-NEDO project "Research and Development of Nanoparticle Characterization Methods" (P06041)-

Risk Assessment of Manufactured Nanomaterials

-Fullerene (C_{60})-

Interim Report issued on October 16, 2009

Executive Summary

Naohide Shinohara · Masashi Gamo · Junko Nakanishi

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CONTENTS of Main document

On the Positioning of Interim Reports Released on October 16, 2009

One of the objectives of the project sponsored by New Energy and Industrial Technology Development Organization (NEDO), "Research and Development of Nanoparticle Characterization Methods", is to develop risk assessment of three different substance groups, TiO_2, C_{60}, and CNTs. The risks to be assessed are human health risks, with a primary focus on occupational risk management since the industries involving nanomaterials are still under development.

The scale of the industries handling nanomaterials at present is small, however, it is expected to be developed extensively in the future. The risk assessment of nanomaterials, therefore, is considerably different from those previously conducted by the National Institute of Advanced Industrial Science (AIST) on the substances with relatively long history of use and published in the Risk Assessment Series. The major difference is the emphasis on the framework to predict risks reflecting future changes of situations rather than presenting the fixed risk values based on the assessment of the available data. The changes of situations include the factors such as production volume, form of manufactured products, production methods, and methods of exposure management. These changes are technically defined as the changes of scenario.

Currently, with limited available data, it is not possible to develop hazard assessment and exposure assessment applicable to all the various scenarios. The only possible approach is to present a framework applicable to a number of substances and situations, with supplemental data generated by manufacturers. Such a framework is proposed in the interim reports.

Interim reports released on October 16, 2009 are the documentation of the current status in the process to develop final risk assessments. The purposes to release these interim reports include; firstly the conclusions obtained so far, though not final, are applicable to the management of occupational environment; and secondly, comments and advices are expected to be obtained on the released reports from many experts outside of the project, which would greatly contribute to improving the final outcomes of the risk assessment.

In these interim reports, the procedures to establish a provisional value of an acceptable exposure concentration in the occupational environment are presented. A method is proposed to establish an acceptable exposure concentration in those situations with a limited number of inhalation exposure studies. With TiO_2, a provisional value of an acceptable exposure concentration in the occupational environment is proposed. In the case of C_{60}, of which data with inhalation exposure studies is limited, only rough figures of acceptable exposure concentrations are estimated based on the comparison of particle burden in the lung between inhalation exposure and intratracheal instillation studies. In the final assessment, it is considered possible to propose standards of acceptable exposure concentrations with greater certainty by quantitative application of the data from intratracheal instillation studies. With CNTs, it has not been possible to

discuss standards of acceptable exposure concentrations in the interim report. The standards proposed in the interim reports are estimated primarily to prevent inflammation in the lung associated with inhalation exposure of particles. As described in “the principles and basic approaches to risk assessment of manufactured nanomaterials”, no review of carcinogenicity studies has been conducted, however, some effort has been made to detect signs of carcinogenicity with various methods. Though it is premature to conclude, the provisional values presented in the interim reports are applicable at this time to risk management, of measures to prevent inflammatory responses in the lung in situations without possible chronic exposures.

With regard to risk management, measures easily taken by manufacturers are those for exposure control. With reference to these interim reports, risk reduction can be achieved through careful and wise control of exposures. It is sincerely hoped that these interim reports contribute to the risk management at manufacturing sites.

Critical reviews and comments on the interim reports are greatly appreciated for the successful completion of our project.

Regrettably, the results of toxicity studies conducted under NEDO Project have not been fully utilized in these interim reports, but should be incorporated into the final reports of risk assessment

October 16, 2009

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Preface (Chapters I and II)

Fullerenes are carbon allotropes composed entirely of carbon, in the form of a hollow sphere, ellipsoid, or tube. The first fullerene was discovered by Prof. Kroto *et al.* in 1985. One of the representative fullerenes, C_{60} molecule has a form like a soccer ball with diameter of approximately 1 nm. Fullerenes have been considered as typical examples of nanomaterials, however, in reality they may exist as crystals much larger than 100 nm, and have many properties somewhat different from other nanomaterials. Since fullerenes are internationally recognized as nanomaterials/nanoparticles, in our risk assessment, all types of fullerenes are considered as nanomaterials irrespective of their status of crystallization and/or aggregation.

Due to their small particle sizes, it has been pointed out that nanoparticles may be systemically translocated in biological organisms after exposure, and persist for a long period without being eliminated by phagocytosis of macrophages. Also, it has been confirmed that inhaled nanoparticles reach pulmonary alveolus and may exert adverse effects in the lung and other organs. It has been reported that fullerenes may induce oxidative stress injuries with production of reactive oxygen species (ROS) such as singlet oxygen with their photoexcitation property, and the possibilities to induce various adverse effects have been suggested. Thus, risk assessment of fullerenes is warranted.

This executive summary includes the selected figures and tables from the main body of the risk assessment document. The numbers of figures and tables are the original numbers used in the risk assessment document, and thus, may not be sequential in this executive summary.

The contents of the main body of the risk assessment document (interim document) are as follows:

Chapter I: Background of the fullerene risk assessment, organization of the document, etc.

Chapter II: Physicochemical properties, production, use, analytical methods, environmental monitoring data, etc. of fullerenes

Chapter III: Exposure assessment of fullerenes by two different methods, i.e., one by exposure estimation based on the several existing exposure investigations, and the other by exposure estimation with the results of a model emission study and the expected future use volume;

Chapter IV: A comprehensive summary of human health hazard assessment, and the provisional NOAELs (No Observed Adverse Effect Level) of fullerenes obtained as the outcome of the human health hazard assessment. The NOAELs obtained with the available data are extremely low since the existing studies with fullerenes were conducted only at low dose levels. It is obvious and apparent that no adverse effects would be induced at these NOAELs, and thus, these levels are defined as provisional NOAELs and used in our risk assessment.

Chapter V: Human health risk assessment of fullerenes with the exposure levels obtained in Chapter III and

the provisional NOAELs obtained in Chapter IV.

Chapter VI: Conclusion of the fullerene risk assessment

Physicochemical Properties of Fullerenes

Representing fullerenes, physicochemical properties of C_{60} and C_{70} are presented in Table II-1.

Table II-1. Physicochemical properties of C_{60} and C_{70}

	C_{60}	C_{70}
Molecular Weight	720.66	840.77
Mass Number	720	840
Molecular Structure [nm] [a)]	0.704 (Frame) 1.002 (Electron Cloud)	0.796 (Transverse Diameter) 0.712 (Conjugate Diameter)
Electron Affinity [eV]	2.65	2.72
Crystal Structure [b), c)]	Face-Centered Cubic Lattice (>260K) Simple Cubic Lattice (Hyphthemic Phase)	Face-Centered Cubic Lattice, Trigonal Lattice, and Hexagonal Close-Packed Lattice at Transitional Phase
Mass Density [g/cm^3] [d)]	1.729 (5K Calculated Value)	1.6926 (Ambient Temperature)
Molecular Density [Molecule/cm^3] [d)]	1.44×10^{21}	No Data
Melting Point [℃] [e)]	1180	No Data
Electric Conductivity (300K) [S/cm] [f), g)]	$10^{-8} \sim 10^{-14}$	No Data
Sublimation Heat [kcal/mol] [h), i)]	40, 38	43, 45
Vapor Pressure [Torr] [i)]	1.9×10^{-5} (400 ℃) 5×10^{-4} (500 ℃) 1×10^{-3} (600 ℃)	1.4×10^{-5} (430 ℃) 2×10^{-4} (500 ℃) 7×10^{-3} (600 ℃)

References: a) Ahmad, 1999; b) Lichtenberger *et al.*, 1992; c) Beckhaus *et al.*, 1992; d) Heiney *et al.*, 1991; e) Fischer & Heiney, 1993; f) Arai *et al.*, 1992; g) Mort *et al.*, 1992; h) Pan *et al.*, 1994; i) Abrefah *et al.*, 1992

C_{60} molecular has a structure similar to a soccer ball with twelve pentagons and twenty hexagons. The length of single bond is 0.146 nm, and that of double bond, 0.140 nm. The diameter of carbon frame is 0.704 nm, and that of π electron cloud area (van der Waals diameter), 1.002 nm (Ahmad, 1999). The inside of C_{60} is approximately 0.4 nm of hollow space.

Dispersion of Aggregated Fullerenes in Solution

C_{60} spontaneously forms stable aggregates/agglomerates of nanoscale (25-500 nm) in solutions including water, acetanitril, ethanol, and acetone (Alargova *et al.*, 2001; Fortner *et al.*, 2005). Various methods to prepare aqueous dispersion of fullerenes are described:

i) Methods to prepare aqueous dispersion of C_{60} by mixing water to the solution of C_{60} in some organic solvents such as tetrahydrofrane (THF) (Deguchi *et al*., 2001), ethanol (Dhawan *et al.*,

2006), toluene and chloroform (Sera *et al.*, 1996) , and subsequently removing the solvent;

ii) Methods to disperse C_{60} mixed with a surfactant such as Tween 80 by ball mill or bead mill [Gharbi *et al.*, 2005; Endoh *et al.*, 2009 (NEDO Project); Shinohara *et al*., 2009 (NEDO Project)];

iii) Method to disperse C_{60} by grinding with sugar candy and polyoxythylene hydrogenated caster oil (Seki *et al.*, 2008); and

iv) Methods to disperse C_{60} by stirring aqueous solution for a long period (longer than two weeks)(Mchedlov-Petrossyan *et al.*, 1997; Brant *et al.*, 2006).

Photoexcitation Property

C_{60} and C_{70} have sequential conjugated π electron systems, and are subjected to π-π* excitation by optical irradiation to form singlet excited state ($^1C_{60}$*), and further to form triplet state ($^3C_{60}$*) by intersystem cross reaction (Arbogast *et al.*, 1991a; 1991b). It has been reported that $^3C_{60}$* is converted to C_{60}^{*-} with one-electron reduction by electron donating compounds (Type I electron transfer reaction), and produces singlet oxygen by energy transfer reaction (Type II energy transfer reaction), and that the yield rate of singlet oxygen with C_{60} is very high (Arbogast *et al.*, 1991a; 1991b). With this property, C_{60} has been applied and developed as n type organic semiconductor being used in solar butteries. At the same time, due to the ability to produce singlet oxygen with $^3C_{60}$* formed by optical irradiation and the reducing efficiency of C_{60}^{*-} , it has been suggested that C_{60} may induce adverse effects to organisms such as oxidative stress or DNA breakage.

