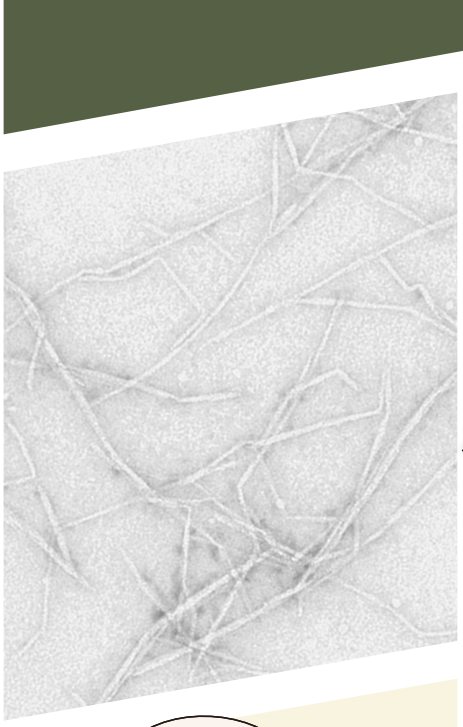
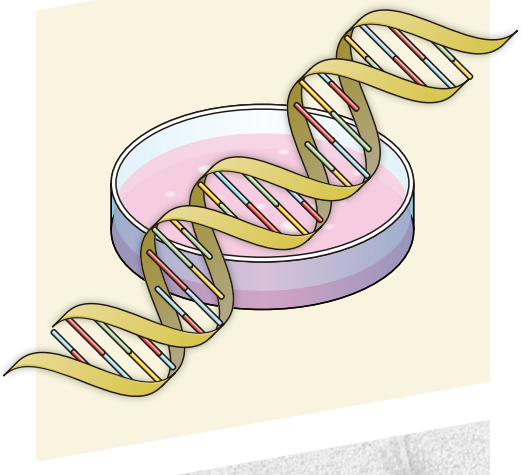
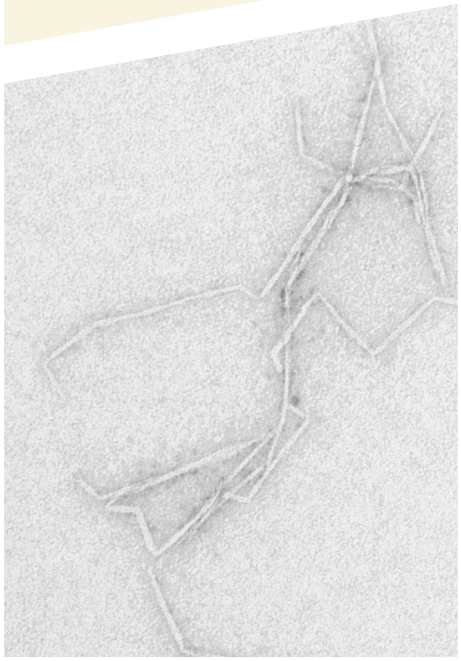
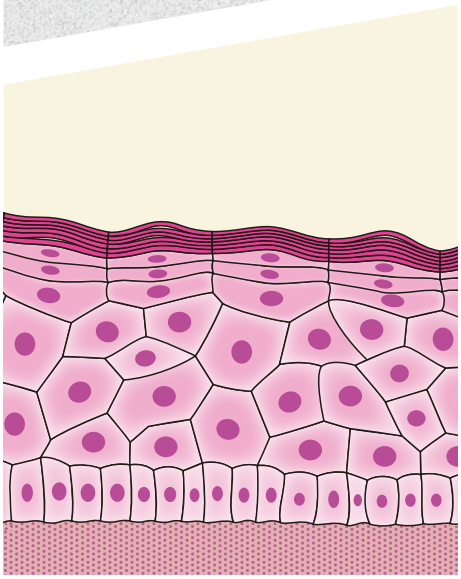
2023

A Manual for Toxicity Testing of Cellulose Nanofibers

Test procedures for sample preparation, characterization, and inhalation effect, transdermal effect, and genotoxicity tests

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This document is based on the results obtained from a project commissioned by the New Energy and Industrial Technology Development Organization (NEDO), “Development of Technologies for Manufacturing Processes of Chemicals Derived from Inedible Plants / Development of Safety Assessment Methods for CNFs (JPNP13006)” and “Cellulose Nanofiber Related Technology Development to Contribute to a Carbon Cycle Society / Development of CNF Use Technology / Development of Hazard Assessment Method and Safety Assessment for Various Product Applications (JPNP20009)”.

PREFACE

In recent years, the development of industrial products composed of cellulose nanofibers (CNFs), also known as cellulose nanofibrils or nanofibrillated cellulose, has been globally increasing. Despite the expectations that this new material will be used for various applications, there have been rising concerns about its potentially toxic effects not being as overtly shown as by other conventional chemical substances, due to its physical characteristics, such as a fibrous structure and ultrafine size. With the rapid technological advance and lagging safety-related knowledge being, it is important for business operators to ensure voluntary safety management as precaution. Plant-derived CNFs are characterized by thixotropic flow property, low thermal stability, and low absorbance; physicochemical characteristics that differ from conventional chemical substances and other manufactured nanomaterials such as carbon nanotubes (CNTs). Thus, it is challenging to handle CNFs using conventional toxicity assessment methods; new methods need to be developed. Furthermore, CNFs are manufactured from a wide variety of wood resources (biomass) and by several procedures such as chemical modification and mechanical defibration, thus their properties are different depending on the raw materials and manufacturing processes. Therefore, when assessing toxicity of CNFs, it is extremely important to characterize their physicochemical characteristics.

To improve the social acceptation of CNFs, the National Institute of Advanced Industrial Science and Technology (AIST), Oji Holdings Corporation, DKS Co. Ltd., and Kyoto University have developed toxicity test methods for CNFs via the New Energy and Industrial Technology Development Organization (NEDO)-commissioned project “Development of Technologies for Manufacturing Processes of Chemicals Derived from Inedible Plants / Development of Safety Assessment Methods for CNFs (JPNP13006)” in FY2017–2019. “A Manual for Toxicity Testing of Cellulose Nanofibers Manual for Toxicity Testing of Cellulose Nanofibers” summarizes the procedures for sample preparation, characterization, and further inhalation, dermal, and genotoxicity testing of CNFs as one of the outcomes of the project. This document also includes some new results obtained from the NEDO project "Development of Cellulose Nanofiber Related Technologies Contributing to a Carbon-Circulating Society / Development of CNF Utilization Technologies / Development of Toxicity Assessment Methods and Safety Assessment for Various Product Applications (JPNP20009)” in FY2020 – 2024 (planned).

The NEDO project also developed CNF detection and quantification methods and CNF discharge and exposure assessment methods (JPNP13006). The outcomes are summarized and published in 2020 as Japanese-language version of documents “Examples of Detection and Quantification of Cellulose Nanofibers” and “Examples of Emission and Exposure Assessments of Cellulose Nanofibers and Their Applied Products”. English-language version of these documents will be published. We would be glad if you would use the present manual as a reference for voluntary safety management of CNFs. These documents are freely available for download on the website of the Research Institute of Science for Safety and Sustainability of AIST (https://riss.aist.go.jp/en/).

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Introduction

As oil consumption expands around the world, we are faced with rising prices, depletion risks, and global warming issues associated with increased CO_2 emissions. In order to overcome the future supply risk of oil resources while realizing a sustainable low-carbon society, it is necessary to switch to various non-petroleum-derived feedstocks such as biomass. Cellulose nanofibers (CNFs) manufactured from inedible biomass are expected to serve as new nanomaterials for versatile applications. On the other hand, there are concerns regarding their impact on human health owing to their ultrafine size, distinct from conventional chemical substances. The "Preventive Measures against Exposure to Nanomaterials," an initiative of the Ministry of Health, Labor and Welfare of Japan, recommends that workers engaged in nanomaterial-related operations to exercise caution to prevent exposure to nanomaterials in accordance with the actual conditions of materials, processes, and the amount handled based on a preventive approach [1)]. To date, a toxicity assessment and appropriate methods for manufacturing nanomaterials, such as titanium dioxide, CNTs, fullerene, and graphene, have been developed [2)-4)]. However, the toxicity of CNF, a new manufactured nanomaterial, is still unclear.

To expedite the practical application and popularization of CNFs, it is important to dispel safety concerns regarding CNFs. The risk of occupational exposure to CNFs is considered high in CNF manufacturing or processing facilities or in consumer products such as sprays containing CNFs. Under exposure to those chemical substances, the first targets are the human respiratory system and skin. Therefore, it is necessary to perform a toxicity assessment of the inhalation and transdermal effects of CNFs as endpoints. Additionally, there is growing concern regarding fibrous manufactured nanomaterials such as asbestos and CNTs causing mesothelioma [4)]. However, few reports have investigated the carcinogenicity and genotoxicity of CNFs, which also have a fibrous structure. Moreover, unlike conventional manufactured nanomaterials, plant-derived CNFs possess specific physicochemical characteristics, such as thixotropy and low thermal stability, low absorbance, *etc*. Therefore, the methods used to test titanium dioxide and CNTs are not suitable, and new test methods for toxicity evaluation must be developed (Figure I).

Accordingly, the NEDO project, "Development of Technologies for Manufacturing Processes of Chemicals Derived from Inedible Plants" (FY2017–2019) considered it a priority to develop test procedures for CNF sample preparation, characterization, and inhalation effect, transdermal effect, and genotoxicity tests. This document summarizes the newly developed test procedures and the evaluation results and findings obtained based on them as the results of the project, and includes some results obtained from the NEDO project "Cellulose Nanofiber Related Technology Development to Contribute to a Carbon Cycle Society / Development of CNF Use Technology / Development of Hazard Assessment Method and Safety Assessment for Various Product Applications" in FY2020–2024 (planned).

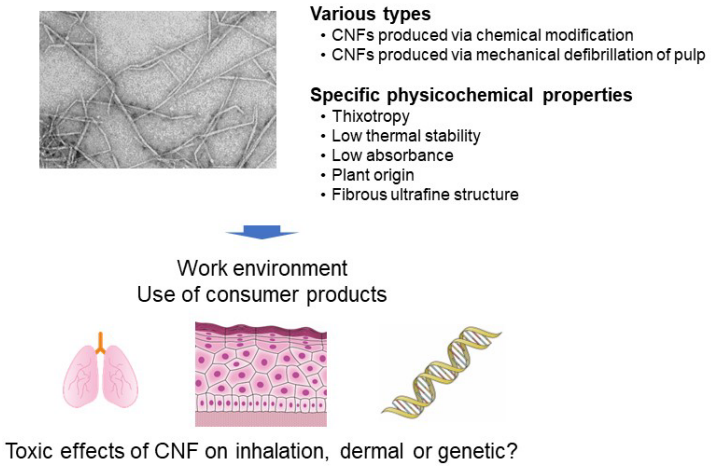

Figure I. Research background

Target readers

This manual is intended for the safety and health management of workers in facilities related to the manufacturing/processing of CNFs, who are most prone to inhalation exposure and transdermal exposure of CNFs. It is expected to result in a human health impact assessment of the respiratory system and skin to assist in the voluntary safety management of business operators handling CNFs. Specifically, this manual focuses on plant-derived CNFs. CNFs have a variety of physicochemical properties owing to their manufacturing technologies, including chemical modification and mechanical defibration. Accordingly, two chemically modified CNFs, i.e., phosphorylated CNFs and TEMPO-oxidized CNFs, and a mechanically defibrated CNFs were selected as representative CNFs.

Structure of the manual

This manual comprises three sections as individual toxicity test items. Chapter 1: “Inhalation Effect Test”, Chapter 2: “Transdermal Effect Test”, and Chapter 3: “Genotoxicity Test ” (Figure II).

