

**December 7-9,2011
9th Meeting of the OECD WPMN
Paris**

**Risk Assessment of Manufactured
Nanomaterials:
Searching for the Critical Factors in
Human Health Risk**

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Thank you, Chairperson. I would also like to thank everyone from WPMN, OECD for giving me this opportunity to discuss our results at such an important meeting.

We were engaged in ' R&D of Nanoparticle Characterization and Risk Evaluation' Research Project which was financed by NEDO for five years and completed it at the end of FY2010. Today, I would like to discuss briefly the objectives and results of the project.

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Nakanishi (2011)

Today I have seven topics as shown on this slide.

1. Introduction

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As an Introduction, I would like to tell you what I presented about nanomaterial's safety at Chemical Committee, OECD before this meeting.

Special Session of the Joint Meeting: Potential Implications of Manufactured Nano-Materials for the Human Health and Environmental Safety

38th Joint Meeting Session, at OECD headquarters, Paris, 7th June, 2005

Approaches to Societal Impacts of Nanotechnologies in Japan

Junko Nakanishi

Director of CRM, AIST

CRM: Research Center for Chemical Risk Management

AIST: National Institute of Advanced Industrial Science and Technology

Nakanishi (2005)⁴

First time is the meeting in June, 2005 in Paris

OECD Workshop on the
Safety of Manufactured Nanomaterials

BUILDING COOPERATION, COORDINATION AND COMMUNICATION
December 7-9, 2005
Washington DC, USA

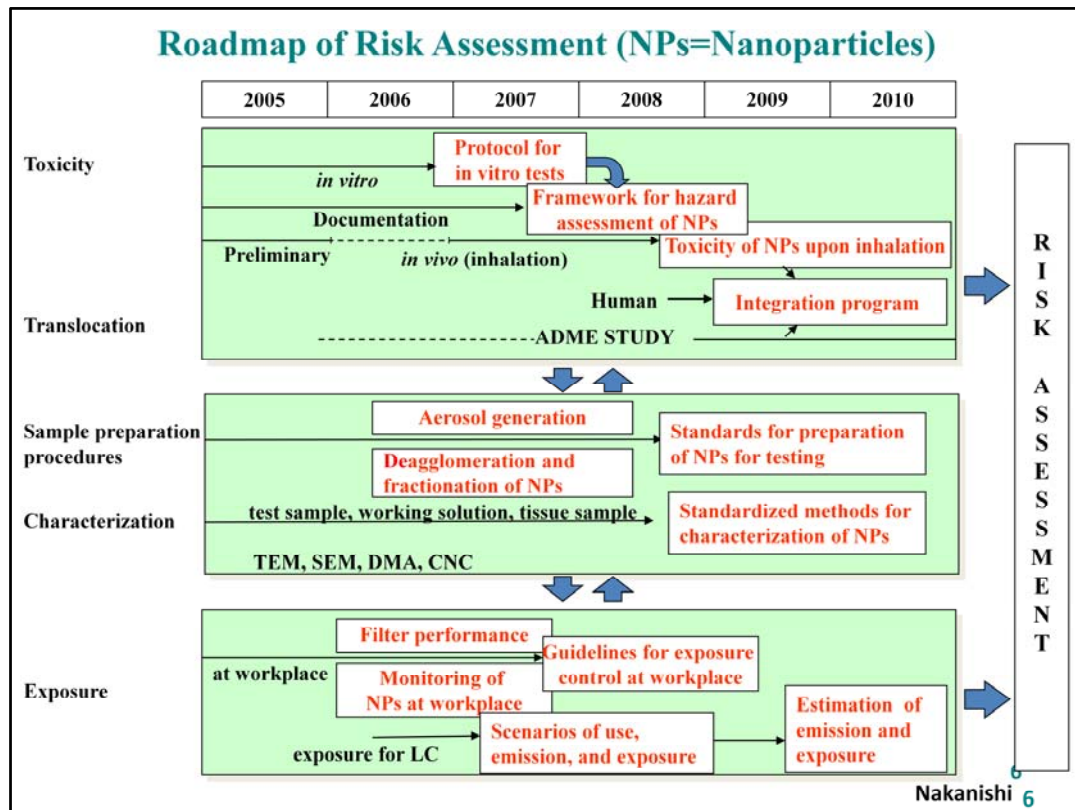
**Japan's Approaches to
Risk Assessment of Manufactured Nanomaterials**

Junko Nakanishi, Dr. of Engineering
Director of CRM, AIST

CRM: Research Center for Chemical Risk Management
AIST: National Institute of Advanced Industrial Science and Technology

Nakanishi (2005)⁵

Second time is this meeting in December, 2005, In Washington DC.