Electronic Property/Radical Capturing Ability

Fullerenes have a high electron-accepting property due to their lowest unoccupied molecular orbital (LUMO) at low energy levels. C_{60} is an electron acceptor which can reversibly accept six electrons. Its oxidation-reduction potential, however, is lower compared to common electron acceptors. Fullerenes have sp2 orbitals spreading on their overall spherical structures. It is suggested that fullerenes easily undergo addition reactions with free radicals, etc. because of their higher reactivity with their strain of spherical shape compared to the plane state, and due to the fact that when one conjugate bond is attacked their structures are maintained with other bonds keeping six π electron systems.

Inclusion Property

Fullerenes have a cage-like structure with a vacuum hollow of 0.4 nm inside the structure, and are capable of trapping various metals. Chai *et al.* (1991) successfully produced a lanthanum-containing fullerene ($La@C_{82}$) by laser vaporization of a lanthanum oxide/graphite composite rod. To date, C_{70}, C_{74}, C_{80}, etc. containing various metals including cerium and calcium (More *et al.*, 1993), titan (Cao *et al.*,

2001), and barium (Reich *et al.*, 2004) have been produced and isolated.

Production, Analytical Method, and Fullerenes in the Environment

Practical application of fullerenes is still limited while various kinds of usage are expected to be developed. At present, it is estimated that approximately two tons of fullerenes are used in Japan annually, of which a larger amount is used for the purpose of research and development and a smaller amount is thought to be commercially distributed in the domestic market. It is predicted, however, that the production of fullerenes will reach 40 tones by 2010, if the future product development is successful. Therefore, the exposure assessment in Chapter III includes the assessment based on the assumed production volume in the future as well as that based on the results of the existing monitoring studies.

Generally, it is difficult to measure amounts of nanomaterials in media. Regarding fullerenes, however, it is possible to apply a method of chemical analysis. With application of both particle measurement and chemical analysis, it is considered possible to evaluate somewhat more precisely the emission, exposure, and kinetics in experimental animals of fullerenes compared to other nanomaterials.

Fullerenes are often considered as a typical example of nanomaterials, however, they are sometimes produced unintentionally, or existed naturally from primordial times as products resulted from meteorite impacts, thunder strokes, etc. (Heymann *et al.*, 1994; Daly *et al*., 1993)

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Exposure Assessment (Chapter III)

Exposures to fullerenes are estimated for risk assessment. First, the lifecycle of the major products are presented, and secondly the existing studies on fullerene exposures in Japan and globally are reviewed and summarized. Thirdly, the following exposure assessments are conducted based on the results of the monitoring at manufacturing sites, and a model emission experiment in a laboratory to simulate the particle emission with rolling dust, and with application of a simple concentration estimation model:

1) Exposure levels at fullerene manufacturing site;
2) Exposure levels at fullerene secondary product manufacturing site; and
3) Exposure levels in the general environment

The actual occupational exposure at the manufacturing site is considered lower than the estimated levels with use of masks, however, the estimation obtained is applied in the risk assessment as a worst-case. Regarding the general environment, a simple estimation is made on the atmospheric concentration around the area near the manufacturing site with the assuming the fullerene emission from the factory at the concentration level estimated in the manufacturing site.

Lifecycle and Emission of Fullerene

Lifecycle of Fullerene

The lifecycle of fullerenes from manufacturing to disposal is shown in Figure III-1. Fullerene exposures at local environment include inhalation and dermal exposures of workers during fullerene manufacturing and processing, and inhalation and dermal exposures of general consumers.

Emissions to the atmospheric environment are considered to include emissions from manufacturing sites during manufacturing and processing, those during consumption of products such as oils, and those during incineration after the product disposal. Emissions to the aquatic environment include water discharge from manufacturing and processing sites, leaching from landfill sites at the time of product disposal or after product incineration. Consumers are exposed via dermal and/or oral route to the fullerenes applied to pharmaceutical and cosmetic products, of which, however, exposures are not assessed as the nanomaterials intentionally applied or ingested are outside of the scope of this risk assessment. C_{60} is stable up to 300˚C even with the presence of enzymes, and thus, it is considered that emissions of C_{60} contained in resins and metals rarely happen except the cases of having physical impacts.

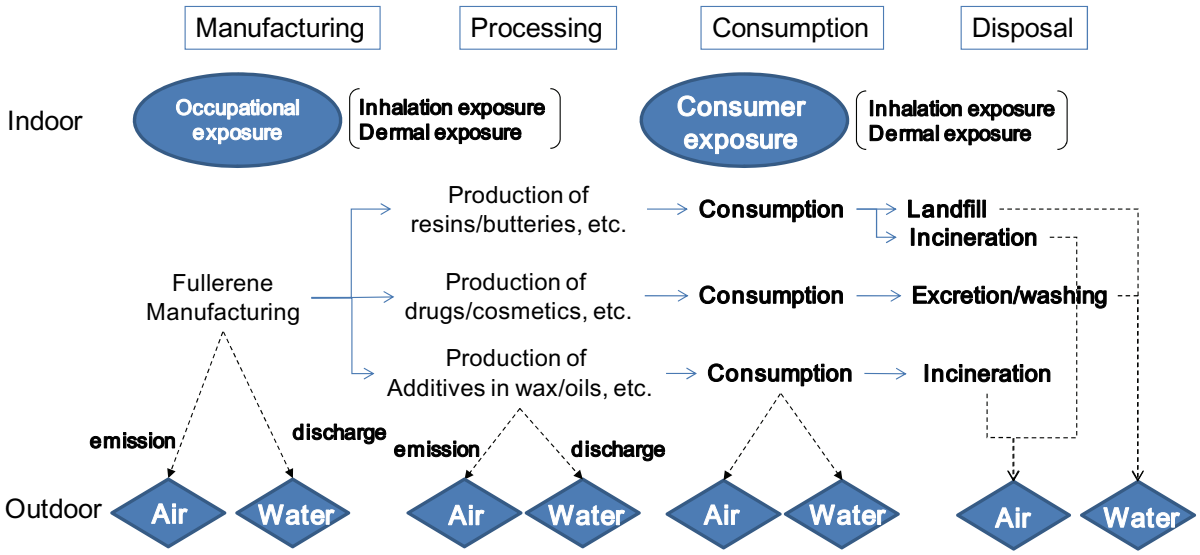

Figure III-1. Lifecycle of fullerenes and anticipated exposures and emissions

Emission and Exposure Scenario of Fullerene during Manufacturing and Processing

Considering the process of manufacturing to shipment of fullerenes or metal-containing fullerenes, as their synthesis is operated in vacuum, and purification under wet condition or within a sealed equipment, possible emissions occur during dust collection, product weighing and packaging, and maintenance and cleaning of equipment. These possibilities have been confirmed in several existing publications [Fujitani *et al.*, 2008; Yeganeh, 2008; Shinohara *et al.*, 2009 (NEDO Project)]. Based on the results of these researches, possible emissions at the fullerene manufacturing sites are summarized in Table III-1. The processes highlighted with yellow are those with possible emissions of fullerenes.

Table III-1. Possible emissions in the occupational environment from manufacturing to shipment of fullerenes

Process	Regular phenomena	Irregular phenomena
Manufacturing	Negligible	Possible during cleaning or maintenance, or if inadequate handling occurs
Collection	Possible (rolling dust): to be assessed based on the monitoring at manufacturing sites	No possibility
Purification	Negligible	No possibility
Collection/Weighing/Packaging	Possible (rolling dust): to be assessed based on the monitoring at manufacturing sites	No possibility
Shipment	Negligible	No possibility

Estimation of Fullerene Exposure Level

Exposure Level of Fullerene at Manufacturing Site (Based on the Existing Monitoring Data)

The exposures associated with the possible emissions presented in Table III-1 are estimated based on the results of monitoring data at manufacturing sites. Among the several reports available, the air concentration data of C_{60} monitored at the metal-containing fullerene manufacturing site in NEDO Project is presented in Table III-6 (Shinohara *et al.*, 2009).

Table III-6. Air concentration of C_{60} captured as particles at the manufacturing site of metal-containing fullerene

Particle Diameter [nm]	Air concentration [μg/m^3]				
	Beside synthesis device			Beside weighing equipment	
	Work hours (6 hours×3 days)	During handling operation (30 minutes)	During synthesis (2 hours)	Work hours (6 hours×3 days)	During handling operation (30 minutes)
< 250 nm	0.0023	0.064	No measurement	0.00083	0.014
250-500 nm	0.0014	Data lost		0.00065	0.0040
500-1,000 nm	0.0038	0.11		0.0029	0.0028
1,000-2,500 nm	0.0040	0.056		0.0032	N.D.
2,500-10,000 nm	0.0092	0.080		0.0049	0.025
All Particles (including >10,000 nm)	0.13	0.22 0.66	0.0045 0.0069	0.040	No measurement

N.D.: Below the limit of detection

There were almost no emissions from the devices used in the process of synthesizing fullerenes or metal-containing fullerenes reported in the existing publications. In contract, particle concentrations of fullerenes increased during the process of collecting the synthesized products from the devices, product packaging, and cleaning equipment, and possibilities of exposures to fullerenes were indicated. In the environment at the manufacturing sites, fullerene particles of nanoscale were rarely found and it is considered that fullerenes exist in the form of micron-size aggregates/agglomerates.

Based on the existing publication reporting the highest possible concentration levels of fullerenes in the occupational environment in the present situations (Fujitani *et al.,* 2008), the highest possible concentrations applied in our exposure assessment are determined to be 2.0μg/m^3 for micron-size particles (>2,000 nm), and 0.004μg/m^3 for nanosized particles (<50 nm). These concentrations, however, are those in the occupational environment, and exposure levels during work hours are reduced to 0.0004 μg/m^3 and 0.2 μg/m^3, respectively with C_{60} particles of <50 nm and 2,000 nm when workers are using a respiratory protective equipment with protective factor of 10.

Estimation of Exposure Levels at the Manufacturing Sites of Fullerene Secondary Products

Exposure levels are estimated based on the results of dustiness test by Ogura *et al.* (NEDO Project), and using two-box model in the occupational environment. The exposure scenario is based on the assumption that 40 units of sport goods containing 300 mg fullerene per unit are produced per day (12 g fullerene/day), i.e., 10,000 units per year. In this scenario, the operation done by a worker is assumed to handle 1.5 g of fullerene for one minute, and that operation is done once per hour, eight times in total per day. The exposure levels for each range of particle diameters are estimated as shown in Table III-7. Reflecting the emission rate for each range of particle diameters obtained in the study by Ogura *et al.*, the particles in the range of 1,000 - 10,000 nm diameters contribute the most to the exposure with the estimated level of 0.54μg/m^3. The estimated levels, however, are in the situations where no engineering measures or respiratory protective equipments are used. When any engineering measures such as to reduce the emission rate of fullerene to 1/10 (e.g., draft chamber with 90% elimination rate, glove box, etc.) are equipped, or respiratory protective equipments with protective factor of 10 are used, the exposure levels are decreased to 1/10 for each case. The exposure levels are assumed to be 1/100 of the estimated values on Table III-7 when both engineering measures and protective equipments are used.

