Research article

Journal of Applied Toxicology

Received: 14 December 2014, Revised: 28 October 2015, Accepted: 28 October 2015 Published online in Wiley Online Library: 29 December 2015

(wileyonlinelibrary.com) DOI 10.1002/jat.3271

Long-term retention of pristine multi-walled carbon nanotubes in rat lungs after intratracheal instillation

Naohide Shinohara[a], Tetsuya Nakazato[a]*, Kumiko Ohkawa[a], Moritaka Tamura[a], Norihiro Kobayashi[a,b], Yasuo Morimoto[c], Takako Oyabu[c], Toshihiko Myojo[c], Manabu Shimada[d], Kazuhiro Yamamoto[a], Hiroaki Tao[a], Makoto Ema[a], Masato Naya[a,e] and Junko Nakanishi[a]

ABSTRACT: As a result of the growing potential industrial and medical applications of multi-walled carbon nanotubes (MWCNTs), people working in or residing near facilities that manufacture them may be exposed to airborne MWCNTs in the future. Because of concerns regarding their toxicity, quantitative data on the long-term clearance of pristine MWCNTs from the lungs are required. We administered pristine MWCNTs well dispersed in 0.5 mg ml^{-1} Triton-X solution to rats at doses of 0.20 or 0.55 mg via intratracheal instillation and investigated clearance over a 12-month observation period. The pristine MWCNTs pulmonary burden was determined 1, 3, 7, 28, 91, 175 and 364 days after instillation using a method involving combustive oxidation and infrared analysis, combined with acid digestion and heat pretreatment. As 0.15- and 0.38-mg MWCNTs were detected 1 day after administration of 0.20 and 0.55 mg MWCNTs, respectively, approximately 30% of administrated MWCNTs may have been cleared by bronchial ciliary motion within 24 h of administration. After that, the pulmonary MWCNT burden did not decrease significantly over time for up to 364 days after instillation, suggesting that MWCNTs were not readily cleared from the lung. Transmission electron microscopy (TEM) showed that alveolar macrophages internalized the MWCNTs and retained in the lung for at least 364 days after instillation. MWCNTs were not detected in the liver or brain within the 364-day study period (<0.04 mg per liver, < 0.006 mg per brain). Copyright © 2015 The Authors *Journal of Applied Toxicology* Published by John Wiley & Sons Ltd.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords: nanomaterial; multi-walled carbon nanotube; intratracheal instillation; pulmonary clearance; toxicokinetics

Introduction

Multi-walled carbon nanotubes (MWCNTs) possess unique physicochemical properties, including rigidity, a large surface area and electrical conductivity or semiconductivity depending on chirality. They are candidate molecules for many industrial (Javey *et al.*, 2003) and medical (Allen and Cullis, 2004; Kam *et al.*, 2005) nanotechnology applications, such as energy conversion and drug delivery. In the future, people working in or residing near manufacturing facilities may be exposed to airborne MWCNTs.

Recently, concern regarding the toxicity of MWCNTs has increased, and this issue has been the focus in many studies (Morimoto 2005). MWCNTs reportedly induced pulmonary symptoms, such as granulomatous inflammation and fibrotic responses, after inhalation exposure or intratracheal instillation in some studies (Muller *et al.*, 2005, Li *et al.*, 2007; Ma-Hock *et al.*, 2009, Pauluhn, 2010), while no toxicity was reported by other studies (Mitchell *et al.*, 2007, Muller *et al.*, 2009, Kobayashi *et al.*, 2010, Morimoto *et al.*, 2012). These differences in toxicity were proposed to be as a result of the physicochemical characteristics of MWCNTs, such as length and diameter, impurities, components of their surface structure such as functional groups and dispersants (Donaldson *et al.*, 2010; Nagai *et al.*, 2011; Nakanishi 2011,).

Biopersistence is one of the most important factors associated with toxicity. If nanoparticles are retained in the lungs for a long period, chronic oxidative stress-induced damage could occur. According to the International Agency for Research on Cancer (IARC, 2002), lung fibrosis and thoracic tumors were often observed when fiber clearance from the lung is slow, and not when it is fast.

*Correspondence to: Tetsuya Nakazato, National Institute of Advanced Industrial Science and Technology (AIST), Onogawa 16–1, Tsukuba, Ibaraki 305–8569, Japan. E-mail: tet.nakazato@aist.go.jp

[a]National Institute of Advanced Industrial Science and Technology (AIST), Onogawa 16-1, Tsukuba, Ibaraki 305-8569, Japan

[b]National Institute of Health Sciences, Kamiyouga 1-18-1, Setagaya, Tokyo 158-0098, Japan

[c]Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Iseigaoka 1-1, Yahatanishi, Kitakyushu, Fukuoka 807-8555, Japan

[d]Graduate School of Engineering, Hiroshima University, Kagamiyama 1-4-1, Higashi Hiroshima, Hiroshima 739-8527, Japan

[e]BioSafety Research Center (BSRC), Shioshinden 582-2, Iwata, Shizuoka, 437-1213, Japan

Therefore, information on the clearance of MWCNTs is required in addition to information on their pulmonary toxicity.

Several studies have been conducted to estimate the biopersistence and biodistribution of carbon nanotubes (CNTs). However, few studies have directly investigated the clearance and kinetics of pristine MWCNTs because these analyzes are problematic. Catalytic cobalt analysis has shown that 90% of tangled MWCNTs found in the lung 1 day after inhalation exposure was still present 6 months later (Pauluhn, 2010). After intratracheal instillation of ^{14}C-taurine-MWCNTs, ^{14}C analysis showed that 78% was detected in the lungs and this had reduced to 28% by day 28 after instillation (Deng *et al.*, 2007). After inhalation exposure to MWCNTs, a study using hybrid markers and high-performance liquid chromatography analysis found that lung retention did not vary between 1 and 56 days after exposure (Ohnishi *et al.*, 2013). Ten to 20% of administered ^{14}C-labeled MWCNTs were retained in lung between 3 and 12 months after administration (Czarny *et al.*, 2014). Based on these previous studies, lung clearance of MWCNTs was considered to occur very slowly. However, the clearance and translocation of MWCNTs could be vary with a different physiological property of MWCNTs; e.g. diameter, length, being tagged or not, and the dispersibility in solution for administration and in the organ after administration. Catalytic metal ions could separate from the CNTs and independently translocate to other organs within the body (Huang et al., 2010). As molecular tagging can alter physicochemical and biological properties (Liu *et al.*, 2008; Marquis *et al.*, 2009; Tamura *et al.*, 2011), the kinetics and toxicity of tagged CNTs may differ from those of pristine CNTs (Leeuw *et al.*, 2007). In the ^{14}C-labeling determination, there are no data whether ^{14}C-labeling could be homogeneously synthesized and/or the isotopic fractionation could not have occurred between exposure sample and a retained sample in the organ. In addition, the clearance could be accelerated or delayed for well-dispersed particles, as the degree of agglomeration affected the cytotoxicity of the carbon nanotube (Wick *et al.*, 2007).

