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Review

Hazard Assessments of Manufactured Nanomaterials

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Abstract: Hazard Assessments of Manufactured Nanomaterials: Yasuo MORIMOTO, *et al*. Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Japan—Background: It has been difficult to make reliable hazard assessments of manufactured nanomaterials, because the nanomaterials form large agglomerations in both *in vitro* and *in vivo* studies. **Objective:** In the New Energy and Industrial Technology Development Organization (NEDO) Project of Japan, the physicochemical properties of many manufactured nanomaterials are being measured, and *in vitro* and *in vivo* studies are being performed to determine which endpoints are correspond to the hazards and risks of nanomaterials. Focusing on titanium dioxide, fullerenes and carbon nanotubes, we introduce findings made in inhalation and intratracheal installation studies overseas, and together with the findings made in the NEDO project, and also assess the hazards presented by manufactured nanomaterials. **Results and Conclusion :** A project by NEDO has succeeded in ensuring the stability of dispersion (nanoscale <100 nm) of manufactured nanomaterials, and is developing hazard assessments of manufactured nanomaterials. In these interim reports, the acceptable exposure concentration of titanium dioxide and fullerene was proposed to be 1.2 mg/m^3 and 0.8 mg/m^3 respirable dust in working environment, respectively.
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The definition of nanomaterials as presented by the International Organization for Standards in ISO/TS 27687 is: nano-objects with at least one of their three dimensions in the range of 1–100 nm and nano-structured materials comprised of such nano-objects[1)]. Structures which are intentionally produced are manufactured nanomaterials, and it can be well-envisaged that their applications will permeate throughout the society in the future. However, it has been reported that fine and ultrafine particles such as particle matter 2.5 (PM2.5) and diesel exhaust particles were found in the organs which were not direct exposure sites, e.g. brain and testes, and that the particles induced the inflammation of the lungs in animal studies[2–5)]. The health effects of manufactured nanomaterials need to be paid attention because of particle size with similarity to PM2.5 and diesel exhaust particles[2–5)]. Furthermore, it has been reported that manufactured nanomaterials induced greater inflammation than submicron particles and that the nanoparticles were also transported to organs receiving no direct exposure[3, 6)].

Direction of Global Research

In risk and hazard assessments of manufactured nanomaterials, the importance of characterization of physicochemical properties is receiving much attention. The various physicochemical properties such as dimension, surface area, primary diameter, agglomerativity, components, solubility, crystalline structure, surface charge and zeta potential have been reported[7–10)]. However, the first report of property with biological effects was by Oberdorster *et al.*[7)], who reported that specific surface area was associated with infiltration of neutrophils in the lung. In their study, rats were intratracheally instilled with titanium dioxide (TiO_2) particles with primary diameters of submicron and nano sizes, and infiltration of neutrophils into the lungs was higher for nanoparticles than for submicron particles at same mass doses. Also, if the specific surface areas of

the particles were calculated in the same experiment, the specific area correlated linearly with the population of neutrophils in bronchoalveolar lavage fluid (BALF) irrespective of the particle size. Furthermore, specific surface area correlated with the incidence of lung tumors in separate long-term inhalation studies of six materials including TiO_2 and carbon black [11]. There are many reports to support of the "nanoparticle paradigm," that the specific surface area of particles regulates the pulmonary response. On the other hand, Warheit *et al.*[12, 13], using TiO_2 and silica in intratracheal installation studies, reported that the pulmonary response is not only dependent on particle diameter and specific surface area, but also on surface activity. Furthermore, Poland *et al.*[14] demonstrated the "fiber paradigm," that the dimensions of manufactured nanomaterials, especially their length, are associated with their hazards. They performed an intraperitoneal injection study using multiwall carbon nanotubes (MWCNTs), and showed that long MWCNT fibers caused the infiltration of inflammatory cells in the peritoneal cavity and the formation of granulomas. In respect of length, hazard research of fibrous materials such as asbestos has been continuing for a long time, and it has demonstrated that the poor solubility of fibers greater than 20 μm in length results in extension of their half-life in the lung, and that these long fibers are correlated with lung disorders[15]. Although it has been reported that some physicochemical properties of nanomaterials are associated with biological effects, the properties are not entirely consistent. For example, short fibers will have the potential with a greater inflammation from the point of nanoparticle paradigm, because short fibers rather than long ones have a larger specific surface area. However, this is the converse of the fiber paradigm. These being the present circumstances, we consider that there is no one parameter that we can explain all of the adverse effects. Accordingly, it is necessary to analyze many physicochemical properties through many *in vitro* and *in vivo* studies, and to examine what endpoints are correspond to pathophysiological responses *in vitro* and *in vivo* studies and what parameters can properly reflect these hazards.