Table III-7. Estimation of exposure levels during handling 1.5 g of fullerenes

Particle diameter [nm]	10-100	100-1,000	1,000- 10,000	>10,000
Exposure level [μg/m^3]	1.2×10^{-8}*	2.5×10^{-3}*	0.54*	7.7×10^{-2}*

* There levels can be reduced to 1/10 with application of respiratory protective equipment of protective factor 10, and futher to 1/10 with applicaiton of elimination equipment of 90% elimination rate.

Estimation of Exposure Levels in the General Environment

The sources of fullerene emissions to the atmospheric environment are the factories manufacturing or using fullerenes, and disposal or incineration sites of products containing fullerenes. It is expected that the fullerene emissions during the use of products containing fullerenes, or those from fullerenes of natural origins, are extremely small. Thus, the possibility is considered extremely low that atmospheric concentrations of fullerenes in the areas other than near the manufacturing sites exceed those around such sites. Therefore, the aim of this risk assessment is to roughly estimate the fullerene atmospheric concentrations near the sites where a large amount of fullerenes are expected to be manufactured or used.

There has been no report of fullerene detection in the outdoor environment, including the areas near the manufacturing sites in the existing monitoring studies. The reasons for no detection is due to the fact that the present usage of fullerenes is still limited, and that the emissions from the factories are managed properly (use of filters, etc.) In order to determine the possibility that the atmospheric concentrations around the manufacturing sites may reach the levels of concern, the concentrations around

the manufacturing sites are estimated assuming no elimination by filters.

The following worst-case scenario is used for the estimation; the estimated future production volume of 40 tons per year (2010) is manufactured and processed in a single factory, and the dust produced in the draft or sealed apparatus during the process is emitted from the factory through the emission system. Here, the estimation is based on the assumption that 0.03% of the emitted gas concentration is released to the air outside of the factory, since the expected elimination rate of HEPA (High Efficiency Particulate Air) filter is 99.97% (with 0.3 μm particles).

Atmospheric concentrations of fullerenes in 500-meter grids around the factory are estimated with one-box model. With the assumption that the behavior of fullerenes does not depend on their particle size, the only highest concentrations near the factory are estimated. The height of the mixed layer is assumed to be 200 meters of daily average with the assumption of unstable air. Further, wind velocity is assumed to be 0.5 m/s, which is in the range generally considered calm (under 1 m/s.) These assumptions are the combination possible in reality, which may yield the highest concentrations.

The atmospheric concentrations in the area around the factory handling 40 tones of fullerenes per year are estimated and shown in Table III-8.

Table III-8. Estimation of exposure levels near the factory manufacturing a large amount of C_{60}

Particle diameter [nm]	10-100	100-1,000	1,000- 10,00	>10,000
Exposure level [μg/m^3]	1.6×10^{-14}	2.2×10^{-9}	4.8×10^{-7}	6.9×10^{-8}

With the case that the unfiltered emission gas is released from the factory due to the malfunction of the emission system, the atmospheric concentration of fullerenes of all particle sizes is estimated to be 1.8×10^{-3} μg/m^3 based on the same assumptions used in the above estimation.

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Hazard Assessment (Chapter IV)

In this chapter, human health hazards of fullerenes (primarily C_{60}) by inhalation, oral and dermal exposures are evaluated. The existing literatures and the results of the NEDO Project, "Research and Development of Nanoparticle Characterization Methods" are reviewed and summarized, based on which a NOAEL (No Observed Adverse Effect Level) of fullerenes is to be determined. Since no epidemiology study is available with fullerenes which have a very limited history of use, the human health hazards are evaluated based on the review of the existing studies with experimental animals (*in vivo*) and cultured cells (*in vitro*).

The primary subject of this risk assessment is C_{60} and the derivatives are not included.

Kinetics and Metabolism of Fullerenes

The existing studies on the kinetics and metabolism of fullerene derivatives and nanoparticles indicate that most of the fullerene nanoparticles exposed orally are excreted or metabolized and that the possibilities of systemic translocation or adverse effects are low (Yamago *et al.*, 1995; Florence & Hussain, 2001).

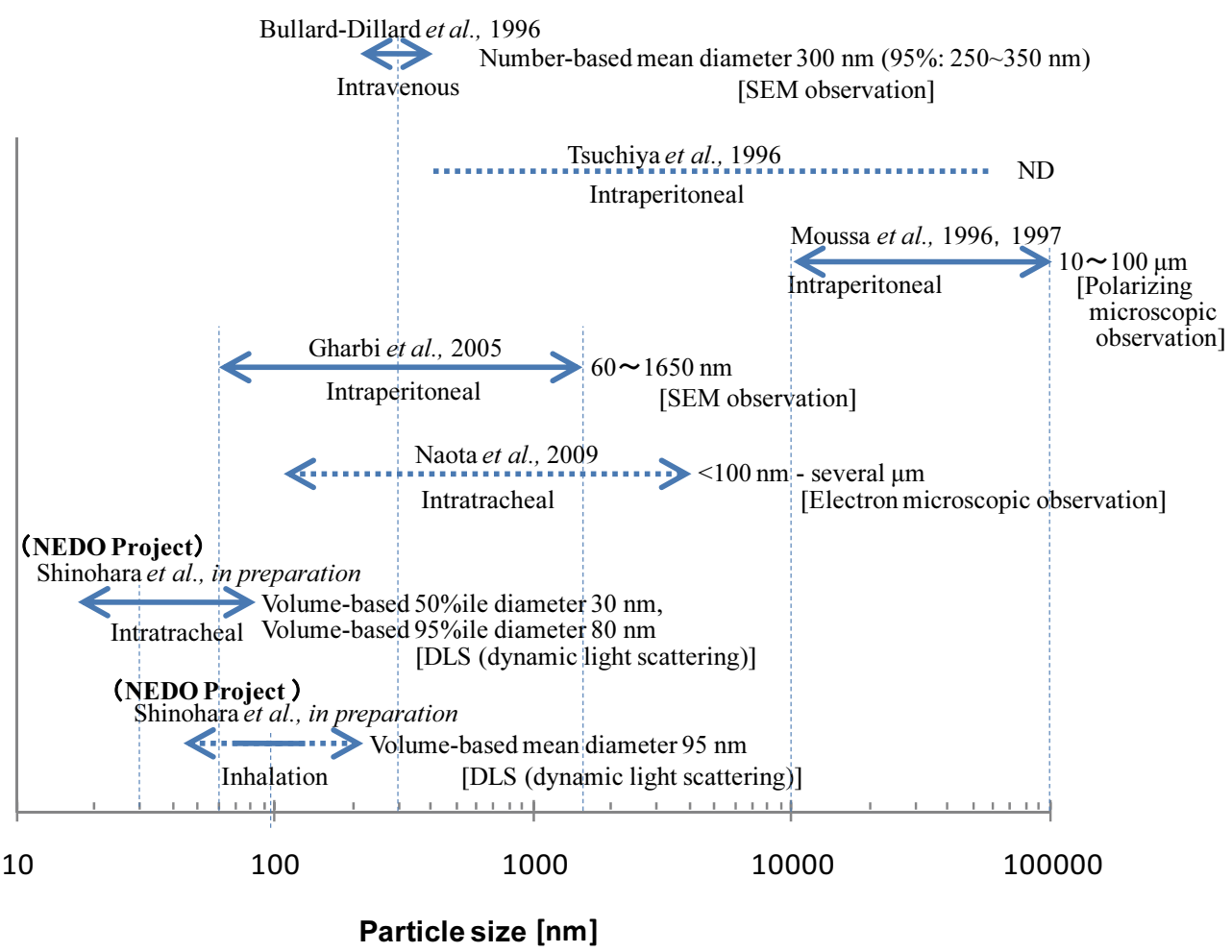
Chemical substances or particles inhaled in the occupational or general environment may be absorbed into the blood circulation by the lung, and systemically translocated to induce some effects, or transferred to the brain through the olfactory bulb. C_{60} administered via intravenous injection is mostly transferred to and accumulated in the liver (92% at one hour after administration, 95.7% at 120 hours after administration) (Bullard-Dillard *et al.*, 1996). Thus, even if C_{60} particles are transferred into blood from the lung following inhalation exposure, it may be possible to assume that there is little translocation of the particles to the organs other than the liver, if the amount transferred to the liver is confirmed to be small. The results of the studies conducted in NEDO Project [Shinohara *et al.* (in preparation)] indicate no translocation of C_{60} to the liver and to the brain with intratracheal instillation test (3.0 mg/kg) and inhalation exposure test (0.12 mg/m^3). Based on these findings, it is considered that the translocation of fullerene nanoparticles exposed via inhalation to the brain and other organs via the lung is negligible. Oberdöster *et al.* (2004) suggested the possibility that a carbon-based nanoparticle of unknown structure is transferred to the brain through the olfactory bulb. In this study, the carbon concentrations per unit organ weight in the olfactory bulb at inhalation exposure of 0.16 mg/m^3 were 0.35 – 0.43μg/g which were 25 to 73 % of those in the lung. In contrast, C_{60} concentrations in the brain per unit organ weight was < 0.0089 μg/g, which was < 0.17 % of that in the lung in the inhalation study of C_{60} nanoparticles in NEDO

Project [Shinohara *et al.* (in preparation)]. Therefore, it is considered that there is almost no transfer of fullerene nanoparticles to the brain through the olfactory bulb with inhalation exposure. Based on these, it is considered that there is almost no possibility that fullerene nanoparticles are systemically translocated after inhalation exposure.

No study on the kinetics and metabolism of fullerenes via dermal exposure has been identified. Based on the available reports on nanoparticles, the possibility of systemic translocation after dermal exposure is considered low. The results of the existing studies on the kinetics and metabolism of fullerenes are summarized in Table IV-5 and Figure IV-1.

