GUIDELINE FOR HAZARD ASSESSMENT TESTING OF INDUSTRIAL NANOMATERIALS IN ANIMALS BY RESPIRATORY EXPOSURE

INTRODUCTION

The results of hazard assessments of industrial nanomaterials can vary from institution to institution. This is probably partly because of differences in the physicochemical properties of the samples administered to test animals. In particular, the nanoparticles constituting industrial nanomaterials have extremely high aggregability; even if dispersed, these nanoparticles are easily prone to massive clumping, producing qualitative and quantitative differences in biologic responses in the exposed body. Therefore, for accurate evaluation of physicochemical properties in the hazard assessment of industrial nanomaterials, it is important that animal exposure tests are conducted with samples in which the dispersion remains stable.

Respiratory exposure tests of animals consist mainly of inhalation exposure tests and intratracheal instillation tests. This guideline defines inhalation exposure test methods, including the procedures required to secure the stability of dispersion of samples, as well as intratracheal instillation test methods, which can be conducted more easily and cheaply than inhalation exposure tests.

1. OUTLINE OF THE TESTS

A suspension of industrial nanoparticles serving as the test article is aerosolized, and then either inhaled by the test animal for 4 weeks or instilled into the trachea of the test animal. The biological effect endpoints, which can be extrapolated to human biological responses ranging from acute to subchronic, are then measured and assessed.

2. Methods of Exposure

2.1. Four-week Inhalation Exposure

2.1.1 Objective

This is a feasible and highly reliable hazard assessment test method for industrial nanomaterials in which the dispersibility of nanoparticles that readily aggregate is secured and respiratory exposure is reproduced. The development of this method was based on existing methods for hazard assessment testing of common chemicals by inhalation exposure in test animals.

The purpose of this test method is to obtain results for industrial nanomaterials that can be extrapolated to human biological responses ranging from acute to subchronic. By performing 4-week animal inhalation exposure, endpoints of biological effects relevant to such results are measured.

2.1.2 Test Method

A suspension of the test article, which is an industrial nanomaterial, is dispersed in air by the spray-dispersion method, and the test animals are exposed to the dispersion by inhalation. First, the test article in dry powder is dispersed into water to produce a sample suspension, which is subjected to pressurized nebulisation to generate aerosols.

Besides the aforementioned wet dispersion method, the dry dispersion method, in which the test article is directly dispersed, is another method for dispersing fine particles in air. The advantages of the wet dispersion method are that 1) aerosols can be dispersed at the nano-size level; and 2) because the test article is in the liquid state until immediately before exposure, the risk of accidental exposure of laboratory workers is minimized. The disadvantage of the wet dispersion method is that, unlike with the dry dispersion method, mass concentrations cannot be increased. However, use of the dry dispersion method distributes large numbers of micro-sized aggregated particles.

The following is a description of the methods and items to be recorded in regard to the test article and test animals and the experimental setup for inhalation, exposure and measurement.

(1) Test Article

The items listed below for assessing the dry powder test article, sample suspension and aerosol should be measured as accurately as possible. The aerosol to be inhaled should be periodically measured during the

exposure period, and the measurement methods, conditions and results should be recorded.

(i) Dry powder test article

Chemical composition, including particle size distribution, shape and morphology; crystallinity; specific surface area; and impurities

(ii) Sample suspension

Particle size distribution, shape and morphology; zeta potential; type and concentration of particle dispersant; type and composition/purity of the vehicle; dispersion stability of the suspension; and endotoxin level of the suspension

(iii) Aerosols to be inhaled

Particle size distribution, shape and morphology; shape measurements such as particle aspect ratio; and mass and number concentrations and stabilities

(2) Test Animals

Male Wistar specific pathogen-free (SPF) rats, aged 8 to 10 weeks at the start of the experiment, should be used as test animals.

(i) Before inhalation exposure

Animals should be randomly divided into three groups: a low-dose exposure group, a high-dose exposure group and a negative control group. The mean weight for each group should be almost the same; the variability in weight in each group should be within ±20%. Animals should be kept in the exposure environment for at least 5 days before the start of the test to allow for acclimatization to laboratory conditions. Animals should be fed a diet for rats — preferably a low-fat feed in compliance with Good Laboratory Practices — and allowed ad libitum access to the feed and to water.

(ii) During inhalation exposure

The cage containing the rats should be placed in the exposure Chamber. Each group is subjected to inhalation exposure for 6 h a day on a 5-day-a-week basis for a period of 4 weeks. Feed and water should be withheld during exposure but allowed ad libitum after the daily exposure period.

