evaluate particle size, based on the Stokes-Einstein equation, by determining the diffusion coefficient of particles based on the fluctuation, over time, of scattered light when light is irradiated onto particle

groups exhibiting Brownian motion.

3.1.5 Cumulant Method

Method used to derive average particle sizes and the polydispersity index by series-expanding the logarithm of the autocorrelation function in a scattered-light electric field derived by DLS. The primary and secondary coefficients of the series expansion correspond to the average particle size and polydispersity index, respectively (refer to Appendix A).

3.1.6 Average Particle Size

Average particle size of secondary particles derived based on the correlation between the fluctuations of scattered light intensity in dynamic light scattering phenomena.

3.1.7 Polydispersity Index

Dimensionless index that indicates the spread of particle size distribution.

3.1.8 Uncertainty

Index expressing the lack of reliability of measurement results.

3.1.9 Fluctuation Fractions

Ratio of scattering intensity (photon quantity) to its initial value.

3.2 Symbols

D_{BM}	Translational diffusion coefficient of particles (Brownian diffusion coefficient), m^2/s
$g^{(1)}(\tau)$	Normalized electric field correlation function of scattered light, dimensionless
PI	Polydispersity index, dimensionless
$\Delta Q_{int,i}$	Fraction of particles weighted with scattered light intensity for particle size x_i, dimensionless
x	Particle size. Diameter of spherical particles, μm or nm
x_{DLS}	Average particle size derived from scattered light intensity-weighted particle size distribution or average particle size derived by the cumulant method, μm and nm
Γ	Decay rate, s^{-1}
$\overline{\Gamma}$	Average value of scattered light intensity-weighted distribution function of the decay rate, s^{-1}
μ_2	Secondary coefficient derived with the cumulant expansion of the particle size distribution of scattered light intensity-weighted, s^{-2}
τ	Correlation time, s and μs

4. Principles

DLS is a method used to optically detect the Brownian motion of particles. The light scattered by particles exhibiting Brownian motion includes those phases that change with time (time-dependent phase). The diffusion coefficient of particles that indicates the random motion of particles D_{BM} can be derived from the change in phase of the scattered light with time over an extended period. The particle size x can be derived using the Stokes-Einstein equation, below:

$$x = \frac{k_B T}{3\pi\eta D_{BM}} \quad (1)$$

where k_B is the Boltzmann constant, T is the absolute temperature and η is the viscosity of the dispersion medium

at that temperature.

5. Calculation of Average Particle Sizes and Polydispersity Indices

The harmonic mean particle size (diameter) of th particle size distribution based on the scattered light intensity is defined as follows:

$$x_{\mathrm{DLS}} = \left[\sum_{i=1}^{N} \frac{1}{x_i} \Delta Q_{\mathrm{int},i} \Big/ \sum_{i=1}^{N} \Delta Q_{\mathrm{int},i} \right]^{-1} \tag{2}$$

where $\Delta Q_{\mathrm{int},i}$ is the fraction of particles based on the scattered light intensity in the particle size interval i and x_i is the representative particle size for the interval i, while N is the total number of particle-size intervals. The average value may vary from that derived by the cumulant method. Other than the above, the volume-based average particle size x_{v}, or number-based average particle size x_{n} is also calculated using the equipment.

The polydispersity index PI that indicates the spread of the particle size distribution is given by the following equation:

$$PI = 2x_{\mathrm{DLS}}^2 \frac{\sum_{i=1}^{N} \Delta Q_{\mathrm{int},i} \left(1/x_i^2 - 1/x_{\mathrm{DLS}}^2 \right)}{\sum_{i=1}^{N} \Delta Q_{\mathrm{int},i}} \tag{3}$$

The value may vary from that derived by the cumulant method.

6. Dynamic Light Scattering Particle Size Analyzer

6.1 Equipment Types and Requirements

The descriptions given in this procedure assume the use of commercially available DLS equipment.

Control the temperature required for the DLS measurement, calculate the average particle size and particle size distribution, and output the correlation functions and/or power spectrum according to the stipulations given in Sections 5.1.3 and 5.1.4 of Chapter IV of the "Procedures for Safety Testing".

6.2 Qualification

The equipment shall be qualified according to stipulations provided in Section 5.1.2 of Chapter IV of the "Procedures for Safety Testing", using reference materials and the like. For instance, certified polystyrene latex reference materials shall be used to evaluate whether the certified values and measured values coincide within the range of uncertainty. The use of monodispersed polystyrene latex reference materials with an average particle diameter of 100 nm is recommended for the qualification of the equipment. The desirable range of deviation for the measured values from certified values is ± 2%, while up to 2%, based on relative experimental standard deviation (coefficient of variation), is desirable for the reproducibility of the repeated measurements (refer to ISO 22412).

7. Reagents and Instruments

Refer to Section 5 of Chapter III "Preparation of Dispersion Liquid Samples for Carbon Nanomaterials" of this document, as well as Sections 6.1 and 6.2 of the "Procedures for Safety Testing" for details on the reagents,

solvents for cleaning, and reference materials for calibration.

Refer to Sections 6.3 to 6.5 of Chapter IV of the "Procedures for Safety Testing" for details on the containers to be used for samples, as well as the digital cameras and viscometers to be used to observe the samples.

8. Operations

8.1 Preparation Prior to DLS Measurements

Refer to Sections 7.1.1 and 7.1.5 of Chapter IV of the "Procedures for Safety Testing" for details on the installation of equipment and the cleaning of instruments.

Refer to Sections 7.1.4 and 7.1.6 of Chapter IV of the "Procedures for Safety Testing" for details on the viscosity, which is a physical value necessary to calculate the particle size as well as the blank measurement of the dispersion medium.

8.2 Storing and Handling Suspensions

Store and handle the carbon nanomaterial suspensions which have been prepared according to the stipulations provided in Sections 7.1.2 and 7.1.3 of Chapter IV of the "Procedures for Safety Testing".

The presence or absence of sediment growth shall be verified visually when a suspension is acquired, as well as before and after DLS measurement. The degree of sedimentation shall be recorded. Images shall be added to the record if possible, using a digital camera or the like.

8.3 Measurement

8.3.1 Skills and Measurement Environments

The skills of the measurement equipment operators, as well as the measurement environments for the DLS shall comply with the stipulations provided in Sections 7.1.7.1 and 7.1.7.2 of Chapter IV of the "Procedures for Safety Testing".

8.3.2 Preparation of DLS Equipment

The equipment shall be warmed up in accordance with the procedure described in Section 7.1.7.3 of Chapter IV of the "Procedures for Safety Testing".

The measurement cells shall be allowed to reach the preset temperature prior to the start of measurement. Furthermore, the cells shall remain within the stipulated temperature range during the measurement operations. The recommended temperature range is ± 0.3 °C.

8.3.3 Measurement and Analysis

Measurements shall be taken and the obtained data shall be analyzed according to the instruction manuals for the respective equipment. It is recommended that the measurements be performed at least three times, to evaluate the repeatability of the measurements.

8.3.4 Records

The experimental conditions and results shall be recorded in accordance with the procedure described in Section 7.1.7.4 of Chapter IV of the "Procedures for Safety Testing".

9. Analysis of Data

9.1 Calculation of Values

The reported value for the average particle size shall be the average of the repeated measurements. Although the calculation method used to derive the average value is not stipulated, how the calculation is performed shall be reported.

9.2 Uncertainty Evaluation

To indicate the validity of the evaluation method, it is recommended that an evaluation of uncertainty be performed for the reported values, according to the stipulations provided in Section 8.2 of Chapter IV of the "Procedures for Safety Testing".

Note: Refer to Appendix B of Chapter IV of the "Procedures for Safety Testing" for details on the evaluation of uncertainty.

10. Reports

Reports shall be made on information relating to the samples, information relating to the DLS equipment, as well as on the measurement conditions of the DLS and the analysis methods according to the stipulations provided in Section 9 of Chapter IV of the "Procedures for Safety Testing".

The following items shall be reported in relation to the results of the particle size measurements:

a) Scattering light intensity weighted average particle sizes, and standard deviations or uncertainties for the repeated measurement

b) Average particle sizes based on number or volume, and standard deviations or uncertainties for the repeated measurement

c) Scattering intensities and its fluctuation fractions, as well as standard deviations or uncertainties for the repeated measurement

Appendix A

Cumulant Method

In the dynamic light-scattering phenomenon, the scattering of light from suspended particles irradiated by laser beam has a random time-dependent phase due to the Brownian motion of the suspended particles.

In the case of monodispersed particles, the autocorrelation function $g^{(1)}(\tau)$ with respect to fluctuation in the scattered light electric field decays exponentially with correlation time τ.

$$g^{(1)}(\tau) = \exp\left(-\Gamma\tau\right) \tag{A-1}$$

where the decay rate Γ is proportional to the diffusion coefficient D_{BM} and the particle size is derived based on the Stokes-Einstein equation.

In the case of polydispersed particles, the autocorrelation function $g^{(1)}(\tau)$ can be regarded as being a combination of autocorrelation functions weighted by the particle size distribution. The logarithm of the autocorrelation function $g^{(1)}(\tau)$ of the electric field is approximated by series expansion to the secondary term with the cumulant method.

$$\ln\left(g^{(1)}(\tau)\right) = -\bar{\Gamma}\tau + \frac{\mu_2}{2}\tau^2 \tag{A-2}$$

The average of the primary coefficient $\bar{\Gamma}$ is the average decay rate, which can be used to derive the average particle size. Furthermore, the polydispersity index *PI* can be derived using the following equation, based on the secondary coefficient μ_2:

$$PI = 2\mu_2 / \bar{\Gamma}^2 \tag{A-3}$$

Appendix B

Practical Example: Evaluation of Particle Size of SWCNT

B.1 Outline

The particle size of SWCNTs fabricated using the super-growth method in the suspension, prepared according to the stipulations provided of Chapter III "Preparation of Dispersion Liquid Samples for Carbon Nanomaterials" of this document, was evaluated.

B.2 Preparation of SG-SWCNT Suspension

B.2.1 Dispersion Medium

An aqueous BSA solution with a concentration of 10 mg/mL was prepared by dissolving BSA in distilled water (Gibco® manufactured by Thermo Fisher Scientific) to prepare the dispersion medium.

B.2.2 Ultrasonic Dispersion

Next, 50 mL of dispersion medium (10 mg/mL of aqueous BSA solution) was added to 70 mg of SWCNTs produced using the super-growth method by AIST (SG-SWCNTs), weighed and collected in a 50 mL beaker. The suspension was then prepared by ultrasonication, according to the procedure described in Section 7.2.2 of Chapter III of this document. The CNT concentration was 1 mg/mL.

B.3 DLS Measurement

A Zetasizer Nano manufactured by Malvern was used for the DLS measurement. The DSL measurement temperature was 25 °C and the measured liquid volume was 700 μL. The CNT concentration of the prepared suspension was 1 mg/mL. Measurements were taken from the sample prepared by diluting the suspension with ultrapure water, since the scattered light intensity was too high. Figure B.1 shows the average particle size (a) and the polydispersity index (b) of the CNT suspension. In the figure, the effects of time in a refrigerator as well as the dilution are shown. The DLS measurements were performed three times. The average is indicated with a key, while the standard deviations are indicated with error bars in the figure.

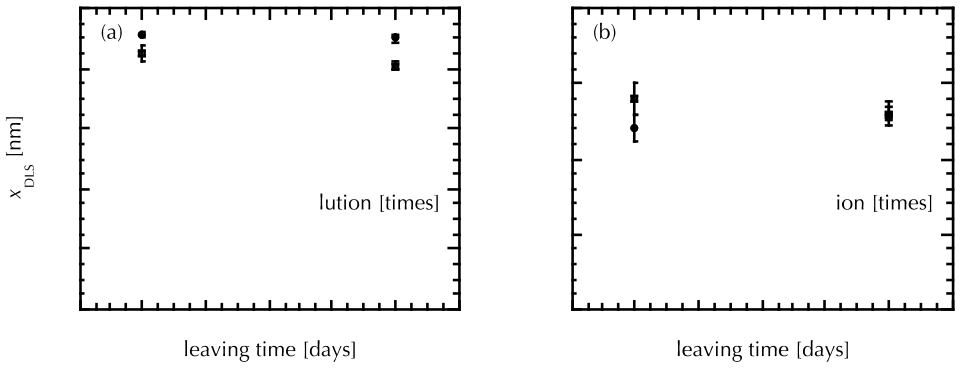

Figure B.1 Example of measuring average particle sizes and polydispersity indices

IV-3. Evaluation of Particle Size and Particle Size Distribution by Differential Centrifugal Sedimentation

Introduction

This section describes the procedure for characterizing a carbon nanomaterial dispersed in a liquid. The characteristic evaluated in this instance is the particle size distribution. Furthermore, the measurement method used in this procedure is the differential centrifugal sedimentation method.

1. Scope of Application

The target of the measurement shall be the carbon nanomaterial aggregates dispersed in a liquid.

2. Referenced Standards and Regulations

This manual refers to the following standards and regulations:

JIS Z8823-1: 2001 Determination Of Particle Size Distribution By Centrifugal Liquid Sedimentation Methods -- Part 1: General Principles And Guidelines

JIS Z8823-2: 2016 Determination Of Particle Size Distribution By Centrifugal Liquid Sedimentation Methods -- Part 2: Photocentrifuge Method

3. Terminology, Definitions, and Symbols

3.1. Terminology and Definitions (Measurement Methods)

3.1.1 Differential Centrifugal Sedimentation (DCS)

A method of particle size classification based on the principle whereby the settling velocity of the particles in a sample depends on the particle diameter, as the particle sample passes through the surface of a solution having a certain density.

3.1.2 Calibration Substance

The particle size to be measured by DCS is corrected by using a standard substance with a known particle size prior to measuring samples. The standard substances used for such corrections are referred to as calibration substances.

3.1.3 Terminal Sedimentation Velocity

The velocity of a particle moving in a static liquid under a combination nof the centrifugal force and the drag acting on the particles.

3.1.4 Particle Size (Stokes' Diameter) *D*

The diameter corresponding to a spherical particle which has the same density and sedimentation velocity as the particle actually settling and which satisfies Stokes' Law.

3.1.5 Particle Size Distribution and Median Size

The particle size distribution is the mass distribution for the particle size. The median size is the particle size at which a cumulative frequency becomes 50% as a result of integrating the obtained mass distribution.

3.1.6 Disk

A chamber in which the size classification of samples is performed in the DCS based on the centrifugal sedimentation velocity.

3.1.7 Density Gradient Fluid

When sample particles with a density greater that that of the solution inside the disk are integrated on the disk, such particles do not sediment according to Stokes' Law and the sample suspension sediments as a homogeneous solution. The density gradient is made for the solution in the disk, to ensure that the sample sediments according to Stokes' Law. Such a solution is referred to as a density gradient fluid.

3.2 Symbols

D	Particle size (Stokes' diameter), µm
D_{50}	Median size, µm

4. Principles

Among the carbon nanomaterials dispersed in a liquid, larger-size particles exhibit a greater sedimentation speed due to the centrifugal force, while particles of smaller sizes have lower sedimentation speeds. By making use of this phenomenon, the difference in the migration time to the detector near the bottom of the disk, which depends on the particle diameter, can be measured in the DCS rotating disk chamber, and particle size classification can be performed. The particle velocity v can thus be expressed by the following equation, by substituting the gravitational acceleration of the Stokes' Equation with the centrifugal acceleration.

$$v = dR/dt = (D^2(\rho - \rho_0)\omega^2 R)\,/18\eta$$

where D is the particle size in the sample, R is the distance in the radial direction, t is the time, ρ and ρ_0 are the particle and liquid densities, respectively, while ω is the angular velocity and η is the liquid viscosity. The particle size can be derived based on the particle migration time from the fluid surface ($R = R_0$) to the detector ($R = R_f$), using this equation.

$$D = \left(\frac{\left(18\eta \ln\left(R_f/R_0\right)\right)}{\left((\rho - \rho_0)\omega^2\right)}/t\right)^{1/2}$$

5. Equipment

5.1 Types of Equipment

The descriptions given in this document assume the use of commercially available DCS equipment.

5.2 Validation

The validity of the equipment shall be verified in advance, using standard substances. For instance, properly quantified polyvinyl chloride latex or polystyrene latex-standard substances shall be measured to evaluate whether the certified values and measured values are within the range of uncertainty. For instance, the use of monodispersed polyvinyl latex standard substances with an average particle diameter of about 500 nm is recommended for performing evaluations for validating the equipment. The desirable range of deviation for the measured values from the certified values is ± 10% and a range of up to 10% based on relative experimental standard deviation (coefficient of variation) is desirable for achieving reproducibility for repeated measurements.

5.3 Output Data

The equipment shall be able to calculate the mass standard particle size distribution and mass standard median size, and shall also have a function to output such results.

6. Reagents and Instruments

6.1. Reagents

The solvents used to clean the disk and dilute solutions shall be clean and shall not contain any fine particles, particularly those that are larger than the particles in the dispersion samples. When the solvent being used for dispersion is water, then the water shall be filtered, to prepare so-called "ultrapure water".

Remarks: "Ultrapure water," as referenced in this procedure manual, shall be defined as "clean water that does not contain any fine particles, which has been purified using an ion-exchange filter or a filter with a pore size of 0.2 μm or less, with an electrical resistivity of 18 MΩ-cm or more and an organic carbon concentration of 5 ppb or less".

6.2 Standard Substances

The use of standard substances that have been declared as having traceability with regards to their characteristic values is desirable.

The use of polystyrene latex of about 500 nm, dispersed in water, is recommended for use as a particle size standard substance. Polystyrene latex cannot be measured under the conditions used for the measurement of carbon nanomaterial aggregates, however, since it has a low specific gravity. Polyvinyl chloride latex with declared traceability shall instead be used for that purpose, under the same measurement conditions. Caution is required when handling both standard substances to ensure that aggregation or sedimentation does not occur as a result of freezing or the like.

Remarks: This standard substance shall be used as a calibration substance prior to taking measurements.

Remarks: When using polystyrene latex as a standard substance, the conditions for a density gradient fluid may be changed for convenience.

6.3 Density Gradient Fluid

A density gradient fluid shall be prepared as a solution in the disk. The solution concentration in the disk shall continuously increase towards the direction of the sample sedimentation. Sucrose water shall be used as the density gradient fluid.