Table IV-5. Published literatures on *in vivo* studies relative to the kinetics and metabolisms of fullerenes

Literature	Test Substance	Particle diameter	Test animal	Administration	Dose (Exposure level)	Observation period	Tissues analyzed and analytical methods	Results
Bullard-Dillard *et al.,* 1996	C_{60} dispersion (Prepared with benzene, THF, acetone) (^{14}C-labeled)	Average 300 nm 95% between 250-350 nm (Before adding PBS and being concentrated)	SD rats (♀,120～200 g)	Intravenous (0.05-0.5 mL)	2.1 μM of C_{60} dispersion is injected after being concentrated to 1/10 - 1/100 and mixed with PBS	Blood: 1-120 mins. after administration Organs: 2 and 120 hours after administration	Brain, blood, adipose, heart, kidney, liver, lung, muscle, skin, spleen, urine [HPLC analysis]	*C_{60} was eliminated from the blood circulation immediately after administration (within 1 min.), and accumulated mostly (90-95 %) in the liver, which remained in the liver even at 120 hours after administration. No oxidation or metabolism of C_{60} was confirmed.
Tsuchiya *et al*., 1996	C_{60}PVP conjugate	No information	Pregnant SLS mice (Gestation day 11)	Intraperitoneal	25-137 mg/kg-bw	18 hours after injection	Vitellicle, embryo (day 11) [Optical microscopic observation]	Distribution of C_{60} PVP conjugate is confirmed with the color in vitellicle and embryo at 50 mg/kg
Moussa *et al.*, 1996	C_{60} dispersion (Tween80/ CMC+NaCl aqueous suspension)	Micron size (Crystal: 2 μm, aggregates: 10-100 μm)	Swiss mice (♂,20±2 g)	Intraperitoneal	50, 80, 100 mg/animal (2500, 4000, 5000 mg/kg-bw)	7 and 14 days after administration	Brain, liver, lung, heart, spleen, kidney [Polarizing microscopic observation]	Deposition of C_{60} in the liver was mainly observed in Kuppfer cells and fat-storing cells, and rare in hepatic cells. Deposition in reticuloendothelial cells was observed in the spleen, lung, heart and kidney. No deposition was observed in the brain.
Moussa *et al.,* 1997	C_{60} dispersion (Tween80/ CMC+NaCl aqueous suspension)	Micron size (Crystal: 2 μm, aggregates: 10-100 μm)	Swiss mice (♂,20±2 g)	Intraperitoneal	50 mg/animal (2500 mg/kg-bw)	1, 3 and 6 days after administration	Blood, liver, spleen [HPLC analysis]	C_{60} blood concentrations at 1, 3 and 6 days after administration were 179, 87.5, 1.10 mg/L, respectively; C_{60} concentrations in the liver, 0.7, 1.0, 0.4%(wt); and C_{60} concentrations in the spleen, 0.5, 2.4, 2.4%(wt).
Gharbi *et al.,* 2005	C_{60} dispersion (0.1%Tween80 aqueous suspension)	60-1650 nm	Wistar rats (♂,200±10 g)	Intraperitoneal	500 mg/kg-bw	1, 2, 3, 4, 5, 6, 7, 9, 14, 21 days after administration	Liver, feces [HPLC analysis, SEM observation]	C_{60} concentration in the liver at 1 week after administration was about 24% of dose, and at 2 weeks and 3 weeks after administration, 5 and 1 % of the concentration at 1 week. Macrophage phagocytosis was observed with SEM.
Naota *et al.*, 2009	C_{60} dispersion (0.1%Tween80 aqueous suspension)	No description (Based on the image, include particles smaller than 100 nm and as large as several μm)	ICR mice (♀,10～11 weeks old, 29～34g)	Intratracheal	0.625, 1.0 mg/animal	Immediately after administration, 5 mins., 1 hour, 6 hours, 24 hours and 7 days after administration	Observation of capillary lumen, pulmonary lymph node, air-blood barrier of the lung [Electron microscopic observation]	At and after 5 mins. after administration, aggregated particles were confirmed within the structures of capillary lumen, pulmonary lymph node and air-blood barrier of the lung.
Shinohara *et al.,* in preparation (NEDO Project)	C_{60} dispersion (0.1%Tween80 aqueous suspension)	50%ile 30 nm 95%ile 70～80 nm	Wistar rats (♂, 9 weeks old, approx. 300 g)	Intratracheal (0.4 mL)	0.1, 0.2, 1.0 mg/animal (0.33, 0.66, 3.3 mg/kg-bw)	1 and 17 hours, 7, 30, 90 and 180 days after administration	Lung, liver, brain [HPLC analysis]	Almost 100% of the intratracheally administered dose was deposited in the lung, and two-phase elimination was suggested. No transfer of C60 particles to the organs other than the lung was observed; with 3.3 mg/kg administration, brain: < 0.018 μg/tissue (<0.0019% of dose), liver: < 0.15 μg/tissue (<0.015% of dose.)
	C_{60} nanoparticle (Mist spray of 0.1%Tween80 suspension)	Average 96 nm		Inhalation exposure (6 hours/day, 5 days/week, 4 weeks)	0.12 mg/m^3 (4.1×10^4 particle/cm^3)	7 and 30 days after termination of exposures		



*C_{60} was eliminated from the blood circulation immediately after administration (within 1 min.), and accumulated mostly (90-95 %) in the liver, which remained in the liver even at 120 hours after administration. [HPLC analysis]

Distribution of C_{60} PVP conjugate in vitellicle and embryo [Optical microscopic observation]

Deposition of C_{60} in the liver was mainly observed in Kuppfer cells and fat-storing cells. No transfer to the brain. [Polarizing microscopic observation]

C_{60} concentration in the liver at 1 week after administration was 24% of dose, and at 2 weeks and 3 weeks after administration, 5 and 1 % of the concentration at 1 week. [HPLC analysis]

Aggregated particles observed in tissues including capillary lumen, pulmonary lymph node and air-blood barrier of the lung. [Electron microscopic observation]

No transfer of C_{60} particles in the organs other than the lung. [HPLC analysis]

No transfer of C_{60} particles in the organs other than the lung. [HPLC analysis]

Figure IV-1. Particle sizes of the test materials in the existing *in vivo* studies relative to the kinetics and metabolism of fullerenes

(Arrows indicate the ranges of particle sizes; dotted lines are the estimations)

Deposition and Clearance of Fullerene Particles in the Lung

Particle deposition in the pulmonary alveolus can be estimated with models including MPPD (Multiple-Path Particle Dosimetry). With application of MPPD model to the data from the existing studies, the deposition fraction of nanosized fullerene particles in the pulmonary alveolus is estimated to be 20 to 30 % in rats and 20 to 40 % in humans.

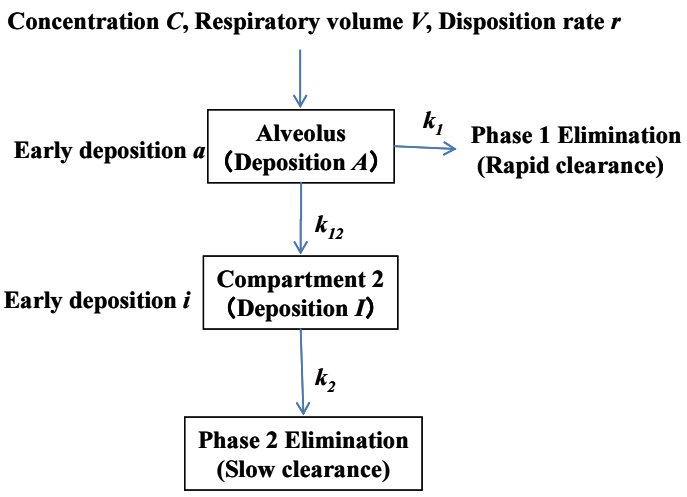


Figure Ⅳ-3. Mechanism of clearance estimated by the model
The assumptions of the model are that the phase 1 elimination directly from the pulmonary alveolus (rapid clearance) is the elimination route from respiratory tract after macrophage phagocytosis, and that the elimination through the compartment 2 (slow clearance) is the elimination route from the lymph nodes via interstitinus or delayed clearance due to adherence to pulmonary epithelium, etc.

Also, in the study by Shinohara *et al.* (in preparation) to analyze the elimination of fullerene particles deposited in the lung after intratracheal instillation, there are discrepancies between the analytical values and those estimated with a first-order rate equation after 90 days, and a good fit is obtained with a second-order rate equation, which indicates that there are a rapid phase and a slow phase in the clearance of fullerene particles in the lung. Therefore, the data of lung deposition is analyzed with the two-phase clearance model shown in Figure IV-3.

When this model is fitted with the analytical data from Baker *et al.* (2008) and Shinohara *et al.* (in preparation), deposition fractions obtained are 0.16 to 0.21, which are similar to the results obtained with MPPD model. As shown in Figure IV-4, with application of the parameters obtained by fitting this model with the analytical values, it is possible to estimate amounts of particles remain in the lung after inhalation exposure and deposition fractions of intratracheal instillation. These results indicate that it is possible to estimate residual amounts of particles in inhalation exposure studies based on the results of intratracheal instillation tests. Results of such estimation, which are not available in this interim ducument, are to be included in the final document.

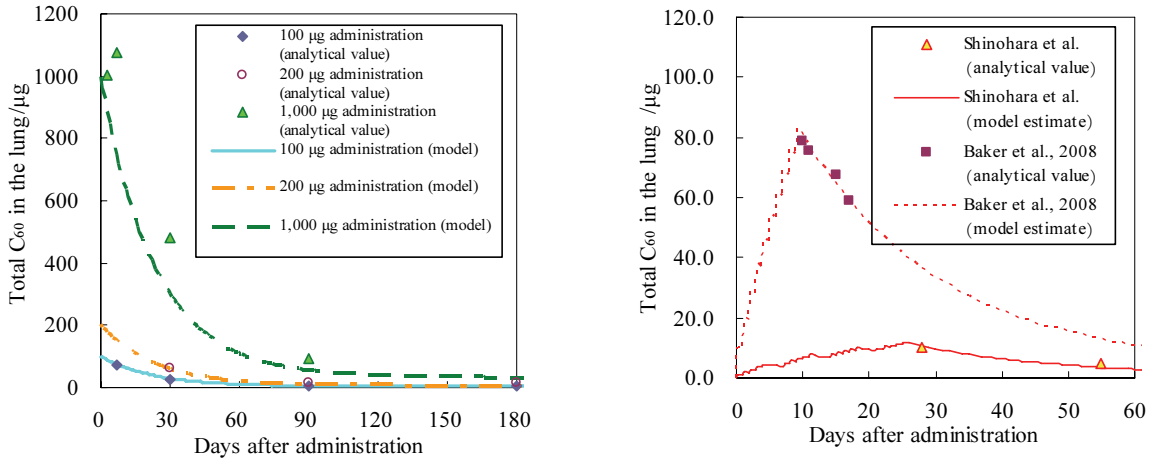


Figure IV-4. Deposition fractions in the lung: analytical values and model fitting curves
Figure on the left: analytical values and model fitting curves in an intratracheal instillation test
Figure on the right (♦, ○, and Δ): estimated and analytical values of residual amounts in the lung in inhalation exposure studies (■ and Δ)

Toxicity Studies via Respiratory System

In vivo studies to evaluate the effects of exposures via inhalation include intratracheal instillation and inhalation exposure tests. Inhalation exposure studies are those in which experimental animals are exposed to test substances at certain levels in exposure chambers in laboratories, which are considered the best approach to evaluate the effects of inhalation exposure (Morimoto & Tanaka, 2008). The number of testing facilities capable of conducting such studies, however, is small due to the need of large, expensive equipments and of a large amount of test substances required. The results of the existing toxicity studies to evaluate adverse effects of fullerenes via application through respiratory system are summarized and presented in Table IV-8 and Figure IV-6, respectively. The results of the studies conducted under NEDO Project are summarized below.