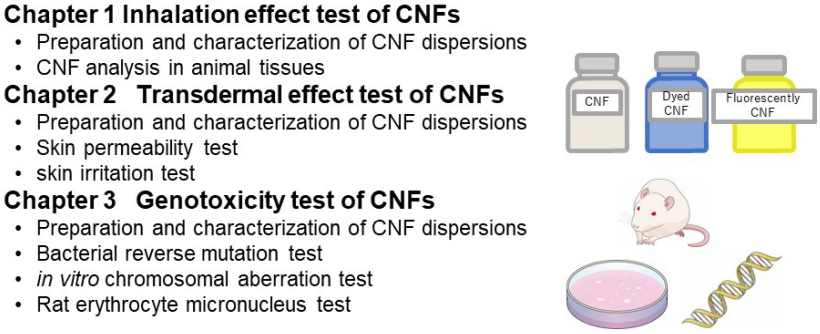

Figure II. Structure of the manual

Overview of each chapter

Chapter 1: “Inhalation Effect Test” describes the preparation and characterization of CNF dispersions, intratracheal instillation test, and CNF analysis in animal tissues. The inhalation exposure test in rodents is available for the toxicity assessment of inhalation effects on the respiratory system. However, it is challenging to perform on all individual test materials as it is costly –time and economic resources–. Alternatively, the intratracheal instillation test, applies diluted chemical test substances directly once or multiple times into rodent’s trachea. Subsequent follow-up examinations can be performed at general animal testing facilities as it requires simple tools and not many test materials. In addition, it can be used for toxicity assessments of many nanomaterials as the dose reaching the lungs can be precisely set. However, there have been few reports on intratracheal instillation studies using CNF as a test material, since CNF is highly viscous in water and requires an appropriate intratracheal instillation method. In the intratracheal instillation test, test materials are mixed in the appropriate solutions and administered into the trachea of rodents. Thus, CNF samples for intratracheal instillation are to be prepared in advance, and a technique for preparing slurry or powder CNFs without affecting their original physicochemical characteristics is required. In addition, as the dose of test materials must be precisely set when assessing the inhalation effects after intratracheal instillation, it is important to determine the CNF concentration in solutions to be administered. Furthermore, it is also critical to select physicochemical characteristics, such as diameter, CNF length distribution, and dispersion viscosity, as parameters associated with inhalation effects and concurrently assess and measure them. Therefore, Subsection 1.1.1, describes the preparation of CNF samples for the intratracheal instillation test and characterization of CNFs in dispersion. The intratracheal instillation test involves direct instillation of CNF dispersions into the trachea of experimental animals via an instillation device and assessment of inhalation effects with subsequent inflammation of pulmonary tissues as index. However, in some cases, the physicochemical characteristics of the administered CNF dispersions may cause suffocation immediately after instillation, and the inhalation effects cannot be assessed appropriately. In addition, due to their thixiotropic flow properties, they may not be uniformly dispersed and administered to the pulmonary tissues of experimental animals. Thus, Subsection 1.1.2, presents a method to confirm the injection state from the instillation device considering the concentration of CNF dispersions. In Subsection 1.1.3, intratracheal instillation tests using dispersions of three types of CNFs (phosphorylated CNFs, TEMPO-oxidized CNFs, and mechanically defibrated CNFs) were administered into rat’s tracheas; the test procedure and results of the BALF test and pulmonary pathological examination up to Day 90 post-instillation are shown. In the intratracheal instillation test, test materials should be uniformly distributed and not localized only to some areas in the lungs after intratracheal instillation. Therefore, as verification of appropriate intratracheal instillation methods, Section 1.2 describes CNF extraction and analysis in each lung lobe following intratracheal instillation of chemically dyed CNFs.

Chapter 2: “Transdermal Effect Test” describes the preparation and characterization of CNF dispersions, the permeability test, and the irritation test. The skin permeability assessment of chemical substances is an important toxicity assessment of transdermal exposure. Currently, to measure the skin permeability

of chemical substances or their concentration in the skin, tests have been conducted using skin from animals such as rats and pigs. However, recently tests using three-dimensional cultured skin models have garnered much attention as an alternative to animal safety tests. As they use human-derived cultured cells, there is no interspecific difference and little variation between lots. Thus, they are advantageous and highly reproducible. In addition, as an alternative to living skin, high-performance artificial synthetic membranes can also be used. Section 2.1 describes a CNF permeability test using high-performance artificial synthetic membranes, and Section 2.2 describes a skin permeability test with 3D cultured skin models using dyed CNFs and fluorescent CNFs. Furthermore, Section 2.3 reports on the results of a CNF skin irritation test using 3D cultured skin models in compliance with the Organization for Economic Co-operation and Development (OECD) TG439.

Chapter 3: "Genotoxicity Test" reports on a CNF genotoxicity test performed according to the methods defined in the OECD Test Guidelines and its usefulness for predicting carcinogenic risks to humans. In this chapter, three tests, namely the bacterial reverse mutation test (commonly known as Ames test), the mouse lymphoma TK assay, the *in vitro* chromosomal aberration test, and the rat erythrocyte micronucleus test, were selected and performed as a battery test.

Note

Please note that the test results in this manual are examples in accordance with the developed test methods and do not guarantee the overall toxicity of CNFs.

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Chapter 1 Inhalation Effect Test

The inhalation exposure test and the intratracheal instillation test have been reported as toxicity assessments of manufactured nanomaterials related to inhalation effects on the respiratory system on rodents, such as rats and mice [1)-3)]. In the intratracheal instillation test, samples are administered once or multiple times directly into the rodents' trachea, with subsequent follow-up observation. Compared to the inhalation exposure test, intratracheal instillation can be performed with simple tools and few test materials. In addition, the dose administered into the lungs can be set precisely; thus, it is utilized in the toxicity assessment of numerous manufactured nanomaterials. However, there are few reports on CNF intratracheal instillation test, and since CNFs dispersions are highly viscous in water, an appropriate intratracheal instillation method needs to be developed [4)]. Furthermore, CNF extraction and analysis methods are required to assess the residual amount and CNF distribution in the lung tissue after intratracheal instillation. Figure 1.1 shows the scheme of CNF inhalation effect test. Section 1.1 describes the confirmation of the injection state of CNF dispersions and the rat intratracheal instillation test, and Section 1.2 CNF analysis in animal tissues.

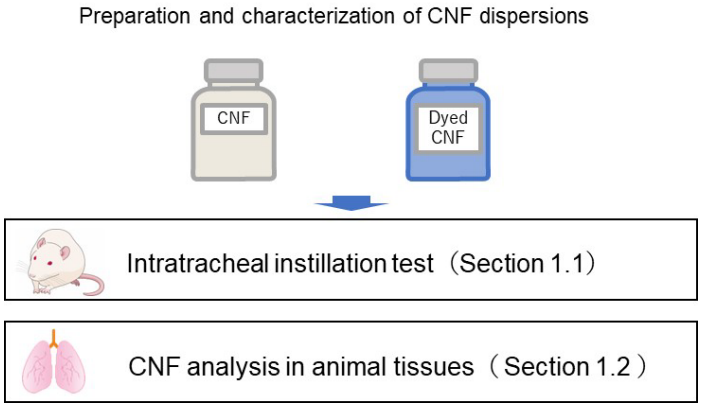

Figure 1.1 Structure of Chapter 1

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1.1 Intratracheal instillation test

The intratracheal instillation test is applied for the toxicity assessment of many manufactured nanomaterials as it can be performed at general animal testing facilities. This section describes the preparation and characterization of CNF dispersions, the confirmation of the injection state of CNF dispersions, and the intratracheal instillation test of CNFs using rats for assessing the inhalation effects of CNF dispersions.

1.1.1 Preparation and characterization of CNF dispersions

CNF dispersions of fixed concentrations for the intratracheal instillation test were prepared by stirring/mixing techniques. In addition, the CNF dispersions were sterilized by adding disinfectants as CNFs are prone to contamination by microorganisms [1]. The prepared CNF dispersions were characterized by microscopic observation, rheological measurement, and pH measurement.

1.1.2 Verification of the injection state of CNF dispersions

The intratracheal instillation test assesses inhalation effects by directly administering CNF dispersions into the trachea of experimental animals via an instillation device; the subsequent inhalation effects are evaluated as an indicator of lung tissue inflammation, among others. However, in some cases, the physicochemical characteristics of the administered CNF dispersions may cause suffocation in experimental animals immediately after instillation, and inhalation effects cannot be assessed appropriately. In addition, highly concentrated CNF dispersions are high viscous and thixotropic, and thus cannot be properly ejected from the test administration device, which may prevent them from being uniformly dispersed into the lung tissue of experimental animals. Therefore, it is necessary to consider the maximum concentration of CNF dispersions that can be intratracheally administered and confirm the injection state from the instillation device in advance.

In this subsection, methods to assess the injection state of samples ejected from the metallic oral probe (hereinafter oral probe) used in the intratracheal instillation test are described using phosphorylated CNF dispersions as test samples. For the intratracheal instillation test, spray probes, which eject instillation solutions in the form of an aerosol, are also available, but oral probes are more easily available and employed at many contract research organizations.

A 20 mg/mL phosphorylated CNF solution prepared with agitation using a vortex mixer, was weighed and sampled with a medicine spoon into a transparent container suitable for agitation, and diluted to 4 mg/mL, 5 mg/mL, and 6 mg/mL by adding distilled water. The prepared phosphorylated CNF dispersions were distributed at 1 mL each to syringes used in the intratracheal instillation test, and an oral probe was attached to each syringe. The syringes were placed on a horizontal stand, and each CNF dispersion was ejected by fully pressing the syringe pump. The distance from the tip of the probe to the tip of the ejected CNF dispersion (i.e., flying distance) was measured and compared with that of distilled water. Figure 1.2 shows a schematic diagram for the measurement of the flying distance of CNF dispersions, and Figure 1.3 the results. The ejection state of the CNF dispersion samples suggested

that the optimal maximum CNF concentration for administration in the intratracheal study was 4.0 mg/mL for the phosphorylated CNF.

To determine the optimal maximum CNF concentration for the intratracheal instillation test, it is extremely important to observe suffocation and behavioral disorders in experimental animals following instillation in a preliminary test. This method is useful for determining the maximum dose and CNF concentration.

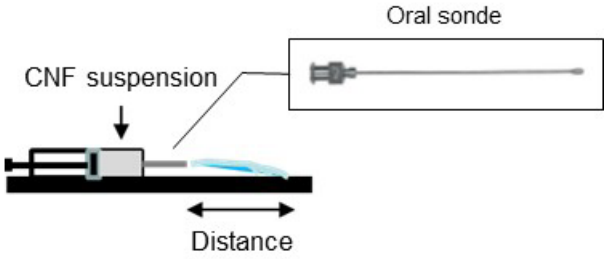

Figure 1.2 Schematic diagram for measuring the flying distance of CNF dispersions

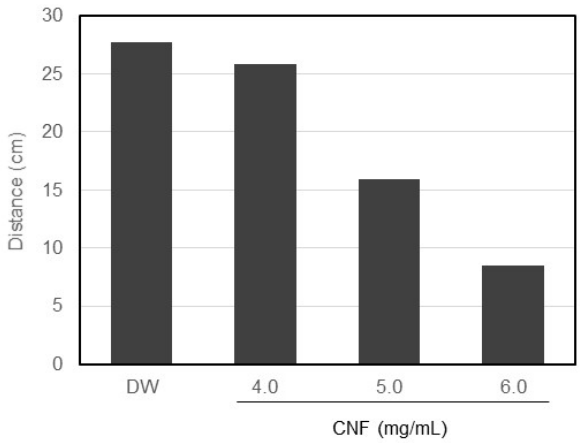

Figure 1.3 Flying distance of phosphorylated CNF dispersions

1.1.3 Rat intratracheal instillation test

An example of rat intratracheal instillation test with dispersions of three types of CNFs (phosphorylated CNF, TEMPO-oxidized CNF, and mechanically defibrated CNF) as test materials is described below. Each CNF dispersion was administered into the trachea of rats at three doses, and the BALF test and pulmonary pathological examination were performed up to Day 90 post-instillation. The procedure and test results are shown below.

Samples

CNF dispersions prepared by dispering three types of CNFs (CNF-A: phosphorylated CNF, CNF-B: TEMPO-oxidized CNF, and CNF-C: mechanically defibrated CNF) in their solvents were used in the rat intratracheal instillation test (concentrations: 0.5 mg/mL, 1.0 mg/mL, and 2.0 mg/mL). Using a

transmission electron microscope (TEM), the distributions of fiber length and diameter of CNF in each dispersion were measured (Table 1.1).