Therefore, the long-term clearance data for well-dispersed pristine MWCNTs are required after the detailed identification of the characteristics in order to elucidate their biopersistence as well as the translocation data for MWCNTs from the lungs to other organs, such as the liver and brain. In the present study, we administered the MWCNTs intratracheally to rats and determined lung, liver, and brain MWCNTs levels for 12 months. To achieve this, we used a recently developed method (Tamura *et al.*, 2011) involving non-dispersive infrared (NDIR) analysis of the CO_2 generated from the decomposition of pristine MWCNTs that was separated from tissues by acid-digestion and heat pretreatment. Pulmonary pristine MWCNTs were also observed over time using optical microscopy and transmission electron microscopy (TEM). The pulmonary clearance of pristine MWCNTs and their translocation to extrapulmonary organs were evaluated using these quantitative and qualitative data.

Materials and methods

Preparation of MWCNTs suspension

To prepare a stable suspension, MWCNTs, which were synthesized using a floating catalyst method (Nikkiso Co. Ltd., Japan), were sonicated (180W) for 30 min in an aqueous solution of 0.5 mg ml^{-1} polyoxyethylene octyl phenyl ether (Triton-X, Wako Pure Chemical Industries Co. Ltd., Osaka, Japan) using an ultrasonic bath (5510-MT; Branson Ultrasonics Co., Danbury, CT, USA) according to previously described methods (Kobayashi *et al.*, 2010; Chen *et al.*, 2011; Morimoto *et al.*, 2012).

The total surface area of raw MWCNTs was measured using the Brunauer–Emmett–Teller (BET) N_2 gas adsorption method, via surface area and pore size analyzers (Autosorb-1-C; Quantachrome Instruments, Boynton Beach, FL, USA). Metal impurities in the raw MWCNTs were determined using inductively coupled plasma–mass spectrometry (ICP–MS) (Agilent 7500a; Agilent Technologies, Inc., Santa Clara, CA, USA) after microwave-assisted acid digestion of the MWCNTs with HNO_3 at 240 °C. The size of the MWCNTs in the suspension was evaluated by TEM (JEM-1010; JEOL Ltd, Tokyo, Japan).

Intratracheal instillation

MWCNTs were suspended in 0.4 ml of 0.5 mg ml^{-1} Triton-X aqueous solution at concentrations of 0.50 and 1.38 mg ml^{-1}. These were intratracheally instilled into male Wistar rats, at a dose of 0.20 or 0.55 mg per rat using tip cutting feeding needle for rat (Cat No. 7204; Fuchigami Kikai Co., Kyoto, Japan). The rats were 8 weeks old with a mean body weight of 196 g (range 186–207) and had been anesthetized by inhalation of sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan). An aqueous solution of 0.5 mg ml^{-1} Triton-X was intratracheally instilled into the control rats. In each of the above 3 groups, 11 rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (Kyoritsu Seiyaku Corporation, Japan) on days 1, 3, 7, 28, 91, 175, and 364 after instillation, and dissection and gross autopsy were performed. Five rats in each group were used for the MWCNTs analysis, 5 rats in each group were used for the optical microscope observations, and 1 rat was used for TEM observation.

All animal handling procedures were conducted in accordance with the guidelines in the Japanese Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee, University of Occupational and Environmental Health, Japan, or by the Institutional Animal Care and Use Committee, National Institute of Advanced Industrial Science and Technology.

Analysis

After drawing blood from the abdominal aorta, the left and right lungs, livers and brains of five rats in each group were dissected, separated and weighed. They were then cut into small pieces with scissors, homogenized in saline (3 ml for the left lung, right lung and brain; 25 ml for the liver) with an electric homogenizer (Polytron RT3100; Kinematica AG, Luzern, Switzerland), and stored at −20 °C prior to analysis.

Quantification of MWCNTs in the lungs, liver and brain was performed by acid digestion, muffle ashing, *in-situ* preheating and combustive oxidation-NDIR measurement, as described by Tamura *et al.* (2011). Three milliliters of 60% HNO_3 was added to approximately 0.1 g of homogenized tissue, and the mixture was digested using a hot plate heat at 120 °C for 8 h. After cooling, the acid-digested samples were filtered with a quartz membrane filter (QR-100; Advantec Toyo Kaisha, Ltd., Tokyo, Japan) which had been preheated at 900 °C for 15 min in a muffle furnace (FP42; Yamato Kagaku Co. Ltd., Tokyo, Japan). The filter containing the MWCNTs was washed with 5 ml of pure water five times and then heated at 400 °C for 15 min. After heating, the MWCNTs on the filter were quantified using a combustive oxidation chamber (SSM-5000A; Shimadzu, Japan) with an NDIR detector (TOC-V CPH, Shimadzu) at a temperature of 900 °C in the

Journal of Applied Toxicology

presence of oxygen gas (purity, > 99.9%). A calibration curve for carbon content was generated using glucose (Wako Pure Chemical Industries Co. Ltd).

The individual variation of left lung MWCNTs burden was much larger than that observed in total lung tissue (Supporting Information Table 1), possibly reflecting differences in MWCNTs deposition in the right and left bronchi during instillation. This left lung variability was caused by the large individual variation in the initial amounts deposited in each lung. We, therefore, analyzed the total lung burden, because these data were not influenced by variability in distribution between the lungs.

Quality assurance and quality control for the determination of MWCNTs

The detection limit and the precision of analysis for pulmonary MWCNTs in the present study were checked in the previous study (Tamura *et al*., 2011). To check the validity of this method, the lung burdens of male Wistar rats (aged 8 weeks, mean body weight 272 g, range 245–299 g) were determined after a 28-day inhalation whole-body MWCNTs exposure (0.37 ± 0.18 mg m^{-3}), and compared with our previously reported results for the same inhalation exposure using different methods (Oyabu *et al*., 2011).

Optical microscope observation and TEM observation

The lungs and lymph nodes of five rats from each group were fixed with 10% formalin, embedded with paraffin, sectioned, and stained with hematoxylin and eosin for optical microscope observation. For TEM, lung tissue was fixed using glutaraldehyde and osmium tetroxide solution, dehydrated in ethanol, and embedded in epoxy resin. Ultrathin microtome sections were cut using a diamond knife. Some sections of the specimen were stained using 2% uranyl acetate solution and 0.5% lead citrate solution at room temperature. Conventional TEM observation was performed by H-7000 (Hitachi, Tokyo, Japan) at an acceleration voltage of 80 kV.

Results

Characteristics of MWCNTs

The surface area of the MWCNTs was 69 ± 37 m^2 g^{-1}, as determined by the N_2 gas adsorption method. With regard to metal impurities, the levels of Li, Al, Ca, Fe and Cd in the MWCNTs were 0.5, 80, 176, 53 and 16 μg g^{-1} respectively, as determined by ICP–MS.