Research in Japan

It is very important to identify the physicochemical properties of nanomaterials, however there is a very big problem. That is the agglomeration of nanoparticles. It is very difficult to disperse the nanomaterials because of Van Der Waals forces and electrostatic effects. If the nanomaterials are not dispersed, it can not be decided whether or not the measured physicochemical properties of manomaterials are accurate, and whether or not exposure to nanomaterials induced reliable pathophysiological responses *in vitro* and *in vivo* studies. Among Japanese national projects on the hazards and risks of manufactured nanomaterials, the New Energy and Industrial Technology Development Organization (NEDO) five-year plan project, mainly involving the National Institute of Advanced Industrial Science and Technology (AIST) and the University of Occupational and Environmental Health Japan (UOEH), began in 2006 [16]. In this project, the physicochemical properties of manufactured nanomaterials are being investigated in detail, and hazard and risk assessments are being developed through *in vitro* and *in vivo* studies using those nanomaterials. Among the physicochemical properties, a particular effort is being made with regard to the dispersibility of particles. Because the nanoparticles have agglomerated in the airways and parts of the lungs and have induced unusual pathophysiological responses in the lungs *in vivo* studies[17, 18]. Additional reasons why dispersed manufactured nanoparticles should be used in hazard assessments are: 1) discrete nanoparticles exist in the gas phase at the time of their creation, even though it is only a short time; 2) nanoparticles are discrete in the organic phase[19]; 3) dispersion of nanoparticles with high efficacy is one of objectives of drug delivery systems[20]; 4) dispersed or individual nanoparticles have been found in organs other than the exposure target[21, 22]; 5) many dispersed nanoparticles have been found in lung tissues obtained from patients with pulmonary fibrosis due to welding work[23]; 6) discrete and dispersed particles are observed in experimental working environments where nanomaterials are handled[24]. On the other hand, there are many reports that agglomerated nanoparticles induced the pulmonary inflammation which is not observed with micron-size particles at same mass dose[25]. However, even if dispersed particles have been found in animal studies exposed to agglomerated nanoparticles, the possibility of insufficient responses against the responses of dispersed nanoparticles cannot be denied. In fact, the bacterial toxicity of individually dispersed carbon nanotubes has been reported to be greater than that of agglomerated carbon nanotubes[26]. Also, there might have been instances in animal studies exposed to agglomerated nanoparticles, in which the transport of nanoparticles to unexposed organs was not observed. However, we can not deny the possibility that the transport of nanoparticles via the blood vessels and lymphatic duct might not be sufficiently done due to their lack of dispersion. Even if the results of experiments using agglomerated particles were the same as those using dispersed particles, these uncertainties cannot be dispelled without performing studies using dispersed particles. For these reasons, researches utilizing dispersed nanoparticles are exceedingly significant in the hazard and risk assessments of manufactured nanomaterials. As many physicochemical properties of manufactured nanomaterials are measured, and the same manufactured nanomaterials were used through *in vitro* and *in vivo* studies in the NEDO project, we proceed to establish the

reliable endpoints, associated with the hazards of the nanomaterials, and to determine the physicochemical properties which reflect the hazard effects. We consider that this hazard assessment system will make it possible to forecast the hazards of newly created manufactured nanomaterials through analysis of their physicochemical properties.