6.4 Instruments

Ordinary containers may be used. There is no problem with using commercially available syringes for the injection of dispersion samples into the disk when taking measurements.

7. Operations

7.1 Installation of Equipment

Equipment shall be installed in a clean environment. Furthermore, electrical noise, mechanical vibrations, and direct sunlight shall be avoided.

Warning: The DCS equipment uses a laser beam, which can be hazardous to the eyes, as its light source. Do not look directly into the incident light.

Furthermore, care shall be taken to ensure that no reflected light enters the eye. Safety codes that are applicable to the location where the equipment is installed shall be observed.

7.2 Cleaning of Instruments Used for DCS Measurements

The use of ordinary disposable syringes is recommended. Any measuring instruments that have been contaminated shall not be used.

7.3 Measurement

7.3.1 Skills

The DCS equipment shall be operated by individuals who have been properly trained and who are capable of appropriate operations. Whether an individual possesses the required measurement skills shall be evaluated in advance by, for instance, examining whether said individual is capable of reporting values that match the certified values of particle size standard substances.

7.3.2 Measurement Environments

The equipment shall be installed in a clean environment but it is also recommended that the environment in which the equipment is handled, as well as lab coats and other such items used by the measurement equipment operatives, also be clean. Caution is required when using water, as it tends to absorb dust which can impede the measurement operations.

7.3.3 Preparation of DCS Equipment

To acquire stable data, the equipment shall be warmed up prior to taking the measurements. The stability of the laser beam varies significantly depending on the laser equipment. However, allowing the equipment to warm up for 15 to 30 min is ordinarily considered necessary to allow the laser beam intensity to stabilize.

7.3.4 Measurements and Analyses

Dispersion samples shall be stored upright in a refrigerator (4 °C) and agitation shall be avoided prior to measurement. When taking measurements, the dispersion sample shall be collected using a syringe from a point about 1 cm below the surface of the liquid, without shaking the centrifuge tube.

Measurements shall be taken and analyses performed according to the instruction manual provided with the equipment.

7.3.5 Records

The following experiment conditions shall be recorded: Information on dispersion sample, time of measurement, duration of measurement, rotational speed of equipment, density gradient fluid used, calibration substance used, etc.

The following results shall be recorded: Measurement results obtained with the equipment (time vs. absorbance), as well as the particle size distribution (particle size vs. mass) derived by analysis, and the median size derived from an integrated particle size distribution curve.

8. Reports

The following items shall be reported:

8.1 General

a) Detailed information for identifying dispersion samples (example: Information on particle samples, acquisition date of dispersion samples, and transportation methods)

b) Measurement date, name of measuring person, and name of measurement organization

c) Information on standard substance used to verify validity of the equipment (including manufacturer name), as well as the date on which the validation was conducted

d) Storage conditions for dispersion sample (example: Storage temperature, storage location, and storage period)

8.2 Equipment, Experiments, and Analyses

a) Equipment name, model name, and manufacturer name.

b) Particle size standard substances used for validation, as well as results of verification

c) Cleaning conditions for the disk and the like

d) Information on reagents used for diluting solutions (if diluted)

e) Particle sample concentration in dispersion sample (when possible)

f) Index of refraction for dispersion liquids (literature information in case of literature data)

g) Capacity of the dispersion sample injected into the disk

h) Methods used to calculate reported values (example: Methods used to derive average values)

8.3. Results

a) Mass standard particle size distribution and mass standard median size

Appendix A

Practical Example: Example of DCS Measurement Results for SWCNT Samples

An example of the particle size distribution in culture medium dispersion samples of eDIPS SWCNTs, derived using the DCS, is shown in Figure A.1. The median size was 0.028 μm.

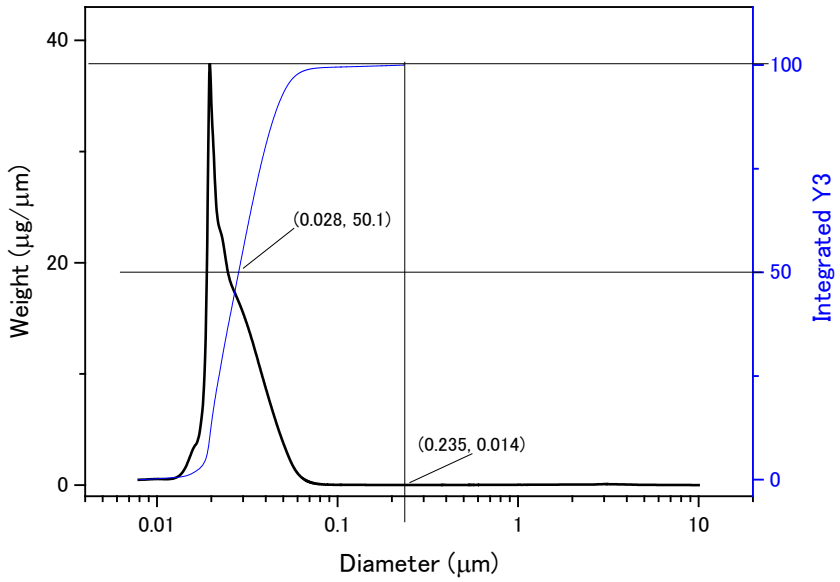

Figure A.1 Example of particle size distribution in culture medium dispersion samples of eDIPS SWCNTs, derived using DCS

IV-4. Evaluation of Carbon Nanomaterial Concentration by Absorbance

Introduction

This section describes the method used to evaluate the concentrations of particles in the suspensions. The particle concentration is one of the important factors affecting hazard testing. Refer to Chapter V "Evaluation of Concentration of Carbon Nanotube Dispersion Liquid Samples" of the "Procedures for Safety Testing" for details.

1. Scope of Application

The target of the evaluation shall be the suspension containing dispersed carbon nanomaterials, as used for hazard testing. This section describes the method for evaluating the concentration of carbon nanomaterials in the suspension, based on the absorbance in the visible range.

2. Referenced Standards and Regulations

This procedure refers to the following standards and regulations:

"Countermeasures for Prevention of Exposure to Nanomaterials" (March 21, 2009) standard, issued by the Ministry of Health, Labour and Welfare, Japan. Publication No. 0331013 (March 31, 2009).

3. Terminology and Definitions

3.1 Absorbance

Quantity that indicates the attenuation of the incident light for a sample liquid, depending on the absorbing medium. When light with a wavelength λ is irradiated onto a sample with an intensity I_0, the absorbance A_λ of a beam transmitted with intensity I shall be defined as follows:

$$A_\lambda = -\log\left(I/I_0\right) \quad (1)$$

where the logarithm is the common logarithm. The absorbance follows the Lambert-Beer Law and is proportional to the concentration of the absorbing medium.

3.2 Ultraviolet-Visible (UV-Vis) Light Absorption Spectroscopy

A measurement method for calculating absorbance according to the Lambert-Beer Law, based on the transmission light observed when ultraviolet (UV) and visible (Vis) light is applied to an absorbing medium.

3.3 Sample Suspension

Suspension containing dispersed carbon nanomaterials.

3.4 Standard Suspension

Suspension with known concentration of carbon nanomaterials. This suspension is used to prepare a calibration curve.

3.5 Dispersion Medium

Liquid medium for suspending carbon nanomaterials. The medium includes dispersion agents for dispersing the carbon nanomaterials. The medium is also used to dilute sample suspensions.

4. Principles

Light irradiated onto nanoparticles dispersed in a liquid, or their clumps, is absorbed at a characteristic wavelength depending on the substance, or scattered/interrupted by the suspending particles, such that the spectrum of the transmission light differs from the incident light. The intensity of the absorbed light in such instances is proportional to the concentration of the substance, according to the Lambert-Beer Law. The absorbance, therefore, is measured and particle concentration *C* can be determined using the Lambert-Beer equation, described below:

$$A_\lambda = \varepsilon C l \qquad (2)$$

where ε is the molar absorption coefficient and *l* is the length of the optical path.

5. Equipment and Instruments

5.1 Carbon Nanomaterial Concentration Measuring Equipment

5.1.1 UV Absorption Spectrometer

This equipment is used to evaluate the carbon nanomaterial concentration based on absorbance, which uses an UV-Vis light range absorption spectrometer. The descriptions given in this procedure document assume the use of a commercially available UV-Vis absorption spectrometer.

5.1.2 Validation

Refer to Section 5.1.2 of Chapter V of the "Procedures for Safety Testing".

5.1.3 Temperature Control

Refer to Section 5.1.3 of Chapter V of the "Procedures for Safety Testing".

5.2 Dispersing Equipment

Equipment used to prepare the standard suspension with a known concentration of carbon nanomaterials. Sufficient dispersion of carbon nanomaterials in the dispersion medium is required in such instances, for which the use of an ultrasonic homogenizer is recommended.

5.3 Instruments

Ordinary containers and dispensing burettes may be used. However, containers and instruments that can change the physiochemical state (such as pH) of the dispersion sample shall not be used. For instance, since ordinary soda glass can potentially change the pH of the suspension, the use of those made of quartz would be desirable.

6. Reagents

6.1 Dispersant

Bovine serum albumin (BSA) shall be used as the dispersant in the preparation of the hazard testing sample for carbon nanomaterials.

6.2 Dispersion Medium and Its Preparation

BSA shall be dissolved in distilled water at a concentration of 10 mg/mL. This aqueous BSA solution shall be sterilized with a 0.22-μm sterilization filter and used as the dispersion medium.

Note: Other than distilled water, water purified with a water purification system, then filtered with an endotoxin removal filter may be used.

6.3 Reference Materials

The use of reference materials that have been declared to have traceability with regards to their characteristic values of UV-vis absorption is desirable. The selection of filters that have a calibration certificate issued by JCSS and which can also be used to calibrate the range in the vicinity of the measurement wavelength is recommended for the filters to be used for calibration.

7. Operations

7.1 Installation of Equipment

Refer to Section 7.1.1 of Chapter V of the "Procedures for Safety Testing".

7.2 Preparation of Standard Suspension

The suspension shall be prepared according to the procedure described below, by referring to Section 7.1.3 of Chapter V of the "Procedures for Safety Testing".

a) Add the prescribed quantity of dispersion medium, prepared according to descriptions provided in Section 6.2, to a beaker containing the prescribed amount of carbon nanomaterial.

b) Thoroughly blend the carbon nanomaterial with the dispersion medium.

c) The ultrasonic transmission horn of the ultrasonic homogenizer shall be inserted directly into the dispersion medium, consisting of an aqueous BSA solution with suspended nanocarbon. Verify visually that the liquid inside the beaker circulates sufficiently by adjusting the position of the horn.

d) The carbon nanomaterials shall be sufficiently dispersed by continuous ultrasonification for the prescribed amount of time.

e) By diluting the standard suspension obtained in Step d) at appropriate ratios, a group of standard suspensions of different concentrations shall be prepared.

Note 1: The optimum ultrasonic output during dispersion operations shall be examined in advance to verify the dispersion effects of carbon nanomaterials, as well as by considering the potential damage to carbon nanomaterials, and wear of the container and the horn.

Note 2: Cool the beaker with ice as appropriate, to suppress the rise in temperature of the dispersion liquid during ultrasonic irradiation.

Warning: The ultrasonic treatment shall be performed on a clean bench, to avoid contamination by airborne particles and exposure to the atomized mist from the suspension. Furthermore, tasks shall be performed while wearing individual protective equipment, including masks and gloves.

7.3 Cleaning of Instruments Used for Measurement

Refer to Section 7.1.4 of Chapter V of the "Procedures for Safety Testing".

7.4 Measurement

7.4.1 General

Details on the skills required of the measurement equipment operatives, as well as the measurement environment, can be found in Sections 7.1.6.1 and 7.1.6.2 of Chapter V of the "Procedures for Safety Testing".

The measurements shall be repeated at least three times to evaluate the repeatability of the measurements. The sample suspension, furthermore, shall be measured under the same measurement conditions (such as bandwidth)

as standard suspensions.

7.4.2 Starting the Equipment and Performing Blank Measurements

The measurement equipment shall be started and operated according to the equipment manual.

a) Start the carbon nanomaterial concentration measuring equipment.

b) Allow the equipment to reach a stable state as described in Section 7.1.6.3 of Chapter V of the "Procedures for Safety Testing."

c) Perform blank measurements as described in Section 7.1.5 of Chapter V of the "Procedures for Safety Testing". Adjust the baseline of the equipment in the measurement wavelength range, using a dispersion medium. Furthermore, when the equipment is a single-beam device, measure I_0 using the dispersion medium as a reference sample.

7.5 Preparation of Calibration Curves

The absorption spectrum in the visible range of the group of standard suspensions, prepared according to the procedure given in Section 7.2, shall be measured with the carbon nanomaterial concentration measuring equipment. The absorbance used for the calibration curve shall be derived according to the following procedure:

a) The transmission light intensity I of the arbitrary standard suspension in the UV-Vis range (wavelength λ: 200 nm to 800 nm) shall be measured. Derive the absorbance A_λ based on I/I_0 within a range of 200 nm to 800 nm.

b) Calculate the average absorbance A_{av} in a wavelength range of λ_1 to λ_2, in which there is no absorption by the dispersion medium. The average absorbance shall be calculated using the following equation.

$$A_{av} = \frac{1}{\lambda_2 - \lambda_1} \int_{\lambda_1}^{\lambda_2} A_\lambda \, d\lambda \qquad (3)$$

c) Prepare a calibration curve, based on the relationship between the carbon nanomaterial concentration of the group of standard suspensions and the absorbance A_{av}.

7.6 Evaluation of Carbon Nanomaterial Concentration in Sample Suspension

Evaluate the carbon nanomaterial concentration in the suspension sample, for which the concentration is an unknown, based on the absorbance in the UV-Vis range. Perform the measurements as described below:

a) Verify that the absorbance of the suspension in the wavelength range of λ_1 to λ_2 is under 3. Dilute the sample using the dispersion medium when the absorbance is equal or higher than 3.

b) Measure the absorption spectrum for the sample suspension by using concentration measurement equipment and calculate the average absorbance A_{av} with a wavelength range of λ_1 to λ_2, using Equation (2).

c) Calculate the carbon nanomaterial concentration using the calibration curve prepared in 7.5. Convert the result to the concentration of the original if the suspension is diluted.

7.7. Records

a) Record the following experiment conditions: Information on dispersion sample, time of measurement, duration of measurement, temperature of suspension, etc.

b) Record the following results: UV-Vis absorption spectrum calculated using the equipment, etc.

8. Analysis of Data

Follow the stipulations given in Section 8 of Chapter V of the "Procedures for Safety Testing" and refer to Appendix C of Chapter V of the "Procedures for Safety Testing" to calculate the concentration values for the sample suspension and to evaluate any uncertainties.

9. Reports

9.1 General Items and Conditions Related to Equipment, Experiments, and Analyses

Report on general items and the equipment, along with the conditions of experiments and analyses as described in Section 9.1 and 9.2 of Chapter V of the "Procedures for Safety Testing".

9.2. Results

Report on the calculated concentration, as well as the repetition standard deviations or uncertainties.

Appendix A

Practical Example: Measurement of CNT Concentration

A.1 Outline

This section describes the preparation of the calibration curve used for evaluating the concentration of samples prepared for the hazard testing of SWCNTs (eDISP-CNT), prepared by the eDIPS method.

A.2 Preparation of Dispersion Medium

The 08587-42 BSA product, manufactured by Nacalai Tesque Co., Ltd., was used as the dispersant.

An aqueous BSA solution with a concentration of 10 mg/mL was prepared by dissolving BSA in distilled water (Gibco® manufactured by Thermo Fisher Scientific).

The dispersion medium was prepared by filtering the aqueous BSA solution, described above, through a sterilization filter with a pore size of 0.22 μm (GP 0.22 μm, manufactured by Merck Ltd.).

A.3 Preparation of Standard Suspension and Preparation of Calibration Curves

A.3.1 Ultrasonic Dispersion

The SWCNTs were eDISP-CNT (B033). A 5 mg sample of eDISP-CNT was corrected in a 50 mL beaker.

The dispersion medium, prepared according to the procedure described in Appendix **A.2** (10 mg/mL aqueous BSA solution), was added to the beaker and the group of standard suspensionswas prepared according to the procedure described in Section **7.2**.

A Branson Sonifier 250 ultrasonic homogenizer was used for the dispersion.

The probe of the ultrasonic homogenizer was inserted into the CNT suspension, and the output control of the homogenizer was set to 4 to perform the ultrasonic treatment.

A.3.2 Preparation of Calibration Curves for CNT Concentration Evaluations

The UV-Vis absorption spectrum of the standard suspension, prepared by diluting the standard suspension with a CNT concentration of 0.1 mg/mL with 10 mg/mL aqueous BSA solution, is shown in Figure A.1 (measured with UV-2550, Shimadzu Corporation).

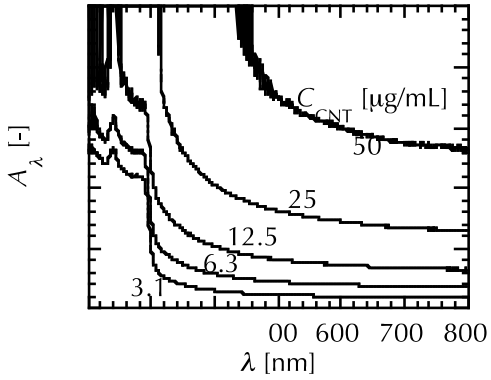

Figure A.1 UV-Vis absorption spectrum of eDIPS-CN suspension

The relationship between the CNT mass concentration and the absorbance of the suspension (calibration curve) was derived based on the average absorbance over a wavelength range of 600 nm to 800 nm (Fig. A.2).

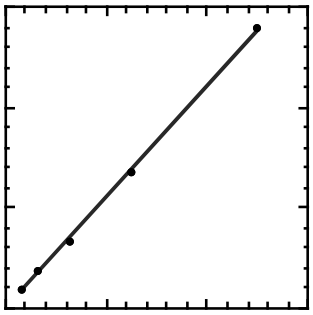

Figure A.2 Relationship between CNT mass concentration and absorbance of suspension (calibration curve)

A linear relationship, which runs through the origin as depicted in the figure, was obtained between the mass concentration C_{CNT} of the eDISP-CNT and the average absorbance A_{av} within a wavelength range of 600 nm to 800 nm. The mass concentration of the CNT was derived using the following relationship:

$$C_{CNT} = 18.1 A_{av} \quad \text{(A-1)}$$

Equation (A-1), was used to evaluate the concentration of the suspension of the eDISP-CNT, for which the concentration is an unknown.