Inhalation Exposure Studies

As a part of NEDO Project, Yokoyama *et al.* (2009) investigated the reducing ability of C_{60} in the lung of ICR male mice (weighing approximately 30 g) exposed via inhalation to C_{60} nanoparticles (number-based geometric mean diameter: 86 nm, number concentration: 1.6×10^5 /cm^3) produced by atomizing C_{60} suspension in 0.1 % Tween 80, with measurement by electron paramagnetic resonance (EPR) imaging at 700 MHz. The results confirmed no reducing ability with C_{60} nanoparticles, while reducing ability was observed with the positive control, nickel oxide (NiO) particles.

As a part of NEDO Project, Tanaka *et al.* (in preparation) conducted a study in which male Wistar rats (9 weeks old, weighing 300g) were exposed via inhalation for 4 weeks (6 hours/day, 5 days/week) to 0.12 mg/m^3 aerosol C_{60} particles [mean particle diameter: 96 nm (mobility diameter), aggregates/agglomerates of particles with diameter of approx. 30 nm, number concentration measured by SMPS: 4.1×10^4] generated by mist spray of C_{60} dispersed in water with 0.1 % Tween 80. Observations were made at 3 days, 1 month and 3 months after completion of the exposure period. The results indicated no changes in the wet weight of the lung, markers of BALF (total cell count, neutrophilic cells), and HO-1 (Heme oxigenase-1) gene (gene related to inflammatory fibrosis) expression in the lung tissues, and were comparable to those of negative control. No abnormality was observed in other tissues examined (cerebrum, cerebellum, nasal cavity, testis, liver, kidney, and spleen). In contrast, significant increases were observed in the group treated with the positive control of NiO in observations of the lung, BALF and HO-1 gene expression in the lung tissues.

Intratracheal Instillation Studies

As a part of NEDO Project, Tanaka *et al.* (in preparation) conducted a study in which male Wistar rats (9 weeks old) were intratracheally instilled a single dose of C_{60} (volume-based 50%ile diameter: 33 nm) dispersed in water with 0.1 % Tween 80 at dose levels of 0.1, 0.2, 1.0 mg/animal (0.33, 0.66, 3.3 mg/kg). Observations were made at 3 days, 1 week, 1 month, 3 months and 6 months after administration. Significant increases in wet weight of the lung were observed only at 1 week after administration in all treatment groups of 0.33, 0.66, 3.3 mg/kg. At 3.3 mg/kg, total cell counts in BALF increased until one week after administration, and a significant increase of neutrophilic cells continued until 3 months after administration. These increases, however, were extremely small compared to those observed with the positive control NiO, and thus, considered negligible changes and of no biological significance. HO-1 gene expression in the lung indicated upregulation until one week after administration. Also, in the quantitative evaluation of pulmonary inflammation in the lung tissues, slight increases were observed at 3 days after administration in 0.33, 0.66 mg/kg groups, which persisted until one week after administration in 3.3 mg/kg group. No increase was observed at later observation periods and the levels were comparable to the negative control. No increases in inflammatory response in blood (leukocyte or neutrophlic cell counts) were observed and no abnormality was found in other organs examined (cerebrum, cerebellum, nasal cavity, testis, liver, kidney, and spleen). In contrast, the group treated with the positive control NiO showed significant changes in all observations except with the other organs examined, and the level of inflammation was significantly greater compared to the C_{60} treated groups.

Summary of Genotoxicity

Positive results of fullerene C_{60} in genotoxicity were reported in the following studies: reverse mutation assay with *Salmonella* under optical irradiation (Sera *et al.*, 1996); comet assay (2.2, 4.2 μg/L, Dhawan *et al.*, 2006); increases polyploids in chromosomal aberration assay (Honma *et al.*, 2008); and point mutation at red/gan gene locus in gpt delta transgenic MEF cells with point mutation detection gene (Xu *et al.*, 2009). Negative results were obtained in the following studies: reverse mutation assay with *Salmonella* with no optical irradiation (Sera *et al.*, 1996); reverse mutation assays with *Salmonella* [Mori *et al.,* 2006; Shinohara *et al.* (in print) (NEDO Project)]; chromosomal aberration assays (Mori *et al.,* 2006, Shinohara *et al.* (in print) (NEDO Project)]; and micronucleus assay [Shinohara *et al.* (in print) (NEDO Project)].

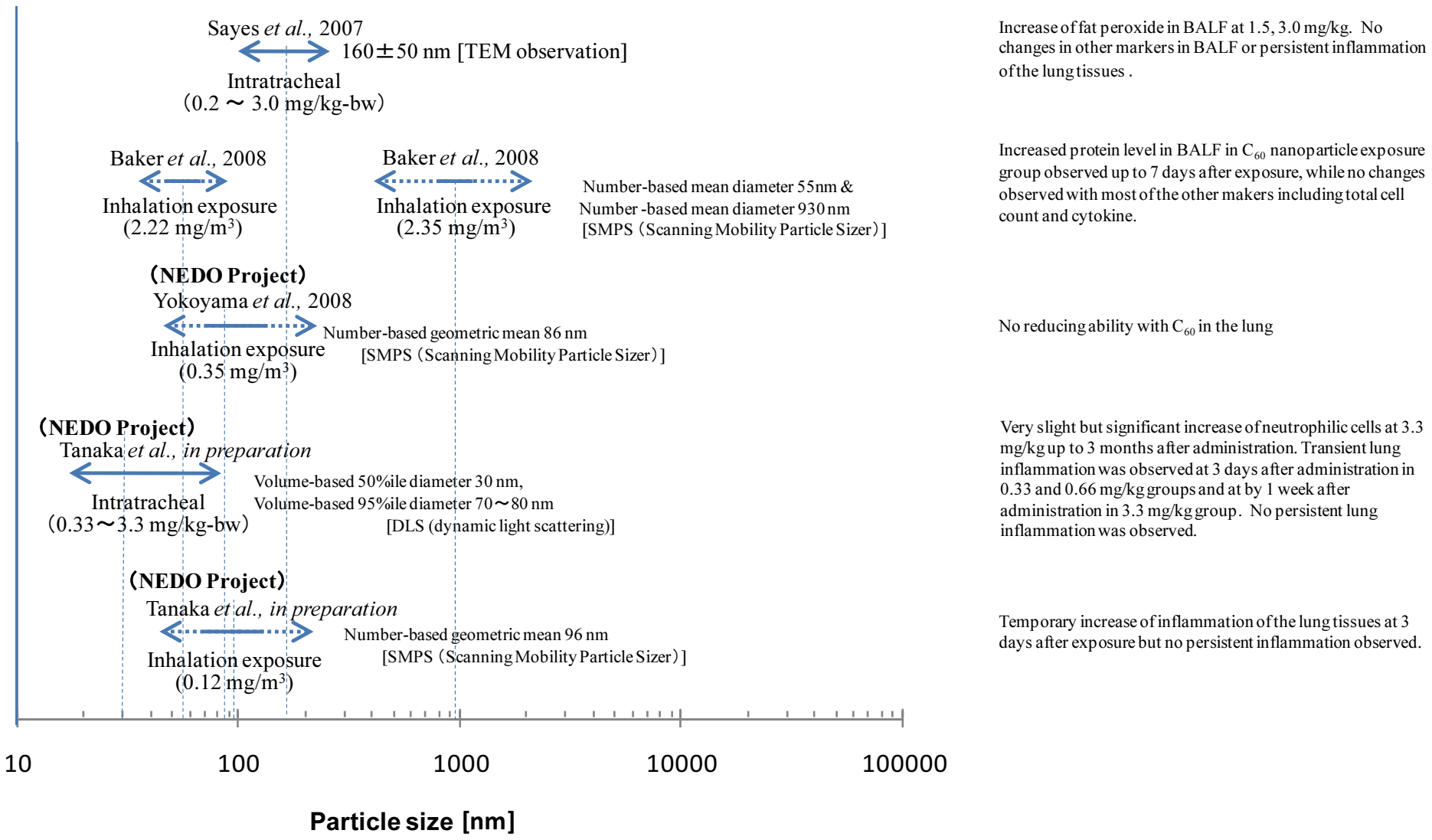
No strong genotoxicity was observed in the reverse mutation assay under optical irradiation or chromosomal aberration assay by Shinohara *et al.* (in print) (NEDO Project), however, Sera *et al.* (1996) observed genotoxicity of fullerene C_{60} under optical irradiation with the strains having stronger optical sensitivity. There have been many publications reporting the specific breakage of guanine group by C_{60} under optical irradiation (Tokuyama *et al.*, 1993; Boutorine *et al.*, 1994), and thus, it is considered highly possible that fullerenes may have some genotoxicity under optical irradiation.

Determination of Critical Endpoint, Provisional NOAELs, and provisional value of acceptable exposure concentration

In general, it is necessary to establish a value to be compared with exposure levels (exposure concentrations) such as NOAEL, which is the highest level without any observed adverse effect in humans or experimental animals, in order to evaluate risks of exposures to chemical substances. An acceptable level of exposure (reference concentration) indicates the ceiling which can be obtained by application of uncertainty factors to such values as NOAEL. As summarized in the previous section, a NOAEL value could not been assessed for fullerene C_{60} as no adverse effects were observed in the inhalation exposure studies. Therefore, for this risk assessment, it was decided to consider the exposure level without any effects (i.e. a provisional NOAEL), and to compare this value with estimated exposure levels. For establishing a NOAEL, a critical endpoint of adverse effects should be determined. It was, therefore, decided to select the adverse effect at the lowest exposure level via the routes possible in humans, observed in the existing toxicity studies, and also those expected to occur based on the properties of fullerenes and nanoparticles. It must be noted, however, that the provisional NOAEL is considered lower than the true NOAEL and thus, is a conservative value.