Hazard and Risk Assessment Reports of Manufactured Nanomaterials

TiO_2 Nanoparticles

TiO_2 nanoparticles, because they have larger surface area per unit mass than microparticles and their catalytic activity is strong, are used in photocatalysts and also in cosmetics because of their high transparency. On the other hand, compared to microparticles, TiO_2 nanoparticles cause greater inflammatory responses and when inhaled nanoparticles are not recognized by alveolar macrophages, suggesting the possibility that nanoparticles remain in the lung for a long time[7, 27]. From the perspective of the nanoparticle paradigm described above, we review some of the original research papers regarding the comparison of the hazards of TiO_2 nanoparticles with that of TiO_2 microparticles. Oberdörster *et al*.[28, 29] performed intratracheal instillation and inhalation studies in rats using TiO_2 particles of two different sizes. They reported that TiO_2 particles with a smaller diameter caused a greater pulmonary inflammatory response at same mass burden, and that their retained amounts in the lungs were also larger. If the doses were expressed as surface area rather than mass in the experiment, the fact that the dose-response relationship appeared as a straight line, led to the opinion that larger surface area is a factor in the strong inflammatory response to TiO_2 nanoparticles[7, 27, 30]. Bermudez *et al*.[31, 32] compared the results of a 13-week inhalation study of TiO_2 nanoparticles in rat, hamster and mouse with previous reports of TiO_2 microparticles. They reported, that at the same mass-based exposures, greater pulmonary inflammatory responses were seen in the groups exposed to TiO_2 nanoparticles than in the groups exposed to TiO_2 microparticles, and concluded that differences in surface area were the cause of this result. Furthermore, Tran *et al*.[33] reported in the results of inhalation studies of $BaSO_4$ and TiO_2, that if surface area is used as the exposure metric, then the dose-response relationships for both compounds were equivalent. Maynard and Kuempel, expressed the opinion that surface area was appropriate as an dose metric for low solubility and low toxicity particles, including TiO_2[34]. United States National Institute for Occupational Safety and Health (NIOSH) has also expressed the same opinion, proposing in November 2005 that the recommended exposure limit (REL) for TiO_2 nanoparticles, 0.1 mg/m^3, which is 15 times more severe than that for TiO_2 microparticles[11]. Subsequent to that proposal, NIOSH researchers reported that biological effects differ with particle size and that these differences can be explained by the differences in surface area[36].

On the other hand, there are some studies in which the hazards posed by TiO_2 nanoparticles to the lungs are comparatively no different from those of TiO_2 microparticles. Warheit *et al*.[36] performed a intratracheal instillation study in rats comparing several types of TiO_2 nanoparticles and microparticles with different sizes, surface areas and crystal structures. In the comparison among the exposure groups, even though the greatest difference in surface areas was of the order of 30-fold, the observed pulmonary inflammatory responses were almost the same, and concluded that the toxicities are not dependent upon particle size or surface area. Furthermore, the same research group, in discussing the results of intratracheal instillation studies in rats using TiO_2 nanoparticles and crystalline silica particles of different sizes and crystal structures, expressed the view that toxicity was dependent on particle surface properties[12, 13]. Also, in an *in vitro* study using human dermal fibroblasts (HDF) and immortalized human lung epithelial cells (A549) exposed to TiO_2 particles of different crystal structures, Sayes *et al*.[37] showed that the influence of size on toxicity was small, and that the crystal structure was the more important factor.

As noted above, many toxicologists are engaged in research into the biological effects of TiO_2 nanoparticles, which has so far produced a diverse range of results and conclusions lacking in definitive agreement. One of the possible reasons for disagreement is that many studies have been performed using particles with different particle size, surface properties, impurities and crystal structures, involving complex interactions among many factors, and it has been difficult to separate the sole effects of particle size and surface area among many factors. For example, in the research of Bermudez *et al*.[31, 32] and Sager *et al*.[35] the TiO_2 nano- and micro-particles used came from different sources and had different crystal structures. Both used the 80% anatase –20% rutile form of TiO_2 particles, generally known by the product name P25, made by Evonik as TiO_2 nanoparticles. On the other side, rutile TiO_2 particles produced by DuPont[32] or Sigma Aldrich[35] were used as TiO_2 microparticles. Also, in some studies, measurements of the particles used in the studies were insufficient, making it all the more difficult to validate the nanoparticle paradigm. For example, there are many reports in which the raw material particle size (primary diameter) is measured, but the actual agglomerated diameter (secondary diameter) in the liquid or air of the experiment is not[10]. Furthermore, even if secondary diameter was measured, the studies were mostly performed using particles agglomerated at micron or submicron sizes. There is uncertainty whether or not the results reflect sufficiently the biological effects of TiO_2

nanoparticles.