Chapter V: Analysis of Carbon Nanomaterials in Animal Tissues

VI-1. Analysis by Ultraviolet Visible Light Absorption Spectroscopy

Introduction

The gaining of an understanding of the behavior of carbon nanomaterials that have entered the body of an organism through paths such as respiratory organs is important to any examination of the safety of carbon nanomaterials. For this reason, the quantitative detection and evaluation of carbon nanomaterials in respiratory organs, which are the primary pathway for entry, is necessary.

This section describes the method used to take spectroscopic measurements of carbon nanomaterials that have entered the lungs of an animal test subject through inhalation or intratracheal injection, as well as carbon nanomaterials that have migrated to biotissues other than the lungs. The decomposition of biotissues by enzymes is further promoted by ultrasonic irradiation for assay, to eliminate the effects of contaminants such as organic carbon and isolate the effects of the carbon nanomaterials.

1. Scope of Application

This section describes a method for measuring the amount of carbon nanomaterial administered into the lungs of rats, based on the absorbance of the decomposition liquid after decomposing in biotissues such as the lungs as a result of enzyme reactions. Furthermore, a method for promoting enzyme reactions and the decomposition of biotissues using ultrasonic irradiation is also described. This method can also be applied to the assay of nanocarbon inside biotissues during inhalation exposure tests on airborne carbon nanomaterials.

2. Referenced Standards and Regulations

This procedure refers to the following standards and regulations:

"Countermeasures for Prevention of Exposure to Nanomaterials" (March 21, 2009) standard issued by the Ministry of Health, Labour and Welfare, Japan. Publication No. 0331013 (March 31, 2009).

3. Terminology

3.1 Proteolytic Enzyme

An enzyme which decomposes biotissues.

3.2 Surfactants

Solvents that dissolve proteins to enzyme solutions during the enzyme decomposition process of biotissues and which promote enzyme reactions.

4. Equipment

4.1 Ultrasonic Bath

Equipment used for the enzyme decomposition of biotissues, which promotes enzyme decomposition through the application of ultrasonic irradiation.

4.2 Containers

Glass vials with a capacity of 50 mL. These are used as sample containers for enzyme decomposition operations.

4.3 Ultraviolet-Visible Range (UV-Vis) Absorbance Measurement Equipment

Equipment used to measure carbon nanomaterial concentration based on the absorption spectrum (absorbance) in the ultraviolet-visible range (UV-Vis) of the decomposition liquid.

4.4 Graduated Cylinder

Cylinder used to measure the volume of decomposition liquid to calculate the content based on the carbon nanomaterial in biotissue decomposition liquid. A cylinder with a capacity of 5 to 50 mL is selected, depending on the amount of biotissue.

5. Reagent for Decomposition

5.1 Surfactants

Aqueous solution with sodium dodecyl sulfate (SDS) dissolved in water output by a purified water production system at an appropriate concentration (100 mg/mL).

5.2 Proteolytic Enzyme

Proteinase K is used. If the surfactant is in the form of an aqueous solution, the enzyme activity shall be 600 U/mL or greater. If the surfactant is in the form of a powder, the enzyme activity shall be 30 U/mg or greater and shall be dissolved in water.

6. Pretreatment of Biotissues: Fragmentation

Weigh the targeted biotissue. Place a lung in a vial and fragment it into small fragments with a pair of scissors or the like.

Warning: Perform all operations on a clean bench to avoid contamination from the air and to prevent the scattering of tissue. Furthermore, tasks shall be performed while wearing individual protective equipment, including masks and gloves.

7. Decomposition of Biotissues

7.1 Addition of Enzymes and Dispersants

Add a prescribed quantity of enzyme solution and surfactant to the vial prepared according to the procedure described in Section **6.** Add the respective items in the quantities determined in advance for the tissues, which are to be the subject of decomposition (refer to Appendix).

Securely close the lid of the vial.

7.2 Decomposition with Ultrasonic Bath

Place the vial in the prescribed location in the ultrasonic bath. The liquid height in the ultrasonic bath shall be adjusted so that the maximum resonance is derived at the surface of the liquid in the vial. Perform enzyme decomposition by applying ultrasonic irradiation for the prescribed duration.

The rise in the temperature of the liquid in the bath occurs due to the ultrasonic irradiation. Control the bath temperature such that it remains between 37 °C and 50 °C, given the relationship between the enzyme kinetics and the enzyme inactivation.

Visually verify the vial for the presence of tissue in the solid state after decomposition. Continue ultrasonic irradiation until such solids can no longer be observed.

Note 1: The vial shall be placed directly above the ultrasonic emitter, such that the vibration of the water in the ultrasonic bath is maximized, thus maximizing the efficacy of the ultrasonic irradiation.

Note 2: Take care to ensure that the lid does not come off the vial during ultrasonic irradiation. Furthermore, carefully examine the container for any damage or wear.

7.3 Measurement of Decomposition Liquid Volume

Measure the decomposition liquid volume V using a graduated cylinder, to enable the calculation of the nanocarbon content in the biotissue.

8. Measurement of Nanocarbon Content

8.1 Outline

The operations described in Sections **8.2** and **8.3,** below, depend on the measurement of absorbance in the UV-Vis range, as described in Section 7.4 of the General Procedure manual "Characterization of Dispersion Liquid Sample of Carbon Nanomaterials in Liquid: Evaluation on Carbon Nanomaterial Concentration Based on Absorbance" (hereinafter referred to as the "Concentration Evaluation Procedure Manual").

8.2 Verification of Background

Decompose biotissues that do not contain any carbon nanomaterials prior to the evaluation of the carbon nanomaterial content in biotissues, according to the procedure described in Section 7. Measure the absorption spectrum of the decomposition liquid using the UV-Vis absorbance measurement equipment, according to the procedure described in Section **8.4.** Derive the absorbance A_b, which is derived from the content of contaminants other than carbon nanomaterials in the decomposition liquid.

8.2 Preparation of Calibration Curves Relating to Carbon Nanomaterial Content

Prepare calibration curves for the absorbance A_C and the carbon nanomaterial concentration C_C in the decomposition liquid in advance, to evaluate the carbon nanomaterial content in the biotissue decomposition liquid. Follow the procedure described in Section 7.2 of the Concentration Evaluation Procedure Manual to prepare the standard suspension with a known carbon nanomaterial concentration used for calibration curve measurements.

Follow the procedure described in Section 7.5 of the Concentration Evaluation Procedure Manual to prepare the calibration curve and derive the following relationship:

$$C_C = kA_C \quad (1)$$

where k is a proportionality coefficient that is dependent on the carbon nanomaterial content.

8.3. Measurement of Carbon Nanomaterial Content in Decomposition Liquid

Decompose the targeted biotissues according to the procedure described in Section 7. Measure the absorbance A of the decomposition liquid using the UV-Vis absorbance measurement equipment, according to the procedure

described in Section 8.4.

8.4 Measurement of Absorbance

Measure the absorption spectrum in the visible range of the decomposition liquid using the UV-Vis absorbance measurement equipment (refer to Section 7.4 of the Concentration Evaluation Procedure Manual).

Follow the procedure described below to measure the absorbance. The measurement equipment shall be operated according to the equipment manual.

a) Start the equipment.

b) Adjust the baseline of the equipment within the measurement wavelength range, using purified water. Furthermore, when using a single-beam device, use the dispersion medium as a reference and measure I_0.

c) Measure the transmission light intensity I in the ultraviolet to visible range (wavelength λ: 200 nm to 800 nm). Derive the absorbance A_λ based on I/I_0 within a range of 200 nm to 800 nm.

d) Calculate the average absorbance A_{av} within a wavelength range of λ_1 to λ_2, in which there is no impact from absorption by the dispersant. The average absorbance in this instance shall be as described below.

$$A_{av} = \frac{1}{\lambda_2 - \lambda_1} \int_{\lambda_1}^{\lambda_2} A_\lambda \, d\lambda \qquad (2)$$

where λ_1 = 700 nm and λ_2 = 800 nm.

e) The average absorbance $A_{av,}$ calculated using Eq. (2), is the absorbance A_b and A, in Sections 8.1 and 8.3, respectively.

8.5 Evaluation of Nanocarbon Concentration

Derive the carbon nanomaterial concentration by substituting in the absorbance derived by subtracting the background A_b from the absorbance A of the decomposition liquid that includes nanocarbons derived according to the procedure described in Section 8.4 of Eq. (1):

$$A_C = A\text{-}A_b \qquad (3)$$

Furthermore, estimate the carbon nanomaterial content M_C based on the decomposition liquid volume, using the following equation:

$$M_C = C_C V \qquad (4)$$

9. Reports

9.1 General

a) Detailed information for identifying biotissue samples (example: Information on biotissue samples, summary of animal testing, acquisition date of biotissue samples, and transportation methods)

b) Storage conditions of biotissue sample (example: Storage temperature, storage location, storage period, etc.)

c) Results of visual observations of biotissue samples (example: Images taken by a digital camera)

9.2 Analyses of Tissues; Experiment and Analysis Conditions

a) Ultrasonic generator, model, and manufacturer.

b) Information on enzymes used for decomposition (enzyme name, enzyme activity, and added dosage)

c) Information on surfactant used for improvement of enzyme activity (surfactant name, concentration, and added dosage)

d) Information pertaining to enzyme decomposition reaction (decomposition time and temperature)

e) Tissue mass and decomposition liquid volume

9.3 Nanocarbon Concentration Measurement Equipment; Experiment and Analysis Conditions

a) Equipment, model, and manufacturer.

b) Reference materials used for validation, as well as results of verification

c) Information on measurement cell

d) Information on reagents used for diluting solutions, if diluted

e) Absorbance of background, as well as average absorbance within a range of 700 nm to 800 nm

e) Average absorbance of decomposition liquid within a range of 700 nm to 800 nm

f) Calculation method for reported values (for instance, calculation method used to determine carbon nanomaterial concentrations)

g) Calculation method for uncertainties and the source of data, if required

h) Number of repeated measurements

9.4. Results

a) Carbon nanomaterial content and repetition standard deviations or uncertainties.

Appendix A

Decomposition of Biotissues and Example of Measurement of Interstitial Carbon Nanomaterial Content

A.1 Outline

The following is an example of the measurement of CNTs inside the lungs, collected during a test in which SWCNTs, prepared by the eDIPS method (eDISP-CNT), are introduced into the trachea of a rat in accordance with the procedure manual.

A.2 Preparation of Surfactant

SDS (Wako Pure Chemical Industries) was used as the surfactant.

Purified water was produced using a purified water production system (Milli-Q manufactured by Millipore). This purified water was used to prepare an aqueous SDS solution with a concentration of 100 mg/mL.

A.3 Proteolytic Enzyme

Proteinase K (Kanto Kagaku) was used. The enzyme concentration was 700 U/mL (an enzyme mass concentration of 20 mg/mL).

A.4 Enzyme Decomposition of Pulmonary Tissues

A.4.1 Pulmonary Tissues and Fragmentation

The left and right lungs of a 9-week-old Wistar rat (male), to which a sample had been administered to the trachea, were the target of the examination.

The targeted lungs were weighed in advance to derive their mass *L*.

The lungs were placed in a 50 mL vial and fragmented using a pair of scissors (Figure A.1).

Figure A.1 Lungs of rat to which CNTs had been administered to trachea, prior to decomposition

A.4.2 Enzymes and Addition of Dispersants

The required dosage of enzyme *E* (mL) was as described below with respect to the lung mass *L* (g):

$$E = L \times 6 \qquad \text{(A.1)}$$

The required dosage *D* (mL) of surfactant with an SDS concentration of 100 mg/mL, prepared according to the procedure described in Appendix A.2, was as described below:

$$D = E / 4 \qquad \text{(A.2)}$$

An enzyme solution and dispersion solution of the quantities described above were added to the vial.

A.4.3 Ultrasonic Enzyme Decomposition of Pulmonary Tissues

The vial described in Appendix A.4.2 was set in the ultrasonic bath (5510-MT manufactured by Branson; 70 W, 42 kHz) in which the water level had been adjusted in advance. Ultrasound was applied for 5 h to decompose the lungs with the enzyme (Figure A.2). The temperature was controlled to ensure that it remained between 37 and 50 °C.

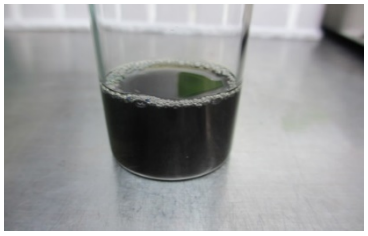

Figure A.2 Pulmonary decomposition liquid after ultrasonic enzyme decomposition

A.5 Evaluation of CNT Concentration

The UV-Vis absorption spectrum for the decomposition liquid was measured using a UV-2550 manufactured by Shimadzu Corporation. The absorption spectra (number of samples: 3) for the pulmonary decomposition liquid, without instillation to the trachea, are shown in Figure A.3 (a). No undecomposed tissue was observed in the decomposition liquid. Furthermore, the average absorbance in the range of 700 nm to 800 nm was extremely low, being only about 0.01. The absorption spectra for the pulmonary decomposition liquid administered in the trachea, at high as well as low dosages, are shown in Figure A.3 (b). No undecomposed tissue was confirmed in any of the decomposition liquids and it is assumed that the absorbance by the solids derived from the tissues can be ignored. The absorbance for the range of 390 to 400 nm is derived from the blood remaining in the lungs (heme complex of hemoglobin). No correlation was confirmed between said absorbance and the average absorbance for a range of 700 to 800 nm, as used for the evaluation of the CNT content. The calibration curve was derived by deriving the CNT concentration in advance, based on the average absorbance for a range of 700 to 800 nm. The figure was converted into the CNT content for each lung.

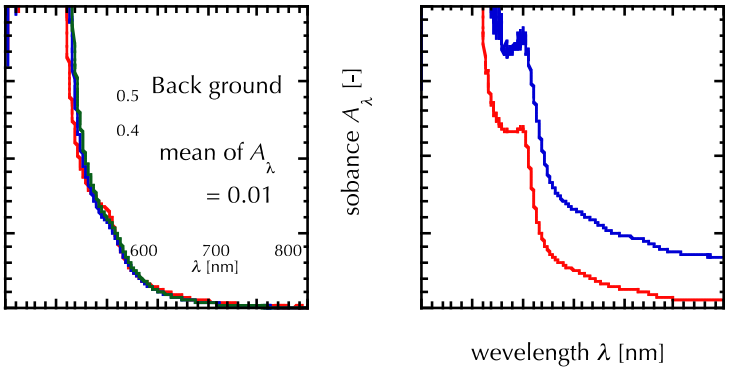

Figure A.3 Absorption spectra for pulmonary decomposition liquids
(a) Lung with no intratracheal instillation; (b) Lung with intratracheal instillation

The results of evaluating the residual quantity of CNTs inside the lungs over time, after the instillation and according to the procedure described above, are shown in Figure A.4. The error bars in this instance indicate the standard deviations for five samples.

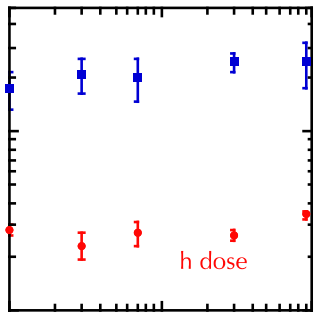

Figure A.4 Estimated value for CNT content in lungs after intratracheal instillation

V-2. Carbon Analysis

Introduction

This section describes the procedure for evaluating the carbon nanomaterial concentration in animal tissue. This procedure involves the measurement of the concentration of the carbon content based on calibration curves prepared using glucose and the like, as well as the measurement of the concentration of the CNT content based on the calibration curves prepared using the targeted CNTs.

1. Scope of Application

The target of the measurements is the CNTs present in animal tissues.

2. Referenced Standards and Regulations

No standards or regulations are either referenced or cited in this document.

3. Terminology and Definitions

3.1 Non-dispersive Infrared Spectrometry

Method for measuring carbon dioxide based on infrared absorption.

4. Equipment and Instruments

4.1 Hot Plate

Apparatus that can heat acidic liquids inside a container for acid treatment, which offers a temperature control performance of about ± 10 °C and which can reach a maximum temperature of 200 °C.

4.2 Microwave Decomposition Equipment

Equipment for decomposing the organic carbon components of samples by directly heating those samples in containers under pressure by irradiating them with microwaves.

4.3 Container for Acid Treatment

A heat- and acid-resistant watch glass, made of quartz glass, was used as a lid for a 100 mL beaker. Alternatively, a heat- and acid-resistant container made of glass that can be used in microwave decomposition equipment (the use of tubes made of PTFE is not desirable, since the CNTs are adsorbed onto the PTFE, and their subsequent removal is difficult).

4.4 Homogenizer

Instrument for preparing a uniform homogenate, by pulverizing and agitating animal tissue.

4.5 Quartz-glass tube

A quartz-glass tube capable of withstanding combustion at temperatures of up to 900 °C, filled with quartz wool for filtering. When a product made of stainless steel (SUS) is used, the inorganic carbon derived from the SUS causes the background level to rise and the lower quantitative limit for analysis rises.

4.6 Combustion Equipment

Equipment for generating carbon dioxide by burning the organic carbon and CNTs in a sample, either in an

oxygen flow or in the presence of a catalyst.

4.7 Carbon Dioxide Measurement Equipment

Equipment for measuring the carbon dioxide generated when samples are combusted. A non-dispersive infrared (NDIR) type is used. However, no restrictions are imposed on the equipment, if it is capable of quantitatively detecting carbon dioxide.

4.8 Centrifuging Tube

Tube with capacity of either 15 mL or 50 mL, used for the storage of organs or for homogenation.

5. Reagents

5.1 Nitric Acid

An acid of a high-purity analysis standard for heavy metal analyses, or of an equivalent or higher level of purity, shall be used. It is used for the decomposition of organic carbon components.

5.2 Sulfuric Acid

An acid of a high-purity analysis standard for heavy metal analyses, or of an equivalent or higher level of purity, shall be used. It is used for the decomposition of organic carbon components.

5.3 Ultrapure Water

Water that has been distilled and passed through an ion exchanger, and which is thus suitable for the microanalysis stipulated by the JIS K 0557 standard, shall be used. No carbon components may be included as impurities.