Table IV-8. Published literatures on the toxicity studies with fullerene via inhalation

Literature	Test substance	Particle diameter	Test animal	Administration	Dose (Exposure level)	Observation	Observation period	Results
Sayes *et al.*, 2007	C_{60} aqueous dispersion, $C_{60}(OH)_{24}$ aqueous dispersion	160 ±50 nm	SD CD rats (♂, approx. 7 weeks old, 220-240 g)	Intratracheal application (single)	0.2, 0.4, 1.5, 3.0 mg/kg-bw	BALF, lung tissues	1 day, 1 week, 1 month and 3 months after administration	Increase of fat peroxide in BALF at 1.5, 3.0 mg/kg. No changes in other markers in BALF or persistent inflammation of the lung tissues observed.
Baker *et al.*, 2008	C_{60} nanoparticle (Aggregates formed after sublimation)	Average 55 nm	Fischer 344 rats (♂, 10 weeks old)	Inhalation exposure (nose only) (3 hours/day, 10 days)	2.22 mg/m^3	BALF, lung tissues	Immediately after exposure, 1, 5 and 7 days after exposure	Increased protein level in BALF in C_{60} nanoparticle exposure group, while no changes observed with most of the other makers including total cell count and cytokine.
	C_{60} micron-particle (Aggregates formed after sublimation)	Average 930 nm			2.35 mg/m^3			
Yokoyama *et al.*, 2009 (NEDO Project)	C_{60} nanoparticle (Mist spray of 0.1%Tween80 suspension)	Number-based geometric mean 86 nm	ICR mice (♂, approx. 30 g)	Inhalation exposure (nose only)	0.35 mg/m^3 (1.6×10^5 particle/cm^3)	Reducing ability in the lung [electron paramagnetic resonance (EPR) imaging (700 MHz)]	2 days and 2 weeks after exposure	No reducing ability with C_{60} while reducing ability in the lung confirmed with positive control, NiO
Tanaka *et al.*, in preparation (NEDO Project)	C_{60} dispersion (0.1%Tween80 aqueous suspension)	50%ile 30 nm 95%ile 80 nm	Wistar rats (♂, 9 weeks old, approx. 300g)	Intratracheal application (single) (0.4 mL)	0.1, 0.2, 1.0 mg/animal (0.33, 0.66, 3.3 mg/kg-bw)	BALF, HO-1 gene expression, pathological evaluation of selected organ and tissues	3 days, 1 week, 1 month, 3 months and 6 months after administration	No difference with negative control in almost all observation items. Transient lung inflammation was observed at 3 days after administration in 0.1 and 0.2 mg/animal groups and at 3 days and 1 week after administration in 1.0 mg/animal group. No persistent lung inflammation was observed.
	C_{60} nanoparticle (Mist spray of 0.1%Tween80 suspension)	Average 95 nm	Wistar rats (♂, 9 weeks old, approx. 300g)	Inhalation exposure (6 hours/day, 5 days/week, 4 weeks)	0.12 mg/m^3 (4.1×10^4 particle/cm^3)	BALF, HO-1 gene expression, pathological evaluation of selected organ and tissues	7 days and 1 month after exposure	No difference with negative control in almost all observation items. No persistent lung inflammation was observed.



Increase of fat peroxide in BALF at 1.5, 3.0 mg/kg. No changes in other markers in BALF or persistent inflammation of the lung tissues .

Increased protein level in BALF in C_{60} nanoparticle exposure group observed up to 7 days after exposure, while no changes observed with most of the other makers including total cell count and cytokine.

No reducing ability with C_{60} in the lung

Very slight but significant increase of neutrophilic cells at 3.3 mg/kg up to 3 months after administration. Transient lung inflammation was observed at 3 days after administration in 0.33 and 0.66 mg/kg groups and at by 1 week after administration in 3.3 mg/kg group. No persistent lung inflammation was observed.

Temporary increase of inflammation of the lung tissues at 3 days after exposure but no persistent inflammation observed.

Figure IV-6. Particle sizes of test samples used in the existing studies relative to hazardous effects of fullerenes via inhalation exposure

Determination of Critical Endpoint for Risk Assessment

It is considered relevant to review adverse effects observed primarily via inhalation exposure for risk assessment. In the studies of kinetics and metabolism of fullerenes summarized in Chapter II, translocation of fullerene particles to organs other than the lung after inhalation exposure has not been reported, and thus, it is considered relevant to determine the adverse effect in the lung as a critical endpoint for risk assessment.

The evaluation in the toxicity studies of fullerenes in the lung included inflammation observed in lung tissue, body weight, organ weights, total cell count, neutrophilic cell, protein, cytokine, HO-1 gene expressions, etc. Inflammation in tissues, which is a direct observation of effects, can be considered as an endpoint. It is considered appropriate that a significant body weight increase compared to the negative control should be regarded as a supplemental index of some other direct effect. Likewise, a significant increase of lung weight compared to the negative control may be induced by some functional disorder in the lung, however, is not a determinant of an adverse effect by itself. According to the mechanism of inducing pulmonary inflammation described in Section 4.4 of Chapter IV of the main body of the risk assessment document, neutrophilic cells and cytokine in BALF, which increase with induction of inflammation, can be indices of inflammation. Increases of protein and total cell count in BALF, which also relate to induction of inflammation, however, are considered not the direct indices of an adverse effect. HO-1 is an antioxidant enzyme which is induced to inhibit inflammation against particles, and thus, considered an indirect index of an adverse effect.

As previously stated, the biological significance of responses observed one day after intratracheal instillation is insignificant, and therefore, more focus is given to the inflammation and changes of biomarkers related to inflammation at, and after, three days of administration.

In order to determine the critical endpoint of adverse affects with fullerenes, inflammation observed in the lung tissues is to be reviewed first, and then the changes of neutrophilic cell and cytokine in BALF are evaluated for overall assessment.

Determination of Provisional NOAELs and provisional value of acceptable exposure concentration

Considering that the exposure expected in real situations is over a period of several years, the toxicity study [Tanaka *et al.* (in preparation)] with the longest exposure and observation periods is selected as the critical study for assessment. The inhalation study with a shorter exposure period (Baker *et al.*, 2008) and the intratracheal instillation test [Tanaka *et al.* (in preparation)] are reviewed as supplemental data.

a) NOAEL with C_{60} Nanoparticle in the Rat Inhalation Exposure Study

NOAEL of C_{60} with adverse effects in the lung of rats is considered greater than 0.12 mg/m^3 based on the results of the study by Tanaka *et al.* (in preparation)which indicated no change in morphological observation of lung tissue, biomarkers in BALF, etc. up to 3 months after inhalation exposure of C_{60} particles (number-based geometric mean diameter; 96 nm, 0.12 mg/m^3) for 6 hours/day, 5 days/week, 4 weeks.

The fullerene particle air concentration of 0.12 mg/m^3 in the study by Tanaka *et al.* (in preparation) is used as the provisional NOAEL in this risk assessment, and is expected to be a very conservative assessment. The exposure and observation periods were shorter in the study by Bakers *et al.* (2008), and in this study rats were exposed to C_{60} nanoparticles for 10 days and observed for 7 days after exposure and no adverse effects were observed at air concentration of 2.22 mg/m^3. Also, in the intratracheal instillation test [Tanaka *et al.* (in preparation)], the lung inflammation was only observed immediately after administration even though the level of lung deposition of the C_{60} nanoparticles was one digit higher (Figure IV-8) than that observed in the inhalation exposure study.

b) Estimation of Provisional NOAEL for Human Inhalation Exposure in the Occupational Environment

Extrapolating the results of Tanaka *et al.* (in preparation) on the effects in the rat lung after exposure, a provisional NOAEL for human inhalation exposure in the occupational environment (*provisional* $NOAEL_{human\_work}$) is estimated with adjustment for the exposure period and species differences. After adjustment of alveolar deposition fraction/respiratory rate/surface area of pulmonary alveolus between Wistar rats and humans (occupational exposure: 8 hours/day, 5 days/week), a provisional NOAEL for humans (occupational environment) is obtained as follows (for this estimation, the ratio of surface area of pulmonary alveolus between rats and humans is used, but it is also possible to use ratio of body weight or lung weight).

$$provisional\ NOAEL_{human-work} = provisional\ NOAEL_{rat} \times \frac{t}{8} \times \frac{5}{5} \times \frac{f_r}{f_h} \times \frac{q_r}{q_h} \times \frac{S_h}{S_r}$$

$$\Rightarrow 0.12[\mathrm{mg/m^3}] \times \frac{6}{8} \times \frac{5}{5} \times \frac{0.192}{0.198} \times \frac{0.301}{36} \times \frac{543200}{3422.5}$$

$$\Rightarrow 116\ [\mu\mathrm{g/m^3}]$$

Here:

t; exposure period [h]

f_r; alveolar deposition fraction in rat [-]

f_h; alveolar deposition fraction in human [-]

q_r; rat respiration rate [m^3/day],

q_h; human respiration rate [m^3/day],

S_r; surface area of rat pulmonary alveolus [m^2],

S_h; surface area of human pulmonary alveolus [m^2]

The exposure period per day in Tanaka *et al.* (in preparation) is used as t, f_r and f_h are estimated with MPPD model, and q_r, S_r, S_h are estimated with the data and equation in U.S. EPA (1994). Respiration rate at light work of 36 m^3/day is used for q_h.

The provisional NOAEL for human inhalation exposure in the occupational environment with fullerene C_{60} nanoparticle of mean diameter 96 nm (primary particle of diameter 33 nm) is estimated to be 116 μg/m^3.

c) Estimation of Provisional NOAEL for Human Inhalation Exposure in the General Environment

Extrapolating the results of Tanaka *et al.* (in preparation) on the effects in the rat lung after exposure, a provisional NOAEL for human inhalation exposure in the general environment (*provisional* $NOAEL_{human\_env}$) is estimated with adjustment of exposure period and species differences. After adjustment of alveolar deposition fraction/respiratory rate/surface area of pulmonary alveolus between Wistar rats and humans, a provisional NOAEL for humans (in the general environment) is obtained as follows in the same way as is estimated for the occupational environment:

$$provisional\ NOAEL_{human\_env} = provisional\ NOAEL_{rat} \times \frac{t}{24} \times \frac{5}{7} \times \frac{f_r}{f_h} \times \frac{q_r}{q_h} \times \frac{S_h}{S_r}$$

$$\Rightarrow 0.12\ [\mathrm{mg/m^3}] \times \frac{6}{24} \times \frac{5}{7} \times \frac{0.192}{0.198} \times \frac{0.301}{20} \times \frac{543200}{3422.5}$$

$$\Rightarrow 49.6\ [\mu\mathrm{g/m^3}]$$

The above equation and the procedure are the same as those used for the occupational environment, except the adjustment of human exposure period is for 24 hours/day and 7 days/week and that the respiration rate used is 20 m^3/day for ordinary life.