Table 1.1 The distributions of fiber length and diameter in CNF dispersions

	Length			Diameter		
	Arithmetric mean [μm]	Geometric mean [μm]	Geometric standard deviation [−]	Arithmetric mean [nm]	Geometric mean [nm]	Geometric standard deviation [−]
CNF-A	0.98	0.80	1.9	8.8	7.9	1.6
CNF-B	1.23	1.00	1.9	8.3	7.6	1.5
CNF-C	2.14	1.70	2.0	26.7	21.2	2.0

Intratracheal instillation

Seven-week-old male SPF-grade Crl:CD (SD) rats were used as experimental animals. Standard environmental variables at the facilities were maintained at 20°C–26°C for room temperature and 35%–70% for humidity. Illumination was automatically regulated for 12 h of light period followed by 12 h of dark period.

After isoflurane anesthesia, the test materials were promptly administered using disposable syringes and metallic oral probes. The instillation volume was 1.0 mL/kg per animal. Doses were set at three levels as follows: low dose: 0.5 mg/kg, medium dose: 1.0 mg/ kg, and high dose: 2.0 mg/ kg. CNF dispersions were checked to ensure the absence of agglomerates. In addition, immediately before instillation, the dispersions were stirred without foaming.

After intratracheal instillation, i.e., instillation Day 0, rats were raised for 90 days, dissected on Days 1, 3, 7, 30, and 90. The BALF test [nucleated cell, neutrophil, eosinophil, and macrophage, total protein, lactate dehydrogenase (LDH)] and pulmonary pathological examination were subsequently performed.

BALF test

CNF-A: Compared with the negative control, the nucleated cell and macrophage counts were higher or tended to be higher in the 0.5, 1.0, and 2.0 mg/kg instillation groups on Days 3–7, 3–30, and 1–30, respectively. In addition, neutrophil and eosinophil ratios were higher or tended to be higher in the 0.5 mg/kg instillation group on Days 1–7 or 1–30 and in the 1.0 and 2.0 mg/kg instillation groups on Days 1–90. In contrast, the macrophage ratio was lower, and tended to be lower in the 0.5 mg/kg instillation group on Days 1–7 and in the 1.0 and 2.0 mg/kg instillation groups on Days 1–90. Neutrophil and eosinophil counts were higher or tended to be higher in all dose groups on Days 1–7 or 1–30. These variations were dose-dependent. No change was observed in BALF recovery volume, lymphocyte, and basophil ratio/counts. In comparison with the negative control, total protein and LDH were higher or tended to be higher in the 0.5, 1.0, and 2.0 mg/kg instillation groups from Day 1. These increments were dose-dependent, with higher values or a tendency for higher values observed in the 0.5 and 1.0 mg/kg

instillation groups up to Day 7 for total protein and LDH, and in the 2.0 mg/kg instillation group up to Day 90 for total protein, and up to Day 30 for LDH.

CNF-B: Compared with the negative control, nucleated cell and macrophage counts were higher in all dose groups on Days 3–7. In addition, in all dose groups on Days 1–7, neutrophil and eosinophil ratios and counts were higher or tended to be higher, and the macrophage ratio was low. These variations were dose-dependent. No change was observed in BALF recovery volume, lymphocyte, and basophil ratio/counts. Notably, the negative control of CNF-B showed a slightly higher neutrophil ratio on Day 1 than the control groups of the other test materials. Additionally, the BALF recovery volume was lower in the 1.0 mg/kg instillation group on Day 1; this change was dose-independent. Compared with the negative control, total protein and LDH were higher or tended to be higher in the 0.5, 1.0, and 2.0 mg/kg instillation groups from Day 1. These increments were generally dose-dependent, with higher values or a tendency for higher values observed in the 0.5 mg/kg instillation group for total protein and LDH up to Day 7, in the 1.0 mg/kg instillation group for total protein up to Day 30, and for LDH up to Day 7, and in the 2.0 mg/kg instillation group for total protein up to Day 90, and for LDH up to Day 7. In addition, compared to the control groups of the other materials, the negative control of CNF-B showed slightly higher LDH on Day 1.

CNF-C: Compared to the negative control, the nucleated cell count was higher or tended to be higher in all dose groups on Days 1–90, and the macrophage count was higher or tended to be higher in all dose groups on Days 7–90. In addition, the neutrophil ratio and count were higher or tended to be higher in all dose groups on Days 1–3, eosinophil ratio and count were higher or tended to be higher in all dose groups on Days 1–7, the macrophage ratio was lower and tended to be lower in the 0.5 mg/kg instillation group on Days 1–3, and in the 1.0 and 2.0 mg/kg instillation groups on Days 1–7. Furthermore, BALF recovery volume was lower in the 2.0 mg/kg instillation group on Day 1. No change was observed in lymphocytes and basophils. In addition, the macrophage count was higher in the 0.5 mg/kg instillation group on Day 1, but this change was dose-independent. A comparison with the negative control showed that total protein and LDH were higher or tended to be higher in the 0.5, 1.0, and 2.0 mg/kg instillation groups from Day 1. These increments were generally dose-dependent, with both higher values and a tendency for higher values observed up to Day 7. Besides, on Day 90, LDH was lower in the 0.5 mg/kg instillation group, whereas total protein was higher in the 1.0 mg/kg instillation group; these changes were dose-independent.

Histopathological examination

CNF-A: In each dose group, increase of intraalveolar macrophages, and degeneration or necrosis of macrophages were observed. Test substances were deposited in the alveoli or macrophages, and over time, intraalveolar deposition tended to decrease, whereas deposition in macrophages tended to increase. Inflammatory cell infiltration (intraalveolar/alveolar wall, blood vessel/peribronchial), bronchial epithelial hyperplasia, and degeneration/necrosis were observed from Day 1 and type II alveolar epithelial hyperplasia was observed from Day 3, but they were attenuated over time. These findings

were dose-dependent. In addition, intraalveolar hemorrhage was occasionally observed in each dose group. In the negative control, increases in intraalveolar macrophages and inflammatory cell infiltration (intraalveolar/alveolar wall, blood vessel/peribronchial) were observed in a small number of cases.

CNF-B: In each dose group, intraalveolar macrophages increased, and test substances were deposited in the alveoli or macrophages. Over time, intraalveolar deposition tended to decrease, whereas deposition in macrophages tended to increase. In addition, granuloma formation was observed on Day 30. Inflammatory cell infiltration (intraalveolar/alveolar wall, blood vessel/peribronchial), bronchial epithelial hyperplasia, and degeneration/necrosis were observed from Day 1 and type II alveolar epithelial hyperplasia was observed from Day 3, but they diminished over time. These findings were dose-dependent. In addition, intraalveolar hemorrhage was occasionally observed in all groups, including the negative control. In the negative control, increases in intraalveolar macrophages and inflammatory cell infiltration (intraalveolar/alveolar wall, blood vessel/peribronchial) were also observed. Compared to the control groups of the other test materials, the number of findings and frequency of the negative control for CNF-B were slightly higher, with one or three cases of inflammatory cell infiltration (intraalveolar/alveolar wall, blood vessel/peribronchial) detected on Days 1–3, and one case of intraalveolar hemorrhage each on Day 1 and Days 7–90.

CNF-C: In each dose group, intraalveolar macrophages increased, and test substances were deposited in the alveoli or macrophages. Over time, intraalveolar deposition tended to decrease, whereas deposition in macrophages tended to increase. In addition, deposition was observed around the terminal bronchiole. Inflammatory cell infiltration (intraalveolar/alveolar wall, blood vessel/peribronchial), bronchial epithelial hyperplasia, and degeneration/necrosis were observed from Day 1 and type II alveolar epithelial hyperplasia was observed from Day 3, but they decreased over time. These findings were dose-dependent. In addition, intraalveolar hemorrhage was occasionally observed in each dose group. In the negative control, inflammatory cell infiltration (intraalveolar/alveolar wall, blood vessel/peribronchial), bronchial epithelial hyperplasia, and type II alveolar epithelial hyperplasia were observed in a small number of cases.

Summary

Three types of CNFs (phosphorylated, TEMPO-oxidized, and mechanically defibrated CNFs) were intratracheally administered to rats. Subsequently, the BALF test and histopathological examination in the lungs were performed up to Day 90. Phosphorylated and TEMPO-oxidized CNFs appeared to reach inside the alveoli and cause a strong inflammation in the lungs. We hypothesize that with the phosphorylated CNF, the inflammatory response was attenuated as macrophages gradually removed the test substance, while with TEMPO-oxidized CNF, and the inflammatory response was attenuated as macrophages phagocytized intraalveolar test substances and formed granulomas. Most mechanically defibrated CNF deposited at the terminal bronchiole, and intraalveolar inflammatory response weakened. Based on the above results, the inflammatory responses caused by each CNF were attenuated over time even if varying among them [2].

References

1) Sai T, Maru J, Obara S, Endoh S, Kajihara H, Fujita K. Screening of preservatives and evaluation of sterilized cellulose nanofibers for toxicity studies. J Occup Health. 2020; 62:e12176.
2) Fujita K, Obara S, Maru J, Endoh S. Pulmonary inflammation following intratracheal instillation of cellulose nanofibrils in rats: comparison with multi-walled carbon nanotubes. Cellulose 2021; 28: 7143-64.

Contributors

Fujita K, Maru J, and Obara S

1.2 CNF analysis in animal tissues

In the intratracheal instillation test in Section 1.1, it is essential to confirm whether CNFs are uniformly distributed in the lungs. In addition, when investigating CNF safety, it is also important to understand the behavior of CNFs invading from the lungs, in terms of their retention or excretion from living tissues. However, it is extremely challenging to measure and assess colorless and transparent CNFs, which are characterized by their low absorbance in living tissues. Therefore, chemically dyed CNF were developed to quantify CNFs in animal tissues based on its absorbance of the visible range. This section describes the method of administering dyed CNF dispersions into the trachea of experimental animals and measuring CNFs by absorbance after removing the contaminant by enzymatically digesting the living tissues, including pulmonary tissues containing CNFs.

Below is an example where dyed phosphorylated CNFs were administered into rats' tracheas; later those dyed CNFs in the extracted lungs were measured. The dye used was Sumifix® Supra, Navy Blue (Sumika Chemtex Co., Ltd.). The concentration of dyed CNF in the administered dispersions was 2 mg/mL. One day after intratracheal instillation, we analyzed the lungs (five lobes) of male Crl:CD (SD) rats (age, 7 weeks). Target lungs were weighed in advance (mass, *L).* The lungs were placed in a 50 mL vial (Figure 1.4) and cut with scissors.

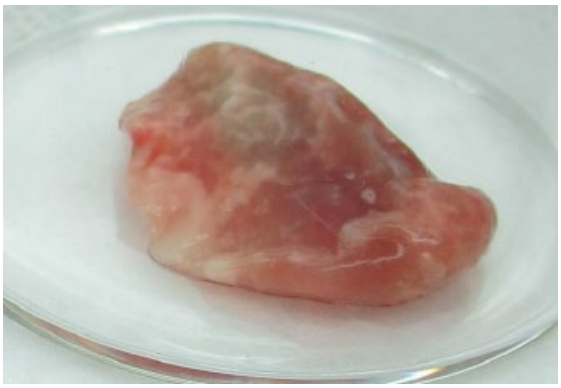

Figure 1.4 Lungs of rats intratracheally administered with dyed CNFs

The amount of 20 mg/mL enzyme added, E, was set according to the lungs' mass, L. A total of 200 mg/mL SDS aqueous solution (FUJIFILM Wako Pure Chemical Corporation) was used as surfactant and Proteinase K (Merck) was used as protease. The enzymatic activity was 600 mAnson-U/mL (20 mg/mL in enzyme mass concentration). The enzyme solution and surfactant were added to the vials corresponding to the lung mass, as follow,

a) 100 mg $\leq L <$ 300 mg: E = 2 mL
b) 300 mg $\leq L <$ 400 mg: E = 3 mL
c) 400 mg $\leq L <$ 500 mg: E = 4 mL
d) 500 mg $\leq L <$ 600 mg: E = 5 mL
e) 600 mg $\leq L <$ 700 mg: E = 6 mL

f) 700 mg ≤L < 800 mg: E = 7 mL

g) 800 mg ≤L < 900 mg: E = 8 mL

h) 900 mg ≤L: E = 9 mL

For 200 mg/mL SDS aqueous solution, D (mL)

$$D = (20/200)\,E \qquad (1.1)$$

The vials were set up in an ultrasonic bath with adjusted water height [BRANSON1510-DTH (70 W, 42 kHz) and BRANSON1800-CPXH (70 W, 40 kHz)] in advance, and sonication was applied for 2–8 h to enzymatically decompose the lungs (Figure 1.5). At this time, the temperature was controlled between 37°C–50°C.