5.4 Glucose

Substance used for preparing calibration curves for carbon content measurements after dissolving and diluting in ultrapure water. Those graded as being of premium class or higher are desirable.

5.5 Protease K

Enzyme for decomposing proteins.

6. Analysis Procedure for CNTs in Sample Solutions

6.1 Preparation of Calibration Curves for Carbon Concentrations

6.1.1 Preparation of Standard Solutions

Weigh a specific amount of glucose and dilute it in a graduated flask to attain the required concentrations when preparing the standard solutions needed to prepare calibration curves.

6.1.2 Addition to Quartz-glass Tubes

Using a syringe, infuse 10 μL of a standard liquid of the respective concentration, prepared according to the procedure described in Section 6.1.1, into each of the quartz-wool segments inside the quartz-glass tubes. Allow it to dry for at least 8 h at 80 °C.

6.1.3 Measurement of Carbon Concentration

Set the quartz tube prepared according to the procedure described in Section 6.1.2 in the combustion equipment and cause it to burn by raising the temperature and measuring the carbon dioxide concentration with the NDIR

equipment. In this instance, combustion shall be performed by raising the temperature at the same rate as when the CNT sample is analyzed.

6.1.4 Preparation of Calibration Curves

Prepare calibration curves by plotting the carbon content [g] in the added glucose on the horizontal axis and the area of the peak (Vs) derived by the NDIR on the vertical axis.

6.2 Preparation of Calibration Curves for CNT Concentrations

6.2.1 Addition of CNTs to Quartz-glass Tubes

Weigh a specific amount of targeted CNTs and add them to a quartz-glass tube. This operation shall be performed under static-free conditions while ensuring that no loss arises due to static electricity.

6.2.2 Measurement of Carbon Concentration

Set the quartz tube prepared according to the procedure described in Section 6.1.2 in the combustion equipment and cause it to burn by raising the temperature. Measure the carbon dioxide concentration with the NDIR equipment. A component that burns at a specific temperature (such as 400 °C) shall be regarded as being a CNT component.

6.1.4 Preparation of Calibration Curves

Prepare calibration curves by plotting the added CNT content [g] on the horizontal axis and the area of the peak (Vs) derived by the NDIR on the vertical axis.

6.3 Pretreatment of Animal Tissue Samples

6.3.1 Preparation of Homogenate

Take an animal tissue sample and add ultrapure water of approximately three times the weight and then homogenize the mixture by using a homogenizer.

6.3.2 Enzyme Treatment

Weigh a portion of the homogenate prepared according to the procedure described in Section 6.3.1 and then add a specific amount of protease K. Then, wash off the sample attached to the wall surfaces with water and allow the solution to stand overnight at 50 °C.

6.3.3 Nitric Acid Treatment

Add a specific amount of nitric acid to the enzyme-treated liquid and decompose the solution using a hot plate or a microwave decomposition system.

6.3.4 Filtering and Washing with Water

Use a Pasteur pipette and a syringe to pass a specific amount of nitric acid through the quartz-glass tube filled with quartz wool. Then, infuse the acidic liquid prepared according to the procedure described in Section 6.3.3. To detach and collect the SWCNTs that attach to and remain on the glass Pasteur pipette and the like when the solution is transferred from a glass container or from a glass container to a quartz-glass tube, wash the containers with purified water and then ethanol, and then pass the liquid through the quartz-glass tube. Finally, pass the purified water through the quartz-glass tube to perform washing filtration.

6.3.5 Sulfuric Acid Treatment

Place a specific amount of sulfuric acid in the quartz-glass tube and allow it to stand for the specified duration to impregnate the filter section. Then, pass the purified water through the quartz-glass tube to perform washing

filtration.

6.3.6 Combustion and Analysis

Eliminate the water in the quartz-glass tube that has been subject to washing filtration according to the procedure described in Section 6.3.5 by drying in a muffle furnace at 80 °C. Then, set the quartz-glass tube in the combustion equipment and heated to a specific temperature while supplying oxygen to the quartz-glass tube to encourage burning. Measure the carbon dioxide concentration to derive the carbon concentration or the CNT concentration.

6.4 Analysis of Blank

Perform acid treatment and analysis of the animal samples of the control group that do not contain any CNTs by performing the same operations as those in the procedure described in Section 6.3. Then, perform measurements on the blanks as well. Derive a value that is equal to 10 times the variance (standard deviation) resulting from taking measurements of the blanks five or more times as the minimum value for determination.

Appendix A

Practical Example: Analysis of CNTs in Rat Organs

A.1 Outline

Calibration curves were prepared for different CNTs (SWCNTs: eDips semiconducting, eDips metal, Meijo Nanocarbon EC1.5-P, SG; MWCNTs: VGCF), and then pretreatment and analysis were performed for the samples that were added to the rat lungs.

A.2 Preparation of Calibration Curves

A specific quantity of CNTs (SWCNTs: eDips semiconducting, eDips metal, Meijo Nanocarbon EC1.5-P, SG; MWCNTs: VGCF) were weighed, placed in a quartz-glass tube, and then burned using the combustion equipment. The calibration curves were prepared based on the peak area derived by NDIR for the components that were burned at temperatures of 400 °C or higher. The temperature-elevating conditions for combustion were set to: 100 °C => (3 °C/min) => 400 °C (120 min)=> (3 °C/min) => 900 °C (60 min).

A.3 Pretreatment

A.3.1 Preparation of Acidic Liquid

Ultrapure water for metal analysis (ultrapure water for ultratrace analysis, manufactured by Wako Pure Chemical Industries), nitric acid (nitric acid 1.42 Ultrapur-100, manufactured by Kanto Kagaku), and sulfuric acid (sulfuric acid for hazardous metal measurement, manufactured by Wako Pure Chemical Industries) were used.

A.3.2 Analysis of Organic Carbon Components in Samples

A 0.4 mL sample of the CNT/rat lung homogenate was placed in a quartz glass container, after which 200 μL of protease K was added. The homogenate attached to the quartz-glass container wall surface was washed off with 500 μL of ultrapure water. The sample was then left overnight at 50 °C, after which 10 mL of nitric acid was added and decomposed at 100 °C for 60 min using a microwave decomposition system (Speedwave 4, manufactured by Berghof, Germany). A Pasteur pipette and a syringe were used to infuse a specific amount of nitric acid through the quartz-glass tube filled with quartz wool, after which the acidic liquid that decomposed the sample was passed through for filtration. To detach and collect the SWCNTs that attach to and remain on the glass Pasteur pipette and the like when the solution is transferred from a glass container or from a glass container to a quartz-glass tube, they were washed with purified water and then the liquid was passed through the quartz-glass tube. Ethanol was also used for washing, by passing it through the quartz-glass tube. As a final step, purified water was passed through the quartz-glass tube to perform washing filtration.

A.3.3 Filtration

The acidic solution after acid treatment was suctioned and filtered using a syringe, while transferring it to a quartz-glass tube with a Pasteur pipette. The glass containers were washed with ethanol and then water, after which the liquids were filtered by passing them through a quartz-glass tube.

A.3.4 Additional Acid Decomposition

Sulfuric acid was placed in the quartz-glass tube described in Appendix A.3.3 and allowed to stand. The filter segment was immersed in sulfuric acid for one hour. Purified water was passed through the quartz-glass tube to perform washing filtration.

A.3.4 Analysis

Any water left in the quartz-glass tube was eliminated by drying in a muffle furnace at 80 °C, then heating to a specified temperature while supplying oxygen gas to the quartz-glass tube to encourage burning. The carbon dioxide concentration was then measured to derive the carbon concentration or the CNT concentration. Analysis was performed with set temperature elevating conditions: 100 °C => (3 °C/min) => 400 °C (120 min) => (3 °C/min) => 900 °C (60 min).

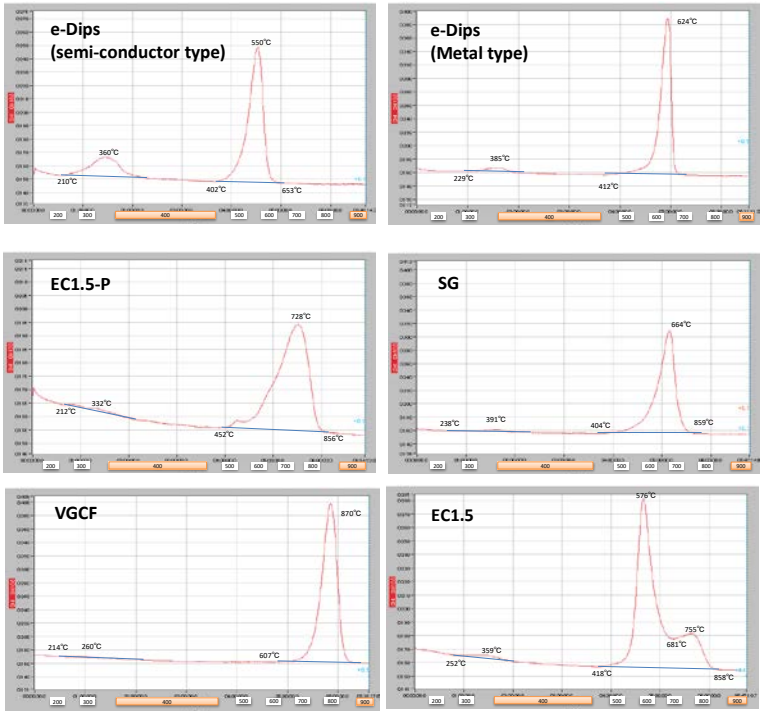

Figure A.1 Temperature elevations and peaks during combustion for respective CNTs

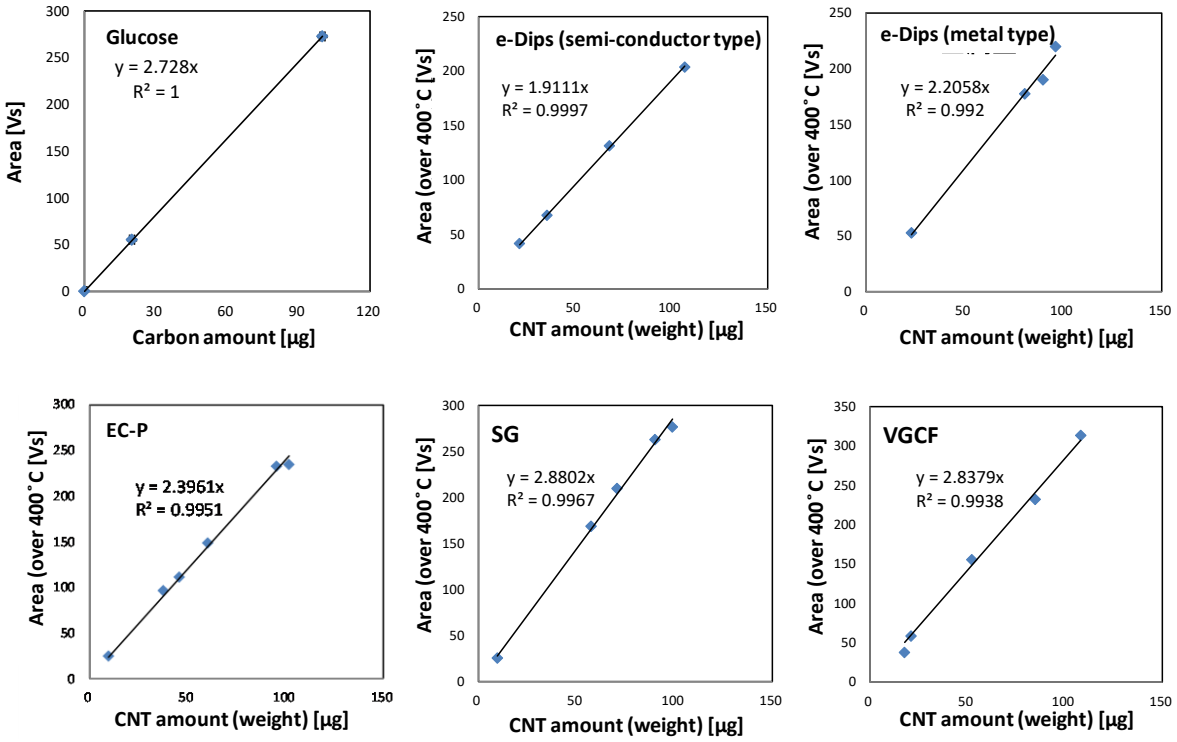

Figure A.2 Calibration curves for glucose and respective CNTs

Chapter VI: In Vitro Cell-based Assays Using Dispersion Liquid Samples of Carbon Nanomaterials

Introduction

This section describes the method for conducting in vitro cell-based assays that are not impeded by carbon nanomaterials that have been prepared and dispersed in cell culture medium by using dispersion samples of carbon nanomaterials in liquid, prepared according to the procedure described in Chapter III "Preparation of Samples for Carbon Nanomaterials" of this document, as test materials and selecting cultured cells that reflect the inflammation of lungs due to inhalation exposure with humans as well as biomarkers (endpoints) as test items.

1. Scope of Application

This section describes the method for performing in vitro cell-based assays for conducting a safety evaluation, by using a cell culture medium of carbon nanomaterials dispersed for in vitro cell-based assays.

2. Referenced Standards and Regulations

None.

3. Terminology, Definitions, and Symbols

3.1 In vitro cell-based assay

Test environment under conditions that have been artificially constituted, such as inside a test tube or in a petri dish, in other words, a test environment in which respective test conditions, such as the medium for cell culture, are artificially controlled. Such tests include those using human cultured cells or animal cultured cells.

3.2 Antibacterial and Antifungal Agent

Liquid mixture with antibiotic and antifungal properties that is added to the culture medium to prevent contamination by bacteria or fungi when cultivating human cultured cells and animal cultured cells. Concentrate liquids are commercially available and are used by being added to the culture medium. In many instances, penicillin and streptomycin and the like are used as antibiotic agents, while amphotericin B and the like are used as antifungal agents.

3.3 Cell Culture Medium

Liquid that provides a suitable growing environment for the culture of human cultured cells and animal cultured cells. The constituents include amino acids, vitamins, salts, and the like that are necessary for the growth of cultured cells. The pH is close to neutral, which is optimally suited for the growth of cultured cells. In many instances, the basal medium described above is supplemented by the addition of about 10% fetal bovine serum that is necessary for the growth of cultured cells. Several representative formulas have been established, such as the Eagle's basal medium, Dulbecco's modified Eagle's medium, and the RPMI1640 culture medium.

3.4. Prepared Stock Suspension

Suspension prepared by dispersing carbon nanomaterials in media such as an aqueous BSA solution.

3.5. Cell Culture Medium

Carbon nanomaterial suspension used for in vitro cell-based assay. Prepare the prepared stock suspension to the prescribed factor using the cell culture medium used for the in vitro cell-based assay.

4. Test Methods

4.1 Cell Strains

Cell strains that can model the respiratory organs targeted for simple and rapid safety evaluation of carbon nanomaterials are used. For instance, adenocarcinomic human alveolar basal epithelial cells (A549) can be used to alveolar type II epithelial cell while rat alveolar cell line (NR8383) can be used as a model for the macrophage (Fujita et al., 2013; Fujita et al., 2015). The storage and preparation of cell strains are performed based on the prescribed methods.

4.2 Cell Culture

Cultured cells of a prescribed concentration in the logarithmic growth phase are placed in a multiwell plate that has as many wells as the number of test items. The use of about four wells for each sample is desirable. After overnight incubation under conditions of optimum temperature, humidity, and carbon dioxide concentration, observe the state of the cells with an inverted microscope or the like to confirm that the cells have been growing appropriately. Remove the cell culture medium using an aspirator or a centrifuge by suctioning and replace or add cell culture medium. The verification of the type, concentration, and the like of the dispersant used for the preparation of the cell culture medium by conducting a preliminary test is desirable, since it has a significant impact on the survival of the cells (Horie et al., 2013). The cell culture medium in such a case is used as the carbon nanomaterial treatment group. However, in addition to these, also prepare a negative control group and a positive control group. Use cells cultured by adding prepared stock suspension that do not contain any carbon nanomaterials to the cell culture medium as the negative control group. Use cells cultured by selecting items for the respective endpoint indices and add these to the cell culture medium as the positive control group. The results of the cell assay for the negative control group and the positive control group become indices for the comparative evaluation of the reliability of the cell assay system as well as the impact of the carbon nanomaterials. Perform the tests described below after performing cultivation for a prescribed amount of time.

4.3 Cell Viability

For a cell viability test, color changes in the formazan dye of tetrazolium salts, as caused by the mitochondrial hydrogenase enzymes in living cells (example: WST-1 method; water-soluble tetrazolium salts) are measured. The cell proliferation ability is measured based on its capacity to form colonies. Select and choose these tests appropriately. Take measurements at the prescribed intervals during cultivation in the cell culture medium. The MTT method (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole), which uses mitochondrial enzyme activity as an endpoint, has been reported as inhibiting colorimetry with CNTs and therefore should be used with caution (Wörle-Knirsch et al., 2006).

4.4 Intracellular Reactive Oxygen Species

The presence of CNTs has been reported to cause reactive oxygen species (ROS) that induce oxidative stress (Fujita et al., 2013). Use cell-permeable fluorescent probes (such as DCFH-DA (2', 7'-Dichlorodihydrofluorescin

diacetate)) for the ROS and measure the fluorescent intensity of the fluorescent probe captured inside the cells (such as DCFH (2′, 7′-Dichlorodihydrofluorescin)), which have been cultivated in the cell culture medium for the prescribed times, to determine the activity of the intracellular ROS.

4.5 Cell Cycle

Carbon nanomaterials have been reported to inhibit specific cell cycles (Sargent et al., 2009). Fluorescent probes that intercalate with the DNA double helix are used for the same reason. These fluorescent probes captured inside cells are measured during the prescribed amount of time the cells are cultivated in the cell culture medium. The measurements are used to reveal the state of the cell cycles, to analyze the inhibition of cell cycles.