The provisional NOAEL for human inhalation exposure in the general environment with fullerene C_{60} nanoparticle of mean diameter 96 nm (primary particle of diameter 33 nm) is estimated to be 49.6 μg/m^3.

d) Significance of Provisional NOAELs and Estimation of provisional value of acceptable exposure concentration

Provisional NOAELs of 116μg/m^3 (occupational environment) and 49.6 μg/m^3 (general

environment) estimated above are derived form the air concentration of fullerene C_{60} of 0.12 mg/m^3 in Tanaka *et al.* (*in preparation*), which are considered very conservative values as explained in the section a). Therefore, these provisional NOAELs should be used for screening purpose to confirm no concern of possible risks rather than to determine any concern of risks. In Chapter V, the main body of the risk assessment document (interim document), risks are assessed by comparing the ratio of provisional NOAELs and estimated exposure levels (i.e., Margins of Exposure; MOEs) with uncertainty factors involved in the risk assessment. However, in certain exposure situations, if the estimated exposure reaches a level for which no concern of possible risk cannot be confirmed with application of these provisional NOAELs, it is suggested to conduct more detailed hazard and exposure assessments and to reconsider the criteria for risk assessment.

Also, the provisional value of acceptable exposure concentrations, which are obtained by dividing the NOAELs by uncertainty factors, should be positioned as guidance levels for screening purpose in risk assessment and risk management. The provisional NOAELs estimated here are considered smaller than the real NOAEL, and are not divided by uncertainty factors. Since no adverse effect was observed at the highest level of particle burden in the lung in the intratracheal instillation test, which was 40 times higher than that in the inhalation study; the provisional value of acceptable exposure concentration is, therefore, estimated to be around 0.8 mg/m^3 in the occupational environment for fullerene C_{60} nanoparticle of mean diameter 96 nm (primary particle of diameter 33 nm), with an uncertainty factor of 6 (please refer to p. 21 for determination of uncertainty factors). Baker *et al.* (2008) reported no adverse effects at a exposure concentration of 2.22 mg/m^3, although the exposure and observation periods were shorter. However, considering that no adverse effects were observed in the intratracheal instillation test with a 6-time higher level of particle burden in the lung compared to the inhalation exposure study, the provisional value of acceptable exposure concentration is estimated to be around 1.3 mg/m^3 in the occupational environment for fullerene C_{60} nanoparticle of 55 nm diameter with an uncertainty factor of 10 (for extrapolation of results from a short-term study to chronic exposure effects). These standards are temporary values, and thus, the standards of higher certainty are to be estimated in the final risk assessment based on the further review of available data and using the results of the intratracheal instillation test.

It is reported by Tanaka *et al.* (in preparation) that the particle diameter of the test sample of fullerene C_{60} nanoparticle was 33 nm in the suspension (volume-based mean diameter measured by DLS), and that the particle diameter of those in the air produced by mist spray of the suspension was 96 nm (geometric mean diameter measured by SMPS). The difference of the particle sizes in the suspension and air is considered due to the different methods of measurement used and/or with aggregation of particles in the air. Both of these values confirm that the study was conducted with nanoscale particles of fullerene C_{60}. Therefore, the provisional NOAELs estimated based on the results of Tanaka *et al.* (in preparation) should be applicable to fullerene particles of nanoscale.

The relationship between particle size and hazard strength has not been fully elucidated, however, the hazard strength depends on two factors. The first factor is the difference in rate of deposition in the pulmonary alveolus with different particle sizes, and the second factor, possible difference of hazard strength per unit weight with different particle sizes. Regarding the second factor, it has been discussed that particle surface area is the relevant index of exposure contributing to adverse effects (Oberdörster *et al.*, 2000, 2005; Donaldson *et al.*, 2001). For the application of the provisional NOAELs estimated in this risk assessment, different arguments can be made based on the definitions of micron-size fullerene particles. There may be two general classifications of micron-size particles (Figure IV-8); a) aggregates/agglomerates of nanosized crystals having the same surface area with the nanosized particle; and b) a single crystal of micron-size having smaller surface area than the nanosized particle. Regarding the particles classified as a), of which the surface area is equivalent to that of a nanosized particles, only the difference in rates of deposition in the pulmonary alveolus should be considered, even if hazard strength depends on surface area. In contrast, with those classified under b), only the difference in rates of deposition in the pulmonary alveolus should be considered if hazard strength depends on particle weight, however, if hazard strength also depends on surface area, their hazard strength per weight would be smaller than that of nanosized particles. Therefore, when applying the provisional NOAELs, estimated in this risk assessment, to those particles classified under b), the risks assessed are conservative estimates even with consideration of the differences in rates of deposition in the pulmonary alveolus. The existing data comparing the adverse effects of C_{60} particles of nanosized and micron-size includes the following two observations: No adverse effects were observed in the short-term inhalation exposure studies at equivalent concentration levels with both nanosized and micronsized C_{60} particles (Baker *et al.*, 2008). Lyon *et al.* (2006) reported that a stronger anti-bacterial activity was observed with C_{60} particles of nanosized compared with the micron-size particles in an *in vitro* study. However, it is not possible to evaluate the difference of adverse effects with C_{60} particles of different particle sizes based on the results of these studies alone. It may become possible to establish a separate NOAEL for micron-size fullerene particles when sufficient data is generated to evaluate the hazard strength of the particles classified under the category b), which is one of the future challenges. In the risk assessment under Chapter V, differences in rates of particle deposition in the lung alveolus with particle sizes will be explicitly considered, however, no additional adjustment is made with the micron-size particles classified under the category b), and only a comment is made that the estimated risks are conservative values.

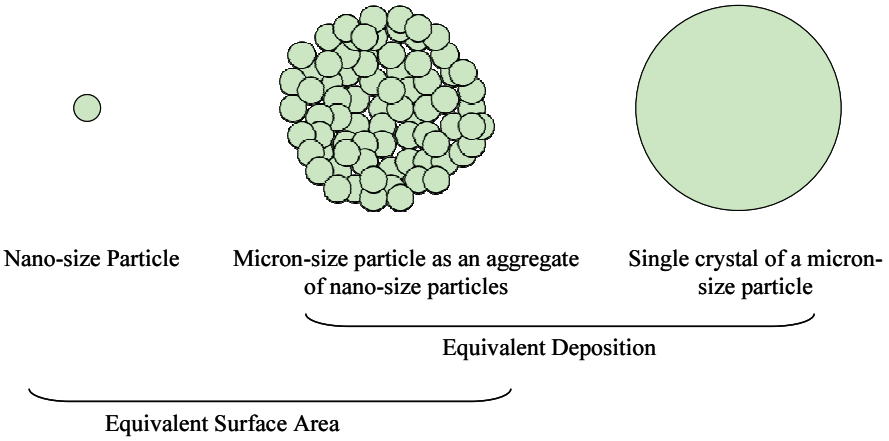


Figure IV-8 Conceptual images of two types of fullerene particles of micron-size

Determination of Uncertainty Factors

The provisional NOAELs obtained in the previous section are estimated with extrapolation of the data obtained in animal studies to humans, however, no consideration has been given of the exposure periods and/or individual differences. The magnitudes of the differences with these factors are related to data uncertainty, and thus, the level of influence of each factor is quantified as an uncertainty factor. The total value of all uncertainty factors multiplied together, is the uncertainty factor of risk assessment. In Chapter V, risks are to be assessed by comparing the ratios of the provisional NOAELs and the estimated exposure levels (i.e., MOE) with this uncertainty factor.

Species differences in kinetics and sensitivity to adverse effects of xenobiotics are expressed with toxicokinetics (TK) and toxicodynamics (TD). Regarding TK, the uncertainty factor between rats and humans is determined to be 1 based on the rationale that adverse effects related to the pulmonary inflammation, the critical endpoint in this risk assessment, are local and depend only on the alveolar deposition fraction of particle. The differences of the deposition fraction and surface area of pulmonary alveolus have been already adjusted in the estimation of the provisional NOAELs. For TD, which is related to the species differences in sensitivity of toxicity induction, the uncertainty factor has been proposed as 1 to 3 by U.S. EPA (2002) or as 2.5 by World Health Organization (WHO) and EC (IPCS, 1994; ECHA, 2008). As it has been suggested that rats have greater sensitivity to particle exposures than humans, the uncertainty factor for TD is determined to be 1 in this risk assessment.

Regarding the uncertainty factors related to the difference of exposure periods, European Commission (EC) proposed to apply 6 for estimation from a subacute study to chronic exposure, and 2 from a subchronic study to chronic exposure (ECHA, 2008). In the study by Tanaka *et al.* (in preparation) rats were observed for three months after an exposure period of four weeks, and thus, the uncertainty factor of 6 is applied for estimation of chronic exposure from the results of a short to medium term study.

For individual differences among humans, the uncertainty factor is determined to be 1, judging that the workers in the occupational environment are healthy, and thus, considered not of a sensitive population. The uncertainty factor for the individual differences of hazard sensitivity among humans in the general environment is determined to be 10.

The resultant uncertainty factor of the provisional NOAEL as multiple of each, is 6 for the exposures among the humans in the occupational environment, and with additional 10 for individual differences, 60 for the exposure among the humans in the general environment.

Summary

In this chapter, information and data on adverse effects of fullerenes are summarized and presented, and provisional NOAELs and uncertainty factors are estimated.

The information on the kinetics and metabolism of fullerenes indicate that systemic translocation of the particles is negligible although the possibility is suggested of uptake in the tissues of pulmonary alveolus after exposures through respiratory tract, which is the primary exposure route in humans. Based on the results of the intratracheal instillation studies, the half-life in the lung is estimated to be 16 to 24 days. With exposures through respiratory system (inhalation exposure and intratracheal instillation studies), a temporary and slight inflammation of the lung tissues was observed with some studies but no sign of persistent inflammation. Furthermore, no evidence of effects in other organs examined was obtained. In the cytotoxicity studies, some results indicated that fullerenes of nanosized particles were more hazardous than those of micron–size. No evidence of adverse effects has been obtained in oral or dermal exposure studies.

For determination of provisional NOAELs for risk assessment, inflammatory responses in the lung is selected as the critical endpoint with the assumption that inhalation is the primary route of human exposure. The inhalation study by Tanaka *et al.* (in preparation), of which the exposure and observation periods were the longest among the available studies, is selected as the critical study to determine a NOAEL.

The provisional NOAELs for nanosized fullerene particles are estimated to be 116 $\mu g/m^3$ in the occupational environment, and 49.6 $\mu g/m^3$ in the general environment. These values, however, are considered very conservative values. The uncertainty factors applicable in risk assessment are considered to be 6 in the occupational environment and 60 in the general environment. The provisional value of acceptable exposure concentration in the occupational environment is estimated to be around 0.8 mg/m^3 (800 $\mu g/m^3$) with consideration to the results of the intratracheal instillation test.

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Risk Assessment (Chapter V)

In this chapter, risks with fullerene exposure are assessed based on the results of the exposure assessment (Chapter III) and the hazard assessment (Chapter IV). Evaluation of risks is done with application of MOE (Margin of Exposure). MOE is obtained with the values including NOAEL (No observed adverse effect level) from human epidemiology studies or experimental animal studies divided by estimated exposure levels in real situations.