The geometric mean diameter of the MWCNTs in the suspension used for the intratracheal instillation test was 48 nm [geometric standard deviation (GSD), 1.1] and the geometric mean length was 2.5 μm (GSD, 2.4), as determined by TEM (Fig. 1). The size of the MWCNTs in the suspension did not change from the raw MWCNT (geometric mean: 44 nm; GSD 1.3).

Quality assurance and quality control for the determination of MWCNTs

The detection limit of the determination method used in the present study, defined as a signal-noise ratio of 3, was 0.0003 mg of MWCNTs, and 0.003 mg per lung, 0.04 mg per liver and 0.006 mg per brain. The precision of analysis (repeatability) for pulmonary MWCNTs determination was 5.6% and the efficiency of recovery was > 95% (Tamura *et al*., 2011).

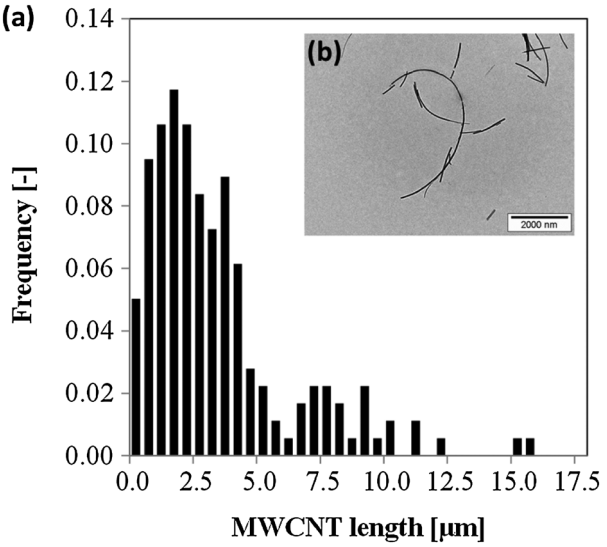

Figure 1. (a) Distribution of the lengths and (b) transmission electron micrographs (TEM) of the multi-walled carbon nanotubes (MWCNTs) suspension used for intratracheal instillation.

The mean pulmonary MWCNTs burdens determined in the present study at 3 days and at 1 month after inhalation exposure to MWCNTs at 0.37 mg m^{-3} were 0.073 ± 0.028 and 0.073 ± 0.012 mg per lung respectively (N = 5). These results were within the same range (±10%) as previously reported pulmonary MWCNT burdens in rats, determined by elemental carbon analysis and X-ray diffraction (Oyabu *et al*., 2011). This validated the accuracy of the methods employed in the present study.

Determination of MWCNTs burden in the lung

The ratio of the MWCNTs burden in the right lung to the total MWCNTs lung burden 1 day after the instillation was 0.80 and 0.91 for the 0.20 mg and 0.55 mg doses, respectively. These ratios were not identical to the ratios of the weight of the right lung to the total lung, which were 0.67 and 0.70 for the 0.20 mg and 0.55 mg doses, respectively (Supplementary Information Table 1). At 3 days to 12 months after the instillation, the MWCNTs burdens in the right lung were also much greater than the corresponding burdens in the left lung (ratio of burden in the right lung to the total were 0.88 and 0.90 for the 0.20 mg and 0.55 mg doses, respectively).

The individual variation of MWCNTs burden in the lung was evaluated using the relative standard deviations (RSDs) of the measured amounts. The left lung RSD values after administration of 0.20 and 0.55 mg MWCNTs were 49%–110% and 43%–130% for the entire period examined in this study, respectively. The equivalent values for total lung were 14%–30% and 20%–34%, respectively. Therefore, the burden values for total lung were used for the following data analysis.

The total lung burdens after intratracheal MWCNTs instillation are shown in Fig. 2. On days 1, 3, 7, 28, 91, 175 and 364 after the instillation of 0.20 mg MWCNTs, the mean ± standard deviation (SD) of total lung MWCNTs burdens were 0.15 ± 0.024, 0.13 ± 0.030, 0.15 ± 0.055, 0.15 ± 0.045, 0.12 ± 0.017, 0.14 ± 0.018, and 0.15 ± 0.028 mg per lung respectively (N = 5). For the 0.55 mg dose, the corresponding pulmonary MWCNTs burdens were 0.38 ± 0.11, 0.30 ± 0.061, 0.30 ± 0.084, 0.28 ± 0.073, 0.37 ± 0.086, 0.28 ± 0.082 and 0.30 ± 0.10 mg per lung (N = 5). As 0.15 and 0.38 mg MWCNTs

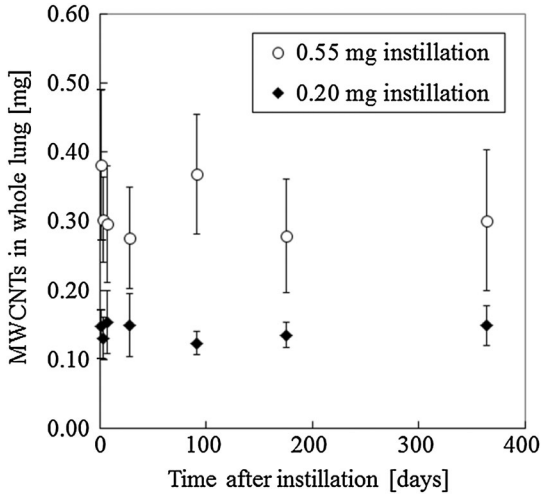

Figure 2. Pulmonary multi-walled carbon nanotubes (MWCNTs) burden by time elapsed after single intratracheal instillation at the doses of 0.20 and 0.55 mg per rat.

were detected 1 day after administration of 0.20 and 0.55 mg MWCNTs, respectively, approximately 30% of administrated MWCNTs may have been cleared by bronchial ciliary motion within 24 h of administration. MWCNTs were not detected in the lungs of control rats (<0.003 mg per lung).

Observation using optical microscopy and TEM

Using optical microscopy, MWCNTs-laden macrophages were observed in the lung interstitium and alveoli throughout the observation period in both dosage groups (Fig. 3). On day 1 after instillation, many of the MWCNTs aggregates observed were within macrophages, with only a small number of extracellular aggregates (Fig. 3a). Three days after instillation, most of the MWCNTs aggregates were observed in macrophages (Fig. 3b). From days 7–90 after instillation, several MWCNTs-laden macrophages accumulated in the lung (Fig. 3c). From days 90–364 after instillation, most of the MWCNTs-laden macrophages were internalized in the alveolar interstitium and formed granuloma (Fig. 3d–f). TEM images of the alveolar macrophages in the lungs of rats