In the NEDO project, intratracheal instillation experiments with nano-TiO_2 particles of different primary sizes (primary diameter: 5, 23 and 154 nm) and agglomerations (secondary diameter: 18, 65 and 300 nm) but the same manufacturer, manufacturing method, and crystalline structure (100% anatase) were conducted in rats to compare the biological responses in the lungs and other tissues of rats exposed to the different particle types. As a result, pulmonary inflammation recovered in all the TiO_2 particle-exposed groups until 1 wk to 1 mo post-instillation. There were no differences in recovery of the pulmonary inflammations between the groups, regardless of particle size. However, when focusing on the short-term effects up to 1 wk post-instillation, small differences between exposure groups were observed, and the inflammatory responses showed a tendency to be greater in the groups exposed to TiO_2 particles with smaller primary diameters; whereas, when the responses of rat groups exposed to TiO_2 particles of the same primary diameter with different agglomerations were compared in the period up to 1 wk post-instillation, almost no differences were found. In summary, pulmonary effects of the primary diameter of the particles were observed, but these effects were limited to results observed in the very short-term, in the period 1 wk after intratracheal instillation. Despite differences in particle surface areas of as much as 30 times, the differences in effect were not large. The results noted above are the assessment results for nano-sized TiO_2 particles from one company. It is difficult to extend these results to all TiO_2 particles. Therefore, we consider that particle size and surface area as a dose metric for hazard assessment cannot solely be used to make a complete hazard prediction, and that case by case assessments are necessary.

In the Risk Assessment of Manufactured Nanomaterials -TiO_2- Executive Summary (Interim Report) compiled by the NEDO project, provisional values of acceptable exposure concentration to TiO_2 nanoparticles in working environments are proposed[39]. The no observed adverse effect level (NOAEL) for experimental animals was determined on the basis of the results of previous TiO_2 nanoparticle inhalation studies, and extrapolated and evaluated for humans. Although responses to TiO_2 nanoparticles differ among different particle types, the acceptable exposure concentration with regard to P25 TiO_2 nanoparticles was estimated to be 1.2 mg/m^3 respirable dust as a time-weighted average in the case of a hypothetical 8-hour day, 5-day working week. Also, it was estimated that the pulmonary inflammatory responses of TiO_2 nanoparticles other than P25 are ranging from 0.03 to 0.3 folds from comparisons of the results of intratracheal instillation studies without no data with inhalation studies, and the acceptable exposure concentration was estimated based on the relative values of these hazards (Fig. 1). We call this hazard assessment methods "by-axial approach." It must be noted, however, that the estimated values and assessment methods of acceptable exposure concentrations in the by-axial approach are provisional measures, and there is a possibility that the values will be changed to take account of methodological improvements or new scientific findings in the Final Report which is scheduled for release in 2011.

Fullerenes

Fullerenes are carbon allotropes with spherical structures[40]. A representative fullerene, the C_{60} molecule, is shaped like a soccer ball and has a diameter of approximately 1 nm. It is one of the representative nanomaterial, but actually it often exists as crystals much larger than 100 nm. Fullerenes are currently used in sports goods and cosmetics and they are expected to have wide applications in other industries such as solar cells and pharmaceuticals.

In initial hazard assessments, there were many reports of fullerenes having high cytotoxicity, but this was due to impurities in tetrahydrofuran (THF) which was used to disperse the fullerenes[41, 42]. Recently, in the hazard test using C_{60} suspensions prepared by continuous stirring for a long duration or grinding with surfactant such as Tween 80 and carboxymethyl cellulose sodium (CMC-Na), no significant pulmonary toxicity or cytotoxicity have been observed.