(iii) During the observation period after the end of exposure

Rats should be maintained at a temperature of 20±5 °C and a

humidity of 50%±20% and allowed at libitum access to feed and water. Each rat given the test article should be visually checked at least once every other day for any systemic change. Body weight should be measured at least once every week. When a significant difference is observed in body weight between the negative control group and the exposure groups, pair feeding should be performed to match weights.

Rats that die during the observation period should be dissected as soon as possible. An effort should be made to determine the cause of death through both histopathological and macroscopic observation.

Moribund rats should be euthanased if they are unlikely to recover. If any macroscopic change is observed in general status or in individual organs, it should be recorded and histopathologically examined.

(iv) Dissection

Dissection is performed on rats selected at random from each group 3 days, 1 month (4 weeks) and 3 months (12 weeks) after the end of exposure. Preferably, at least five animals should be dissected at each time point.

(3) Inhalation Exposure System

The inhalation exposure system consists of an aerosol generator, an exposure chamber, and an aerosol-measuring system. The Figure schematically shows the experimental setup of the inhalation exposure system.

排気処理装置へ　Exhaust
切替弁　Valve
清浄空気　Clean air

エアロゾル発生装置 Pressurized nebuliser
懸濁液タンク Suspension tank
イオン Ionised air
ヒーター Heater
ドレン Water purge

暴露チャンバー Exposure chamber
排水 Drain
排気処理装置へ Exhaust

インラインエアロゾル解析装置 In-line aerosol analysis system
SMPS または ELPI SMPS or ELPI

オフラインエアロゾル解析装置 Off-line aerosol analysis system
フィルタの質量測定または化学分析 Mass measurement or chemical analysis of the filtrate

SEM 観察用粒子捕集装置 Precipitator for SEM observation
高圧電源 H.V.
静電粒子捕集装置 Electrostatic precipitator

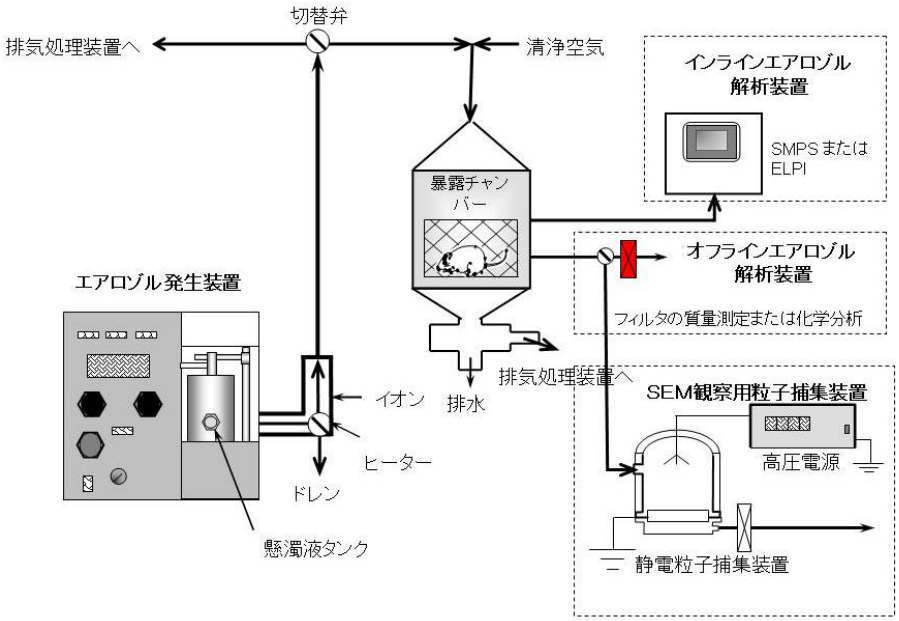

Figure: Schematic diagram of the experimental setup

(i) Pressurised nebuliser

The test article is aerosolized by using an apparatus that nebulises suspensions under pressure and then dries them to generate aerosol particles.

Preferably, the aerosol generator is capable of generating the test aerosol continually for more than 6 h from one sample instillation.

By determining the consumption of suspensions under the operating

conditions specified for the generator, the amount of suspension required during the exposure period should be determined.

The sample suspension should be visually examined in advance to ascertain that the particles are dispersed in it.

In order to ascertain that the dispersant for the suspension has no effect, aerosolized dispersant only should be provided to the negative control group.

(ii) Exposure chamber

Three large whole-body exposure chambers (effective volume about 350 L) capable of accommodating 40 rats are used: one for supply of high-concentration test aerosols; another for low-concentration test aerosols; and the third for the dispersant aerosol given to the negative controls.