In this meeting, I presented the Roadmap for Risk Evaluation Research of Nanomaterials for five to six years.

In these two meeting, I stressed the importance of characterization of test materials and also the importance of risk evaluation and risk management study, but not merely hazard evaluation study.

The NEDO Project Launched in 2006

**Research and Development of Nanoparticle
Characterization Methods**

**- Evaluating Risks Associated with
Manufactured Nanomaterials -**

FY2006 - FY2010

Project Leader : Junko Nakanishi

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Immediately after the Workshop at Washington DC, the Nedo Project launched. The objective of this R&D is “Evaluating Risks Associated with Manufactured Nanomaterials”

Fund is approximately US\$ 2 million. I was charged with its project leader. Today, I will tell you achievements of this project.

2. Our Research Strategy

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Let me go to the second topics, Our Research Strategy

Points to Be Noted Regarding Nanoparticle Characterization

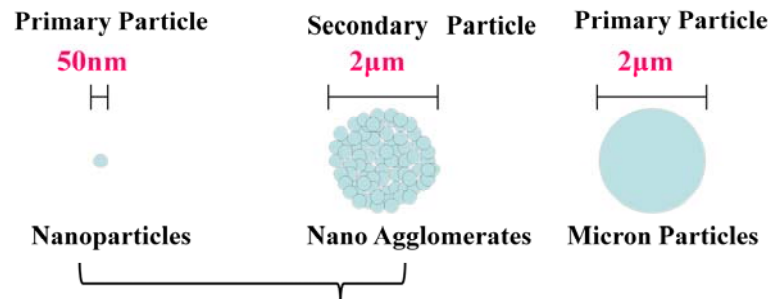
- 1. To identify the size and shape of the primary particle and the secondary particle, respectively.**
- 2. To conduct toxicity testing using test samples whose secondary particles are nanoscale.**

Nakanishi (2009)

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I repeated in many occasions that it is important to measure the size and shape of test nanomaterials. Furthermore, I added two points to be noted regarding nanomaterial characterization as shown in this slide, when I began this project.

To Identify the Primary Particles and the Secondary Particles, Respectively



	Nanoparticle		Micron
	Dispersed	Agglomerates	Dispersed
Specific Surface Area (BET)	<i>X</i>	<i>X</i>	<i>Smaller than X</i>
Deposition Rate	<i>Larger than Y</i>	<i>Y</i>	<i>Y</i>

Nakanishi (2009)

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Using the this slide, let me explain the first point. The left circle is a primary particle of 50nm diameter. The middle is an agglomerate of the primary particles., and if it exists as it is, it is called a secondary particle.

The right one is primary particle of micron size. Considering the BET specific surface area, which is a very important character , its value of the primary particle and agglomerated secondary particle are almost same. However, BET for the micron particle is much smaller than those.

Regarding the deposition rate which is also important , the values of the agglomerated particles (middle) and of micron particles(right) are almost same.. But, that of the primary nanoparticles (left) is much greater than those. This is why I say, we must identify and characterize the primary and secondary particles , respectively.

To Conduct Toxicity Testing Using Test Samples Whose Secondary Particles Are Nanoscale

Because:

- 1. Otherwise we will not be able to answer the questions from the general public.**
- 2. Although many nanomaterials are aggregated, there is a possibility that they exist in a single, dispersed state.**
- 3. We need to clarify the relationship with size and shape of the material.**

Therefore we:

- 1. Developed a method to prepare the dispersed nanoscale test particles.**
- 2. Developed a method to transport the nanoscale dispersed aerosol into the exposure chamber.**
- 3. Measured the particle size and shape of the nanoscale particles.**

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Nakanishi (2011)

So, We have come up with our policy to conduct toxicity testing using test samples whose secondary particles are nanoscale.

The reason are three written on the slide. The biggest one is, otherwise we will not be able to answer the question from the general public.

About this, I will explain more using the next slide.

To carry out this objective, we developed three technical methods listed. The most difficult task is to prepare the dispersed nanoscale test particles.