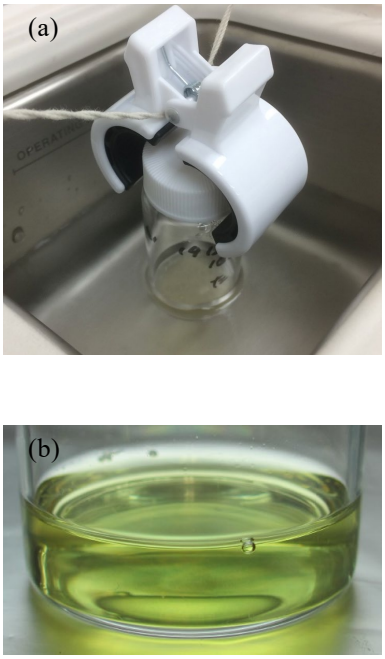

Figure 1.5 Ultrasonic-enzymatic digestion (a) and lung digestion solution (b)

CNF concentration was measured by the absorption spectrum of the digestion solutions. Figure 1.6 shows the absorption spectrum of lung digestion solutions not intratracheally administered (negative control), and that of the instillation groups. No undigested tissue was observed in any digestion solution. The absorption by the dyed CNFs was observed ranging between 610–630 nm wavelength, the characteristic absorption wavelength of the dye. The absorbance for 610–630 nm was very low, approximately 0.01, for the negative control, whereas the absorption was confirmed for the digestion solutions of the instillation groups. The absorption at 390–400 nm could be due to residual blood (hemoglobin's heme complex) in the lungs. No correlation was found between the absorbance at 390–400 nm. Thus, the absorbance at 610–630 nm was used in for CNF content assessment.

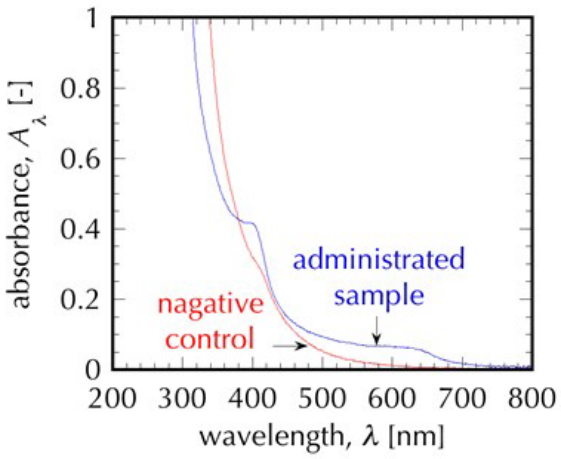

Figure 1.6 Absorption spectra of lung digestion solutions

A standard curve (Figure 1.7) based on the absorbance at the absorption wavelength, 616 nm, most sensitive to the dyed CNFs was generated. Based on it, the dyed CNF concentration in digestion solutions was determined and converted to dyed CNF content in the lungs based on the volume of the lung digestion solutions. For instance, the CNF content in the left lung after instillation was 0.236 mg as shown in Figure 1.6.

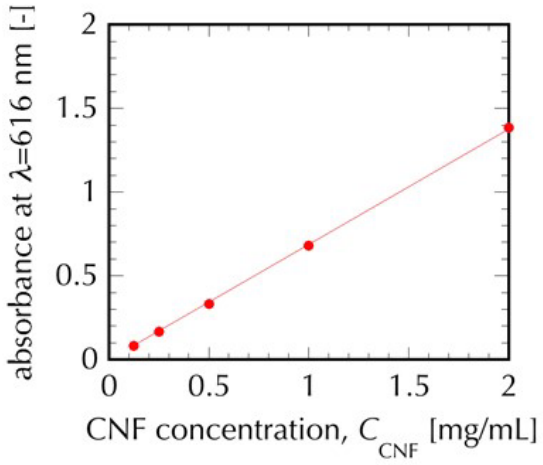

Figure 1.7 Standard curve used in dayed CNF mass concentration assessment

Summary

CNF dispersions chemically dyed in advance were administered to experimental animals. Subsequently, digestion solutions without the influence of contaminants were obtained after extracting living tissues, such as lung tissues, and enzymatically digesting them using sonication on top. A standard curve was generated from the absorbance at the absorption wavelength most sensitive to the dyed CNFs, which served to determine the dyed CNF concentration in the digestion solutions. As a result, the CNF content in the lungs immediately after instillation was almost the same as that administered. This method can be

useful for verifying the uniformity of the CNFs distributed in the lungs of experimental animals after intratracheal instillation.

Contributors

Endoh S, Maru J, and Fujita K

Chapter 2 Transdermal Effect Test

Transdermal exposure management is one of the safety and health management requirements for workers at CNF manufacturing or processing facilities. The skin permeability test and the skin irritation test are considered important for the transdermal toxicity assessments. Currently, to measure the skin permeability of chemical substances or their concentration in the skin, tests have been conducted using skin from animals such as rats and pigs. However, recently tests using three-dimensional cultured skin models have garnered much attention as an alternative to animal safety tests. As they use human-derived cultured cells, there is no interspecific difference and little variation between lots. Thus, they are advantageous and highly reproducible. In addition, as an alternative to living skin, high-performance artificial synthetic membranes can also be used. Section 2.1 describes a CNF permeability test using high-performance artificial synthetic membranes, and Section 2.2, describes a skin permeability test with 3D cultured skin models using dyed CNFs and fluorescent CNFs. Furthermore, Section 2.3, reports on the results of a CNF skin irritation test using 3-dimensional (3D) cultured skin models in compliance with the OECD Test Guideline No. 439, "*In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method," a test method developed for an *in vitro* skin irritation test using 3D cultured skin models consisting of normal human epidermal keratinocytes. Figure 2.1 shows the chapter structure.

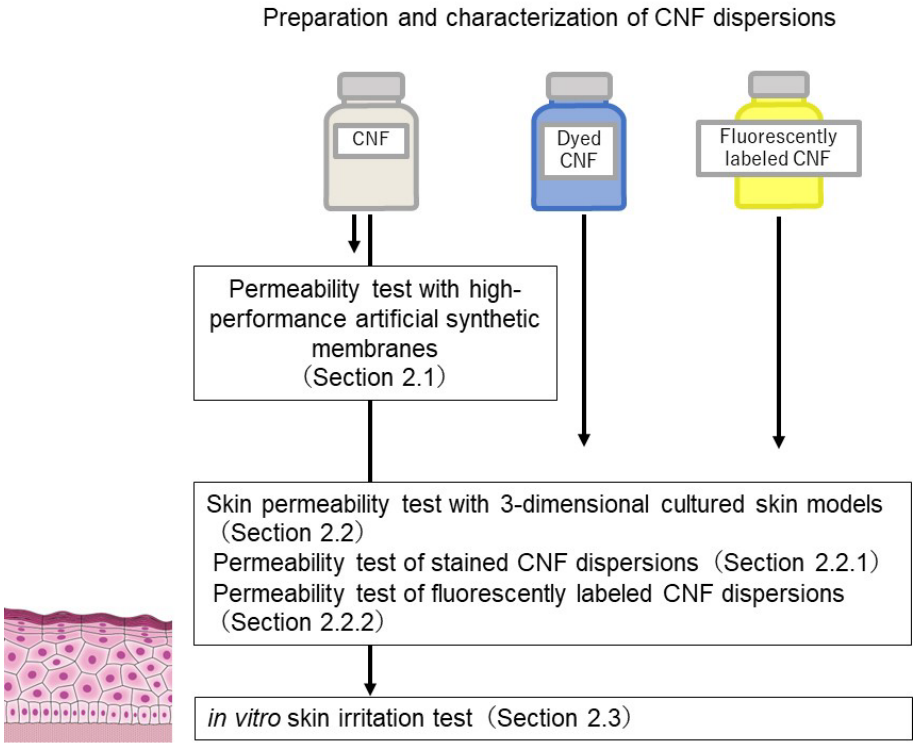

Figure 2.1 Structure of Chapter 2

2.1 Permeability test with high-performance artificial synthetic membranes

A skin permeability test using 3Dcultured skin models is attractive as an alternative to animal safety tests. 3D cultured skin models are advantageous as they provide highly reproducible test results since they use human-derived cultured cells. However, they are costly and have limitations with respect to delivery and expiration date. Conversely, a skin permeability test using high-performance artificial synthetic membranes, which are relatively cheaper and more convenient, might be more convenient as an alternative to living skin [1)]. This section describes a test method using CNFs and glucose, an element of CNFs, to investigate the usefulness of CNF permeability testing using high-performance artificial synthetic membranes.

2.1.1 Example of quality control of permeability test using caffeine

This subsection shows an example where caffeine was used as positive –quality– control saple for the permeability test using high-performance artificial synthetic membranes. Two conditions for quality control on the permeability test were used. 1) both donor and receptor solutions are phosphate buffered saline (PBS), and 2) both donor and receptor solutions are pure water. In addition, the results for 2 days (twice) under the PBS condition are shown.

A total of 0.2 g of caffeine (Special Grade, FUJIFILM Wako Pure Chemical Corporation) was weighed and dissolved by adding a 50-fold volume of commercially-available PBS; FUJIFILM Wako Pure Chemical Corporation) at pH 7.4 using a vortex mixer. Similarly, 0.2 g of caffeine was weighed and dissolved in a 50-fold volume of pure water using a vortex mixer.

The high-performance artificial synthetic membrane (Strat-M, Merck) was set in a static Franz diffusion cell (diffusion area: 1.77 cm^2), and the receptor solution was stirred with a magnetic stirrer at 32°C during the permeability test. At the beginning of the test, 1 mL of caffeine solution (20 mg/mL) was added to the static Franz diffusion cell as donor. Then, a fixed amount (400 μL) of receptor solution was collected from the sampling port every hour up to 6 h later, and the same volume of PBS or pure water was added to maintain constant the volume of receptor solution.

The caffeine solution (5 mg/mL) was weighed as appropriate, then serially diluted with PBS or pure water to prepare caffeine standard solutions for generating standard curves. The caffeine concentration in standard solutions was measured using absorption spectrophotometry. The relationship between caffeine concentration and absorbance (standard curve) was determined from the mean absorbance at 273 nm (Figures 2.2, 2.3) (JASCO V-660, JASCO Corporation).

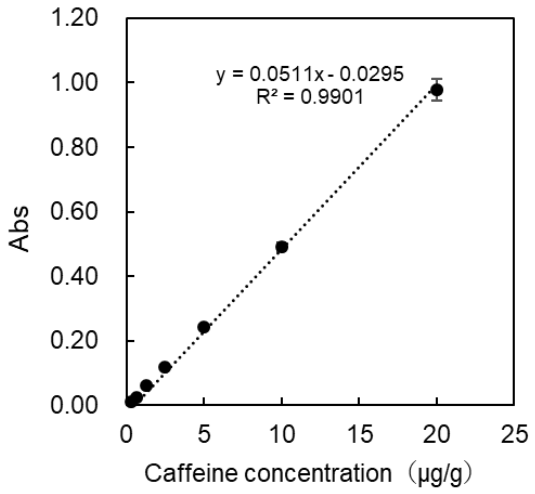

Figure 2.2 Relationship between caffeine concentration (solvent: PBS) and absorbance (standard curve)

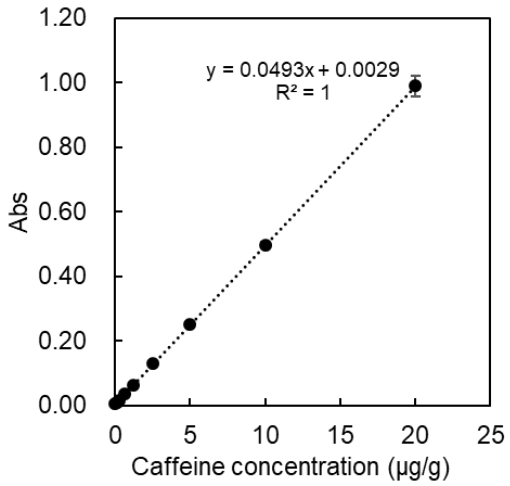

Figure 2.3 Relationship between caffeine concentration (solvent: pure water) and absorbance (standard curve)

The caffeine concentration in the receptor solution was measured by spectrophotometry. Figure 2.4 shows the permeability time and mean permeability coefficient under the PBS and pure water conditions, and Figure 2.5 shows the permeability time and mean permeability coefficient using PBS on another day.