Ventilation should be maintained at 20 or more air changes per hour (ventilation flow rate: 120 L/min). The temperature inside the chambers should be maintained at 20±5 °C and the humidity at 50%±20%. The exhaust from the exposure chambers should be cleared of deleterious material by using high-efficiency particulate air (HEPA) filters or activated carbon.

(iii) Aerosol-measuring system

The details of the measuring system are described below in (4). This system consists of a real-time aerosol-measuring system, for confirming particle size distribution etc. and evaluating the stability of concentrations during inhalation exposure, and a filter sampler for measuring the mass concentration of the test article.

(4) Measuring system

Aerosols in the exposure chambers should be measured by the real-time aerosol-measuring system and filter sampler.

(i) Real-time aerosol-measuring system

The particle size distribution of the aerosol is measured several times on the day of exposure with a scanning mobility particle sizer, an electrical low-pressure impactor, etc. It is preferable to perform serial measurements of relative concentration and number concentration during exposure, by using a dust meter and a condensation particle counter.

(ii) Filter sampler

During the exposure period, a fixed volume of air is suction-filtered from the exposure chamber and particles are collected from the filter. The

mass concentration of the sample collected is obtained and chemical analysis is performed. Furthermore, samples for electron microscopic observation are harvested by using the filter or the electrostatic precipitator, and the particle morphology and size distribution are measured.

(5) Operating Conditions for the Exposure System

Exposure should be performed under the operating conditions of the exposure system, which should be defined in advance. The conditions should be confirmed and recorded as the experiment proceeds.

(i) Time

Start time of exposure and end of exposure

(ii) Quality of the air in the exposure chamber during operation

Temperature, humidity, ventilation air volume and internal pressure

(iii) Nebuliser operation

Pressure of supplied air, rate of air flow from the nebuliser, temperature of the heating device used for drying, and consumption of suspensions

(iv) Sampler for measurement of mass concentration

Amount of air to be sampled and times of start and end of sampling

2.2 Administration by Intratracheal Instillation

2.2.1 Objective

Intratracheal instillation is an exposure technique by which a test article can be directly instilled into the lungs via the respiratory tract. It conducted more easily and cheaply than inhalation exposure. Furthermore, because the volume administered can be defined, it is possible to clarify the dose–response relationship between this volume and the response. However, it should be noted that because the test article is delivered by forced injection, great care should be taken to ensure that the particles are evenly distributed in the suspension.

The purpose of this method of testing industrial nanomaterials is to obtain results that can be extrapolated to human biological responses ranging from acute to subchronic. After intratracheal instillation of the test article, the endpoints of the biological effects relevant to such results are measured.

2.2.2 Test Method

(1) Test Article

The items listed below for assessing the dry-powder test article and sample suspension should be measured as accurately as possible.

(i) Dry powder test article

Chemical composition, including particle size distribution, shape and morphology; crystallinity; specific surface area; and impurities

(ii) Sample suspension

Particle size distribution, shape and morphology; zeta potential; pH; type and concentration of particle dispersant; type and composition/purity of vehicle; dispersion stability of suspension; and endotoxin level of suspension

When these physicochemical properties have been measured, if any item is found to be inappropriate for the samples to be instilled, an effort should be made to prepare samples again from the original powder so that all the items adequately fulfil the requirements for samples.

(2) Test Animals

Male Wistar SPF rats aged 8 to 10 weeks at the start of the experiment are used as test animals.

(i) Before the start of instillation

Animals should be randomly allocated so that the mean body weight in each group is almost the same. If possible, it is preferable to have two or more groups receiving different doses of the test article, together with a negative control group receiving vehicle only. The variability in weight in each group should be within ±20%. Animals should be kept in the exposure environment for at least 5 days before the start of the test to allow for acclimatization to laboratory conditions.

(ii) Administration

The test article in the vehicle should preferably be administered at two or more different dosage levels, ranging from low to high doses. The negative control group should receive vehicle only, at the same volume.

Animals should be deeply anesthetized by inhalation of diethyl ether vapour. The mouth is then opened with a laryngoscope and a cannula inserted into the trachea, into which the sample solution contained in the

syringe is instilled as a single dose. All instruments should be sterilized before use.

(iii) Observation period after administration

Animals should be maintained in a sterile cage at a temperature of 20±5 °C and a humidity of ±20%. Each rat given the test article should be visually checked at least once every other day for any systemic change. Body weight should be measured at least once every week. When a significant difference is observed in body weight between the negative control group and the exposure groups, pair feeding should be performed to match weights.

Rats that die during the observation period should be dissected as soon as possible. An effort should be made to determine the cause of death through both histopathological and macroscopic observation.