4.6 Inflammatory Cytokines

Carbon nanomaterials have been reported to produce inflammatory cytokines (Herzog et al., 2009). Inflammatory cytokines can be a useful index as they are a causal factor that triggers a variety of inflammatory symptoms in an organism. Furthermore, such inflammatory cytokines can be used as a common biomarker for validating the safety evaluation methods used in animal tests with rodents such as rats. There are a number of different inflammatory cytokines (IL-1a, MCP-1, MIP-1a, IL-1b, IFN-g, IL-18, GRO/KC/CINC, TNF-α, SPP-1, etc.) that depend on the animal species (human, rat, etc.). Select the most suitable from among these and use single or multiple types of cytokines to take measurements of the respective inflammatory cytokine in BALF, either independently or simultaneously, using the supernatants from cell cultures that have been cultivated over prescribed times in the cell culture medium.

4.7 Gene Expression Analysis

Genes activated or regressed by carbon nanomaterials are considered useful for estimating endpoints and can be used as common biomarkers in the validation of the safety evaluation methods used for animal tests with rodents such as rats. Use cells that have been cultivated over prescribed times in a cell culture medium, select the total RNA, and use the quantitative polymerase chain reaction (qRT-PCR)) based on this, as well as the DNA microarrays, to analyze a specific or comprehensive gene expression.

4.8 Cell Sample Observation by Electron Microscopy

The incorporation of carbon nanomaterials into cells, as well as characteristics such as the shapes of the incorporated carbon nanomaterials, are considered to serve as useful data for the observation of respective endpoints. Use cells that have been cultivated over prescribed times in the cell culture medium, wash the carbon nanomaterials with a phosphate buffer, prefix with glutaraldehyde, wash and postfix with osmium tetraoxide followed by dehydration treatment, embed the cells in resin, prepare ultrathin fragments, stain and reinforce. Then, observe these cell samples using an electron microscope.

5. Reports

The following items shall be reported.

5.1 General

a) Detailed information for identifying dispersed carbon nanomaterial samples for conducting in vitro cell-based assays (example: Information on particle samples, dispersion preparation method, information on the dispersion medium, acquisition date of dispersion samples, transportation methods, characterization of the dispersion

samples in solution)

b) Measurement date, name of person performing the measurements, and name of measurement organization

c) Date on which the equipment was validated

d) Storage conditions of dispersion sample (example: Storage temperature, storage location, and storage period)

e) Results of visual observation of precipitation formation (example: Images taken by a digital camera)

5.2 Equipment and Measurement Reagents, Experiment and Analysis Conditions

a) Equipment name, model name, and manufacturer name.

b) Positive control substances used for validation and results of verification

c) Measurement reagent name and manufacturer name

d) Name of software used for analysis, as well as manufacturer name

5.3. Results

5.3.1 Cell Viability

Calculate the cell survival rate of the carbon nanomaterial treatment group compared with the negative control group and the repetition standard deviations.

5.3.2 Intracellular Reactive Oxygen Species Measurement

Calculate the absolute value of the intracellular ROS level in all groups (example: DCF value) or otherwise calculate the intracellular ROS level in the carbon nanomaterial treatment group relative to the negative control group and relevant repetition standard deviations.

5.3.3 Cell Cycle

Calculate the proportions for each cell cycle (G1 period, S period, G2 period, and M period) in all groups and their repetition standard deviations.

5.3.4 Inflammatory Cytokines

Calculate the value of each inflammatory cytokine in all groups and the relevant repetition standard deviations.

5.3.5 Gene Expression Analysis

Calculate the gene expression variable ratio for the carbon nanomaterial treatment group, relative to that of the negative control group. Analyze the significant difference in the expression gene functional group analysis, clustering such as hierarchical clustering, or otherwise analyze the network with the pathway database as needed.

5.3.6 Cell Sample Observation by Electron Microscopy

Observation records for cell samples in all groups.

5. References

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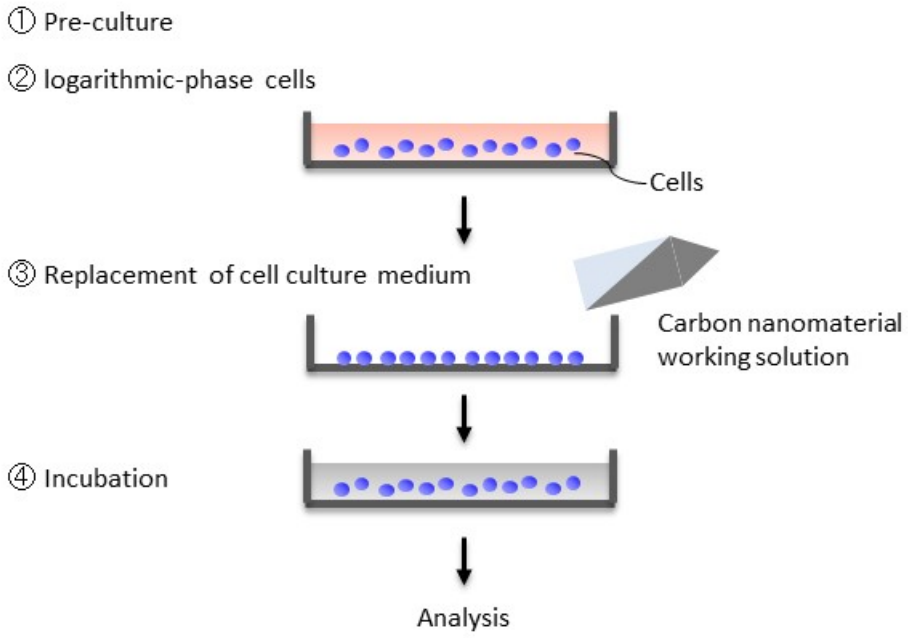
Appendix A

System Diagram of In Vitro Cell-based Assay for Safety Evaluations



Appendix B

Practical Example: In vitro cell-based assay for SWCNTs using rat alveoli macrophage cells

B.1 Outline

This section describes an example involving an in vitro cell-based assay of rat alveolar cell line (NR8383). The cell culture medium of SG-SWCNTs (concentration: 0.1 mg/mL) was exposed to the NR8383 cell strain for 24 h and then subjected to a cell viability test, intracellular ROS measurement test, comprehensive gene expression analysis, and cell shape observation using a TEM (Fujita et al., 2015).

B.2 Preparation and Characterization of Used Samples

Short SG-SWCNTs in a cell culture medium (geometric mean length: Approx. 0.36 μm) and long SG-SWCNTs in a cell culture medium (geometric mean length: approx. 1.52 μm) were prepared according to the sample preparation procedure for carbon nanomaterials described in Chapter III of this manual and used for the cell culture medium. The characterization of the respective samples in the prepared stock suspension and cell culture medium complied with the characterization of the dispersion samples in the liquid for carbon nanomaterials, described in Chapter IV of this manual.

B.3 Cell and Culture Conditions

The NR8383 cell strain (ATCC) was used for the in vitro cell-based assay. The cells were cultured in 5% CO_2 at 37 °C in the F-12K cell culture medium to which 10% FBS had been added. The culture preparation liquid described above was exchanged and this instant was assumed to be exposure time 0, after which the cells were cultured for 24 h. The code names of the respective samples are abbreviated as follows:

No.	Sample	Code
1	Short SG-SWCNTs in cell culture medium	CNT-1
2	Long SG-SWCNTs in cell culture medium	CNT-2
3	Negative control culture medium (including BSA)	Control

B.4 Cell Viability Test

The impact on cell survival of SG-SWCNTs (exposure for 24 h) was measured using WST-1 (Takara Bio, Inc.) with the mitochondrial hydrogenase activity of living cells taken as an indicator. The results indicated that CNT-1 or CNT-2 had little effect on the cell viability after 6 h. However, the NR8383 cell viability was significantly decreased following a 24-h exposure to CNT-2. A positive control group consisting of items such as 100-μM Mitomycin C was established in respective tests to verify the test systems. The test systems were verified as being problem free.

B.4 Intracellular Reactive Oxygen Species Measurement Test

The levels of intracellular ROS were measured in cells exposed to CNT-1 or CNT-2 for 6 or 24 h using the DCFH-DA (2', 7'-Dichlorodihydrofluorescin diacetate) assay within cells using flow cytometry (Merck Millipore). Significantly elevated expression levels of the pro-inflammatory cytokine MIP-1α were observed 6 h post-treatment with CNT-1 or CNT-2, and the levels at 24 h in CNT-2-treated cells were significantly higher than those of the controls.

B.6 Inflammatory Cytokine Measurement

A multiplex system for multiple protein items (Luminex 200^{TM} xPONENT, manufactured by Merck Millipore) was used to measure the MIP-1a, which is a type of inflammatory cytokine in cell suspension, 24 h after exposure to examine the inflammation triggered by the SG-SWCNTs. The results confirmed a significant increase in the MIP-1a in the case of the CNT-1 group after exposure for 6 h, relative to the control. Furthermore, a significant increase in the MIP-1a was confirmed with the CNT-2 group after exposure for 6 h and 24 h, relative to the control. The extent of these increases was more significant with the CNT-2 group in comparison with the CNT-a group.

B.7 Comprehensive Gene Expression Analysis

The effects of SG-SWCNTs on the comprehensive gene expression (exposure for 24 h) was analyzed using a DNA microarray. The total RNA was extracted using a RNeasy Mini kit (Qiagen) after cultivation. RNA was quantified using a Nanodrop 2000 (Thermo) and Bioanalyzer (Agilent Technologies). Cyanine-3-labeled cRNA was prepared from RNA using a One-Color Low RNA Input Linear Amplification PLUS Kit (Agilent Technologies), according to the manufacturer's instructions. Each labeled cRNA probe was used separately for hybridization to a 4 × 44 K Whole Rat Genome Microarray Kit (G4131F; Agilent), and hybridization was performed at 65 ℃ for 17 h. Hybridized microarray slides were washed according to the manufacturer's instructions and were scanned using an Agilent DNA Microarray Scanner with a 5-μm resolution. The scanned images were analyzed numerically using the Agilent Feature Extraction Software, version 10.7.3.1. The results confirmed the significant expression of the main genes involved in inflammation, oxidative stress, extracellular degrading enzymes, and the like. The extent of these increases was more significant with the CNT-2 group, relative to the control group. Details of these results have been submitted to the gene expression information database, Gene Expression Omnibus (GEO) at the U.S. National Center for Biotechnology Information (NCBI), accession number: GSE61319.

B.8 Cellular Morphology and Phagocytic Uptake of SWCNTs Observed by TEM

After treatment, cells were fixed sequentially in 1.2% (v/v) glutaraldehyde for 1 h at 20 °C and in 1% osmium oxide solution for 1 h at 4 °C, dehydrated in ethanol, and then embedded in a commercial epoxy resin (TAAB Laboratories Equipment Ltd., Reading, England). Samples were transferred to fresh resin in capsules and then polymerized at 60 °C for at least 48 h. A TEM system operating at 75 kV (H-7000; Hitachi, Tokyo, Japan) was used to observe the intracellular distribution of the SWCNTs and morphologic changes in the NR8383 cells. The

results confirmed that for CNT-1, relatively thin bundles of SWCNTs with a short linear shape, and for CNT-2, relatively thick bundles of SWCNT with a long linear shape (Figure B.1), were observed in the phagosomes of those cells exposed to SWCNTs for 24 h. These results suggested that SWCNTs were translocated to the phagosomes, while maintaining their physical structure. No SWCNTs were observed within the nucleus in any of the samples. Furthermore, no abnormal cell shapes were observed in any of the groups.

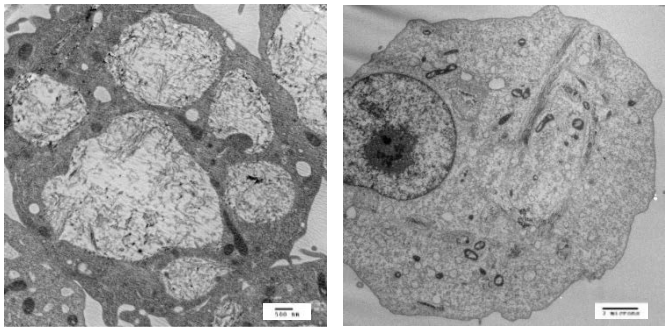


Figure B.1 SG-SWCNTs inside NR8383 after exposure for 24 h, as observed by TEM (left figure: CNT-1 exposure group; Right figure: CNT-2 exposure group).

Appendix C

Practical Example: In vitro cell-based assay of exfoliated graphene using rat alveoli macrophage cells

C.1 Outline

This section describes an example involving an in vitro cell-based assay of rat alveoli macrophage cells (NR8383). The cell culture medium of exfoliated graphene (concentration: 0.001 mg mL) was exposed to the NR8383 cell strain for 24 h, after which a cell viability test and cell shape observation were conducted using a TEM. The exfoliated graphenes vary in their physiochemical characteristics, depending on the solvent being used. Two types of solvents were used to perform the dispersion treatment to prepare the required liquids and they were respectively used to conduct the in vitro cell-based assays.

C.2 Preparation and Characterization of Samples

The exfoliated graphenes dissolved and dispersed using solvents A0 and B0 are referred to as preparation liquid Grp-A0 and Grp-B0, respectively. These were further supplemented with 10% FBS and diluted to 1/10 in the F-12K culture medium to prepare cell culture media Grp-A and Grp-B for use. The stability evaluation of the exfoliated graphene in the preparation liquid was performed by conducting measurements of the particle size distributions of the exfoliated graphenes over time, by using a laser diffraction particle diameter distribution measurement system (model SALD-2300, manufactured by Shimadzu Corporation) up to 72 h after preparation. The results indicated that the particle size distributions for Grp-A0 and Grp-B0 were same up to 72 h after preparation and both were determined to be stable.

C.3 Cell and Culture Conditions

The NR8383 cell strain (ATCC) was used for the in vitro cell-based assay. The cells were cultured in 5% CO_2 at 37 °C in the DMEM cell culture medium to which 10% FBS added. The cell culture medium described above was switched and this instant was assumed to be exposure time 0. Cell cultivation was performed for 24 h after this point. The code names of the respective samples were abbreviated as follows:

No.	Sample	Code
1	Exfoliated graphene cell in culture medium (including solvent A0)	Grp-A
2	Exfoliated graphene cell in culture medium (including solvent B0)	Grp-B
3	Negative control in cell culture medium (including solvent A0 or solvent B0)	Control

C.4 Cell Viability Test

The effect of the exfoliated graphene (exposure for 24 h) on cell survival was measured using a WST-1 system (Takara Bio, Inc.) with the mitochondrial hydrogenase activity of living cells as an indicator. The results of measuring the cell growth potential for respective groups using the WST-1 confirmed no significant reduction

with the NR8383 cells in 0.001 mg/mL of the Grp-A and Grp-B groups, relative to the control. A positive control group consisting of the likes of 100-μM Mitomycin C was established in respective tests to verify the test systems. The test systems were verified as being problem-free.

C.5 Cellular Morphology Observed by TEM

The shapes inside cells, as well as the incorporation of exfoliated graphene after exposure (exposure for 24 h) were examined using a TEM (H-7000, manufactured by Hitachi Ltd.). The results confirmed the presence of several aggregated substances, considered to be exfoliated graphene, in the cytoplasm of both the Grp-A and Grp-B groups, 24 h after exposure (Arrow in Figure C.1).

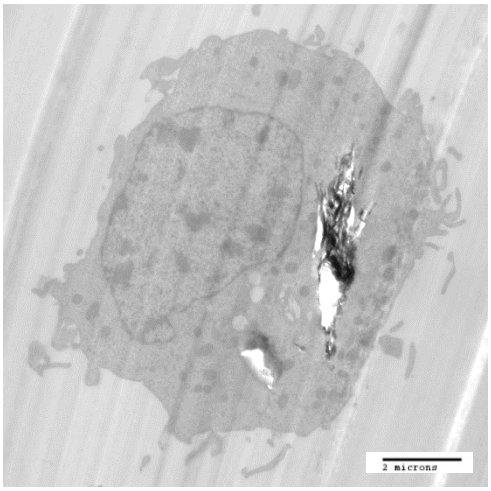


Figure C.1 NR8383 (Grp-A exposure group) after exposure for 24 h, as observed by TEM

C.6. Reference

Fujita K, Fukuda M, Endoh S, Kato H, Maru J, Nakamura A, Uchino K, Shinohara N, Obara S, Nagano R, Horie M, Kinugasa S, Hashimoto H, Kishimoto A. Physical properties of single-wall carbon nanotubes in cell culture and their dispersal due to alveolar epithelial cell response. Toxicol Mech Methods. 2013, 23(8):598-609.

Chapter VII: Animal Testing Using Dispersion Liquid Samples of Carbon Nanomaterials

VII-1. Intratracheal Instillation Test

Introduction

This section describes the method to be used when conducting intratracheal instillation tests that are not impeded by carbon nanomaterials that were prepared and dispersed during instillation in cultured cells when using the dispersion samples prepared according to the procedure described in Chapter III "Preparation of Samples for Carbon Nanomaterials" of this document as test materials, as well as when selecting animal indices that reflect the inflammation of lungs due to inhalation exposure, with humans as well as biomarkers (endpoints) as test items.

1. Scope of Application

This section describes the method for performing the intratracheal instillation test using animals for conducting safety evaluations, by using an instillation liquid of carbon nanomaterials dispersed for the intratracheal instillation tests.

2. Referenced Standards and Regulations

None.

3. Terminology, Definitions, and Symbols

3.1 Intratracheal Instillation Test

Test intended to perform impact evaluation primarily on pulmonary tissues and living organisms after instillation, by inserting an instillation instrument into the trachea through the oral cavity of anesthetized animals to administer carbon nanomaterials dispersed in liquid on either a single occasion or on multiple occasions. This test can be performed relatively easily and at low cost, since there is no need for large equipment or facilities, and the maintenance work that they would incur, when compared with an inhalation exposure test. The verification of size is necessary to ensure that samples are not contaminated with non-inhalant particles, since the carbon nanomaterials are forcibly injected.

3.2 Suspension

Suspension prepared by dispersing carbon nanomaterials in media such as aqueous BSA solution. The solution is prepared by diluting to a prescribed concentration.

4. Test Methods

4.1 Animals

Rodents that can be used as models for evaluating inflammation of the lung through inhalation exposure of

carbon nanomaterials are used. These tests are widely used for safety tests, with rats of the CD (SD) lines, the Wistar lines, or F344 lines, for instance, being used due to their resistivity to sensitivity to known chemical substances and sensory ailments, genetic stability, and ease of implementation (Kobayashi et al., 2010; Fujita et al., 2015). Animals are handled according to the prescribed animal testing related regulations.