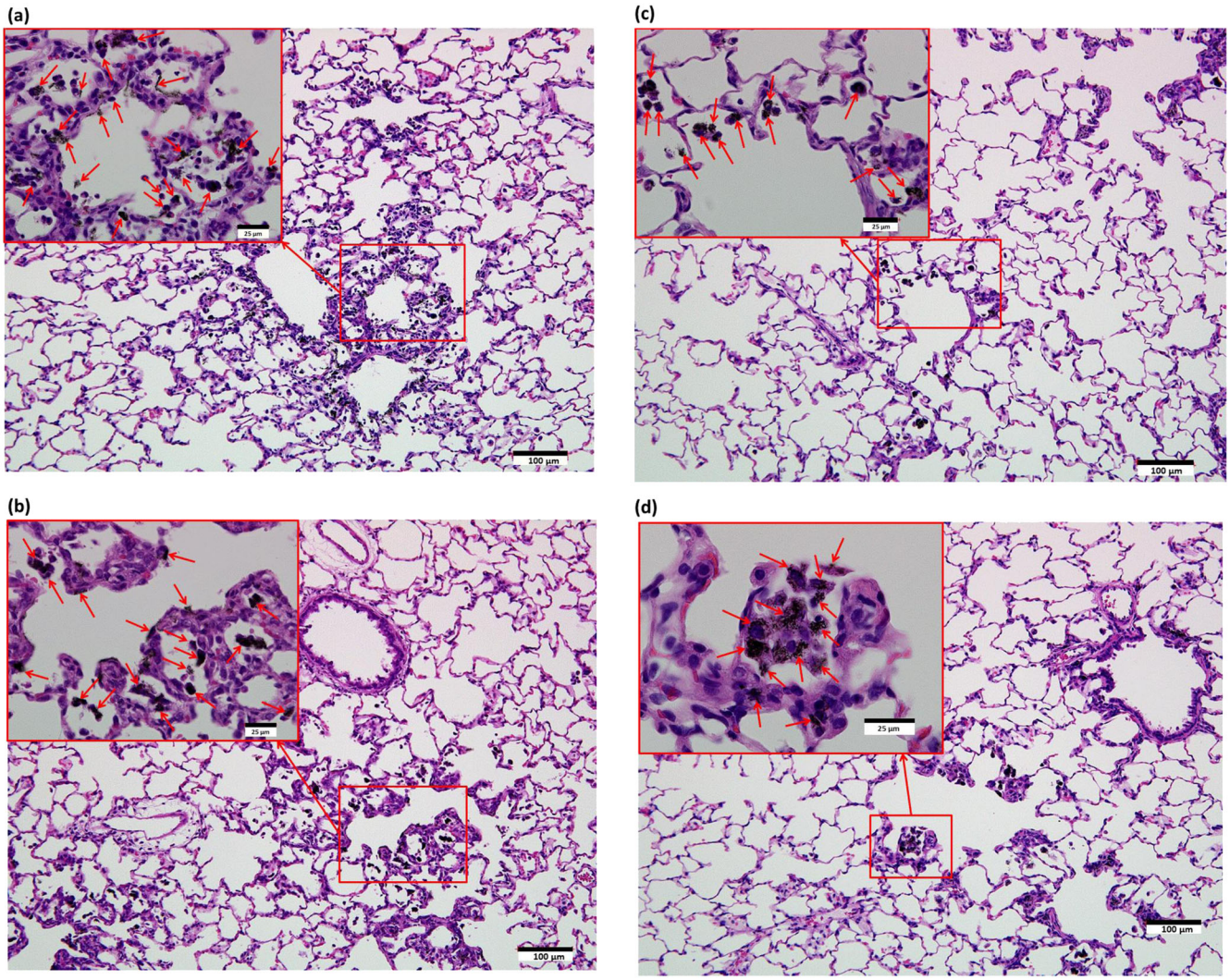

Figure 3. Multi-walled carbon nanotubes (MWCNTs)-laden macrophages in the interstitium and alveolus at (a) day 1, (b) day 3, (c) day 7, (d) day 28, (e) day 91, (f) day 175, and (g) and (h) day 364 after instillation in rats that were administered a high dose (0.55 mg per rat).

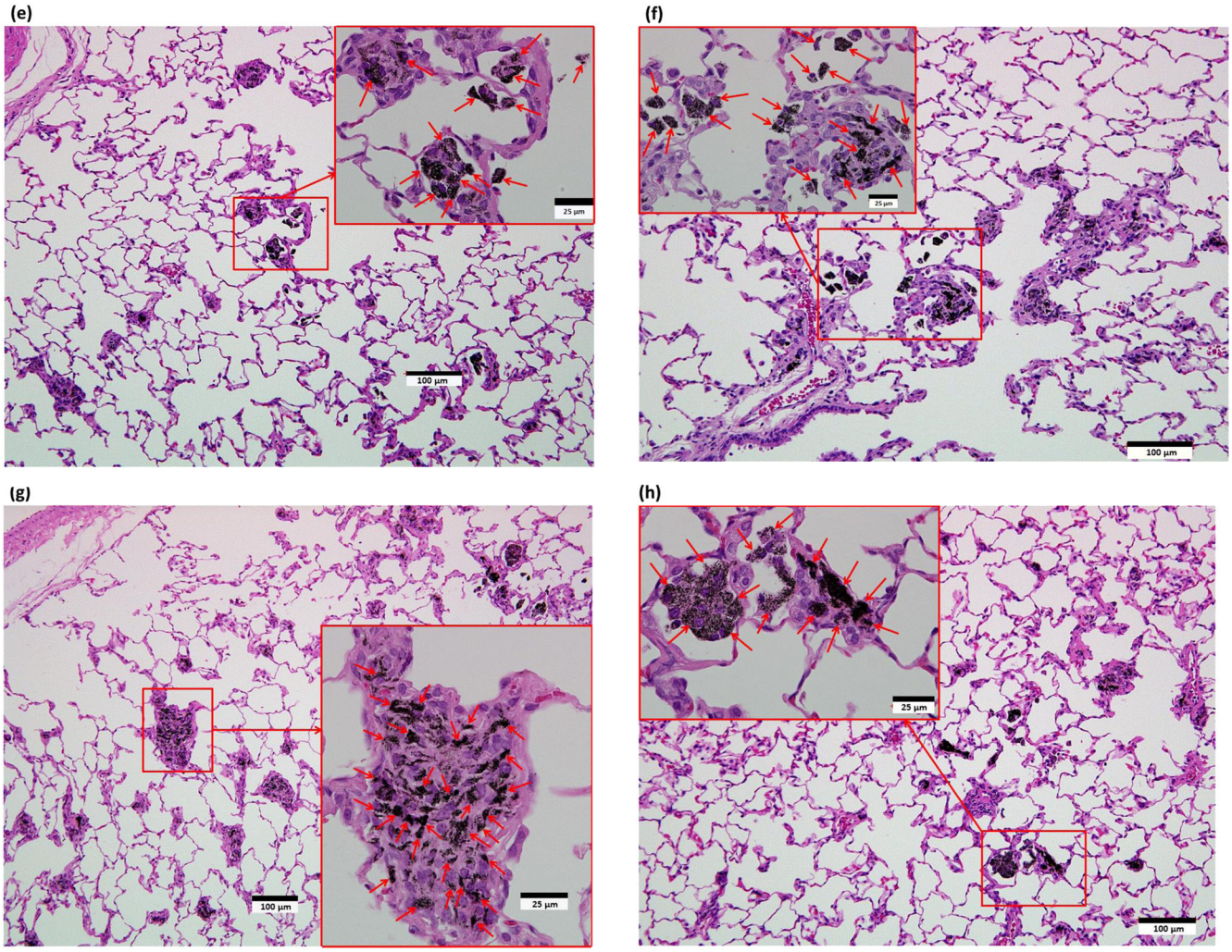

Figure 3. (Continued)

obtained on days 7 and 364 after the instillation of 0.20 mg MWCNTs are shown in Fig. 4. While MWCNTs were observed in phagolysosomes in alveolar macrophages, they were not found in the nuclei or other intracellular organelles. The internal multilayered structure of the MWCNTs was maintained.