Moribund rats should be euthanased if they are unlikely to recover. If any macroscopic change is observed in general status or in individual organs, it should be recorded and histopathologically examined.

(iv) Dissection

Dissection is performed in the low-dose exposure group, high-dose exposure group and negative control group 3 days, 1 week, 1 month, 3 months and 6 months after instillation. If possible, perform additional dissections at 12 and 24 months. Preferably, at least five animals should be dissected at each time point.

3. Evaluation Points and Evaluation Method

3.1 Outline of Evaluation

In respiratory tract exposure tests, the hazard assessment of the test article should include a comprehensive analysis of the results of the following:

(i) Definition of a number of endpoints — such as responses that can be extrapolated to human pathological conditions or highly reliable surrogate markers for determining these pathological conditions — among the biologic responses induced by the test article.

(ii) Comparison of each end point with that in the negative control group (or non-exposure group); determination of qualitative and quantitative responses; and determination of the relationships between such

responses and the concentration of the exposure article and the duration of administration, as well as the 'dose quantity–effect relationship'.

(iii) Examination of consistency among end points.

3.2 Evaluation Points

The following points should be evaluated.

(1) Lung tissue

Evaluation should be made from periodic histopathological observations, e.g. for the presence or absence of inflammatory changes, fibrotic change and tumorigenesis.

(2) Bronchoalveolar lavage fluid (BALF)

Cellular and non-cellular components are recovered from BALF. In the cellular component, the number of neutrophils serves as a marker of infiltration of inflammatory cells. In the non-cellular component, the protein concentration is a marker of the permeability of the alveolar–capillary barrier; the level of lactate dehydrogenase release serves as a marker of cytotoxicity; and quantification of cytokine concentrations as inflammatory markers is useful.

(3) Tissues from other organs

Usually, the following organs and tissues are selected for examination for organ weight determination and histopathology; however, fewer, or more, tissues may be examined as appropriate, depending on the results of macroscopic observations etc.

For organ weight determination: lungs, liver, spleen, kidney, testes and brain.

For histopathology: hilar and mediastinal lymph nodes, heart, liver, spleen, kidney, testes and brain.

Animals that die during the administration period should be immediately necropsied. Organ weighing and histopathology, as well as macroscopic observation of organs and tissues, should be performed as necessary to clarify the cause of death and the degree of toxicity at the time of death.

3.3 Examination Process

Examinations should be conducted in the order of lung weight

determination → collection of BALF → fixation of lung tissue→ removal of organs other than the lungs and organ weighing. Details are given below.

(1) Lung weight determination

Both lungs should be removed and their wet weights determined.

(2) Collection of BALF

For BALF collection, after a catheter is inserted into the respiratory tract, the bronchus is clamped with e.g. forceps. Saline from a syringe is instilled into the right lung via the catheter.

When a rat is the test animal, the volume instilled into one lung is about 5 to 10 mL. After the lung is almost fully inflated, free-flowing BALF is collected. For protein analysis of BALF, the supernatant from 15 mL of lavage fluid recovered at the start of collection should be used. For analysis of cell numbers, a total of 50 mL should be recovered for use in subsequent measurements of cell numbers and cell fractionation.3) Fixation of Lung Tissue

After the end of BALF collection, the bronchus is unclamped. Then, the tissue is fixed under constant pressure by pouring in a fixative (10% neutral formalin or 4% paraformaldehyde) via a cannula at a pressure of about 25 cm H_2O. This constant-pressure fixation enables observation of the lung tissue with the alveoli inflated in a natural manner. After instillation of the fixative, the trachea is ligated and fixed for 24 h to allow penetration of the fixative.

It should be noted that the left lung is used for histopathological preparation.

Additionally, for molecular biological examination, BALF is not collected; after the wet weight determination, the tissue is homogenized to extract RNA and protein.

(4) Removal and Weighing of Organs

Each organ is removed and the adhering adipose tissue etc. eliminated. The organ is then weighed. The tissues are then fixed with a fixative such as formalin. For testicular biopsy, Bouin's fixative can be used instead, in which case the fixation time should be 24 h, as with formalin.

3.4 Histopathological Evaluation of the Lung

Fixed specimens are washed to remove the fixative and then dehydrated and defatted with ethanol etc. Subsequently, the alcohol is

removed with a clearing agent (e.g., xylene, chloroform) before paraffin impregnation (melting point: 58 to 60 ℃). Specimens are then embedded in paraffin blocks.

From the paraffin-embedded lung tissue, 3-μm-thick sections are cut and mounted on glass slides. Paraffin sections are deparaffinised with xylene and alcohol and then stained with haematoxylin and eosin for assessment of the degree of inflammation or with Masson's trichrome and/or Elastica van Gieson stain for assessment of fibrosis.