Sayes *et al.*[43] reported on single intratracheal instillations of C_{60} suspensions (160 ± 50 nm diameter) and $C_{60}(OH)_{24}$ suspensions (unspecified size) at 0.2, 0.4, 1.5 and 3.0 mg/kg to rats. Increased neutrophil numbers in BALF and significant inflammation were observed at 24 h after administration of either C_{60} or $C_{60}(OH)_{24}$, but the responses were not persistent. As for lipid peroxidation in BALF, significant increases were observed at 1 day and 3 mo after C_{60} administration at doses of 1.5 and 3.0 mg/kg, whereas no significant effects were observed for $C_{60}(OH)_{24}$. Park *et al.* [44] reported intratracheally instilled C_{60} (2 mg/kg) increased in the expression of proinflammatory cytokines and Th1 cytokines in BALF. In an inhalation study, Baker *et al.*[45] performed a pulmonary toxicity assessment on rat for a 7-day observation period after 10 days inhalation exposure using nano-sized (2.22 mg/m^3, 55 nm) and micron-sized (2.35 mg/m^3, 930 nm) C_{60} particles prepared by sublimation and agglomeration. For the nano-sized fullerenes, no effects were observed in cell types and cytokines in BALF other than a rise in protein concentration. Their report is the only report of a nano-scale exposure study of C_{60}, and is very valuable as the examination of toxicity with short-term observation.

Analysis of the kinetics of nanoparticles inside the body is also an important factor for the toxicity assessment. The percentage of airborne C_{60} nanoparticles deposited

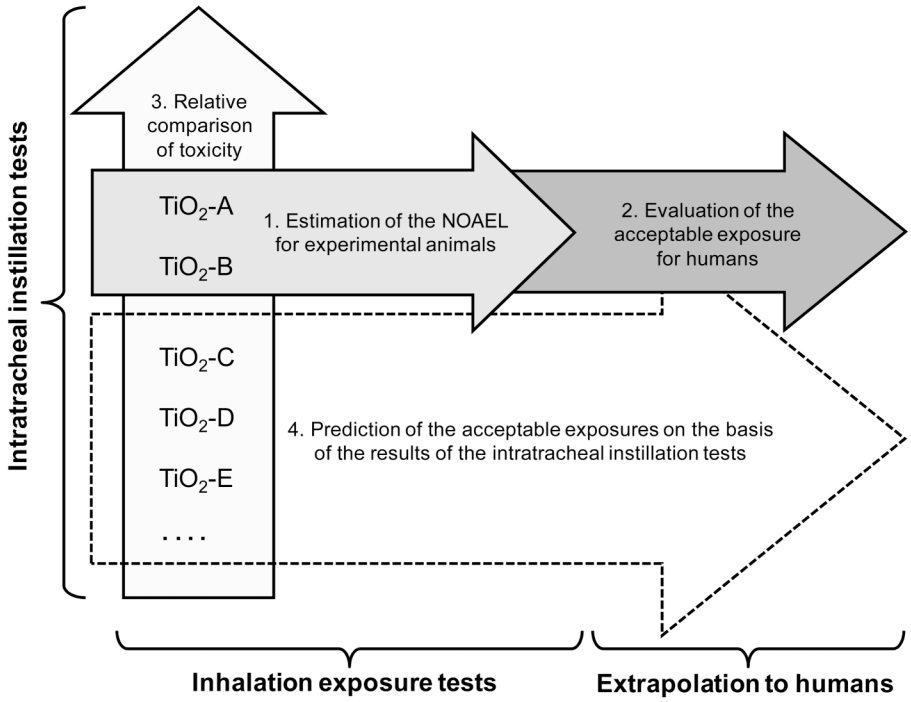

Fig. 1. The by-axial approach to estimate the acceptable exposure concentrations. In this approach, the first step is to estimate the NOAEL for experimental animals on the basis of the results of the inhalation exposure tests for certain types of TiO_2 nanomaterials (1). The second step involves the estimation of the acceptable exposures of TiO_2 nanomaterials for humans by extrapolating the NOAEL for experimental animals to humans taking into consideration the uncertainty factor (UF), which will be described later (2). Next, information on the relative values of toxicity of TiO_2 particles is obtained by comparing the test results of TiO_2 particles for which there are results from the intratracheal instillation test but not from the inhalation exposure test (3). The final step is to combine the data on acceptable exposures of certain types of TiO_2 particles for humans (obtained in 3) and relative values of toxicity (obtained in 3) to calculate provisional values of the acceptable exposures of TiO_2 nanomaterials for which there are only results from the intratracheal instillation test (4).

on the alveolar area and their transport from the lungs to the other organs have been calculated using the aerosol deposition model and the analysis of the kinetics. In some reports, C_{60} particles were detected in the liver and spleen after intraperitoneal or intravenous injection[46–48], but there are no reports on the translocation of C_{60} particles to the brain. Clearance of C_{60} from the blood after an intravenous injection was extremely fast, taking place within 1 min, and most of cleared C_{60} was accumulated in the liver, where it remained for over 120 h[48].