4.2 Intratracheal Instillation

Intratracheal instillation is performed by directly injecting carbon nanomaterials suspended in a liquid with a feeding needle or spray needle to sufficiently anesthetized rodents such as rats.

4.3 General State Observations of Animals

The general state of the animals is observed once a day after an intratracheal instillation, throughout the period of animal observation.

4.4 Animal Body Weight Measurements

Animal body weight measurements are conducted daily or once a week and recorded prior to and after the intratracheal instillation and during the observation period. The body weight of animals that are subjected to autopsy will be measured on the day of the autopsy, while the body weight is measured and recorded when an animal is found with an animal that experienced a sudden death or an otherwise dead animal.

4.5 Bronchoalveolar Lavage Fluid (BALF) Inspection

4.5.1 Collection of BALF

Insert a cannula into the trachea and repeatedly inject and suction out physiological saline solution or a phosphate buffer heated to about 37 °C. The collected liquid is considered to be BALF. Repeat this operation several times and record the amount of BALF collected. Handle the collected BALF by storing it in ice.

4.5.2 Cell Examination of BALF

Centrifuge the collected BALF and use the sediments for the cell examination. Add measurement reagent to the sediment and prepare a suspension of constant volume and use general hematology examination equipment to measure the number of nucleated cells in the BALF. Add a phosphate buffer to the acquired sediment set aside for the calculation of the cell ratio and use the constant-volume liquid to prepare a suspension for use in the preparation of smear samples. Perform Pappenheim staining or the like, and then conduct a microscopic examination, calculate the cell ratios, and count the respective numbers of cells of heterophilic leucocytes, lymphoid corpuscles, macrophages, acidocytes, basocytes, and the like.

4.5.3 Biochemical Examination of BALF

Centrifuge the collected BALF and use the supernatant for biochemical examination.

4.5.3.1 Total Protein

The total protein concentration in the BALF serves as a useful index for a variety of inflammations in the pulmonary tissue. Measure the total protein concentration in the BALF by using colorimetric analysis according to the Lowry method or the like, as well as infrared analysis equipment.

4.5.3.2 Lactate Dehydrogenase Activity

The lactate dehydrogenase (LDH) in the BALF serves as a useful index for a variety of cell damage in the pulmonary tissue. Measure the LDH activity isolated in the BALF using colorimetric analysis or the like.

4.5.3.3 Inflammatory Cytokine Measurement

Carbon nanomaterials have been reported to produce inflammatory cytokines (Herzog et al., 2009). Inflammatory cytokines can be a useful index as they are a causal factor that triggers a variety of inflammatory symptoms in an organism. Furthermore, such inflammatory cytokines can be used as a common biomarker for validating the safety evaluation method applied to tests on cell cultures derived from rodents such as rats and mice. There are a number of different inflammatory cytokines (IL-1α, MCP-1, MIP-1α, IL-1β, IFN-γ, IL-18, GRO/KC/CINC, TNF-α, SPP-1, etc.) that depend on the animal species. Select the most suitable from among these and use a single type or multiple types of cytokines to take measurements for respective types of inflammatory cytokine in the BALF, either independently or simultaneously.

4.6 Pleural Cavity Lavage Fluid (PCLF) Examination

4.6.1 Collection of PCLF

Open a section of the diaphragm on the sternum side and use a cannula to inject and suction out phosphate buffer heated to about 37 °C. The collected liquid is considered to be pleural cavity lavage fluid (PCLF). Record the amount of PCLF collected. Handle the collected PCLF by storing it on ice. Set aside 1 mL of the collected PCLF to measure the number of nucleated cells and retain the remainder to calculate the cell ratio.

4.6.2 Cell Examination of PCLF

Centrifuge the collected PCLF and use the sediments for cell examination. Add the measurement reagent to the sediment to prepare a suspension of constant volume and use general hematology examination equipment to measure the number of nucleated cells in the PCLF. Add the phosphate buffer to the acquired sediment set aside for the calculation of the cell ratio and use a liquid of constant volume to prepare the suspension needed to prepare the smear samples. Perform Pappenheim staining or the like, and then conduct a microscopic examination, calculate the cell ratios, and count the respective number of cells of heterophilic leucocytes, lymphoid corpuscles, macrophages, mastocytes, acidocytes, basocytes, and the like.

4.6.3 Biochemical Examination of PCLF

Centrifuge the collected BALF and use the supernatant for the biochemical examination.

4.6.3.1 Total Protein Concentration Measurement

The concentration of total protein in the PCLF serves as a useful index for a variety of inflammations in the pulmonary tissue. Measure the total amount of protein in the PCLF by using colorimetric analysis according to the Lowry method or the like, as well as infrared analysis equipment.

4.6.3.2 Lactate Dehydrogenase Activity

The released LDH in the PCLF serves as a useful index for a variety of types of cell damage in the pleural area. Measure the LDH isolated in the PCLF using colorimetric analysis or the like.

4.6.3.3 Inflammatory Cytokine Measurement

Carbon nanomaterials have been reported to produce inflammatory cytokines (Herzog et al., 2009). Inflammatory cytokines can be a useful index as they are a causal factor that triggers a variety of inflammatory symptoms in an organism. Furthermore, such inflammatory cytokines can be used as a common biomarker for validating the safety evaluation method applied in tests using cell cultures derived from rodents such as rats and mice. There are a number of different inflammatory cytokines (IL-1α, MCP-1, MIP-1α, IL-1β, IFN-γ, IL-18, GRO/KC/CINC, TNF-α, SPP-1, etc.) that depend on the animal species. Select the most suitable from among these and use a

single type or multiple types of cytokines to take measurements of the respective types of inflammatory cytokine in the PCLF, either independently or simultaneously.

4.7 Pathological Examinations

Perform observations of animal organs and tissues with the naked eye (autopsy) as well as taking organ weight measurements and performing pathological tissue examinations as pathological examinations. Cut the abdominal aorta of anesthetized animals scheduled for dissection to attain euthanasia through bleeding, and then conduct the autopsy of the animal.

Prepare pathological samples for pulmonary tissues according to the usual procedure, that is, performing paraffin embedding, slicing thinly, and then performing hematoxylin-eosin staining. Use such samples for pathological tissue examinations to identify and record respective observations, including the types and extents of the lesions.

4.8 Gene Expression Analysis

Genes activated or regressed by carbon nanomaterials are regarded as being useful for estimating biomarkers for inflammation of pulmonary tissues. Using cells that have been cultivated over prescribed times in a culture medium preparation solution, the total RNA is selected by using pulmonary tissue from which the blood has been drained and are used to analyze specific or comprehensive gene expressions based on the qRT-PCR and DNA microarrays.

4.9 Biotissue and Cell Sample Observation by TEM

The incorporation of carbon nanomaterials into biotissues such as lungs or cells in alveoli, as well as characteristics such as the shapes of incorporated carbon nanomaterials, are regarded as being useful for evaluating the safety of carbon nanomaterials. Use biotissues as well as the cells in the BALF and PCLF, by washing with a phosphate buffer, prefixing with glutaraldehyde, washing and postfixing with osmium tetraoxide, followed by dehydration treatment. Then, embed the cells in resin, prepare ultrathin slices, stain, and then reinforce. Then, observe these biotissues and cell samples using an TEM.

5. Reports

The following items shall be reported.

5.1 General

a) Detailed information for identifying dispersed carbon nanomaterial samples after performing an intratracheal instillation test (example: information on particle samples, the dispersion preparation method, information on the dispersion medium, acquisition date of the dispersion samples, transportation methods, characterization of the dispersion samples in solution)

b) Measurement date, name of person performing the measurements, and name of measurement organization

c) Date on which the equipment was validated

d) Storage conditions of dispersion sample (example: Storage temperature, storage location, and storage period)

e) Results of visual observations of precipitate formation (example: Images taken by a digital camera)

5.2 Equipment and Measurement Reagents, Experiment and Analysis Conditions

a) Equipment name, model name and manufacturer name.

b) Positive control substances used for validation and results of verification

c) Measurement reagent name and manufacturer name

d) Name of software used for analysis, as well as manufacturer name

5.3 General State Observations for Animals

a) The general state of animals shall be observed and recorded once a day after an intratracheal instillation, throughout the period of animal observation.

5.4 Animal Body Weight Measurements

a) Measure and record the animal body weight daily or once a week, prior to and after the intratracheal instillation and during the observation period.

b) Measure the body weight of animals that are subjected to autopsy on the day of the autopsy, and record the measurements when an animal is found with animals that have experienced a sudden death or otherwise dead animals.

5.5 BALF Examinations

a) Measure the number of nucleated cells in the BALF, using a general hematology examination system.

b) Perform Pappenheim staining or the like on smear samples, and then conduct a microscopic examination. Calculate the cell ratios and count the number of respective cells of heterophilic leucocytes, lymphoid corpuscles, macrophages, acidocytes, basocytes, and the like.

c) Measure the total protein in the BALF by using colorimetric analysis according to the Lowry method or the like, as well as by using infrared analysis equipment.

d) Measure the LDH isolated in the BALF by using a colorimetric analysis or the like.

e) Measure the production of inflammatory cytokines in the BALF.

5.6 PCLF Examinations

a) Measure the number of nucleated cells in the PCLF, using a general hematology examination system.

b) Perform Pappenheim staining or the like on smear samples, and then conduct a microscopic examination. Calculate the cell ratios and count the respective numbers of cells of heterophilic leucocytes, lymphoid corpuscles, macrophages, mastocytes, acidocytes, basocytes, and the like.

c) Measure the total protein in the PCLF by using colorimetric analysis according to the Lowry method or the like, as well as by using infrared analysis.

d) Measure the LDH isolated in the BALF by using colorimetric analysis or the like.

e) Measure the production of various inflammatory cytokines in the PCLF.

5.7 Pathological Examinations

Record the respective observations, including the types and extents of lesions, using pathological tissue samples.

5.8 Gene Expression Analysis

Calculate the gene expression variable ratio for the carbon nanomaterial treatment group, relative to that of the negative control group. Analyze any significant difference in the expression gene functional group analysis, clustering such as hierarchical clustering, or otherwise analyze the network with the pathway database when needed.

5.9 TEM Observation

Observation records of biotissues and cell samples in all groups.

6. References

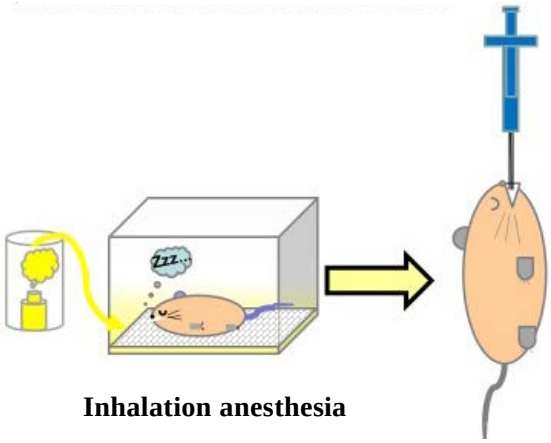
Kobayashi N, Naya M, Ema M, Endoh S, Maru J, Mizuno K, Nakanishi J. Biological response and morphological assessment of individually dispersed multi-wall carbon nanotubes in the lung after intratracheal instillation in rats. Toxicology. 2010, 276(3):143-53.

Fujita K, Fukuda M, Endoh S, Maru J, Kato H, Nakamura A, Shinohara N, Uchino K, Honda K. Pulmonary and pleural inflammation after intratracheal instillation of short single-walled and multi-walled carbon nanotubes. Toxicol Lett. 2016, 257(22):23-37.

Herzog E, Byrne HJ, Casey A, Davoren M, Lenz AG, Maier KL, Duschl A, Oostingh GJ. SWCNT suppress inflammatory mediator responses in human lung epithelium in vitro. Toxicol Appl Pharmacol. 2009, 234(3):378-390.

Appendix A

Outline of Intratracheal Instillation

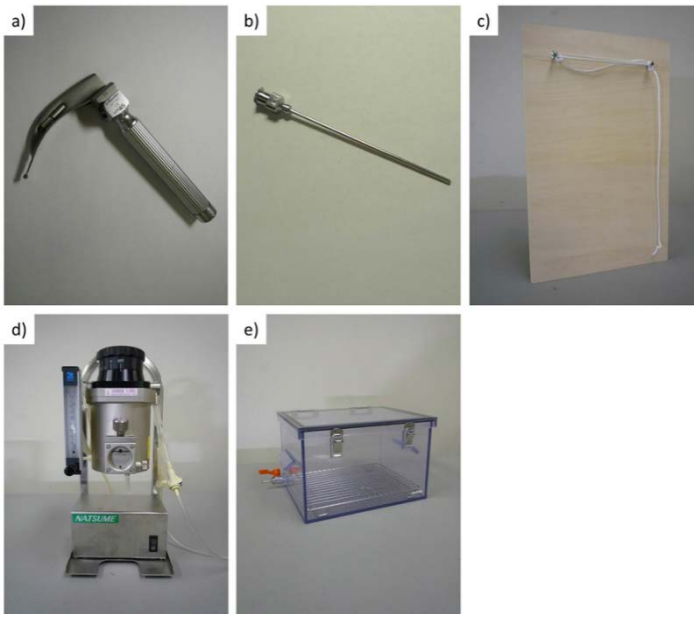


Inhalation anesthesia

Intratracheal Instillation

Appendix B

Equipment Used for Intratracheal Instillation



a) Laryngoscope

b) Intubation tube

c) Board

d) Simple inhalation anesthesia apparatus for small animals

e) Box for induction of anesthesia

Appendix C

Practical Example: Examination of Intratracheal Instillation Technique that Impacts Lungs

C.1 Outline

The gold standard test for the hazard evaluation of inhaled chemical substances, including nanomaterials for industrial applications, is the inhalation exposure test. It is a costly test as it requires the use of larger-scale equipment and skilled operators. For that reason, it is not practical to implement hazard tests on all nanomaterials for industrial applications by conducting inhalation exposure tests. On the other hand, the intratracheal instillation test injects known substances directly into the lungs via the trachea and, as such, enables the identification of dose-dependent relationships. Therefore, it is hoped that this test will provide a means for the hazard evaluation screening of nanomaterials for industrial applications. There are, however, a variety of instillation methods for intratracheal instillation tests, including the use of a cannula that is retained inside the trachea, a feeding needle, or a spray needle. All these have an impact on the distribution of the samples in the lungs. Since the impact of the body positions of animals at the time of instillation also affects the outcomes, an examination was performed to examine whether there is any variation in the inflammation caused by the intratracheal instillation of nanomaterials in the lungs, according to the cannula and animal body positions.

2. Test methods

C.2.1 Samples

MWCNTs suspended in liquid based on BSA or the like were used as samples. The mass concentration was set to 1.0 mg/mL as well as 2.5 mg/mL.

C.2.2 Animals

Fischer 344 male rats (free of virus antibody (SPF)), 12 weeks old, were used as the animal test subjects. To acclimatize the animals, they were purchased two weeks prior to the instillation and kept in sterilized cages at the animal facility. The temperature inside the facility was 24 ± 1 °C and the humidity was 50 + 20% RH. The lighting was controlled by a timer, with light and dark cycles set to a 12:12 ratio.

C.2.3 Intratracheal Instillation

Isoflurane was injected into the simple handling inhalation anesthesia apparatus for small animals and the Fischer 344 male rats were placed in the box to induce anesthesia. Isoflurane was passed into the box to induce anesthesia and a check was made to ensure that the animals were sufficiently anesthetized.

Three methods were used for the intratracheal instillation, namely, (1) an ordinary feeding tube with the board oriented vertically for injection; (2) a spray tube with the board erected vertically for injection; and (3) a spray tube with board inclined at 45°. A rat was set on the board and then its mouth was opened using a laryngoscope. The glottis located under the epiglottis was verified and a suspension of MWCNTs was injected by inserting a feeding tube or spray tube. A suspension of nanomaterials for industrial applications was then injected via a tube. The amount of the injection was set to two doses, that is, 0.04 mg and 0.2 mg per rat. A 10% BSA solution was

used as the negative control. Once the animal recovered from the anesthetic, it was returned to its cage. The animals were dissected one day after the injection, as well as three days after the injection.

The suspension was verified on the day of the intratracheal instillation to ensure that there were no aggregates and ultrasonic treatment was conducted for a few minutes immediately prior to the instillation.

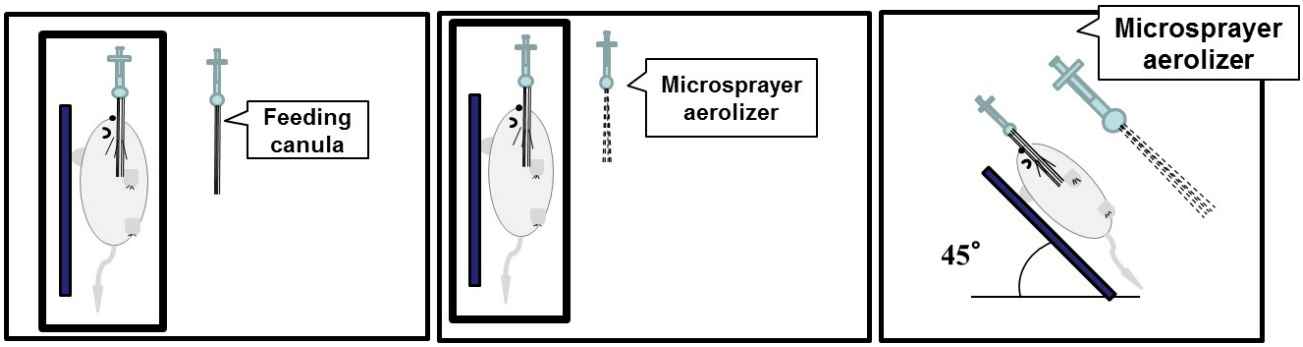


Figure C.1 Intratracheal instillation methods with varying instillation techniques

C.2.4 Dissection

The rats were abdominally administered with anesthetic, and then verified to ensure that they were sufficiently anesthetized (based on nociceptive stimuli) before being bled to death. A hole was made in the diaphragm and a feeding tube fitted to a syringe containing 5 mL of physiological saline solution. This syringe was used to inject the solution into both the left and right pleural cavities. The same feeding tube was used to recover the physiological saline solution from the pleural cavities. The recovery rate was about 90%. The lungs were then extracted and cleaned. The bronchus of the left lung was clamped, while bronchoalveolar lavage was performed on the right lung. A total of 15 mL of physiological saline solution was injected and collected, using two or three operations. A 10% formaldehyde solution was dripped from a water column height of about 25 cm and then stabilized at a specific pressure. The pulmonary tissue could be observed under conditions in which the pulmonary alveoli were naturally spread. Once the fixing solution was injected, the trachea was ligated and impregnated with the fixing solution by immersing it in the solution for 24 h. The recovered BALF was centrifuged for 15 min at 1500 rpm and the supernatant was collected. The cell pellet was again suspended in 1 mL of PBS and the total number of cells was measured using a cell counter.