Pathological observations should be described at each observation (dissection) point: inflammatory cell infiltration; collagen deposition in the stroma and pleura; the presence of tumours or hypermorphosis of respiratory epithelial cells and alveolar epithelial cells; emphysematous change, etc.

Inflammatory changes and fibrotic changes can be described by using the point-counting method (see Appendix) for quantitative evaluation.

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APPENDIX

POINT-COUNTING METHOD

1. Example of Quantification of Degree of Inflammation of Lung Tissue

Digital images taken of tissue specimens are analysed by using a light microscope linked to a digital camera and a personal computer. It is desirable to capture at random at least six images of lung specimen stained with hematoxylin and eosin under light microscopy at a magnification of 100 (Appendix Figure 1A)

When taking images, it is desirable to obtain images of only the alveolar region selectively and randomly throughout the lung field by adjusting the field of view so that the air spaces found in e.g. relatively large vessel lumens and bronchial lumens are excluded as far as possible.

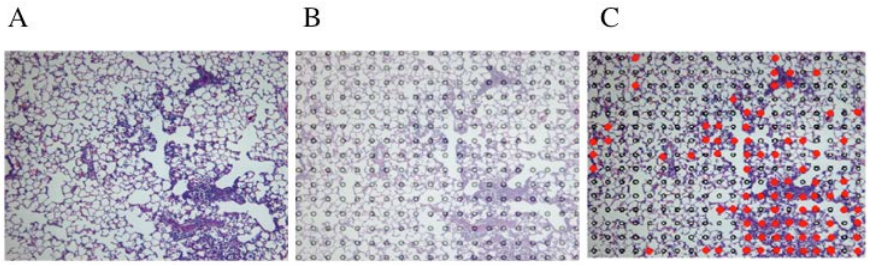
A 300-point grid is placed over each captured image on the computer screen (Appendix Figure 1B), and the number of grid points falling on inflammatory areas is counted (Appendix Figure 1C). Inflammatory areas are exemplified by the following:

(1) Infiltration of inflammatory cells (neutrophils, eosinophils, lymphocytes, monocytes, etc.)

(2) Aggregated foci of alveolar macrophages (including phagocytic macrophages)

(3) Granulomas

The degree of inflammation of lung tissue is defined as the value obtained by dividing the number of points on inflammatory areas by 300, which is the total number of grid points. Scores should preferably be evaluated by more than one assessor at every time point.



Appendix Figure 1 Quantification of the Degree of Inflammation of Lung Tissue (Example)

2. Example of Quantification of Extent of Fibrosis (Collagen Deposition) by the Point-counting Method

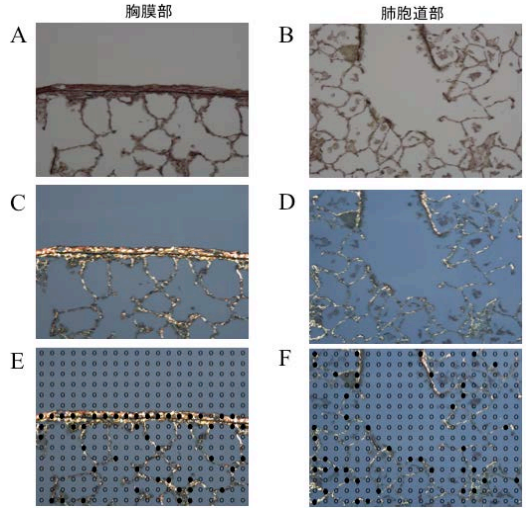
Elastica-van-Gieson-stained lung tissue is divided into a pleural region and an alveolar duct region. It is desirable to take at least two images of each specimen under light microscopy at a magnification of 400 (Appendix Figure 2A and B)

By illuminating each captured region with polarised light under microscopy, images with enhancement of collagen-positive regions are recaptured (Appendix Figure 2C and D).

On the computer screen, a grid is laid on the collagen-enhanced image of each region. The number of grid points falling on the collagen-positive region and pulmonary interstitial region is counted. The total number of grid points is 1200 (Appendix Figure 2E and F).

To evaluate the extent of fibrosis (collagen deposition) in the pleural region and alveolar duct region, the number of points in the collagen-positive region is divided by the number of points in the pulmonary interstitial region. Scores should preferably be evaluated by more than one assessor at every time point.

Pleural Region　　Alveolar Duct Region



Appendix Figure 2 Quantification of Extent of Fibrosis of Lung Tissue by the Point-counting Method (Example)