MWCNT translocation to extrapulmonary organs

MWCNTs were not detected in the liver or the brain after intratracheal instillation ($<$0.04 mg per liver, $<$ 0.006 mg per brain). However, optical microscopy revealed the presence of MWCNTs in the peribronchial lymph nodes (Fig. 5).

Discussion

Well-dispersed MWCNTs were used in the present study. The biological behaviors of MWCNTs, such as toxicity and biodistribution, could depend on their aggregation state. In contrast to aggregated MWCNTs, dispersed MWCNTs can penetrate mesothelial cells and further induce cell injury (Nagai *et al*., 2011). Smaller nanoparticles showed more widespread organ distribution (De Jong *et al*., 2008). Exposure to small aggregates of TiO_2 nanoparticles produced increased oxidative stress effects and cytotoxicity than exposure to large aggregates of these nanoparticles (Noël *et al*., 2013). Therefore, well-dispersed MWCNTs might be more toxic and show more widespread organ distribution than aggregated MWCNTs, although published data have not indicated that dispersion affected the toxicity of TiO_2 nanoparticles (Kobayashi *et al*., 2009). However, there are a few previous studies that have investigated the biodistribution of well-dispersed MWCNTs for observation periods of over 6 months (Mercer *et al*., 2013; Czarny *et al*. 2014,). In the present study, TEM identified no large MWCNT aggregates and only a few fibers aggregate in the instilled suspension (Fig. 1).

The present study directly determined the lung retention of pristine MWCNTs after intratracheal instillation. Most of the previous studies analyzed the organ distributions of MWCNTs indirectly, for example by analyzing catalytic metal (Pauluhun 2010) or functionalized MWCNTs (Deng et al. 2007, Liu et al. 2007). Pauluhun (2010) estimated pulmonary MWCNT burdens in rats for 26 weeks after a 13-week inhalation exposure by analyzing the catalytic Co. Catalytic metal can distribute throughout the body (Huang *et al*., 2010). In a study of the biodistribution of ^{99m}Tc-radiolabeled carbon nanoparticles, ^{99m}Tc or its oxides could enter the blood circulation independently of the nanoparticle (Mills *et al*., 2006). Deng *et al*. (2007) estimated the pulmonary ^{14}C-taurine-functionalized MWCNT burdens in mice after intravenous injection, intratracheal instillation and stomach intubation for 12 h to 90 days after administration. Functionalization could change the biodistribution of

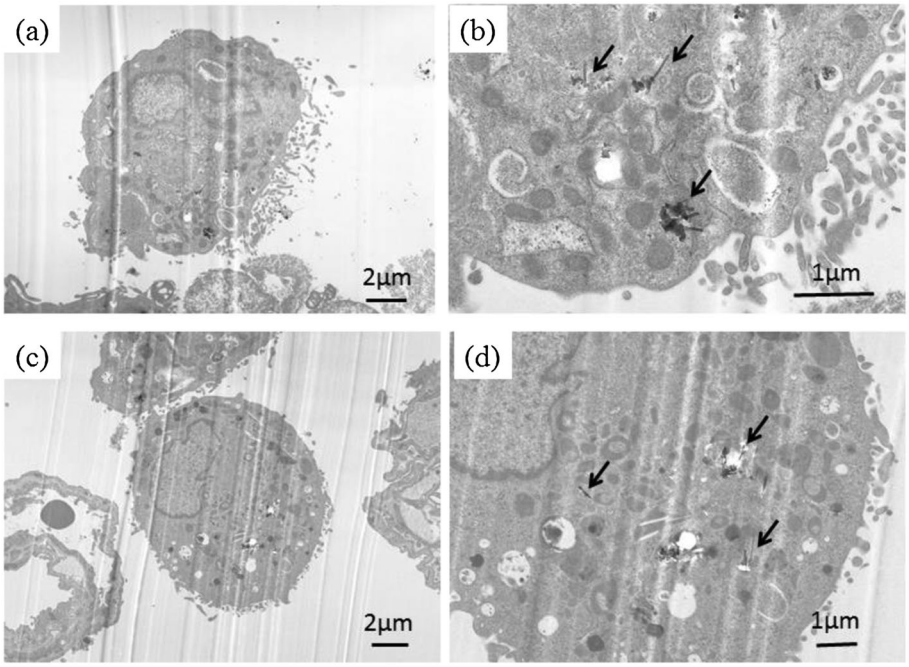

Figure 4. Transmission electron micrographs (TEM) images of alveolar macrophages. (a) and (b) 7 days after instillation of a low dose (0.20 mg per rat) of multi-walled carbon nanotubes (MWCNTs) and a magnified image, and (c) and (d) 364 days after instillation of a low dose (0.20 mg per rat) of MWCNTs and a magnified image. Endocytosed MWCNTs are indicated by arrows.

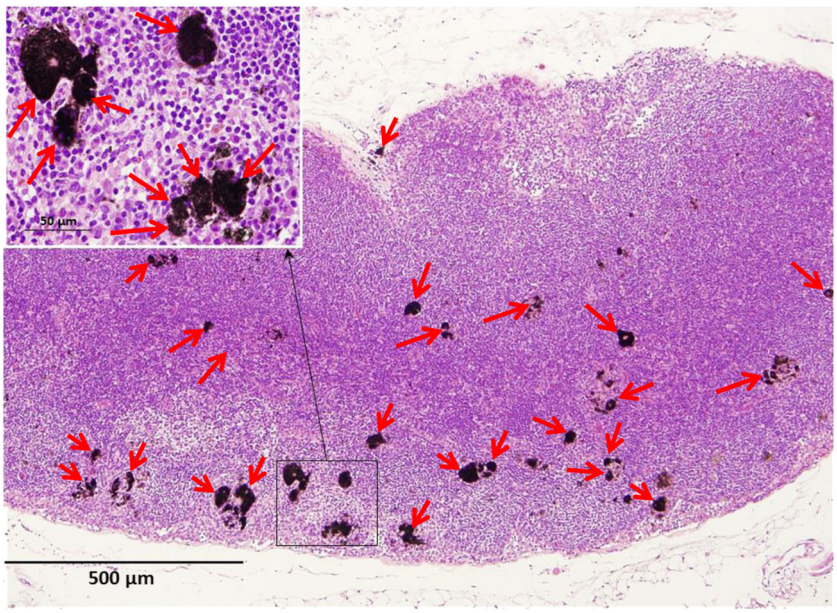

Figure 5. Multi-walled carbon nanotubes (MWCNTs)-laden macrophages in the peribronchial lymph node at day 364 after instillation in rats that were administered a high dose (0.55 mg per rat).