Confused Understanding regarding Nanoparticle

Diverse understanding:

The general public: Being *nano* means that the size of secondary particle is nanoscale. Therefore, nano is toxic.

Specialists: Being *nano* means that the size of primary particle is nanoscale. However, it exists as aggregated state, and it is not nanoscale, therefore nano is not toxic.

Common understanding:

Nano may or must be toxic, if the secondary particle is nanoscale. (Is it right?)

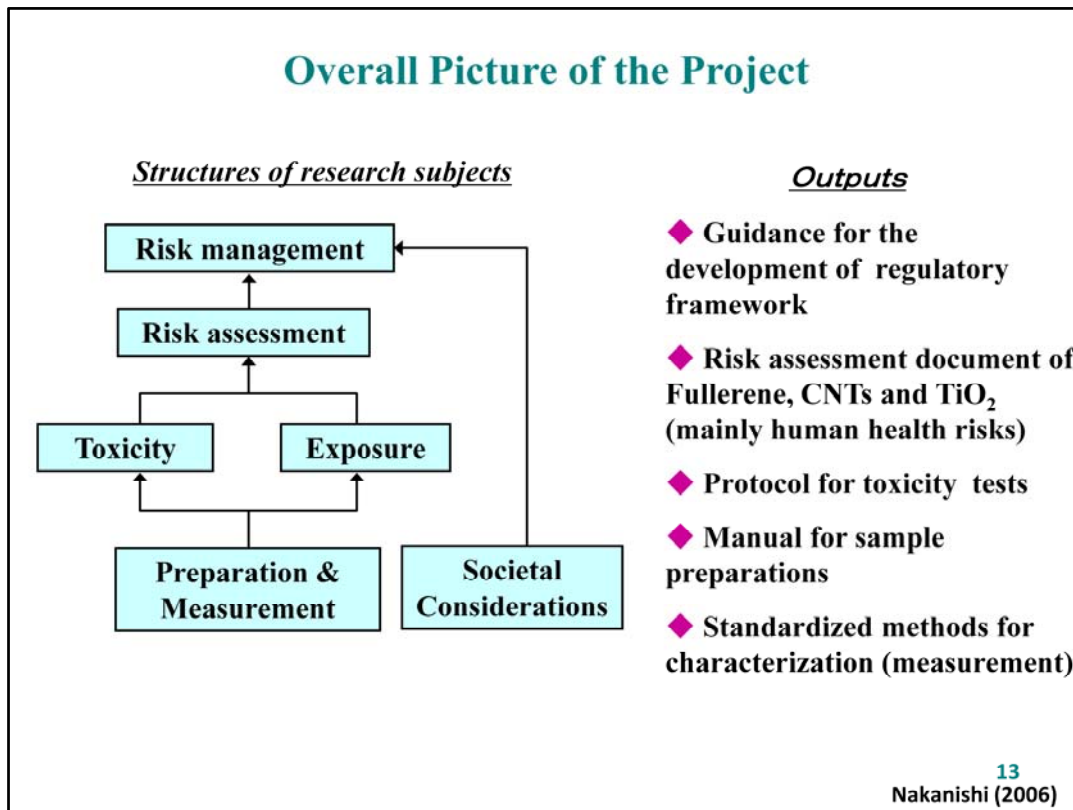
Nanoscale: 1~100 nm

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Nakanishi (2011)

Next, I have paid attention to the reason why people think nano is dangerous. And I noticed that general public and the specialists have different opinions as shown on the slide.

Their opinion looks different, However, there is one thing in common between the two which is indicated here. The common thing is that nano may or must be toxic if the secondary particle is nanoscale.

Is this assumption true or not? I think that will be the key for solving the questions regarding the nanoscale toxicity or nanomaterial toxicity. Especially from the viewpoint of risk communication.



Let me go on to talk about the Overview Picture of the project. This represents the interrelationship between the four subjects.

Exposure assessment and toxicity assessment. And based on them, there is risk assessment, and risk management.

I think this is a familiar diagram. As was mentioned earlier, preparation and the measurement is included. This is unique to nanomaterial research project. This is where we had greatest focus.

These are expected out puts. And this time, we come up with an assessment document of three nanomaterial groups (Fullerene, CNTs and TiO₂) and other manuals and proposal.