C.2.5 BALF Examinations

A smear sample was prepared from 100 µL of resuspension using a Cytospin (5 min centrifugation at 1300 rpm) and then Diff-Quik stain was used to determine the cell fractionation. The recovered pleural cleaning solvent was also centrifuged for 15 min at 1500 rpm, after which the supernatant was collected. The cell pellet was again suspended in 1 mL of PBS and the total number of cells was measured using a cell counter. Furthermore, a smear sample was prepared from 100 µL of the resuspension using the Cytospin (5 min centrifugation at 1300 rpm), after which Diff-Quik stains were used to calculate the cell fractionation.

a) Total cell count in BALF

This figure generally increases with particles that have a high pulmonary toxicity. Care shall be taken, however, since the increased number of cells may not be confirmed in some instances when there is extensive cell damage due to the excessive pulmonary toxicity. A high leucocyte ratio is often indicated in such instances, which can be used as a reference.

b) Neutrophil cell count in BALF

This figure generally increases with particles that have a high pulmonary toxicity. Care shall be taken, however, since the number can rapidly increase regardless of the toxicity when the particles have a high level of solubility. The strength of the toxicity is related to the sustained increase in the number of neutrophils and, as such, it shall be observed until the chronic phase (3 to 6 months after injection).

c) Percentage of neutrophils in BALF

This ratio generally increases with particles that have a high pulmonary toxicity. Care shall be taken, however, since the number can rapidly increase regardless of the level of toxicity with particles that have a high solubility. The intensity of the toxicity is related to the sustained increase in the percentage of neutrophils and, as such, it shall be observed until the chronic phase (3 to 6 months after injection).

C.2.6 Pulmonary Pathological Observations

Wash the fixing liquid off those samples fixed for the preparation of pathological tissue samples of the lungs, and then dehydrate and degrease with ethanol or the like. Then, dealcoholize with a mediator (such as xylene, chloroform, or the like) prior to paraffin infiltration. Allow the paraffin to impregnate the sample, and then embed the sample as a block. Place a section of lung tissue, 3 to 5 μm thick and embedded in paraffin, on a microscope slide, use xylene and alcohol to remove the paraffin, and then perform HE staining.

The inflammatory cell infiltration can be confirmed, primarily with the neutrophils and alveolar macrophages in the alveoli region, around the peripheral airway. Care shall be taken, however, since in the same way as with the number of neutrophils in the BALF or percentage of neutrophils, a rapid increase can occur during the acute phase, regardless of the toxicity of those particles that have a high level of solubility and since the degree of the toxicity is related to the sustained inflammation, it shall be observed until the chronic phase (3 to 6 months after injection).

The variations in the inflammation in the lungs according to the method of intratracheal injection, and the evaluation of the hazard presented by the targeted nanomaterials, is regarded as being possible when basic data is established for the approaches by comprehensively judging the observations of the BALF and pulmonary pathology.

C.2.7 Precautions Related to Bronchoalveolar Lavage and Pathological Observations

Inflammation observations related to the intratracheal instillation test are similar in terms of the responses to approaches taken for the intratracheal instillation. This does not indicate similarities in the results for the inhalation exposure tests. Acute reactions tend to occur with the intratracheal instillation tests, relative to comparison with inhalation exposure tests, and the tests are often subject to the effect of non-inhalant particles. This means that determining whether the injected particles in a suspension can be inhaled when conducting an intratracheal instillation test, and then performing injection and evaluation during the chronic phase with the physiological instillation dose, would make it possible to reflect the findings on the results of the inhalation

exposure tests.

Among the three approaches, the use of the spray tube results in the confirmation of nanomaterials that have arrived in the pleural cavity starting one day after injection. The possibility of nanomaterials being able to penetrate the pleural cavity if the positive pressure of the spray were high enough cannot be denied. The reactions in the pleural cavity shall be reexamined, since there is the potential for variance depending on the selected approach.

C.3 Results

C.3.1 Percentage of neutrophils in BALF

The percentage of neutrophils in the BALF increased one day after the instillation using all three approaches. While it was significantly higher than the positive control three days after the instillation, there was a declining trend compared to one day after the instillation. The percentage of neutrophils for all three approaches indicated similar trends and therefore did not reveal any clear difference between these approaches.

C.3.2 Physiological Observations of Lungs

The physiological observation of the lungs using the three approaches, one day after the instillation, revealed that the inflammatory cell infiltration can be confirmed, primarily from the neutrophils and alveolar macrophages. There was also a slight hypertrophy with the peripheral interstitial tissues. A similar trend was indicated three days after the instillation. Regarding the distribution in the lungs, rats that were injected with MWCNTs through an ordinary feeding tube with the board set in a vertical position did not have any carbon nanomaterials in the vicinity of the pleural cavity, but there was a uniform distribution both in the upper and lower lungs. Rats that were injected with MWCNTs using a spray tube, with the board oriented either vertically or at 45°, indicated that MWCNTs reached only the alveoli in the direction of the injection, and reached the pleural cavity one day after instillation.

The total cell count in the BALF obtained from the rats that had MWCNTs injected through an ordinary feeding tube with the board oriented vertically, as well as those rats that had MWCNTs injected with a spray tube with the board oriented either vertically or at 45°, exhibited an increase one day after the instillation. A declining trend was observed with all three approaches, three days after the instillation. This means that the total number of cells exhibited an increasing pattern in all three cases, and no clear difference was observed between the approaches.

C.4 Conclusion

No difference in the inflammation cells in the alveolar space was confirmed with those rats that were administered nanomaterials through an ordinary feeding tube while the board was oriented vertically, or with those rats that were administered with nanomaterials using a spray tube with the board oriented either vertically or at 45°. This means that no difference was confirmed with regards to the inflammation reactions in the lungs, regardless of the technique used.

C.5 References

Morimoto Y, Izumi H, Yoshiura Y, Fujisawa Y, Fujita K. Significance of intratracheael instillation tests for the

screening of pulmonary toxicity of nanomaterials. J UOEH 2017, 39(2): 123-132.
Morimoto Y, Izumi H, Yoshiura Y, Fujisawa Y, Yatera K, Fujita K, Maru J, Endoh S, Honda K. Basic study of intratracheal instillation study of nanomaterials for the estimation of the hazards of nanomaterials. (Submitted)

Appendix D

Practical Example: Intratracheal Instillation of SWCNTs and MWCNTs Into Rats

D.1 Outline

This section describes an example of a rat intratracheal instillation test using an instillation liquid of SWCNTs and MWCNTs as test materials. The instillation liquid of SWCNTs and MWCNTs (concentration: low dosage of 0.08 mg mL; high dosage of 0.8 mg mL) was intratracheally administered to rats. A BALF examination, PCLF examination, physiological examination of the lungs, gene expression analysis of the lungs, as well as TEM observations of the mediastinal lymph node, were conducted up to the 90th day after the instillation (Fujita et al., 2016).

D.2 Test methods

D.2.1 Samples

The SWCNTs and MWCNTs suspended in liquid based on BSA or the like were used as samples. The instillation liquid was prepared according to the method described in Chapter III "Preparation of Samples for Carbon Nanomaterials" of this document. The results of the respective characterizations are listed in Table D.1. A BSA solution of the same concentration, prepared with carbon nanomaterials, was used for the Vehicle control group of intratracheal instillation.

Table D.1 Characterization of intratracheal instillation liquid

Sample	CNT concentration (mg/mL)	G/D ratio	Fe (μg/mL)	Particle size (nm)	Length (nm)	Tube diameter (nm)
SWCNT Low-dose (0.15 mg/kg)	0.08	NT	NT	NT	NT	1.7-2.1
SWCNT High-dose (1.5 mg/kg)	0.81	10.7	49	557	0.5	NT
MWCNT Low-dose (0.15 mg/kg)	0.08	NT	NT	NT	NT	60-100
MWCNT High-dose (1.5 mg/kg)	0.84	8.8	3.9	622	1.81	NT

NT: not tested

D.2.2 Animals

Wistar male rats, 9 weeks old, were used as the animal test subjects. The temperature inside the facility was 22.5 ± 0.6 °C and the humidity was 45.5–65.9%. The lighting was controlled by a timer, with the light and dark cycles

set to a 12:12 ratio.

D.2.3 Intratracheal Instillation

The instillation of the test materials was performed rapidly after anesthetization with Isoflurane, using disposable syringes and feeding tubes for the intratracheal instillation. The dose was set to 0.4 mL per animal (low dose: Approx. 0.15 mg/kg; high dose: Approx. 1.5 mg/kg). The suspension was verified on the day of the intratracheal instillation, immediately before instillation, to ensure that there were no aggregates and ultrasonic treatment was conducted for a few minutes immediately prior to the instillation. Furthermore, the solution was agitated by rotating to prevent bubbling at the time the instillation liquid was collected.

D.2.4 Intratracheal Instillation

The animals were kept for 90 days after the intratracheal instillation, and autopsies were conducted 1 day, 3 days, 7 days, 30 days, and 90 days after the instillation, involving BLF examination, PCLF examination, a physiological examination of the lungs, a gene expression analysis of the lungs, as well as TEM observations of the mediastinal lymph node.

D.3 Results

D.2.5 BALF Examinations

The inflammation reaction caused by the contamination was assumed to be caused by an increase in the number of nucleated cells in the leucocytes and macrophages of the low-dose group of SWCNTs, as well in as the high-dose group. There was an increasing trend with the acidocyte, as well as the number of lymphoid corpuscles and macrophages from 30 days after the instillation, which suggested the potential of a delayed immune response. A significant elevation in comparison with the vehicle control group with the addition of MIP-1a, MCP-1, IL-18, and SPP-1 was confirmed with the high-dose group. No significant elevation in the total number of nucleated cells or the leucocytes was confirmed in either the low-dose or high-dose groups of the MWCNTs, which suggested the possibility of a lymphocyte response immune reaction. A significant elevation, relative to the Vehicle control group, of the SPP-1 was confirmed with the high-dose group of MWCNTs; however, no significant elevation was confirmed with the MIP-1a or the MCP-1.

D.3.2 PCLF Examinations

A significant elevation, relative to the Vehicle control group, in the total number of nucleated cells in the PCLF, as well as in the number of lymphocytes or leucocytes was not confirmed with either the low-dose group or the high-dose group of SWCNTs. However, no significant elevation in the MIP-1a or the MCP-1 was confirmed. A significant elevation, in comparison with the vehicle control group, in the total number of nucleated cells, as well as the lymphocytes, leucocytes, or macrophages was confirmed with both the low-dose and high-dose groups of MWCNTs. No significant elevation in the total proteins, IL-18, or SPP-1 was confirmed with the high-dose group of SWCNTs. A significant elevation in these was confirmed with the high-dose group of MWCNTs.

D.3.3 Pathological Examinations of Lungs

A change in the local inflammation (inflammatory cell infiltration in alveoli, fibrosis and hyperplasia of surrounding alveoli walls, and the like), as well as neutrophilic infiltration in the alveoli and eosinophil infiltration in the surroundings of the blood vessels, foaming macrophages, and granuloma were confirmed from

7 days after the instillation, with the high-dose group of SWCNTs not exhibiting full dissipation until 90 days after the instillation. Furthermore, foaming macrophages were observed from 30 days after the instillation. No alveolitis (localized inflammation) or infiltration of the leucocytes into the alveoli was confirmed until 3 days after the instillation with the high-dose group of MWCNTs. Foaming macrophages were observed from 7 days after instillation, but no granuloma was seen.

D3.4 Gene Expression Analysis of Lungs

High levels of various genetic expressions relating to inflammatory reactions (Ccl2, Ccl7, Ccl9, and Ccl12) occurred, in addition to matrix metalloproteinase (Mmp7, Mmp12, and Spp1) after instillation. Their increases with time were confirmed with the high-dose group of SWCNTs. High levels of such genetic expressions occurred with the high-dose group of MWCNTs up to 3 days after the instillation. However, the level gradually declined thereafter.

D.3.5 Electron Microscope Observation of Mediastinal Lymph Node

The localized presence of mediastinal lymph nodes was confirmed on the 90th day after instillation with the high-dose group of SWCNTs, as well as with the MWCNTs.

D.4 Conclusion

SWCNTs and MWCNTs, prepared with the same weight concentration and of about the same short length, were administered intratracheally to rats. A BLF examination, PCLF examination, physiological examination, gene expression analysis of the lungs, as well as TEM observations of the mediastinal lymph node were conducted up to the 90th day after the instillation. The results confirmed an accentuation of the inflammation in the lungs due to chronological contamination reactions following the instillation of the SWCNTs. The inflammation in the pleural cavity can be considered small. On the other hand, inflammation of the lungs was confirmed with MWCNTs immediately after instillation but a gradual chronological recovery was also confirmed. Furthermore, the accentuation of the inflammation was confirmed in the pleural cavity. SWCNTs and MWCNTs, of shorter lengths, are assumed to give rise to inhalation effects due to the effects of a different mechanism. The SWCNTs and MWCNTs were found to be present in the mediastinal lymph node after the instillation, suggesting that they migrate to other organs from the lungs via the lymphatic system.

D.5 Reference

Fujita K, Fukuda M, Endoh S, Maru J, Kato H, Nakamura A, Shinohara N, Uchino K, Honda K. Pulmonary and pleural inflammation after intratracheal instillation of short single-walled and multi-walled carbon nanotubes. Toxicol Lett. 2016, 257(22):23-37.

Appendix E

Practical Example: Intratracheal Instillation of Semiconducting and Metallic SWCNTs, and MWCNTs Using Rats

E.1 Outline

The project consigned by NEDO, entitled "Innovative carbon nanotubes composite materials project toward achieving a low-carbon society" (P10024) was conducted to perform research and development on the topic of "Technology for Efficient Separation of Metallic and Semiconducting Metallic SWCNTs". This is intended to establish leading-edge materials technology by developing a technology for separating semiconducting and metallic SWCNTs with a high degree of purity and with a great yield, while doing so at low cost and in large volumes, based on the gel column chromatography separation method. This is a completely new separation method for SWCNTs that was developed by AIST. This makes it possible to use pure semiconducting SWCNTs as a raw material for transistors and sensors, while metallic SWCNTs can be used for transparent conductive films. Significant energy and resource savings are expected, particularly through applications to printable electronics.

The amount of available information relating to the hazards associated with semiconducting- and metallic-SWCNTs is very limited at present (Wang et al., 2016). Verifying the differences in the hazards presented by semiconducting and metallic SWCNTs shall be done at an early stage in response to the demands of society. Furthermore, doing so will encourage innovation by business operators such as manufacturers developing products, so that application to industrial use can proceed with peace of mind.

This section describes a case example of a rat intratracheal instillation test using an instillation liquid containing semiconducting and metallic SWCNTs. The instillation liquid of the semiconducting and metallic SWCNTs and MWCNTs (concentration: 1.0 mg mL) was intratracheally administered to rats. A BALF examination, PCLF examination, physiological examination of the lungs, gene expression analysis of the lungs, as well as TEM observations of the mediastinal lymph node were conducted up to the 180th day after the instillation.

E.2 Test methods

E.2.1 Samples

Semiconducting and metallic SWCNTs suspended in a liquid based on BSA were used as samples. The instillation liquid was prepared according to the method described in Chapter III "Preparation of Samples for Carbon Nanomaterials" of this document. The respective characterizations are listed in Table E.1. A BSA solution with same concentration as that used to prepare the CNTs was used for the vehicle control group of intratracheal instillation.

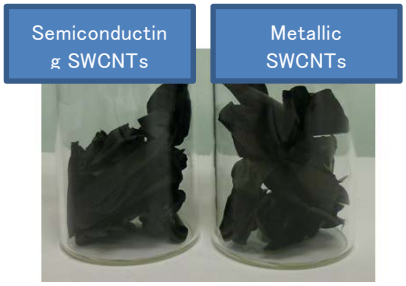


Figure E.1 Semiconducting and metallic SWCNTs prior to preparation

Table E.1 Characterization of intratracheal instillation liquid

Sample	CNT concentration (mg/mL)	Fe (μg/mL)	Particle size (nm)	Length (nm)	Tube diameter (nm)
Semiconducting SWCNTs	1.0	33	361	0.547	NT
Metallic SWCNTs	1.0	44	533	0.927	1.7-2.1

NT: not tested

E.2.2 Animals

Wistar male rats, nine weeks old, were used as the animal test subjects. The temperature inside the facility was 22.5 ± 0.6 ℃ and the humidity was 45.5–65.9%. The lighting was controlled by a timer, with the light and dark cycle set to a 12:12 ratio.

E.2.3 Intratracheal Instillation

The test materials were instilled rapidly after the animals were anesthetized with Isoflurane, using disposable syringes and feeding tubes for the intratracheal instillation. The dose was set to 0.4 mL per animal (Approx. 1.8 mg/kg). The suspension was verified on the day of the intratracheal instillation, immediately before instillation, to ensure that there were no aggregates. Ultrasonic treatment was conducted for a few minutes immediately prior to the instillation. Furthermore, the solution was agitated by rotating to prevent bubbling at the time the instillation liquid was collected.

E.2.4 Intratracheal Instillation

The animals were kept for 180 days after the intratracheal instillation and autopsies were conducted after 1 day, 3 days, 7 days, 30 days, 90 days, and 180 days after the instillation. The autopsy involved a BLF examination, PCLF examination, physiological examination of the lungs, gene expression analysis of the lungs, as well as TEM observations of the mediastinal lymph node.

E.3 Results

E.3.1 BALF Examinations

A chronological increase in the total protein was confirmed with the semiconducting and metallic SWCNT groups,

relative to the vehicle control group. Furthermore, high values were indicated for the inflammatory cytokine for both instillation groups. A sustained effect is believed to occur up to 180 days after the instillation, based on the results described above.