In this assessment, first the particles are classified into a number of ranges to reflect the differences in the rates of deposition in the lung tissues, and *MOE* is estimated for each range of particle sizes as the ratio of the provisional NOAEL and the estimated exposure level. Then, the estimated *MOE*s are totaled with the equation below to yield MOE_{total} for comparison with the uncertainty factor.

$$\frac{1}{MOE_{total}} = \sum_{d} \frac{1}{MOE_{d}}$$

Risks assessed are with: 1) exposure levels in the occupational environment at a fullerene manufacturing site; 2) exposure levels in the occupational environment at a fullerene secondary product manufacturing site; and 3) exposure levels in the general environment (area near the fullerene manufacturing factory).

Exposure Levels

Exposure Levels in the Occupational Environment at Fullerene Manufacturing Site

The exposure levels in the occupational environment at the fullerene manufacturing site estimated with the results of monitoring studies available in the published literatures are < 2.0 μg/m^3 with particles of all sizes and with only nanosized particles, < 0.004 μg/m^3. These levels are considered the highest limits in the occupational environment.

Table V-1. Fullerene exposure levels obtained based on the resuts of monitoring at the manufacturing site

Particle diameter [nm]	10 - 50	2,000 – 10,000
Exposure level [μg/m^3]	<0.004*	<2.0*

* With use of respiratory protective equipments of protective factor 10, these levels can be reduced to 1/10.

Exposure Levels during the Process at the Fullerene Secondary Product Manufacturing Site

Based on the data of the rolling dust study and using two-box model, exposure levels of fullerene particles in the occupational environment are estimated to be 1.2×10^{-8}, 2.5×10^{-3}, and 0.54 μg/m^3, for the ranges of particle sizes of 10 - 100 nm, 100 - 1,000 nm, and 1,000 - 10,000 nm, respectively. These levels, however, are the estimates with the condition that no technical measures (use of a draft chamber, glove box, etc.) or respiratory protective equipments are applied.

Table V-2. Estimation of exposure levels during handling 1.5 g of fullerenes (Same as Table III-7)

Particle diameter [nm]	10-100	100-1,000	1,000- 10,00	>10,000
Exposure level [μg/m^3]	1.2×10^{-8}*	2.5×10^{-3}*	0.54*	7.7×10^{-2}*

Exposure Levels in the Environment (Near Factories)

With the results of the rolling dust study, the atmospheric concentration in the area near the factory which handles 40 tons per year of fullerenes, the production estimate for 2010, is estimated to be 5.6×10^{-4} μg/m^3.

This estimation is obtained through a simple calculation with one-box model assuming meteorological conditions to yield higher atmospheric concentrations. The estimated concentration is considerably lower than the estimated level in the occupational environment, and thus, concentrations of fullerenes in the general environment, in areas other than near the manufacturing factory, are considered even lower than this estimate.

Table V-3. Estimation of exposure levels near the factory manufacturing a large amount of C_{60} (Same as Table III-8)

Particle diameter [nm]	10-100	100-1,000	1,000- 10,00	>10,000
Exposure level [μg/m^3]	1.6×10^{-14}	2.2×10^{-9}	4.8×10^{-7}	6.9×10^{-8}

Provisional NOAEL and Uncertainty Factors

The provisional NOAEL with fullerene C_{60} particle of mean diameter 96 nm for humans (occupational environment) is 116 μg/m^3, and the total uncertainty factor is 6. In the general environment, the provisional NOAEL is 49.6 μg/m^3, and the total uncertainty factor is 60.

These NOAELs are derived from the results of the study conducted with the test sample of mean

particle diameter of 96 nm. As described in Chapter IV, the rate of deposition in the pulmonary alveolus depends on the particle size. Thus, the provisional NOAELs reflecting the differences of deposition fraction are obtained with the equation as follows:

$$provisional\ NOAEL_{d=x} = provisional\ NOAEL \times \frac{r_{d=96}}{r_{d=x}}$$

Here;

provisional NOAEL; the provisional NOAEL obtained in Chapter IV [mg/m^3]

provisional NOAEL$_{d=x}$; provisional NOAEL for the particle with particle diameter of *x* nm [mg/m^3]

$r_{d=96}$; deposition fraction of the particle with particle diameter of 96 nm [-]

$r_{d=x}$; deposition fraction of the particle with particle diameter of *x* nm [-]

For particle diameter *x*, a typical value in each range used for estimation of exposure level is applied.

The deposition fraction of the particles of mean diameter of 96 nm in the inhalation study is estimated to be 0.19 assuming that the rate is equal to that of single dispersed particles.

The ranges of nanosized particles in the occupational exposure assessment at C_{60} manufacturing site based on the monitoring data are 10 - 50 nm and 2,000 - 10,000 nm, and the deposition fractions are estimated to be 0.29 and 0.083, respectively. With these values, the provisional NOAEL for each particle size range is estimated to be 52 μg/m^3 for 10 - 50 nm, and 182 μg/m^3 for 2,000 - 10,000 nm.

On the other hand, in the occupational exposure assessment of the site handling $C_{60,}$ and in the general atmospheric environment of the area near the factory, ranges of nanosized particles are estimated to be 10 - 100 nm, 100 - 1,000 nm, and 1,000 – 10,000 nm, and the deposition fractions, 0.29, 0.074, and 0.079, respectively. With these values, the provisional NOAELs for these particle size ranges are estimated to be 22, 86, and 80 μg/m^3, for 10 - 100 nm, 100 - 1,000 nm, and 1,000 - 10,000 nm, respectively.

The estimated values are summarized in Table V-4.

Table V-4. Provisional NOAEL for each range of concentrations for exposure assessment

Value from toxicology studies	Particle diameter [nm]	96		
	Provisional NOAEL [μg/m^3]	116		
Exposure in the occupational environment	Particle diameter [nm]	10-100	100-1,000	1,000- 10,000
	Provisional NOAEL [μg/m^3]	77.2	301	282
	Particle diameter [nm]	10-50	2,000-10,000	
	Provisional NOAEL [μg/m^3]	77.2	270	
Exposure in the general environment	Particle diameter [nm]	10-100	100-1,000	1,000- 10,000
	Provisional NOAEL [μg/m^3]	32.7	129	119

Risk Estimation

Risk Estimation in the Occupational Environment at Fullerene Manufacturing Site

MOE for each range of particle sizes is estimated as follows:

$$MOE_{50-100nm} = \frac{provisional\ NOAEL_{10-50nm}}{Exposure_{10-50nm}} = \frac{77.2}{0.004} = 19300$$

$$MOE_{2,000-10,000nm} = \frac{provisional\ NOAEL_{2,000-10,000nm}}{Exposure_{2,000-10,000nm}} = \frac{270}{2.0} = 135$$

MOE for the total exposure of all particles, MOE_{total} is calculated as the sum of above values:

$$\frac{1}{MOE_{total}} = \frac{1}{MOE_{10-50nm}} + \frac{1}{MOE_{2,000-10,000nm}}$$

$$= \frac{1}{19300} + \frac{1}{135}$$

$$= \frac{1}{135}$$

$$MOE_{total} = 1.35 \times 10^2 > UF(=6)$$

MOE_{total} is larger than UF, and thus, no concern of risk is confirmed.

Risk Estimation during the Process at the Fullerene Secondary Product Manufacturing Site

MOE for each range of particle sizes is estimated as follows:

$$MOE_{10-100\,nm} = \frac{provisiona\,l\ NOAEL_{10-100\,nm}}{Exposure_{10-100\,nm}} = \frac{77.2}{1.2\times10^{-8}} = 6.43\times10^{9}$$

$$MOE_{100-1,000\,nm} = \frac{provisiona\,l\ NOAEL_{100-1,000\,nm}}{Exposure_{100-1,000\,nm}} = \frac{301}{2.5\times10^{-3}} = 1.20\times10^{5}$$

$$MOE_{1,000-10,000\,nm} = \frac{provisiona\,l\ NOAEL_{1,000-10,000\,nm}}{Exposure_{1,000-10,000\,nm}} = \frac{282}{0.54} = 5.22\times10^{2}$$

MOE for the total exposure of all particles, MOE_{total} is calculated as the sum of above values:

$$\frac{1}{MOE_{total}} = \frac{1}{MOE_{10-100nm}} + \frac{1}{MOE_{100-1,000nm}} + \frac{1}{MOE_{1,000-10,000nm}}$$

$$= \frac{1}{6.43\times10^{9}} + \frac{1}{1.20\times10^{5}} + \frac{1}{5.22\times10^{2}}$$

$$= \frac{1}{5.20\times10^{2}}$$

$$MOE_{total} = 5.20\times10^{2} > UF(=6)$$

MOE_{total} is larger than *UF*, and thus, no concern of risk is confirmed with the estimated exposure level during the process such as adding fullerenes to resins at the manufacturing sites of the secondary products.

Risk Estimation in the General Environment (Near Manufacturing Site)

MOE for each range of particle sizes is estimated as follows:

$$MOE_{10-100\,nm} = \frac{provisiona\,l\ NOAEL_{10-100\,nm}}{Exposure_{10-100\,nm}} = \frac{32.7}{1.6\times10^{-14}} = 2.04\times10^{15}$$

$$MOE_{100-1,000\,nm} = \frac{provisiona\,l\ NOAEL_{100-1,000\,nm}}{Exposure_{100-1,000\,nm}} = \frac{129}{2.2\times10^{-9}} = 5.86\times10^{10}$$

$$MOE_{1,000-10,000\,nm} = \frac{provisiona\,l\ NOAEL_{1,000-10,000\,nm}}{Exposure_{1,000-10,000\,nm}} = \frac{119}{4.8\times10^{-7}} = 2.48\times10^{8}$$

MOE for the total exposure of all particles, MOE_{total} is calculated as the sum of above values:

$$\frac{1}{MOE_{total}} = \frac{1}{MOE_{10\text{–}100nm}} + \frac{1}{MOE_{100\text{–}1{,}000nm}} + \frac{1}{MOE_{1{,}000\text{–}10{,}000nm}}$$

$$= \frac{1}{2.04\times10^{15}} + \frac{1}{5.86\times10^{10}} + \frac{1}{2.48\times10^{8}}$$

1 $$= \frac{1}{2.47\times10^{8}}$$

$$MOE_{total} = 2.47\times10^{8} >> UF(=60)$$

2

3 MOE_{total} is considerably larger than *UF*, and thus, no concern of risk is confirmed with the estimated
4 exposure level in the areas near the factory handling a large amount of fullerenes (40 tons per year) in
5 future.

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The original executive summary and the main body of the risk assessment document (in Japanese) are available for download from AIST-RISS website (http://www.aist-riss.jp/main/?ml_lang=ja).