Key Points of the Project

- 1. To assess risk, not merely toxicity.**
- 2. To determine the particular effects due to the small size (nanoscale).**
 - •• To prepare test samples as dispersed as possible.**
- 3. To measure and understand the physical and chemical characteristics of the primary and secondary particles of test samples as much as possible.**
- 4. To grasp the whole picture of a material group rather than focusing on one particular material.**
- 5. To develop a method which can become the risk assessment model for new materials.**

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Nakanishi (2011)

I summarized the Key Points of the Project. I already talked about Number 1, Number 2 and Number 3.

Here, as in Number 4, we aspired to grasp the whole picture of a material group rather than focusing on one particular material.

And, lastly, we developed a method which can become the risk assessment model for new materials.

3. Approach to Toxicity Assessment

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Let me then go on to talk about how we have carried out the toxicity assessment.
Approach to Toxicity Assessment.

Process of Toxicity Assessment

1. To focus on the impact of the inhalation exposure pathways.
2. To calculate rat NOAEL of a representative nanomaterial from the result of inhalation exposure tests, choosing inflammation as the endpoint.
3. To estimate human OEL (occupational exposure limit) from the rat NOAEL.
4. To examine carcinogenicity separately.
5. To adopt simplified test methods and extrapolation methods in order to grasp the whole picture of NMs toxicity.

IH test: Inhalation Exposure test

IT test: Intratracheal Instillation test

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Nakanishi (2011)

This is a process that we have employed. Firstly, we focused on the impact of the inhalation exposure pathways. Let me stress the fifth point.

Adoption of the Three Innovative Methods

- ◆ **To conduct the IT test with up to a 2-year observation period in order to understand the effects of long-term exposure related to tumor.**
- ◆ **To use IT test which takes pulmonary retention into account in order to extrapolate a 13-week NOAEL from the 4-week NOAEL for IH test.**
- ◆ **To introduce the biaxial approach in order to compare different materials within the same material group. The IT test was employed here as well.**

IH test: Inhalation Exposure test
IT test: Intratracheal Instillation test

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Nakanishi (2011)

Now in order to achieve this specifically, we have adopted the three innovative experimentation and analysis methods.

First of all, we conducted the IT test.....

Secondly, we conducted 4-week inhalation exposure test and obtained the NOAEL and based on that we extrapolated 13-week sub-chronic NOAEL. We chose 4-week inhalation test, because of time, fund and experimental instrument shortage and technical difficulty.

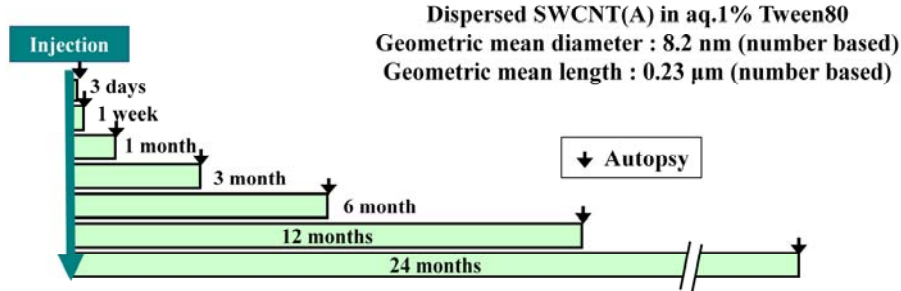
For extrapolating a 13-week NOAEL, the IT test to examine the appropriateness of the exposure duration extrapolation was conducted and UF of 2 was introduced.

We introduced

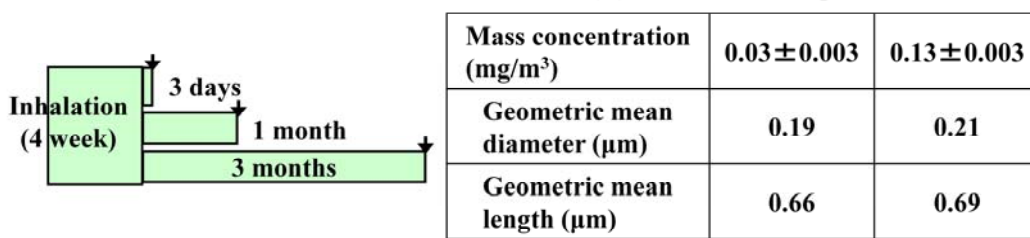
Let me give you the details of test methods using the following some slides.