E.3.2 PCLF Examinations

The leucocyte ratio was found to increase slightly, 1 day after the instillation, relative to other examination days with the negative control group, as well as the semiconducting and metallic SWCNT instillation groups. High values were confirmed for the number of macrophages from 1 day to 30 days after the instillation with the semiconducting and metallic SWCNT instillation groups, as a result of a comparison with the negative control group.

E.3.3 Pathological Examinations of Lungs

A change in the local inflammation (inflammatory cell infiltration in alveoli, fibrosis and hyperplasia of surrounding alveoli walls and the like), as well as neutrophilic infiltration in the alveoli and eosinophil infiltration in the surroundings of the blood vessels, an increased number of alveoli macrophages, aggregation of foaming macrophages and cell debris, as well as test-substance-devouring macrophages were observed in all the semiconducting and metallic SWCNT instillation groups. A change in the local inflammation was confirmed in all cases on the day after instillation and the extent intensified chronologically. Intra-alveolar neutrophilic infiltration, as well as perivascular neutrophilic infiltration, was confirmed in all cases in the early phases following the instillation, and between 1 and 7 days after the instillation. All instances of the test-subject-devouring macrophages were observed upon autopsy from 3 days after the instillation. Practically all instances of the increase in the number of alveoli macrophages occurred from 7 days after the instillation, while aggregation of the foaming macrophages and cell debris were observed 30 days after the instillation.

E3.4 Gene Expression Analysis of Lungs

High levels of genetic expressions relating to inflammatory reactions (Ccl2, Ccl7, Ccl9, Ccl12, Cxcl2, Cxcl6, Cxcl10, etc.) and matrix metalloproteinase (Mmp7, Mmp12, and Spp1) were confirmed with all the CNT groups. No significant difference in the expression patterns is thought to exist between the semiconducting and metallic SWCNTs.

E.4 Conclusion

Semiconducting SWCNTs and metallic SWCNTs, prepared with the same weight concentration and of about the same length, were administered intratracheally to rats. A BLF examination, PCLF examination, physiological examination, gene expression analysis of the lungs, as well as TEM observations of the mediastinal lymph node were conducted up to the 180th day after the instillation. The results confirmed an accentuation of the inflammation in the lungs due to chronological contamination reactions, similar to the instillation of SWCNTs conducted previously, occurring with semiconducting and metallic SWCNT instillation.

E.5 Reference

Wang X, Mansukhani ND, Guiney LM, Lee JH, Li R, Sun B, Liao YP, Chang CH, Ji Z, Xia T, Hersam MC, Nel AE. Toxicological Profiling of Highly Purified Metallic and Semiconducting Single-Walled Carbon Nanotubes in the Rodent Lung and E. coli., ACS Nano. 2016,10(6):6008-6019.

VII-2. Genotoxicity Test

Introduction

In 2016, MWNT-7 and NT-7K, which are MWCNTs, were distinguished from other CNTs and added to the list of designated substances under the "Guideline for Prevention of Health Impairment Due to Chemical Substances Designated by Minister of Health, Labour and Welfare Base on Stipulations Under Section 3 in Article 28 of the Industrial Safety and Health Law" (the so-called "Carcinogenicity Guidelines"). One of the reasons why these have been determined as presenting a "threat of causing severe health impairments such as cancer and others" is because, according to the carcinogenicity evaluation by the International Agency for Research on Cancer conducted in 2014, MWNT-7 and NT-7K are classified as Class 2B (posing carcinogenic threat) while other CNTs are classified as Class 3 (no classification available for carcinogenicity) (Grosse et al., 2014). The other reason is a research report announced at a review meeting conducted by the Ministry of Health, Labour and Welfare in 2015, which indicated that a portion of rats, kept over two years in an environment with a high concentration of MWNT-7, developed lung cancer (Kasai et al., 2016). There is still insufficient research data on other CNTs, as well as carbon nanomaterials, although a consensus has been formed that dictates that caution is needed with regards to carcinogenicity with certain MWCNTs, although the effect on humans remains uncertain. Various genotoxicity tests have been developed to examine the carcinogenicity of chemical substances. The Organization for Economic Co-operation and Development (OECD) has established safety evaluation test methods that are common internationally and for which many test methods for genotoxicity have been stipulated (http://www.oecd.org/env/ehs/testing/). Each of these genotoxicity tests are implemented using the endpoints of different species. Thus, individual tests are not able to determine all aspects of genotoxicity but rather, several genotoxicity tests are generally combined to detect the genotoxicity of chemical substances (this is referred to as battery testing). This section describes standard battery testing, consisting of a selection including a reverse mutation test using bacteria (Ames test) included in the OECD test guidelines, as well as a test addressing chromosomal aberration with mammalian cultured cells, as well as a genotoxicity test using samples of carbon nanomaterial dispersed in liquid as a test material, by selecting a micronucleus test using mammalia as a genotoxicity test using a dispersion sample of carbon nanomaterials in liquid as a test sample. It should be noted that the reverse mutation test using bacteria is a type of cell culture test but, for convenience in this document, it has been featured in the section that covers animal testing using carbon nanomaterials dispersed in liquid as a test material, in Chapter VII.

1. Scope of Application

The administration liquid of carbon nanomaterials in a dispersed preparation is used to perform the reverse mutation test using bacteria test on chromosomal aberration with mammalian cultured cells, as well as a micronucleus test using mammalia, to evaluate safety.

2. Referenced Standards and Regulations

The reverse mutation test using bacteria complies with OECD Test Guideline 471, the test on chromosomal

aberration with mammalian cultured cell complies with OECD Test Guideline 473, and the micronucleus test using mammalia complies with OECD Test Guideline 474.

3. Terminology, Definitions, and Symbols

3.1 Preparation Liquid

Suspension containing dispersed carbon nanomaterials in a medium. The solution is prepared by diluting to a prescribed concentration and is then used in the genotoxicity tests.

4. Test Methods

4.1 Reverse Mutation Test Using Bacteria (Ames Test)

This test is conducted in compliance with OECD Test Guideline 471 (OECD Guideline for the Testing of Chemicals 471 (21 July 1997: Bacterial Reverse Mutation Test, DOI: 10.1787/9789264071247-en). 11.10.

4.2 Chromosomal Aberration with Mammalian Cultured Cells

The test is conducted in compliance with OECD Test Guideline 473 (OECD Guideline for the Testing of Chemicals 473 (26 Sep. 2014: In Vitro Mammalian Chromosome Aberration Test, DOI: 10.1787/9789264224223-en).

4.3 Micronucleus Test Using Mammalia Mammalian erythrocyte micronucleus test

The test is conducted in compliance with OECD Test Guideline 474 (OECD Guideline for the Testing of Chemicals 474 (26th Sep. 2014: Mammalian Erythrocyte Micronucleus Test, DOI: 10.1787/9789264224292-en).

5. Reports

Reports are made in compliance with the OECD Test Guidelines.

6. References

Grosse Y, Loomis D, Guyton KZ, Lauby-Secretan B, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, Guha N, Scoccianti C, Mattock H, Straif K; International Agency for Research on Cancer Monograph Working Group. Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. Lancet Oncol. 2014 15(13):1427-1428.

Kasai T, Umeda Y, Ohnishi M, Mine T, Kondo H, Takeuchi T, Matsumoto M, Fukushima S. Lung carcinogenicity of inhaled multi-walled carbon nanotube in rats. Part Fibre Toxicol., 2016 13:54.

Appendix A

Practical Example: Reverse Mutation Test Using Bacteria for Exfoliated Graphene

A.1 Outline

This section describes an example of a reverse mutation test that used Salmonella typhimurium TA100, TA1535, TA98, and TA1537 strains, and *Escherichia coli* (*E. coli*) WP2uvrA strain to examine the genetic mutagenicity of exfoliated graphene developed by the TASC. The test was compliant with OECD Test Guideline 471 and was conducted using the preincubation method that involved both the metabolic activation system non-existent process using rat liver S9 (-S9 treatment) and the metabolic activation system existent process (+9S treatment). Exfoliated graphenes vary in their physiochemical characteristics, depending on the solvent used. For this reason, two different types of solvents were used to perform the dispersion treatment to prepare the stock suspension for the reverse mutation tests.

A.2 Preparation and Characterization of Samples

The exfoliated graphenes, dissolved and dispersed using solvent A0 and solvent B0 and respectively referred to as preparation the stock suspension Grp-A0 and Grp-B0, respectively, were used for the reverse mutation test. The stability evaluation of the stock suspension was performed by conducting measurements on the particle size distributions of exfoliated graphenes over time, by using a laser diffraction particle diameter distribution measurement system (SALD-2300; Shimadzu Co., Kyoto, Japan) up to 72 h after preparation. The results indicated that the particle size distributions for Grp-A0 and Grp-B0 were the same up to 72 h after preparation and both were determined to be stable.

A.3 Test methods

Bacterial reverse mutation test was conducted according to the OECD Guideline for Testing of Chemicals 471 ‘Bacterial Reverse Mutation Test’ (OECD, 1997). The histidine-requiring Salmonella typhimurium strains TA98, TA100, TA1535, and TA1537 and the tryptophan-requiring *E. coli* mutant WP2uvrA were cultured in a nutrient broth at 37 °C with shaking. The test was performed with pre-incubation in the presence and absence of metabolic activation using a rat liver S9 fraction pretreated with the enzyme-inducing agents phenobarbital and 5, 6-benzoflabone and mixed with cofactor-I (Oriental Yeast Co. Ltd., Tokyo, Japan), to give a final concentration of 10% (v/v) S9. In a preliminary cytotoxicity test, all strains were tested at concentrations ranging 0.313, 0.625, 1.25, 2.5, 5.0 and 10.0 μg per plate of exfoliated graphene to determine the appropriate concentration range. The main test was conducted at concentrations ranging 0.625, 1.25, 2.5, 5.0 and 10.0 μg per plate based on the results of the preliminary test. The preliminary and main test were conducted using duplicate plates for each concentration, respectively. The results were judged positive if a 2-fold or more increase above the concurrent negative control in the number of revertant colonies was observed in the exfoliated graphene-treated groups with a dose–response relationship.

A.3.1 Strains

The following five strains were used.

No.	Strain		Mutation type
1	Salmonella typhimurium	TA100	Histidine-requiring base substitution type
2	Salmonella typhimurium	TA1535	Histidine-requiring base substitution type
3	Escherichia coli	WP2uvrA	Tryptophan-requiring base substitution type
4	Salmonella typhimurium	TA98	Histidine-requiring frame shift type
5	Salmonella typhimurium	TA1537	Histidine-requiring frame shift type

A.3.2 Positive Control

The following positive control substance solutions, prepared in advance, were used: AF-2, 9-AA, and 2-AA solutions were prepared using dimethyl sulfoxide (DMSO) and a NaN_3 solution was prepared using water for injection. The respective prepared solutions were injected in quantities of 0.5 mL each, then frozen for storage (at -80 °C). Those that were confirmed as being within the period for which the effectiveness has been verified were used for the test.

Metabolic activation system non-existent: -S9 treatment

No.	Positive control		Concentration (μg/plate)	Strain
1	AF-2	2-(2-furyl)-3-(5-nitro － 2 － furyl) acrylamide	0.01	Salmonella typhimurium: TA100
2	NaN3	Sodium azide	0.5	Salmonella typhimurium: TA1535
3	AF-2	2-(2-furyl)-3-(5-nitro － 2 － furyl) acrylamide	0.01	Escherichia coli: WP2uvrA
4	AF-2	2-(2-furyl)-3-(5-nitro － 2 － furyl) acrylamide	0.1	Salmonella typhimurium: TA98
5	9-AA	9-aminoacridine hydrochloride	80	Salmonella typhimurium: TA1537

Metabolic activation system existent: +S9 treatment

No.	Positive control		Concentration (μg/plate)	Strain
1	2-AA	2-Aminoanthracene	1	Salmonella typhimurium: TA100
2	2-AA	2-Aminoanthracene	2	Salmonella typhimurium: TA1535
3	2-AA	2-Aminoanthracene	10	Escherichia coli: WP2uvrA
4	2-AA	2-Aminoanthracene	0.5	Salmonella typhimurium: TA98
5	2-AA	2-Aminoanthracene	2	Salmonella typhimurium: TA1537

A.4 Results

The maximum dose was set to 10.0 μg/plate and dose setting tests were conducted with six doses of between 0.313 and 10.0 μg. No increase in the reverse mutation colonies of double or more than the negative control group was confirmed with the exfoliated graphene dispersed in solvent A0 and solvent B0 for both the -S9 and +S9 treatments. The test was performed with five doses between 0.625 and 10.0 μg/plate for all strains, based on this result. No increase in the reverse mutation colonies to double or more of that of the negative control group was confirmed with the exfoliated graphene dispersed in solvent A0 or solvent B0 for both the -S9 and +S9 treatments as a result of the test. This confirmed the reproducibility of the test results. The positive control substances indicated clear mutation-inducing effects on the respective test strains. Exfoliated graphene dispersed in solution A0 and solution B0 (Grp-0A and Grp-B0) was determined not to indicate any genetic mutation inductibility (negative) for bacteria under the applicable test conditions, based on the results described above.

A.5 Reference

OECD guidelines for the testing of chemicals. Test No. 471: Bacterial reverse mutation test. 1997. (adopted on July 21, 1997). DOI: 10.1787/9789264071247-en

Appendix B

Practical Example: Mammalian erythrocyte micronucleus test of exfoliated graphene

B.1 Outline

Graphene and related materials (GRM) have novel optical and thermal characteristics and are expected to be adopted for industrial applications; however, there are concerns with respect to the safety of carbon nanomaterials. In addition to direct interaction with the cell membrane, the proposed mechanisms of toxicity development include ROS generation and direct action on the DNA, but these remain to be clarified. A report on the genotoxicity of graphene oxide has been published, based on mammalian erythrocyte micronucleus test using mouse bone marrow cells (Liu et al., 2013). ISO/TS 12901-2:2014 is a guideline for managing the risks associated with nano substances as well as aggregates of nano substances measuring 100 nm or greater and occupational exposure to aggregates, by control banding that can be applied even when knowledge pertaining to the toxicity or quantitative exposure estimates are limited or even non-existent. In cases where positive results are found through in vivo genotoxicity tests, the hazard classification (Hazard Group Allocation) becomes HB5 (Hazard band 5: very high_Severe hazard requiring a full hazard assessment by an expert) and the control banding that corresponds to that is CB4 (Control band 4: full containment) or CB5 (Control band 5: full containment and review by a specialist). The recommended environment for use, therefore, becomes extremely difficult to consider. An exfoliated graphene dispersed preparation solution, developed by the Graphene Division at TASC, was used as the test material in compliance with OECD Test Guideline 474 to verify the presence of the chromosomal damage inductibility of the test substance in living organisms, based on the micronucleus inductibility in mouse bone marrow cells. Exfoliated graphenes vary in their physiochemical characteristics, depending on the solvent used. For this reason, two different solvents were used to perform the dispersion treatment for the working solution. These were used to conduct the mammalian erythrocyte micronucleus test.

B.2 Preparation and Characterization of Samples

The exfoliated graphenes dissolved and dispersed using solvents A0 and B0 were respectively referred to as the stock suspension Grp-A0 and Grp-B0 for the mouse red blood cell micronucleus test. A stability evaluation of the exfoliated graphene preparation liquid was performed by conducting measurements of the particle size distributions of exfoliated graphenes over time, by using a laser diffraction particle diameter distribution measurement system (SALD-2300; Shimadzu Co., Kyoto, Japan) up to 72 h after the preparation. The results indicated that the particle size distributions for Grp-A0 and Grp-B0 were the same up to 72 h after preparation and both were determined to be stable.

B.3 Test methods

Mammalian erythrocyte micronucleus test were conducted according to the OECD Guideline for Testing of Chemicals 474 ‘Mammalian Erythrocyte Micronucleus Test’ (OECD, 2014). Eight-week-old Crlj:CD1 (ICR)

mice purchased from Charles River Laboratories, Japan, Inc. (Yokohama, Japan) were divided into 5 experimental groups of six male animals each, and housed within metal cages at a controlled temperature of 23 ℃ (52 ± 10% humidity) and fed a chow diet *ad libitum*. We determined that male mice were used for the experiments because no clear gender difference was observed in the toxic action in a preliminary test. Three group mice were administered intravenously with exfoliated graphene working solution at 0.5, 1.0 and 2.0 mg/kg once a day for five consecutive days, corresponding respectively to the total doses of 2.5, 5.0 and 10 mg/kg. The other two group mice were respectively received 0.4 wt% Tween-20® and cyclophosphamide (50 mg/kg) as the negative and positive controls. Femur bones of mice were excised and the bone marrow flushed into test tubes, and then the micronucleus assay was performed following standard protocols. The frequency of micronucleated poychromatic erythrocytes (MNPCE) was calculated based on observations of 2000 polychromatic erythrocytes (PCE) per animal. The proportion of PCE among all erythrocytes was determined by counting 500 erythrocytes for each mouse. Significant differences in the frequency of MNPCE between the negative control and the other groups were determined using comparison of the Kastenbaum-Bowman test. The proportion of PCE among the total erythrocyte population was analyzed by Dunnett's test. All procedures and animal handling were approved by the Institutional Animal Care and Use Committee of TASC, AIST, and the Public Interest Incorporated Foundation at the BioSafety Research Center (Shizuoka, Japan).

B.4 Results

No statistically significant increase in the emergence frequency of the MNPCE was confirmed in comparison with the respective negative control group in all those groups to which the Grp-A0 and Grp-B0 administration liquids were administered (2.5, 5, and 10 mg/kg). No statistically significant reduction in the ratio of the polychromatic erythrocyte (PCE) was confirmed relative to the negative control groups, for both administration liquid A and administration liquid B. The emergence frequency of the MNPCE for the untreated control group was within the standard value derived from the background data and the MNPCE for the positive control group was confirmed to exhibit a statistically significant increase in comparison with the untreated control group. Thus, the test was deemed to have been conducted appropriately. Exfoliated graphene materials (administration liquid Grp-0A and Grp-B0) were determined not to exhibit any micronucleus induction to mouse bone marrow cells under the applicable test conditions, based on the results described above.

B.5 References

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General Procedure for Safety Tests on Carbon Nanomaterials

Procedures for Sample Preparation, Characterization, In Vitro Cell-Based Assays and Animal Tests on Carbon Nanomaterials

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