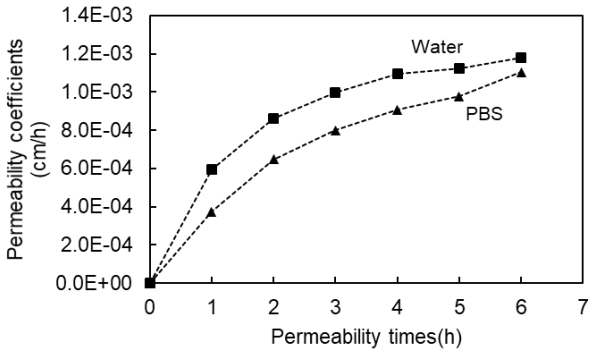

Figure 2.4 Permeability time and mean permeability coefficient of permeability tests with PBS and pure water

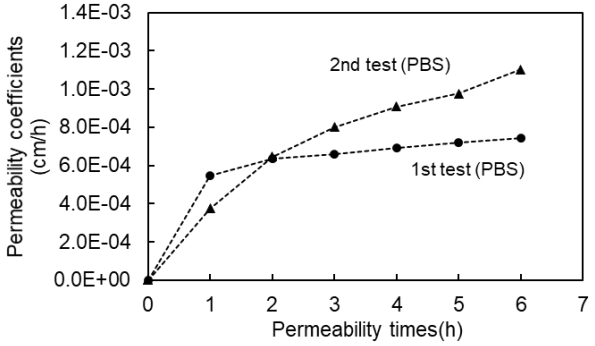

Figure 2.5 Permeability time and mean permeability coefficient of the permeability test using PBS

Based on the above results, the mean permeability coefficient 6 h after the beginning of the permeability test under the PBS condition was 1.1×10^{-3} to 7.4×10^{-4} cm/h, and that after 6 h under the pure water condition was 1.2×10^{-3} cm/h. These values were close to the mean permeability coefficient of caffeine at 1.7×10^{-3} cm/h in the study of Uchida et al. [1] performed under the PBS condition.

Summary

For quality control of the permeability test using the static Franz diffusion cell and high-performance artificial synthetic membranes, we prepared a caffeine standard solution for generating the standard curve in advance, and the caffeine concentration in the receptor solution was measured via absorption spectrophotometry. The mean permeability coefficient of caffeine 6 h after starting the permeability test was close to that in a previous report, suggesting that this permeability test was valid. This method can be useful for the quality control of permeability tests using high-performance artificial synthetic membranes.

Reference

1) Uchida T, Kadhum WR, Kanai S, Todo H, Oshizaka T, Sugibayashi K. Prediction of skin permeation by chemical compounds using the artificial membrane, Strat-M™. Europ J Pharmac Sci. 2015; 67:113-8.

Contributors

Oguri T and Obara S

2.1.2 Permeability test of saccharide

The permeability test of the aqueous sugar solution was performed using high-performance artificial synthetic membranes; the mean permeability coefficient was calculated by measuring the sample concentration in the receptor solution.

D-glucose, D (+)-Cellobiose, and D-maltotriose were dissolved in ultrapure water to prepare a 1.77 wt% aqueous solution. The high-performance artificial synthetic membrane (Strat-M, Merck) was set in a static Franz diffusion cell (diffusion area: 1.77 cm^2), and the receptor solution was stirred with a magnetic stirrer at 32°C during the permeability test. Under the PBS condition, PBS was used as solvent for both donor and receptor solutions; under the pure water condition, pure water was used as solvent for both donor and receptor solutions. When the test started, 1 mL of the aqueous sugar solution was added to the static Franz diffusion cell as donor. After starting the test, a fixed amount (400 μL) of receptor solution was collected from the sampling port every hour up to 6 h later, and the same volume of PBS or pure water was added to maintain constant the volume of receptor solution. The permeability test was conducted for 24 h [1)].

Sample concentration of each sugar in the receptor solution after the 24 h permeability test was measured using CNF concentration quantification by a colorimetric assay; all were below the detection limit. Thus, the permeation of sugar in high-performance artificial synthetic membranes is slow, or it may not permeate. As this is a permeability test using high-performance artificial synthetic membranes, further studies using 3D cultured skin models or animals are necessary to predict the permeability in human skin. However, as the mean permeability coefficient is a useful index for examining skin permeability, considering that its usefulness for future studies, it was calculated from the detection limit determined in this test (Table 2.1).

Table 2.1 Results of high-performance artificial synthetic membrane permeability test of saccharide

		D-Glucose	D (+)-Cellobiose	D-Maltotriose
Receptor	Donor concentration (permeability time: 0 h) (ppm)	1.77	1.77	1.77
	Sample concentration in receptor concentrate (ppm)	Below the detection limit (<0.707)	Below the detection limit (<0.614)	Below the detection limit (<0.625)
	Concentration factor of receptor solution	10	10	10
	Sample concentration in the receptor solution (ppm)	Below the detection limit (<0.071)	Below the detection limit (<0.061)	Below the detection limit (<0.063)
	Receptor volume (mL)	12	12	12
	Sample volume in the receptor solution (μg)	Below the detection limit (<0.848)	Below the detection limit (<0.737)	Below the detection limit (<0.750)
Membranes	Area (cm^2)	1.77	1.77	1.77
	Permeability time (h)	24	24	24
Results	Quantitation of the permeation ($\mu g/cm^2$)	<0.479	<0.416	<0.424
	Mean permeation rate ($\mu g/cm^2/h$)	<0.020	<0.017	<0.018
	Mean permeability coefficient (cm/h)	1.13×10^{-6}	9.80×10^{-7}	9.97×10^{-7}

Reference

1) Uchida T, Kadhum WR, Kanai S, Todo H, Oshizaka T, Sugibayashi K. Prediction of skin permeation by chemical compounds using the artificial membrane, Strat-M™. Europ J Pharmac Sci. 2015; 67:113-8.

Contributor

Goi Y

2.2 Skin permeability test with 3D cultured skin models

Methods to examine the skin permeability of chemical substances include 3D cultured skin models. Compared to artificial membranes, skin models are characterized by a structure extremely close to human tissues. This section describes the methods to examine the skin permeability of dyed and fluorescently labeled CNF dispersions using 3D cultured skin models.

2.2.1 Permeability test of dyed CNF dispersions

2.2.1.1 Preparation and characterization of dyed CNF dispersions

Dyed CNF dispersions were prepared using chemical dyes. The prepared CNF dispersions were characterized by microscopic observation, rheology test, and pH measurement.

2.2.1.2 Skin permeability test of dyed CNFs using 3D cultured skin models

CNF analysis methods include capillary electrophoresis and fluorescently labeled and dyed CNF dispersions. As CNFs, which originally have no absorption in the visible range, show characteristic absorption in the visible range after dying, it is possible to quantitate CNFs from the absorbance at the receptor chamber that collects the permeate in the static Franz diffusion cell used in the permeability test. In addition, it is also possible to observe 3D cultured skin models with an optical microscope. This subsection shows an example of a permeability test with 3D cultured skin models using dyed TEMPO-oxidized CNFs (TOCNs). After exposing previously characterized dyed TOCN dispersions to 3D cultured skin models, the absorbance of the permeate was measured, and 3D cultured skin models were observed with an optical microscope to confirm CNF skin permeability.

TEMPO-oxidized CNF dispersions (TOCN-D-C) were prepared after dying CNF with Sumifix Supra [Navy Blue]. The characterization was performed by rheological measurement and TEM observation. Table 2.2 shows the samples used in the skin permeability test. CNF mass concentration in the dispersions was 1 mg/g.

Table 2.2 Test materials used in the skin permeability test

Sample	Abbreviation	CNF concentration	Remarks
Negative control	Blank	-	Milli-Q water
Dye aqueous solution	Dye	-	The dye concentration was prepared so that the absorbance at l=616 nm was equal to 1 mg/g TOCN-D-C-D1.
Undyed CNF dispersions	TOCN-D-C	1 mg/g	
Dyed CNF dispersions	TOCN-D-C-D1	1 mg/g	

The 3D cultured skin model (MatTek Corporation, US) was set in a static Franz diffusion cell (diffusion area: 1.77 cm^2), and each test material, including Blank, Dye, TOCN-D-C, and TOCN-D-C-D1, was exposed for 24 h.

Two types of 3D cultured skin model, namely EpiDerm™ EPI-606 (standard model) and EPI-606X (the model with enhanced barrier function, recommended for transdermal absorption tests), were selected. The receptor solution was stirred with a magnetic stirrer at 32°C during the permeability test. After exposing the test materials for 24 h, the receptor solution was collected and the absorbance measured, and the 3D cultured skin models were observed with an optical microscope.

Figure 2.6 shows the absorption spectra of the dyed CNF dispersions (TOCN-D-C-D1) at 600–620 nm. To investigate the effect of the mass ratio of dye to CNFs for preparation, the relationship between CNF concentration and absorbance at 616 nm, A_{616}, was determined (Figure 2.7). At a ratio >0.1, there was no considerable difference, and the dying reaction almost reached equilibrium. The lower concentration detection limit was 0.02–0.04 mg/g.

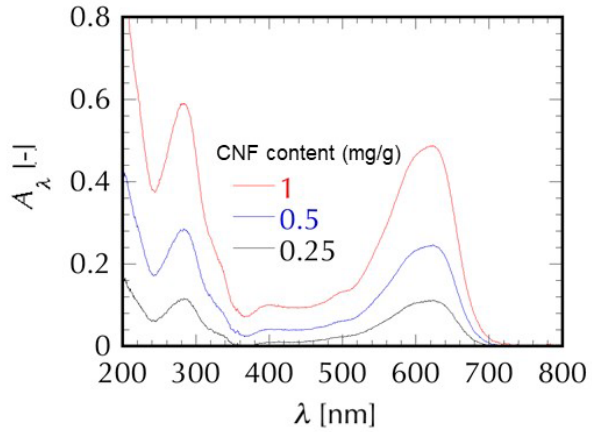

Figure 2.6 Absorption spectra of dyed CNF dispersions

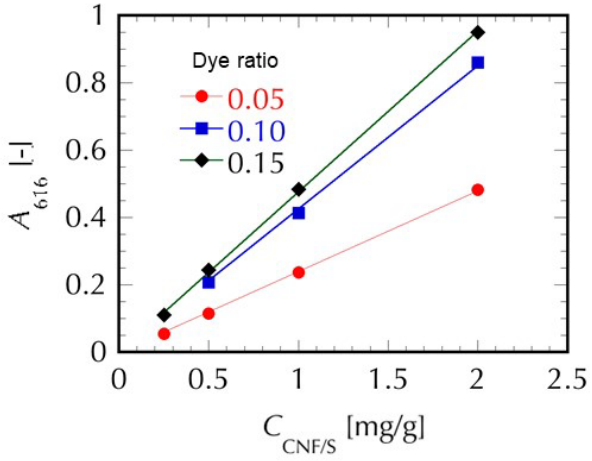

Figure 2.7 Relationship between dye preparation ratio and absorbance at 616 nm, A_{616}

Figure 2.8 shows the viscosity curve of the dyed CNF dispersions (TOCN-D-C-D1) prepared at 1 mg/g as rheological characteristics of the dyed CNF dispersions. Compared to undyed CNF dispersions (TOCN-D-C), no significant difference was found.