Basic Design of the Pulmonary Toxicity Test

Intratracheal Instillation



Inhalation Exposure



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Morimoto, Shimada (2011)

This is a basic design of the pulmonary toxicity test. The upper chart is Intratracheal Instillation, and the lower chart is Inhalation Exposure test.

It is characteristic of our method that the observation period is long up to two years in the IT test. 4-week inhalation exposure is basic for this project. SWCNT(A) is a representative of single-wall carbonnanotube.

Summary in inhalation study

	MWCNT(N)	SWCNT(A)	NiO
	0.37 mg/m ³	0.13 mg/m ³	0.12 mg/m ³
Lung weight	↑ →	→	↑
Cell analysis in BALF	→	→	↑
Inflammation/fibrosis related gene -HO-1-	→	→	↑
Inflammation in lung	→	→	↑
Fibrosis in lung	→	→	→
8OH-dG in urea	→	→	→
Other organs	(-)	(-)	(-)
Final Judge	NO	NO	YES

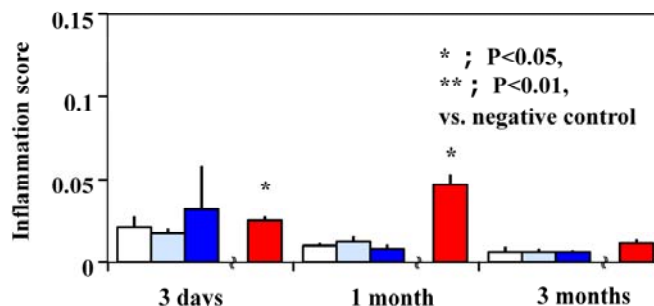
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Nakanishi (2011)

This is a brief diagnosis results for the two test runs in which it was judged that no adverse effects are seen. We choose the persistent inflammation by histopathological diagnosis as an endpoint of the toxicity evaluation and the histopathological diagnosis is double-checked by the external experts.

Inflammation Score in the SWCNT(A) Inhalation Exposure Test

Inhalation study

Tween-exposed
Low con. of SWCNT(A)
High conc. of SWCNT(A)
NiO-exposed



SWCNT(A) aerosol in the exposure chamber

	SWCNT(A)		NiO
	Low Dose	High Dose	
Mass concentration (mg/m³)	0.03 ± 0.003	0.13 ± 0.003	0.12 ± 0.03

Morimoto (2011)

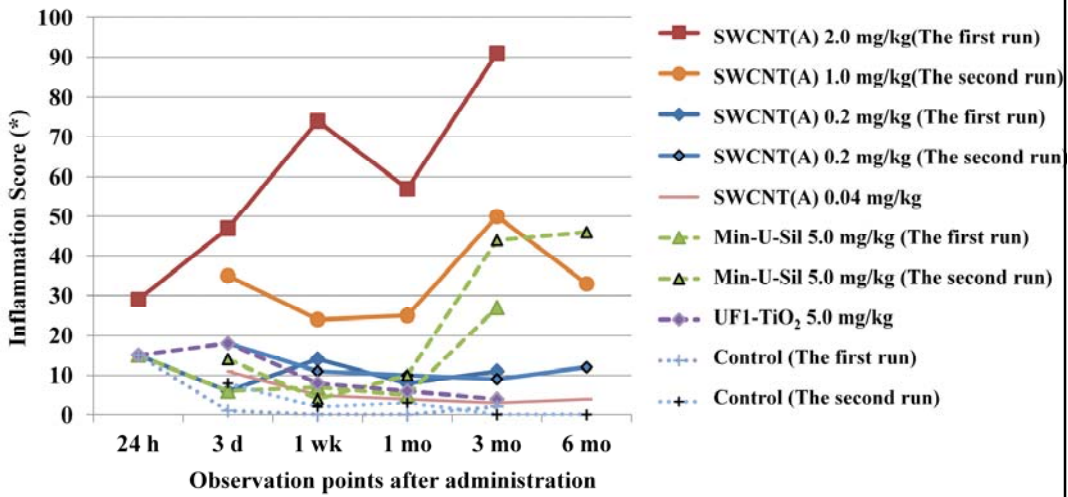
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This is a result of inhalation exposure test on SWCNT(A) with two dosages, 0.03 and 0.13 mg/m³. SWCNT(A) is a representative of single-walled carbon nanotube.