In the NEDO project intratracheal instillation and inhalation exposure studies on rat have been performed using C_{60} suspension stably dispersed as nanoparticle (90%ile particle size based on volume: <100 nm). Fullerenes are suspended in aqueous Tween 80 solution, stirred and ground in a bead mill under nitrogen, centrifuged and filtered[49]. These fullerenes have a crystal structure with a volume median particle diameter of 33 nm. Their size and concentration were confirmed to be stable for 3 wk. Morimoto *et al.*[50] performed single intratracheal instillations of fullerenes at 0.1, 0.2 or 1.0 mg (0.33, 0.66 or 3.3 mg/kg) to Wistar rats followed by observation for 6 mo using the C_{60} suspension. At the high dose (3.3 mg/kg), pulmonary inflammation mainly by neutrophils was observed in BALF and lung, and the expression of heme-oxygenase-1 (HO-1) was also increased, but both findings were transient and mild in severity. At the low doses (0.33 and 0.66 mg/kg), pulmonary inflammation was not observed. Furthermore, at all doses, there were no findings of granuloma, fibrotic change, or emphysematous change.

Inhalation exposure studies of C_{60} on mice and rats have also taken place by Yokoyama *et al.*[51] and Morimoto *et al*[50]. Using the same dispersed fullerene suspension as used in the intratracheal instillation study, Shimada *et al.*[52] succeeded in stabilizing the particle concentration and nanoscale sizes of airborne fullerenes with a pressurized nebulizer. In the inhalation test of an aerosol of C_{60} (geometric mean diameter, 86 nm; particle concentration, $1.6 \times 10^5/cm^3$) on ICR mice, the electron paramagnetic resonance at 700 MHz confirmed the oxidation-reduction ability Nickel oxide nanoparticles as

a positive control, but not that of C_{60}[51]. Furthermore, Morimoto *et al.* [50] exposed Wistar rats to 0.12 mg/m^3 C_{60} aerosol (mean geometric diameter, 96 nm; particle concentration, 4.1×10^4/cm^3) for 6 h/day, 5 days/wk for 4 wk, followed by 3 mo observation. In the lungs there was only slight transient inflammation. Changes in the expression of HO-1 in lung and pathological changes in the other organs were not observed, being the same as the negative control.

In order to determine the kinetics of nanoparticles in the body, a high sensitivity analysis method for C_{60} has been developed, and applied to observations of internal migrations of C_{60} nanoparticles in rats following inhalation and intratracheal instillation[53]. In the results, airborne C_{60} nanoparticles showed a deposition fraction in the alveoli of 14%, and it was found that the amounts of inhaled and intratracheally instilled C_{60} nanoparticles migrating to the other organs from the lungs, such as the liver and brain, were below the detection limits: less than 0.2% of the administration dose for the liver, and less than 0.02% for the brain[53].

Using the results of the NEDO project inhalation studies, provisional acceptable exposure concentration for C_{60} nanoparticle was calculated [53]. Since any adverse effects in biomarkers were not seen in the 3 months after inhalation of 0.12 mg/m^3 C_{60} particles, the NOAEL on rat lungs was considered to be greater than 0.12 mg/m^3. Correcting for the exposure period and species differences, the working environment NOAEL for humans (*provisional* $NOAEL_{human\_work}$) was derived as follows:

$$\begin{aligned} provisional\,NOAEL_{\ human-work} &= NOAEL_{rat} \times \frac{t}{8} \times \frac{5}{5} \times \frac{f_r}{f_h} \times \frac{q_r}{q_h} \times \frac{S_h}{S_r} \\ &\Rightarrow 0.12[\mathrm{mg/m^3}] \times \frac{6}{8} \times \frac{5}{5} \times \frac{0.192}{0.198} \times \frac{0.301}{36} \times \frac{543200}{3422.5} \\ &\Rightarrow 0.12\ [\mathrm{mg/m^3}] \end{aligned}$$

where t is the exposure time in hours, f_r is the deposition fraction in rat lungs (dimensionless), f_h is the deposition fraction in human lungs (dimensionless), q_r is the rat respiration rate (m^3/day), q_h is the human respiration rate (m^3/day), S_r is the rat lung surface area (m^2), and S_h is the human lung surface (m^2). The exposure time, t, was the daily exposure period of Morimoto's experiment[50]; f_r and f_h are values calculated utilizing a multi-path particle dosimetry (MPPD) model; q_r, S_r and S_h were taken and calculated from United States Environmental Protection Agency data (1994); and q_h is the respiration rate for light work of 36 m^3/day.