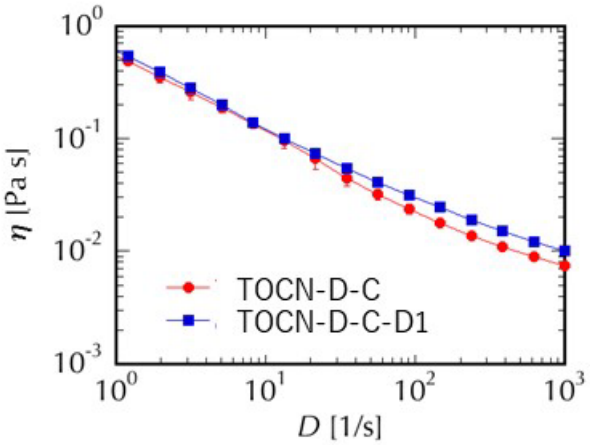

Figure 2.8 Rheology of undyed CNF dispersions and dyed CNF dispersions

Figure 2.9 shows transmission electron microscope (TEM) images of undyed (TOCN-D-C) and dyed CNF dispersions (TOCN-D-C-D1). No significant differences were found.

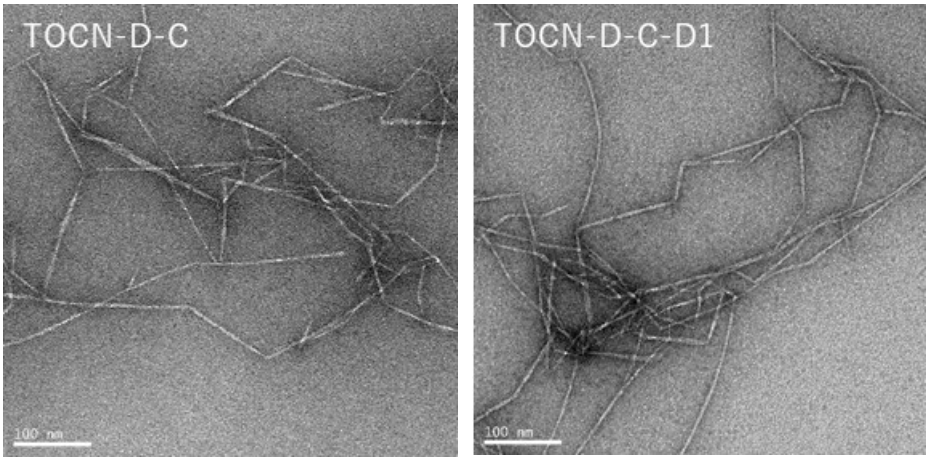

Figure 2.9 TEM images of undyed CNF dispersions and dyed CNF dispersions

Figure 2.10 shows the absorption spectra of the permeate in the 3Dcultured skin model EPI-606X. The absorption spectrum of EPI-606 showed the same trend as that of EPI-606X; no difference was observed in CNF permeation with different cultured skin models. In addition, none of the permeates showed absorption in the visible range but they did in the ultraviolet region. The absorption of the other receptors, except for the undyed CNF dispersions (TOCN-D-C), was almost the same and similar to that of the Blank. The absorption spectrum of the diluted TOCN-D-C dispersion was observed between 230 nm and 270 nm, and corresponded to absorption of additives in the CNF dispersions. In the dyed CNF dispersions (TOCN-D-C-D1), these additives were washed off during the dying process; thus, absorption in these ultraviolet regions was not observed.

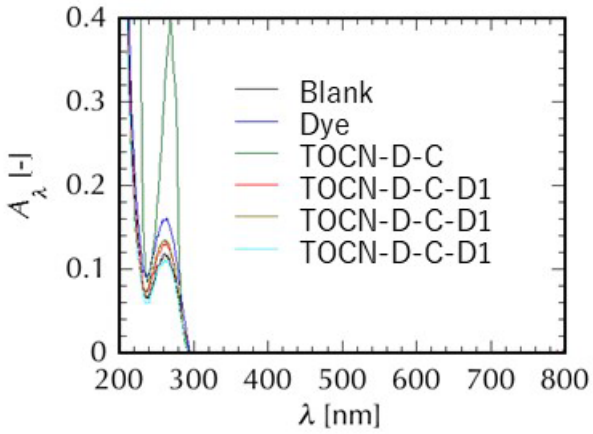

Figure 2.10 Absorption characteristics of permeates in EPI-606X

Figure 2.11 shows the optical microscopic observation of the 3D cultured skin model EPI-606X. The addition of dyed TOCN dispersions had no effect on human skin models. Similar results were obtained for the 3D cultured skin model EPI-606.

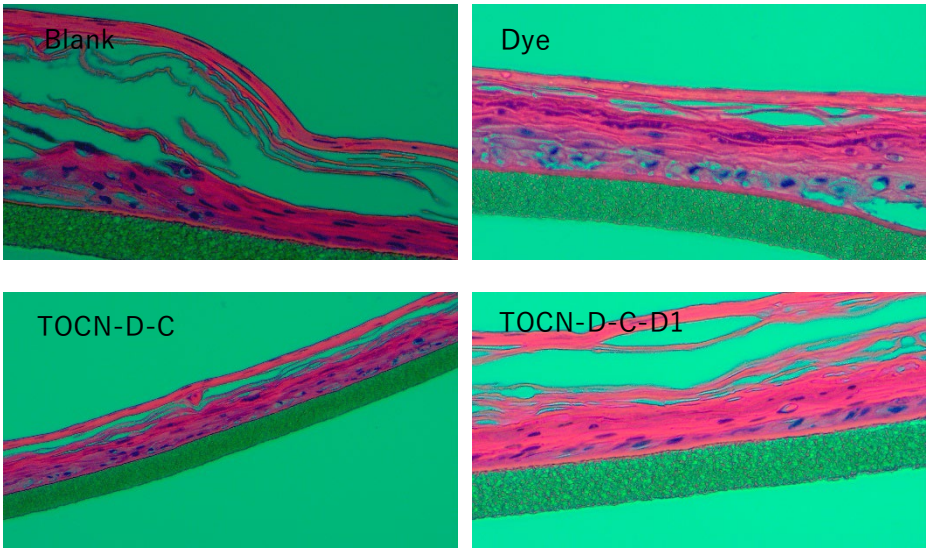

Figure 2.11 Optical microscopic observation of EPI-606X

Summary

A permeability test using two types of 3D cultured human skin models was performed using dyed TEMPO-oxidized CNFs. Rheology and TEM observation revealed no differences between undyed TOCN and dyed TOCN dispersions. In addition, the evaluation of permeates based on the absorbance of the dyed CNFs at 616 nm revealed no skin model permeation. Optical microscopic observation revealed that exposure to dyed TOCN dispersions had no effect on human skin models. The above results suggest that dyed TEMPO-oxidized CNFs did not permeate a 3D cultured human skin model.

Contributors

Fujita K, Obara S, Maru J, and Endoh S

2.2.2 Permeability test of fluorescently labeled CNF dispersions

3D cultured skin models serve to examine the skin permeability of materials. Compared to artificial membranes, skin models have a structure extremely close to human tissue. This subsection describes how to prepare fluorescently labeled CNF dispersions, and examines the skin permeability of said CNFs using 3D cultured skin by quantitatively measuring their fluorescence in receptor solutions and by directly observing 3D cultured skin models using a confocal laser scanning microscope.

2.2.2.1 Preparation and characterization of fluorescently labeled CNF dispersions

Fluorescently labeled CNF dispersions were prepared via fluorescent labeling mediated by hydroxy groups. The prepared CNF dispersions were evaluated by microscopic observation, rheology, and pH measurement.

2.2.2.2 Skin permeability of fluorescently labeled CNFs in 3D cultured skin models

The permeability test of fluorescently labeled CNFs was performed using 3D cultured skin models. The mean permeability coefficient was calculated from fluorescence measurements in the receptor solution.
The concentrations of the fluorescently labeled CNF dispersions used were 798 ppm (0.798 mg/g) and 2991 ppm (2.991 mg/g). The EpiDerm^{TM} epidermis model (MatTek) EPI-606X (a model with enhanced barrier function, recommended for transdermal absorption tests, diameter: 22 mm) was used as 3D cultured skin model. The permeability test was performed over 24 h with the sample solution volume at 1 mL, D-PBS volume at 12 mL, and D-PBS temperature at 32°C in the receptor chamber [1)].
The quantitation of fluorescently labeled CNF permeation, showed levels under the detection limit in all conditions (Table 2.3). In addition, for future verification of CNF permeability in 3D cultured skin models, the mean permeability coefficient calculated from the detection limit was $<1.66 \times 10^{-6}$ cm/h for a CNF concentration of 798 ppm, and $<4.44 \times 10^{-7}$ cm/h for a CNF concentration of 2991 ppm.
The stratum corneum of human skin desquamates layer by layer every day; the desquamation rate is approximately 10^{-6} cm/h. If the permeation rate of a substance is slower than the desquamation rate of the stratum corneum, the latter will desquamate before the substance permeates the skin [2)]. Assuming this value as the non-permeability coefficient, all the above values would be lower than the non-permeability coefficient of 3.6×10^{-6} cm/h.

Table 2.3 Results of the skin permeability test of fluorescently labeled CNFs

Donor	Donor	Fluorescently labeled CNF	
	Donor concentration (permeability time：0h) (ppm)	798	2991
Receptor	CNF concentration in the receptor solution (ppb)	Below the detection limit (<4.63)	Below the detection limit (<4.63)
	Receptor volume (mL)	12	12
	Sample volume in the receptor solution (μg)	Below the detection limit (<0.056)	Below the detection limit (<0.056)
Membranes	Area (cm^2)	1.77	1.77
	Permeability time (h)	24	24
Results	Quantitation of the permeation ($\mu g/cm^2$)	<0.031	<0.031
	Mean permeation rate ($\mu g/cm^2/h$)	$<1.33 \times 10^{-3}$	$<1.33 \times 10^{-3}$
	Mean permeability coefficient (cm/h)	$< 1.66 \times 10^{-6}$	$< 4.44 \times 10^{-7}$

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2.2.2.3 3D cultured skin model observation by confocal microscopy

To verify the usefulness of fluorescently labeled CNFs in skin tissue, their permeability in 3D cultured skin models was observed with confocal microscopy.

Fluorescently labeled TEMPO-oxidized CNFs (initial CNF concentration, 0.1 wt% or 0.5 wt%) were exposed to 3D cultured skin models (EPI-200SIT, MatTek) for 24 h. After moistening the recovered 3D cultured skin models with PBS, a confocal microscope (FV-1000, Olympus) with the following settings: objective lens, 10 × (UPLSAPO 10X2 NA:0.40); fluorescence (Ex/Em), 473 nm/490–590 nm; differential interference, 559 nm; XY scan, 512 × 512 pixels (2.485 × 2.485 µm/pixel); and Z scan, 101 slices (3.0 µm/slice) was used for observation. Fluorescence images show XZ and XY images, whereas differential interference images show the XZ axes. It is possible to recognize the approximate position of cultured skin models in fluorescent images with differential interference images.

In 0.3 wt% fluorescently labeled TEMPO-oxidized CNFs at an initial concentration of 0.5 wt%, fluorescence was detected only on the upper regions of the cultured skin models. However, although weak fluorescence was noted in the lower part of the cultured skin models, it appeared to originate from the basal membrane. As no fluorescence was observed in the middle portion of the cultured skin models, it is highly likely that the fluorescently labeled TEMPO-oxidized CNFs did not permeate the 3D cultured skin models remaining in the epidermis (Figure 2.12).