As shown in the slide, no inflammation score in the two dosages. We were not able to conduct the inhalation exposure test with higher than 0.13 doses due to technical difficulty and limited experimental capacity;

Therefore, we supposed temporarily the dose of 0.13 to be no-adverse-effect-level. And we conducted the IT tests to make up our weakpoint, which we cannot conduct IH test with higher than 0.13 dose.

Drastic Change in Inflammatory Reaction according to the Dosage in the IT Test

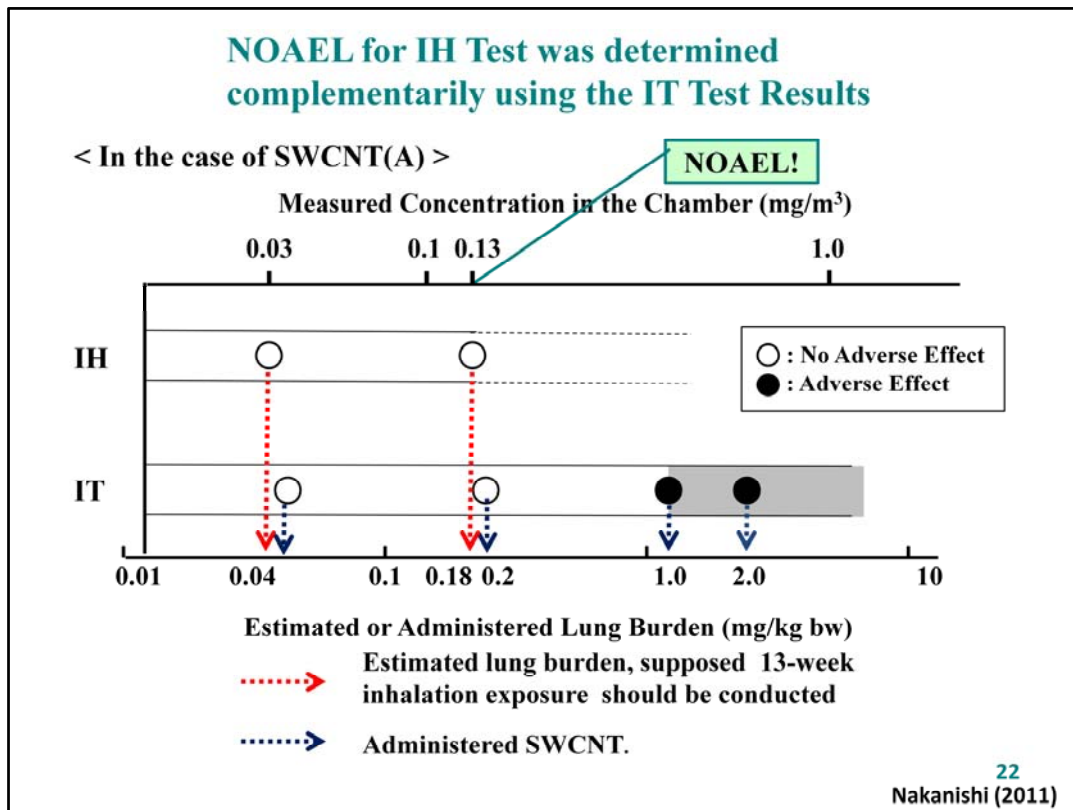


(*) Inflammation score is calculated, being based on the histopathological diagnosis

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Nakanishi (2011)

That is, we conducted IT tests with varying dose.

This shows the results of IT tests on SWCNT(A) with varying dose. Vertical Axis is Inflammation Score. As shown in this slide, the whole picture drastically changes according to the dose.