inhaled particles depending on the type of functional group. Functionalized MWCNTs were phagocytosed more effectively than pristine MWCNTs in a study (Fraczek-Szczypta *et al.*, 2011), although functionalized MWCNT and pristine MWCNTs showed no differences in clearance in a study (Silva *et al.*, 2014). Therefore, analysis of a catalytic metal or functionalized MWCNTs may produce distribution data that cannot be extrapolated to pristine MWCNTs. Although the translocation of MWCNT after aspiration in mice using ^{14}C-labeled MWCNTs, it is difficult to apply the determination of many kinds of MWCNTs owing to the synthesizing cost. Our direct determination of pristine MWCNTs was more suitable to determine the lung clearance and biodistribution of pristine MWCNTs. We also determined the burden of the total lung to accurately evaluate the pulmonary clearance but not one side lung. MWCNT burdens were actually higher in the right lung than in the left lung in the present

Journal of Applied Toxicology

study. In humans, aspiration pneumonitis tends to occur in the right lung (Kouno *et al*., 2006), indicating that this lung could be exposed more to ingested substances because the main right bronchus is shorter, thicker and less angled than the main left bronchus (Kouno *et al*., 2006). This difference in the right and left bronchi is due to the position of the heart on the left side of the body (Kouno *et al*., 2006). As the rat heart apex is also placed slightly to the left (Hiraiwa et al. 1960), there could be a difference between rat right and left bronchi. Therefore, we considered it is more accurate to determine the total lung burden when evaluating pulmonary clearance.

In the present study, approximately 70% of the MWCNTs were detected in lung tissue 1 day after the intratracheal instillation. Particles trapped on the trachea and bronchioles can be cleared by ciliary motion within 1 day because bronchial ciliary motion rates were 7.5–13.6 mm min^{-1} (Lightowler and Williams 1969) and the length of trachea and bronchioles (until terminal bronchioles) were 5.1 mm in the rat (Yeh *et al*., 1979). Therefore, it can be presumed that 30% of the instilled MWCNTs might be trapped on the trachea and bronchioles and might be cleared by ciliary motion.

On day 1 after instillation, only a small number of extracellular aggregates of MWCNTs were observed although aggregates were not observed in suspension. One possible reason is that macrophage gathered them in a day. The other possible reason is that a part of dispersed MWCNTs aggregated when the suspension mixed with bio-surfactant in the alveolar region.

In the present study, MWCNTs were rarely cleared from the lung within 1 year after intratracheal instillation. Previous inhalation exposure tests using MWCNTs have also low clearance rates. After inhalation exposure to tangled MWCNTs (6.0 mg m^{-3}), pulmonary retention at 26 weeks was >90% of that observed 1 day after exposure [0.1 mg m^{-3}, half-time 5 month; 0.4–6.0 mg m^{-3}, half-time 10 to > 13 months (Pauluhun, 2010)]. After inhalation exposure and intratracheal instillation to original and functionalized MWCNTs, no clearance was observed within 21 days (Silva *et al*., 2014). After inhalation exposure, 25% and 35% cleared at 168 and 336 days post-exposure compared with the burden at 1 day post-exposure in rats (Mercer *et al*., 2013). In contrast, spherical C_{60} nanoparticles were cleared from the lungs much faster than MWCNTs [half-life < 1 month (Shinohara *et al*., 2010)] although C_{60} and CNTs are both carbon allotropes, and both have a graphene structure. For other poorly soluble fibers, the pulmonary clearance was similarly slow to that observed in the present study [crocidlite and amosite, half-life > 13 months (Hesterberg and Hart, 2000); silicon carbide whisker, half-life 16 months (Akiyama *et al*., 2007)]. These reports comprehensively indicate that water-insoluble fibers such as MWCNTs showed slow clearance from the lung.

Another factor in elucidating the slow clearance, that is, the length of MWCNT, the pulmonary macrophage cytotoxicity, overload of instillation amount of MWCNTs, and dispersion of the CNTs, was examined.

We considered the possibility estimated that the MWCNTs length could be the cause of their cytotoxicity to macrophages. Previous studies indicated that longer fibers could not be fully phagocytosed by macrophages, and induced cytotoxicity [>15 μm in length (Searl *et al*., 1999; Poland *et al*., 2008)]. However, the MWCNTs used in the present study were much shorter (average length = 2.4 μm; 96%, < 0.5–10 μm 96%; 2.8%, 10–15 μm; 1.1%, > 15 μm) than those shown to block phagocytosis. In addition, our TEM observations did not observe any MWCNTs piercing macrophages. Therefore, these findings indicated that the length of the MWCNTs was not the cause of their lung retention in the present study.

The macrophage cytotoxicity of MWCNTs could also cause their slow clearance. There have been many studies showing that MWCNTs are cytotoxic to macrophages (Hirano *et al*., 2008; Cheng *et al*., 2009; Boncel *et al*., 2011; Chen *et al*., 2011; Zhang *et al*., 2012; Luo *et al*., 2012). The viability of mature human macrophages decreased after their exposure to, and uptake of MWCNTs (Cheng *et al*., 2009; Boncel *et al*., 2011; Zhang *et al*., 2012). MWCNTs have been reported to disrupt the macrophage plasma membrane integrity (Hirano *et al*., 2008), inhibit their migration (Hirano *et al*., 2010), increase markers of oxidative stress (Chen *et al*., 2011), and cause ultrastructural and morphological changes (Luo *et al*., 2012). In contrast, no significant macrophage toxicity was observed for C_{60} (Jia *et al*., 2005). Therefore, the MWCNT cytotoxicity might delay the macrophages' pulmonary clearance.