Since the significant adverse effects of C_{60} were not observed even in intratracheal instillation studies when the intrapulmonary amounts found were more than 40 times those seen in inhalation studies, and applying this as a correction factor, as well as an uncertainty factor of 6 for estimation of a long-term index from short- or medium-term indices, the *provisional Threshold Level* (*TL*) was estimated as

$$\begin{aligned} provisionalTL &= \frac{provisionalNOAEL_{human}}{Uncertainty\ \ factor} \times correction\ \ \ factor \\ &= \frac{0.12\,[\mathrm{mg/m^3}]}{6} \times 40 \\ &= 0.8\,[\mathrm{mg/m^3}] \end{aligned}$$

implying an acceptable exposure concentration of 0.8 mg/m^3 in working environments for C_{60} fullerene nanoparticles having a geometric mean diameter of 96 nm.

It must be noted, however, that these estimation methods for acceptable exposure concentration to C_{60} fullerenes, as well as the estimated values, are provisional undertakings for the Interim Report, and it is possible that the values will be changed in the Final Report to take account of methodological improvements or new scientific findings.

Carbon Nanotubes

CNTs are fibrous materials formed from honeycomb crystal lattice layers of graphite wrapped into a tube shape either as a single layer or multi layers, which are respectively called single-wall carbon nanotubes (SWCNTs), and multi-wall carbon nanotubes (MWCNTs). CNTs, are classified as a type of nanomaterial because they have nanoscale diameters, and since CNTs have many outstanding physical and chemical properties, their applications and uses in various fields are being explored all over the world.

The main concerns regarding the hazards of CNTs arise not only from their nano-sized structures, but also from the fact that those structures are fibrous. Specifically, there is a concern that carbon nanotube may have hazards similar to asbestos.

At the present time, although there are many results for *in vivo* studies with MWCNTs, there are as yet few research reports for SWCNTs. In particular, there are several researches of *in vivo* inhalation studies, which are the "gold standard" for inhalation toxicity assessments[54–57], but for SWCNTs there is only one[58]. From the perspective of the fiber paradigm, we introduce the existing findings related to the hazards of CNTs, focusing on MWCNTs research reports.

In Japan, concerns rose about whether CNTs might have asbestos-like toxicities were raised by the publication of reports by Poland *et al.*[14] and Takagi *et al.*[59] in 2008. Takagi *et al.*[59] intraperitoneally administered a dose of 3 mg/mouse of MWCNTs in heterozygous p53+/– mice, and reported that in the

following 180 days, 14 of the 16 mice died as a result of malignant mesothelioma. However, because neither the dose used nor the administration route resembled actual exposure conditions, it cannot be determined whether or not MWCNTs have asbestos-like toxicities under realistic exposure conditions from these results. Poland *et al.* [14] made intraperitoneal administrations of 50 μg of MWCNTs, asbestos and carbon black in mice. They reported that at 1 day and 7 days following administration, significant inflammation was observed in the groups dosed with MWCNT with fibers more than 15 μm long, whereas inflammation was not observed in the groups dosed with MWCNTs not containing long fibers.

On the other hand, also in an intraperitoneal injection study, Muller *et al.*[60] obtained results which differed from those of Takagi *et al.*[59]. After intraperitoneally administering 2 or 20 mg/rat of MWCNTs, Muller *et al.* [60] observed the post-administration course for 2 yr at the longest, and reported no differences in the incidences of malignant mesothelioma between the MWCNT-exposed groups and the negative control. In the study 2 mg/rat of UICC crocidolite was administered as a positive control, and the incidence of malignant mesothelioma increased significantly in the asbestos-exposed group, showing differences in the biological responses between the MWCNTs and asbestos-exposed groups. The authors considered that the results may differ according to animal species and type of CNTs, and concluded that further research was necessary.