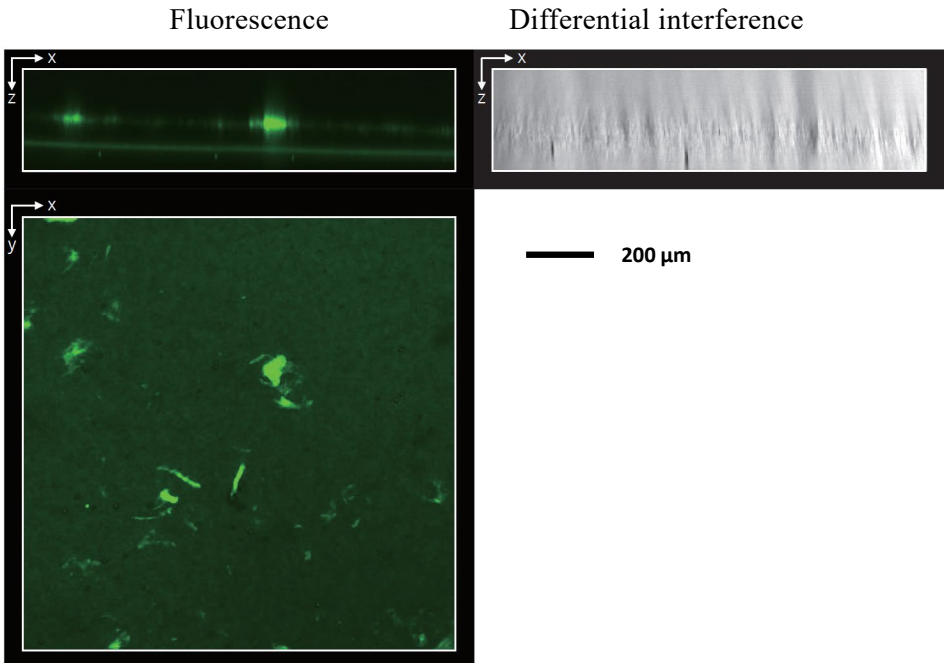

Figure 2.12 Permeability of fluorescently labeled TEMPO-oxidized CNFs in 3D cultured skin models

For fluorescently labeled mechanically defibrated CNF dispersions (initial CNF concentration of 0.5 wt%) diluted to 0.3 wt%, a weak fluorescence seemingly originating from the basal membrane was

observed in the lower part of 3D cultured skin models. The fluorescence was observed in the upper part of the cultured skin models. Since no fluorescence was observed between them, it was very likely that the fluorescently labeled mechanically defibrated CNFs did not permeate the cultured skin models but remained in the epidermis (Figure 2.13).

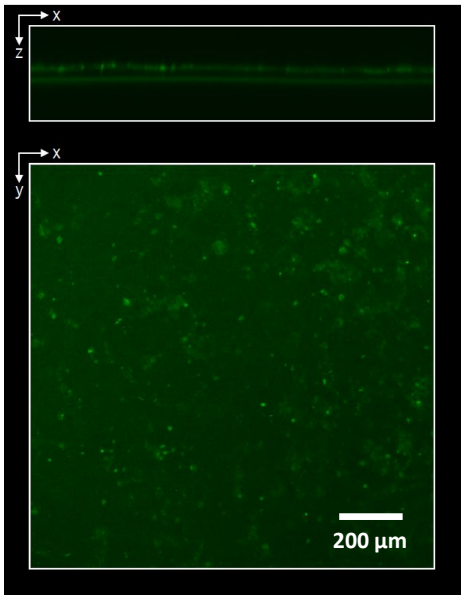

Figure 2.13 3Dcultured skin model permeability of fluorescently labeled mechanically defibrated CNFs

In summary, using 3D cultured human skin models, we assessed the skin permeability of fluorescently labeled TEMPO-oxidized CNFs and mechanically defibrated CNFs with a confocal microscope. Fluorescence was observed in the upper part of the cultured skin models, which implies a high likelihood that the fluorescently labeled TEMPO-oxidized CNFs and mechanically defibrated CNFs did not permeate the cultured skin models but remained in the epidermis.

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2.3 *In vitro* skin irritation test

In the OECD Test Guideline No. 404 "Acute Dermal Irritation/Corrosion" [1], experimental animals are used for assessing dermal irritation/corrosion of chemical substances. However, a hierarchical test using validated *in vitro* and *ex vivo* methods is recommended from the standpoint of animal protection. The OECD Test Guideline No. 439, "*In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method," [2] describes a method for *in vitro* skin irritation test using 3D cultured skin models consisting of reconstructed human epidermis (RHE), imitating the biochemical and physiological characteristics of human epidermis, or normal human epidermal keratinocytes, as an alternative to Test Guideline No. 404.

Skin irritation triggered by chemical substances manifests as erythema and edema owing to swelling of vascular endothelial cells and increased permeability, resulting from the penetration and infiltration of the stratum corneum by chemical substances. The *in vitro* skin irritation test is a method to determine cell viability in 3D cultured skin models by the MTT assay. When cell viability is <50%, it is considered irritating (classified as UN GHS Category 2), and when cell viability is >50%, it is considered non-irritating (classified as beyond the UN GHS categories). The OECD Test Guideline No. 439 introduces a method using four currently commercially-available 3D cultured skin models (EpiSkin™, EpiDerm™ SIT, SkinEthic™ RHE, and LabCyte EPI-MODEL24).

This section shows an example of *in vitro* skin irritation test using 3D cultured skin models to examine skin irritation of TEMPO-oxidized CNF dispersions as test materials. The test complies with the OECD Test Guideline No. 439 (adopted on July 26, 2013) and used EpiDerm™ (EPI-200SIT) and SkinEthic™ RHE.

2.3.1 Preparation and characterization of CNF dispersions

TEMPO-oxidized CNF (TOCN) dispersions were prepared by setting the CNF mass concentration in the dispersion to 1 mg/g and 5 mg/g. The prepared CNF dispersions were evaluated by microscopic observation, rheology, and pH measurement.

2.3.2 Skin irritation test

In compliance with the OECD Test Guideline No. 439, EpiDerm™ (EPI-200SIT) (MatTek Corporation, US) and SkinEthic™ RHE (Episkin, Lyon) were selected as 3D cultured skin models, and the test was conducted according to each manufacturer's protocols. After exposing each of the test materials, namely, 0.1 wt% and 0.5 wt% TOCN, TOCN solvent, D-PBS (Dulbecco's-PBS, negative control (NC)), and 5 wt% SDS (Sodium Dodecyl Sulfate, positive control (PC)), to 3D cultured skin models (n = 3) for a predetermined time (60 min for EpiDerm™; 42 min for SkinEthic™ RHE), the MTT assay was performed. Cell viability in comparison with the negative control was measured, and irritation was judged based on the mean cell viability.

The mean cell viability of the 0.1 wt% and 0.5 wt% TOCN groups exposed to EpiDerm™ and SkinEthic™ RHE, respectively, were both >50%. In addition, based on the mean cell viability of the

positive control for each 3D cultured skin model, this test was considered appropriately conducted. Figure 2.14 shows the test results using EpiDerm™.

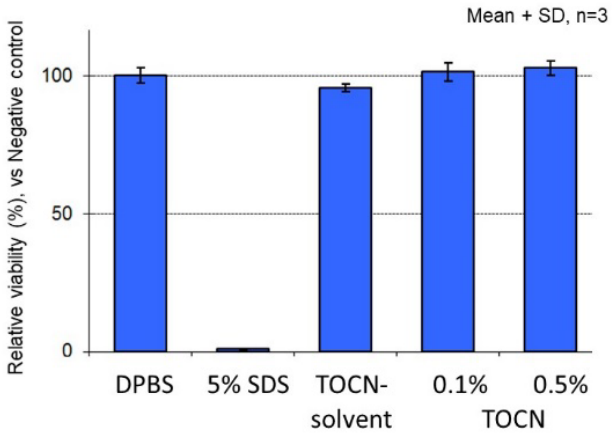

Figure 2.14 Survival rate of TEMPO-oxidized CNFs in 3Dcultured skin model (EpiDerm™)

Summary

In compliance with OECD Test Guideline No. 439, a skin irritation test was performed on two types of 3D cultured skin models. The MTT assay was performed after exposing the 0.1 wt% and 0.5 wt% TOCN groups to the respective 3D cultured skin model and mean cell viability was measured. In all cases, it was >50% compared to the negative control. Based on the above results, TEMPO-oxidized CNFs were judged as non-irritating (classified as beyond the UN GHS categories) [3].

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Chapter 3 Genotoxicity Test

In 2016, MWNT-7 and NT-7K, a type of multi-walled carbon nanotube (MWCNT) were distinguished from other CNTs and added to the list of target substances of the "Guideline to prevent health impairment caused by chemical substances" stipulated by the Minister of Health, Labor and Welfare based on the provisions of Article 28, paragraph (3) of the Industrial Safety and Health Act (the so-called "Carcinogenicity Guideline"). One of the reasons for this decision was "having the risk of causing cancer and other severe health impairments in workers" based on the carcinogenicity assessment by the International Agency for Research on Cancer conducted in 2014, which determined both were Class 2B, while other CNTs were Class 3 (cannot be classified as carcinogenic) [1]. Another reason was the report by the Ministry of Health, Labor and Welfare of Japan at a review meeting in 2015, that some rats raised for two years under a high-concentration MWNT-7 atmosphere had developed lung cancer [2]. Thus, although the impact of specific MWCNTs on humans is still uncertain, there is a consensus to exercise caution because of their potential carcinogenicity. However, there is still insufficient research on other CNTs, nanocarbon materials, CNFs, and other fibrous manufactured nanomaterials like CNTs.

Various genotoxicity tests have been developed for the carcinogenicity assessment of chemical substances. The OECD has established guidelines for internationally common safety assessment test methods for chemical substances and has stipulated dozens of test methods related to genotoxicity (http://www.oecd.org/env/ehs/testing/). Each genotoxicity test uses different species and endpoints. Therefore, it is common to use a battery of genotoxicity tests. The genotoxicity of manufactured nanomaterials such as MWCNTs and exfoliated graphene has been evaluated by such tests [3), 4)]. This chapter describes the bacterial reverse mutation test (commonly known as Ames test), the mouse lymphoma TK assay, the chromosomal aberration test using mammalian cultured cells, and the micronucleus test in mammalian cells, selected as genotoxicity battery tests for CNFs, and performed in compliance with OECD guidelines (Figure 3.1).

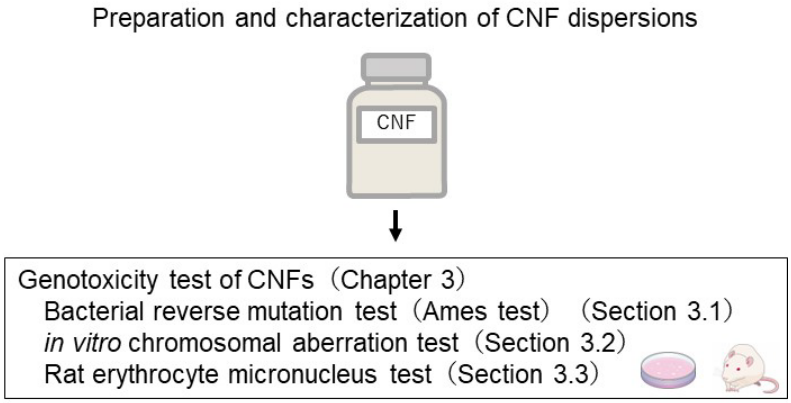

Figure 3.1 Structure of Chapter 3

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3.1 Bacterial reverse mutation test (Ames test)

We performed a reverse mutation test using the *Salmonella typhimurium* TA100, TA1535, TA98, and TA1537 strains, and the *Escherichia coli* WP2*uvrA* strain, to investigate the genetic mutagenicity of dispersions of three types of CNFs (phosphorylated CNF, TEMPO-oxidized CNF, and mechanically defibrated CNF) as test materials. In compliance with OECD Test Guideline No. 471, the test was performed by the preincubation of two treatments, in the presence or absence of a metabolic activation system (+ or −S9 treatment) using rat liver S9 fraction [1]. Each CNF dispersion was prepared by dissolving each CNF in its respective solvent. Flow characterizations of each CNF preparation were assessed using a rheometer (RheoCompass, Anton Paar).