Lung overload could be also the cause of delay MWCNT clearance from the lung. As overload occurred after administration of the exceeding threshold volume of MWCNTs (Pauluhn, 2010), we discuss the volume of MWCNTs employed in the present study. Assuming the density of MWCNT in the present study was a true density of 1.72 g cm^{-3} because the suspension was well dispersed, pulmonary MWCNTs was estimated to be only 0.12 μl g^{-1} per lung, which is within the range of minimal lung overload for tangled MWCNTs [0.1–0.3 μl g^{-1} per lung (0.1 mg m^{-3}); Pauluhn, 2010]. In the present study, however, only 0.12 μl g^{-1} per lung of MWCNTs induced a complete delay in clearance. In Pauluhn (2010), complete lung overload occurred at a much higher volume of tangled MWCNTs [1.2–12 μl g^{-1} per lung (1.5–6.0 mg m^{-3})]. There are two possible explanations for this difference between Pauluhn (2010) and the present study. One possibility relates to the severity of cytotoxicity, as the toxicity of well-dispersed rigid MWCNTs could be more severe than that of tangled MWCNTs. In a previous *in vitro* test, greater cytokine induction was observed in macrophages after phagocytosis of long needle-like CNTs than after phagocytosis of long-tangled CNTs (Palomaki *et al*., 2011). The other possibility relates to the administration method resulting in different local doses in the lung, as the overload threshold could be higher for inhalation exposure than for intratracheal instillation. Brain *et al*. (1976) reported that intratracheal instillation resulted in less even lung distribution than inhalation exposure. Therefore, even if the total lung burden after intratracheal instillation and inhalation exposure is the same, the local burden per unit lung area at some points could be higher after intratracheal instillation than that after inhalation exposure. More research is required to study the relationship between cytotoxicity/overload and delayed clearance.

A recent study showed that MWCNT, which synthesized by a chemical vapor deposition process, were cleared by 80%–90% at 90–360 days post-aspiration in mice (Czarny *et al*., 2014). Their MWCNT size (3.9 μm of length and 40 nm of diameter) and dose per weight [1 mg kg^{-1} per body weight (BW)] was similar to our study (2.5 μm of length and 48 nm of diameter; 1.0 and 2.8 mg kg^{-1} per BW of dose). Therefore, the difference in pulmonary clearance between two studies might not be due to the size and dose.

In conclusion, MWCNTs were retained in the lung, even 1-year post-instillation. However, these MWCNTs in pulmonary macrophages seemed to form stable granuloma and no tumors were observed for up to 1-year post-instillation. Although the long-term retention observed in the present study might have the possibility of chronic MWCNT toxicity, our findings indicated that there was a low possibility of MWCNTs inducing severe chronic adverse effects on the lung.

Declaration of interest

This study was funded by a grant, 'Evaluating risks associated with manufactured nanomaterials' (no. P06041), from the New Energy and Industrial Technology Development Organization (NEDO) of Japan. Intratracheal instillation tests were performed at the Kashima Laboratory, Mitsubishi Chemical Medience Corp, Kashima, Japan, and inhalation exposure tests for the validation of analysis were performed at the Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Kitakyusyu, Japan. The authors report no conflicts of interest.

References

Akiyama I, Ogami A, Oyabu T, Yamato H, Morimoto Y, Tanaka I. 2007. Pulmonary effects and biopersistence of deposited silicon carbide whisker after 1-year inhalation in rats. *Inhal. Toxicol.* **19**: 141–147.

Allen TM, Cullis PR. 2004. Drug delivery systems: entering the mainstream. *Science* **303**: 1818–1822.

Boncel S, Muller KH, Skepper JN, Walczak KZ, Koziol KKK. 2011. Tunable chemistry and morphology of multi-wall carbon nanotubes as a route to non-toxic, theranostic systems. *Biomaterials* **32**(30): 7677–7686.

Brain JD, Knudson DE, Sorokin SP, Davis MA. 1976. Pulmonary distribution of particles given by intratracheal instillation or by aerosol inhalation. *Environ. Res.* **11**(1): 13–33.

Chen B, Liu Y, Song WM, Hayashi Y, Ding XC, Li WH. 2011. *In vitro* evaluation of cytotoxicity and oxidative stress induced by multiwalled carbon nanotubes in murine RAW 264.7 macrophages and human A549 lung cells. *Biomed. Environ. Sci.* **24**(6): 593–601.

Cheng C, Muller KH, Koziol KKK, Skepper JN, Midgley PA, Welland ME, Porter AE. 2009. Toxicity and imaging of multi-walled carbon nanotubes in human macrophage cells. *Biomaterials* **30**(25): 4152–4160.

Czarny B, Georgin D, Berthon F, Plastow G, Pinault M, Patriarche G, Thuleau A, L'Hermite MM, Taran F, Dive V. 2014. Carbon nanotube trans location to distant organs after pulmonary exposure: insights from *in situ* ^{14}C-radiolabeling and tissue radioimaging. *ACS Nano* **8**: 5715–5724.

De Jong WH, Hagens WI, Krystek P, Burger MC, Sips AJAM, Geertsma RE. 2008. Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. *Biomaterials* **29**: 1912–1919.

Deng X et al. 2007. Translocation and fate of multi-walled carbon nanotubes in vivo. *Carbon* **45**(7): 1419–1424.

Donaldson K, Murphy FA, Duffin R, Poland CA. 2010. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. *Part Fibre Toxicol* **7**: 5.

Fraczek-Szczypta A, Menaszek E, Stanislaw B. 2011. Some observations on carbon nanotubes susceptibility to cell phagocytosis. *J. Nanomater.* **473516**: 1–8.

Hesterberg TW, Hart GA. 2000. Lung biopersistence and in vitro dissolution rate predict the pathogenic potential of synthetic vitreous fibers. *Inhalation Toxicology* **12**: 91–97.

Hiraiwa Y, Uchida A, Yoshida H. 1960. *Reproduction and Anatomy of Rat*. Nakayama Shoten Co. Ltd., Tokyo, Japan. [in Japanese]

Hirano S, Kanno S, Furuyama A. 2008. Multi-walled carbon nanotubes injure the plasma membrane of macrophages. *Toxicol. App. Pharmacol.* **232**(2): 244–251.

Hirano S, Fujitani Y, Furuyama A, Kanno S. 2010. Uptake and cytotoxic effects of multi-walled carbon nanotubes in human bronchial epithelial cells. *Toxicol. App. Pharmacol.* **249**(1): 8–15.

Huang YW, Wu C, Aronstam RS. 2010. Toxicity of transition metal oxide nanoparticles: recent insights from *in vitro* studies. *Materials* **3**: 4842–4859.

IARC. 2002. Man-made vitreous fibres. *IARC Monographs on the Evaluation of Carcinogenic Risk to Humans*. Vol. 81, International Agency for Research on Cancer (IARC), Lyon, France.

Javey A, Guo J, Wang Q, Lundstrom M, Dai H. 2003. Ballistic carbon nanotube field-effect transistors. *Nature* **424**: 654–657.

Jia G, Wang HF, Yan L, Wang X, Pei RJ, Yan T, Zhao YL, Guo XB. 2005. Cytotoxicity of carbon nanomaterials: Single-wall nanotube, multi-wall nanotube, and fullerene. *Environ. Sci. Tech.* **39**(5): 1378–1383.

Kam NW, O'Connell M, Wisdom JA, Dai H. 2005. Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction. *Proct. Natl. Acad. Sci. USA* **102**(33): 11600–11605.

Kobayashi N, Naya M, Endoh S, Maru J, Yamamoto K, Nakanishi J. 2009. Comparative pulmonary toxicity study of nano-TiO_2 particles of different sizes and agglomerations in rats: Different short- and long-term post-instillation results. *Toxicology* **264**(1–2): 110–118.