When pulmonary inflammatory responses are used as the toxicological endpoint, conflicting results have also been obtained for the relationship between the length of CNTs and their toxicities. Muller *et al.*[17] performed intratracheal instillation study of MWCNTs grounded by a ball mill, and reported that ground MWCNTs caused a greater inflammatory responses than raw MWCNTs at the same dose. In the results of the characterization of the MWCNT samples, the authors noted that the lengths of individual MWCNTs were greatly altered by grounding, from 5.9 μm to 0.7 μm, but the other main characteristics such as tube diameter and specific surface area were not changed by the operation. In addition, in a study by Mercer *et al.*[61], mice were subjected to pharyngeal aspiration exposure to dispersed SWCNTs (particle size, 0.69 μm) and non-dispersed SWCNTs (particle size, 1.52 μm), and it was reported that thickening of the alveolar wall was found only in the groups exposed to dispersed SWCNTs. The authors considered that the dispersed SWCNTs would more easily penetrate the interstitium. Because the dispersed SWCNTs would not be easily recognized by alveolar macrophages, it would suggest that dispersed and non-dispersed SWCNTs show qualitative differences in pulmonary effect.

As described above, there have been several investigations of the relationship between the length of CNTs and their toxicities. The results of these studies are disparate, and cannot all be explained by the fiber paradigm. Also, we can not say that the measurements of the carbon nanotube materials were sufficient in many of the previous hazard studies of CNTs. We consider it necessary to accumulate data from experiments using adequately characterized and size-controlled test materials in order to clarify the toxicity paradigm of CNTs. However, since there are big differences in properties due to the different production methods of CNT manufacturers, we consider that it will be difficult to arrive at one toxicity paradigm that will explain the hazards of all CNTs, rather that it will be necessary to perform individual assessments for each material.

In the Risk Assessment of Manufactured Nanomaterials –carbon nanotube- Executive Summary (Interim Report) of the NEDO project, the weighted time average acceptable exposure concentration for one type of MWCNT in working environments was estimated to be 0.21 mg/m^3, assuming exposures of 8 h/day, 5 days a week[62]. This value was based on the results of an original NEDO project inhalation study of aerosolized MWCNTs, dispersed as much as possible, which had been kept in a stable condition for one month[62]. Also, based on the methodology of the by-axial approach described above, from the performance of a relative comparison using the results of an intratracheal instillation study, a similar acceptable exposure concentration was inferred for another type of MWCNT in a study conducted in the NEDO project. However, these presumptive values for acceptable exposure concentrations derived using this method are provisional estimates as of October 2009, and there is a possibility that methodological improvements or new scientific findings will result in them being changed in the Final Report due for release in 2011. In addition, at present there is insufficient hazard data for CNTs, especially SWCNTs, and in order to perform quantitative risk assessments, it is desirable to accumulate more data from studies using adequately characterized test materials.

Japanese and Global Regulation

Even though manufactured nanomaterials have the same chemical composition as existing materials in the United States of America (USA) and European Union (EU), their manufacture and export is in the process of being regulated as new chemical substances. In the USA, four items, SWCNT, MWCNT, silica and aluminum nanoparticles, are being requested through 90-day inhalation studies[63] based on OECD guidelines under the Toxic Substances Control Act, administered by the Environmental Protection Agency. In the EU, the European Commission is indicating that manufactured nanomaterials will be regulated under the Registration,

Evaluation, Authorisation and Restriction of Chemicals (REACH), the same as ordinary chemical substances.

In Japan, the management of manufactured nanomaterials is indicated in reports, official notices and guidelines from the Ministry of Economy, Trade and Industry, the Ministry of Health Labour and Welfare, and the Ministry of the Environment. However, it is not an overstatement to say that there is no exposure index of high reliability for hazard and risk assessments of manufactured nanomaterials. In the NEDO Project, the physicochemical properties of many manufactured nanomaterials are being measured, and *in vitro* and *in vivo* studies are being performed to determine which endpoints are correspond to hazards and risks. It is thought that it will be possible to establish a highly reliable exposure index for manufactured nanomaterials through these efforts. We expect that this would greatly contribute to the establishment of acceptable exposure concentrations and administrative levels for manufactured nanomaterials.

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