The bacterial reverse mutation test was performed in compliance with OECD Test Guideline No. 471. As a titration study (preliminary test), the inhibitory growth effect of each strain was identified in three types of CNF dispersions at different concentrations and their solvents as test materials. The test validity conditions were (1) the mean counts of reverse mutated colonies of the negative and positive controls are within the reference value determined by background data, and (2) the mean count of reverse mutated colonies of the positive control is more than double of the mean count of reverse mutated colonies of the negative control. The test was valid when both above conditions (1) and (2) were satisfied. When the mean count value of reverse mutated colonies increased to more than double that of the negative control, and the increase was dose-dependent or reproducible, it was considered as a positive. The five types of strains used are shown in Table 3.1.

Table 3.1 Strains used in the bacterial reverse mutation test

#	Strains		Mutation type
1	*Salmonella typhimurium*	TA100	Histidine-requiring base pair substitution type
2	*Salmonella typhimurium*	TA1535	Histidine-requiring base pair substitution type
3	*Escherichia coli*	WP2*uvrA*	Tryptophan-requiring base pair substitution type
4	*Salmonella typhimurium*	TA98	Histidine-requiring base pair substitution type
5	*Salmonella typhimurium*	TA1537	Histidine-requiring base pair substitution type

Positive control substance solutions were prepared in advance (Table 3.2). AF-2, 9-AA, and 2-AA solutions were prepared in dimethyl sulfoxide (DMSO), and the NaN_3 solution was prepared in water for injection. Each preparation was dispensed at 0.5 mL each, then cryopreserved (−80°C), and those within the validity period were used in the test.

Table 3.2 Positive controls used in the bacterial reverse mutation test

in the absence of the S9 mix

#	Positive control	Concentrations（μg/plate）	Strain
1	AF-2	0.01	*Salmonella typhimurium*：TA100
2	NaN_3	0.5	*Salmonella typhimurium*：TA1535
3	AF-2	0.01	*Escherichia coli*：WP2*uvrA*
4	AF-2	0.1	*Salmonella typhimurium*：TA98
5	9-AA	80	*Salmonella typhimurium*：TA1537

in the presence of the S9 mix

#	Positive control	Concentrations（μg/plate）	Strains
1	2-AA	1	*Salmonella typhimurium*：TA100
2	2-AA	2	*Salmonella typhimurium*：TA1535
3	2-AA	10	*Escherichia coli*：WP2*uvrA*
4	2-AA	0.5	*Salmonella typhimurium*：TA98
5	2-AA	2	*Salmonella typhimurium*：TA1537

AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide; NaN_3: sodium azide; 9-AA: 9-Aminoacridine hydrochloride; 2-AA: 2-Aminoanthracene.

The maximum dose was set at 100 μg/plate; the titration study was performed at six doses between 3.13–100 μg/plate. The count of reverse mutated colonies of CNFs dispersed in each solvent did not increase more than double that of the negative control under both −S9 and +S9 treatments. Based on this result, the test was conducted on all strains at the six doses. The positive control substance demonstrated a clear mutagenic effect on each test strain.

Summary

The bacterial reverse mutation test was performed in compliance with OECD Test Guideline No. 471. None of the three types of CNFs demonstrated genetic mutagenicity (negative) on the test strains under the test conditions.

3.2 Mouse lymphoma TK assay

We performed *in vitro* mammalian cell gene mutation tests (mouse lymphoma TK assay: MLA assay) to investigate the genetic mutagenicity of dispersions of two types of CNFs (TEMPO-oxidized CNF and mechanically defibrated CNF) as test materials. The test complies with OECD Test Guideline No. 490 [2]. The basic medium, designated as R-0, consisted of RPMI 1640 medium supplemented with 200 μg/ml sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Growth medium, designated as R-10, consisted of R-0 with 10% (v/v) heat-inactivated horse serum. The cloning medium used for colony formation measurement, designated as R-20, consisted of basic medium with 20% (v/v) heat-inactivated horse serum. The mouse lymphoma cell line L5178Y tk+/−3.7.2C was cultured in R-10 in a humidified incubator with 5% CO2 at 37°C. Methyl methanesulfonate was used as the positive control for short-term exposure (3 h, in the absence of S9 mix) and continuous exposure (24 h, in the absence of S9 mix) experiments. Cyclophosphamide hydrate (CP) was used as the positive control for short-term exposure experiments (3 h, in the presence of S9 mix). The test chemical solvent was used as the negative control. A preliminary test was performed to determine the cytotoxicity of CNFs at concentrations of 3.13–100 μg/mL using diluted 1.0 mg/mL aqueous suspension. The cytotoxicity was determined by the relative suspension growth (RSG) and relative total growth (RTG). In the main study, the maximum concentration was set at 10%–20% RTG based on the OECD test guidelines. Cells were plated at a density of 104 cells/mL in 96-well plates to evaluate cell cloning efficiency (CE) after incubation at 37°C for 12 days. The ratio of CE in each treatment group to the negative control group was calculated as the relative cloning efficiency (RCE), after which RTG was calculated by multiplying RSG and RCE. Two more replicates per experimental group were exposed to 4 μg/mL trifluorothymidine (TFT) for mutation analysis. Plates were incubated at 37°C for 12 days. The mutant colonies of each plate were counted using the naked eye. The colony size (small or large) was estimated in a similar manner to that described in the OECD Guideline for Testing of Chemicals 490. Induced mutant frequency (IMF) was calculated by subtracting the negative control (or untreated control) MF from the test culture MF. The global evaluation factor (GEF) was then defined as 126×10^{-6}.

The main study demonstrated that the IMF did not exceed a GEF value of 126×10^{-6} at any concentration of two types of CNFs for continuous treatment or short-term (6 h) treatment in the presence or absence of S9 mix. IMF and CE of the negative control group were both within an acceptable range (IMF: 50–170 × 10^{-6}; CE: 0.65–1.20). Positive control MMS and CP showed high mutation frequency compared with negative control groups for all treatments.

Summary

A mouse lymphoma TK assay (MLA assay) was conducted using the mouse lymphoma cell line L5178Y tk+/−3.7.2C in compliance with OECD Test Guideline No. 490. As a result, we determined that none of the two types of CNF tested induced *in vitro* mammalian cell gene mutation under our test conditions.

3.3 *In vitro* chromosomal aberration test

This subsection shows an example of *in vitro* chromosomal aberration test using the Chinese hamster lung fibroblast cell line (CHL/IU cells) and three types of CNF dispersions (phosphorylated CNFs, TEMPO-oxidized CNFs, and mechanically defibrated CNFs) as test materials. In this test, chromosomal aberration induction is assessed by the frequency of appearance of cells with structural chromosomal abnormalities and polyploid cells. The test complies with OECD Test Guideline No. 473[3)].

Three types of CNF dispersions were prepared by dissolving each of the three types of CNFs in their respective solvent. Flow characterization of each CNF preparation was performed using a rheometer (RheoCompass, Anton Paar).

CNF dispersions or control substances were added to CHL/IU cells under the following conditions. 1) Short-time treatment, test substances are treated for 6 h in the absence of a S9 mix, followed by a recovery time of 18 h (−S9 treatment); 2) short-time treatment, test substances are treated for 6 h in the presence of S9 mix, followed by a recovery time of 18 h (+S9 treatment); 3) continuous treatment, test substances are treated for 24 h in the absence of S9 mix (24 h treatment). Respective solvents of CNF dispersions were used as negative controls, while mitomycin C (MMC) and cyclophosphamide monohydrate (CP) were used as positive controls.

Based on the results of the preliminary cell proliferation suppression test, all treatments for CNF dispersions were examined up to a treatable concentration (100 μg/mL). For the 24 h treatment of phosphorylated CNF dispersions, chromosomal abnormalities were observed with a microscope at three concentrations at appropriate intervals with the observable concentration (25.0 μg/mL) as maximum concentration because the number of observable division images reduced at concentrations >50.0 μg/mL. For other treatment methods, chromosomal abnormalities were observed with a microscope at three concentrations at appropriate intervals with the highest concentration tested (100 μg/mL) as maximum concentration for observation.

In none of the treatment methods for any CNF treatment groups, did the appearance frequency of cells with structural chromosome abnormalities and polyploid cells significantly increase compared to the negative control. Both the −S9 treatment and 24 h treatment by MMC and the +S9 treatment by CP induced structural chromosome abnormalities at a frequency higher than the negative controls and showed a statistically significant increase compared with the negative control. In addition, the appearance frequency of abnormal chromosome cells in both the no-treatment and positive controls was within the reference value determined from preliminary data, while no significant difference was observed between the no-treatment and negative controls. Furthermore, structural chromosome abnormalities were induced at a significantly higher frequency in the positive control compared with the negative control. The test validity conditions were therefore satisfied.

Summary

An *in vitro* chromosomal aberration test was conducted using the Chinese hamster lung fibroblast cell line (CHL/IU cells) in compliance with OECD Test Guideline No. 473. As a result, we determined that

none of the three types of CNF tested induced chromosomal abnormalities (negative) under our test conditions.

3.4 Rat erythrocyte micronucleus test

This subsection shows an example of the rat erythrocyte micronucleus test using micronucleus induction in bone marrow cells as index to investigate chromosomal aberration induction of three types of CNF dispersions (phosphorylated CNF, TEMPO-oxidized CNF, and mechanically defibrated CNF) as test materials. In compliance with the OECD Test Guideline No. 474 [4], we intratracheally administered test materials to rats once daily with a 24 h interval for 2 consecutive days, and evaluated the frequency of appearance of micronucleated immature erythrocytes in bone marrow cells of the femur extracted 24 h after the final instillation.

CNF dispersions prepared by dissolving each of the three types of CNFs in their respective solvent were used and flow characterization of each CNF preparation was performed using a rheometer (RheoCompass, Anton Paar).

The rat erythrocyte micronucleus test was performed in compliance with the OECD Test Guideline No. 474. As titration study (preliminary test), three types of CNF dispersions at different concentrations and their solvents were intratracheally administered to rats, and death or severe toxic symptoms were evaluated. Crl:CD1 (SD) (SPF) rats were used as experimental animals. Test substance instillation began in rats aged 8 weeks. The titration study was conducted using three female and three male rats to determine sex differences in toxic effects. Based on the titration study, no clear sex differences were observed. This test was performed using three doses (0.5, 1.0, and 2.0 mg/kg), with 2.0 mg/kg as maximum dose. Additionally, a no-treatment control group was set. For the positive control, a CP solution prepared using saline was administered by forced oral instillation (dose: 10 mg/kg) once. To have a final count of five rats for assessment, six rats in each group received the treatments (only five rats were part of the no-treatment control group). If rats were alive, five rats were used for assessment in the descending order of animal number. The femur was extracted 24 h after the final instillation. After preparing bone marrow smears, micronucleated polychromatic erythrocytes (MNPCEs) were microscopically observed, analyzed, and weighed. The test was valid under the following conditions: (1) The appearance frequency of micronucleated immature erythrocytes of the no-treatment control group is within the reference value determined from the background data of the negative control. (2) The appearance frequency of micronucleated immature erythrocytes of the positive control is significantly higher than that in the no-treatment control group. A significant difference between the appearance frequency of MNPCEs between the negative control and the test substance treatment groups was considered a positive result.

The appearance frequency of MNPCEs did not increase significantly in any administered CNF groups (0.5, 1.0, and 2.0 mg/kg) when compared to each corresponding negative control, nor did the ratio of MNPCEs. As the appearance frequency of MNPCEs of the no-treatment control group was within the reference value determined from the background data of the negative control, and a significant increase compared to the no-treatment control group was observed in the MNPCEs in the positive control, this test was appropriately performed.

Summary

The rat erythrocyte micronucleus test was conducted in compliance with OECD Test Guideline No. 474; none of the three types of CNFs showed micronucleus induction in rat bone marrow cells (negative) under the test conditions.

3.5 Comprehensive assessment of genotoxicity

As shown in Sections 3.1, 3.2, 3.3 and 3.4, none of the three types of CNFs used in the tests showed genotoxicity (negative) as they did not demonstrate genetic mutagenicity, chromosomal aberration induction, or micronucleus induction [5].

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A Manual for Toxicity Testing of Cellulose Nanofibers

Test procedures for sample preparation, characterization, and inhalation effect, transdermal effect, and genotoxicity tests

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