Kobayashi N et al. 2010. Biological response and morphological assessment of individually dispersed multi-wall carbon nanotubes in the lung after intratracheal instillation in rats. *Toxicology* **276**(3): 143–153.

Kouno K; Ito R, Sakamoto H. 2006. *Anatomical Science*. Ishiyaku Pub, Inc., Tokyo, Japan. [in Japanese]

Leeuw TK et al. 2007. Single-walled carbon nanotubes in the intact organism: near-IR imaging and biocompatibility studies in Drosophila. *Nano Lett.* **7**(9): 2650–2654.

Li JG et al. 2007. Comparative study of pathological lesions induced by multiwalled carbon nanotubes in lungs of mice by intratracheal instillation and inhalation. *Environ. Toxicol.* **22**(4): 415–421.

Lightowler NM, Williams JR. 1969. Tracheal mucus flow rates in experimental bronchitis in rats. *Brit. J. Exp. Pathol.* **50**: 139–149.

Liu Z et al. 2007. *In vivo* biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. *Nat. Nanotechnol.* **2**(1): 47–52.

Liu Z et al. 2008. Drug delivery with carbon nanotubes for *in vivo* cancer treatment. *Cancer. Res.* **68**(16): 6652–6660.

Luo M, Deng XY, Shen XZ, Dong L, Liu YF. 2012. Comparison of cytotoxicity of pristine and covalently functionalized multi-walled carbon nanotubes in RAW 264.7 macrophages. *J. Nanosci. Nanotech.* **12**(1): 274–283.

Ma-Hock L et al. 2009. Inhalation toxicity of multiwall carbon nanotubes in rats exposed for 3 months. *Toxicol. Sci.* **112**(2): 468–481.

Marquis BJ, Love SA, Braun KL, Haynes CL. 2009. Analytical methods to assess nanoparticle toxicity. *Analyst* **134**(3): 425–439.

Mercer RR et al. 2013. Extrapulmonary transport of MWCNT following inhalation exposure. *Part. Fibre. Toxicol.* **10**: 38.43.

Mills NL, Amin N, Robinson SD, Anand A, Davies J, Patel D, de la Fuente JM, Cassee FR, Boon NA, MacNee W, Millar AM, Donaldson K, Newby DE. 2006. Do inhaled carbon nanoparticles translocate directly into the circulation in humans? *AM. J. Respir. Crit. Care. Med.* **173**(4): 426–431.

Mitchell LA et al. 2007. Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes. *Toxicol. Sci.* **100**(1): 203–214.

Morimoto Y. 2005. Inhalation Toxicity Assessment of Carbon-Based Nanoparticles. *Acc. Chem. Res.* **46**(3): 770–781.

Morimoto Y et al. 2012. Pulmonary toxicity of well-dispersed multi-wall carbon nanotubes following inhalation and intratracheal instillation. *Nanotoxicology* **6**(6): 587–599.

Muller J et al. 2005. Respiratory toxicity of multi-wall carbon nanotubes. *Toxicol. Appl. Pharmacol.* **207**(3): 221–231.

Muller J et al. 2009. Absence of carcinogenic response to multiwall carbon nanotubes in a 2-year bioassay in the peritoneal cavity of the rat. *Toxicol. Sci.* **110**(2): 442–448.

Nagai H et al. 2011. Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis. *Proct. Natl. Acad. Sci. USA* **108**(49): E1330–E1338.

Nakanishi J (ed.) 2011. Risk Assessment of Manufactured Nanomaterials: Carbon Nanotubes (CNT). Final report issued on August 17, 2011. NEDO project (P06041) "Research and Development of Nanoparticle Characterization Methods."

Noël A, Charbonneau M, Cloutier Y, Tardif R, Truchon G. 2013. Rat pulmonary responses to inhaled nano-TiO_2: effect of primary particle size and agglomeration state. *Part. Fibre Toxicol.* **10**: 48.

Ohnishi M, Yajima H, Kasai T, Umeda Y, Yamamoto M, Yamamoto S, Okuda H, Suzuki M, Nishizawa T, Fukushima S. 2013. Novel method using hybrid markers: development of an approach for pulmonary measurement of multi-walled carbon nanotubes. *J. Occup. Med. Toxicol.* **8**: 30.

Oyabu T et al. 2011. Biopersistence of inhaled MWCNT in rat lungs in a 4-week well-characterized exposure. *Inhal. Toxicol.* **23**(13): 784–791.

Palomaki J, Valimaki E, Sund J, Vippola M, Clausen PA, Jensen KA, Savolainen K, Matikainen S, Alenius H. 2011. Long, needle-like carbon nanotubes and asbestos activate the NLRP3 inflammasome through a similar mechanism. *ACS Nano* **5**(9): 6861–6870.

Pauluhn J. 2010. Subchronic 13-week inhalation exposure of rats to multiwalled carbon nanotubes: toxic effects are determined by density of agglomerate structures, not fibrillar structures. *Toxicol. Sci.* **113**(1): 226–242.

Poland CA et al. 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nat. Nanotechnol.* **3**(7): 423–428.

Searl A et al. 1999. Biopersistence and durability of nine mineral fibre types in rat lungs over 12 months. *Ann. Occup. Hyg.* **43**(3): 143–153.

Shinohara N et al. 2010. Clearance kinetics of fullerene C_{60} nanoparticles from rat lungs after intratracheal C_{60} instillation and inhalation C_{60} exposure. *Toxicol. Sci.* **118**(2): 564–573.

Silva RM, Doudrick K, Franzi LM, Teesy C, Anderson DS, Wu ZQ, Mitra S, Vu V, Dutrow G, Evans JE. 2014. Instillation versus inhalation of multiwalled varbon nanotubes: exposure-related health effects, clearance, and the role of particle characteristics. *ACS Nano* **8**(9): 8911–8931.

Tamura M et al. 2011. A determination method of pristine multiwall carbon nanotubes in rat lungs after intratracheal instillation exposure by combustive oxidation-nondispersive infrared analysis. *Talanta* **84**(3): 802–808.

Wick P, Manser P, Limbach LK, Dettlaff-Weglikowska U, Krumeich F, Roth S, Stark WJ, Bruinink A. 2007. The degree and kind of agglomeration affect carbon nanotube cytotoxicity. *Toxicol. Lett.* **168**: 121–131.

Yeh HC, Schum GM, Duggan MT. 1979. Anatomic models of the tracheo-bronchial and pulmonary regions of the rat. *Anat. Rec.* **195**: 483–492.

Zhang T, Tang M, Kong L, Li H, Zhang T, Zhang SS, Xue YY, Pu YP. 2012. Comparison of cytotoxic and inflammatory responses of pristine and functionalized multi-walled carbon nanotubes in RAW 264.7 mouse macrophages. *J. Hazard. Mater.* **219**: 203–212.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.