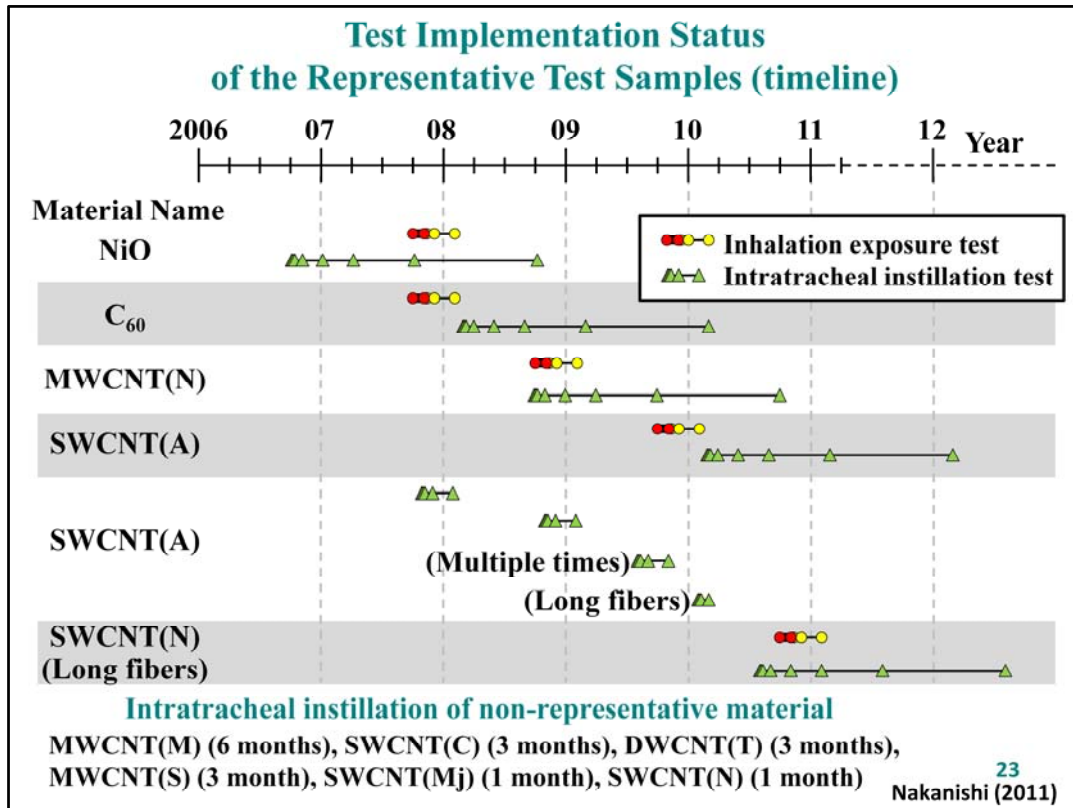


Organizing the IT and IH test results in terms of CNT lung burden, this chart is obtained. Upper scale indicates the concentration level of the chamber in IH test, and the lower scale is the CNT lung burden.

We estimated the SWCNT(A) lung burden after 13-week exposure, supposing that inhalation exposure test should be conducted for 13-week. In the case of 0.03 mg/m³, estimated body burden is 0.04 mg/kg bw. And at higher dose, 0.18.

Furthermore, the lung burden in IT tests was plotted. In IT test, the amount administered is amount of lung burden. In these area non-adverse-effect, whereas in those area adverse effects exist.

Comparing the lung burden of IH with that of IT, we concluded the temporal NOAEL of 0.13 to be final NOAEL for IH test. This is our experimental method.



This is the timeline of the tests of representative materials. These are the representative of each group.

Red is the inhalation test and green is the intratracheal instillation test. Initially, we tested nickel oxide as the positive control and next year, fullerene, and then multi-walled CNT(N), single-walled CNT(A), and then single-walled CNT(N) long fibers.

Those are the materials for which we conducted inhalation test over the 5-year period.

Calculation of NOAEL, and then OEL for Representative Materials

Material	NOAEL (rat) mg/m ³		OEL (human) mg/m ³
	4-week	13-week	
MWCNT(N)	0.37	0.185	0.08
SWCNT(A)	0.13	0.065	0.03
C ₆₀	Intratracheal instillation test	---	0.39
TiO ₂	Not tested in NEDO project	2.0*	0.6

* Bermudez et al. 2004

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Let me talk about the conclusive results of pulmonary toxicity of three representative materials. I will explain the details later. I am just going to give you the results.

We first had a 4-week test to derive a NOAEL for persistent inflammation endpoint and then we extrapolated to a 13-week NOAEL. We conducted Four-week testing of multi-walled, single-walled and fullerene.

A little bit different method was applied between CNT and fullerene. For titanium dioxide, we did not inhalation exposure test as a part of a NEDO project, but we determined NOAEL for titanium oxide based on the results from Professor Bermudez et al.

Based on the NOAEL we extrapolated OEL. Then, we have extrapolated to derive the human occupational exposure limit.

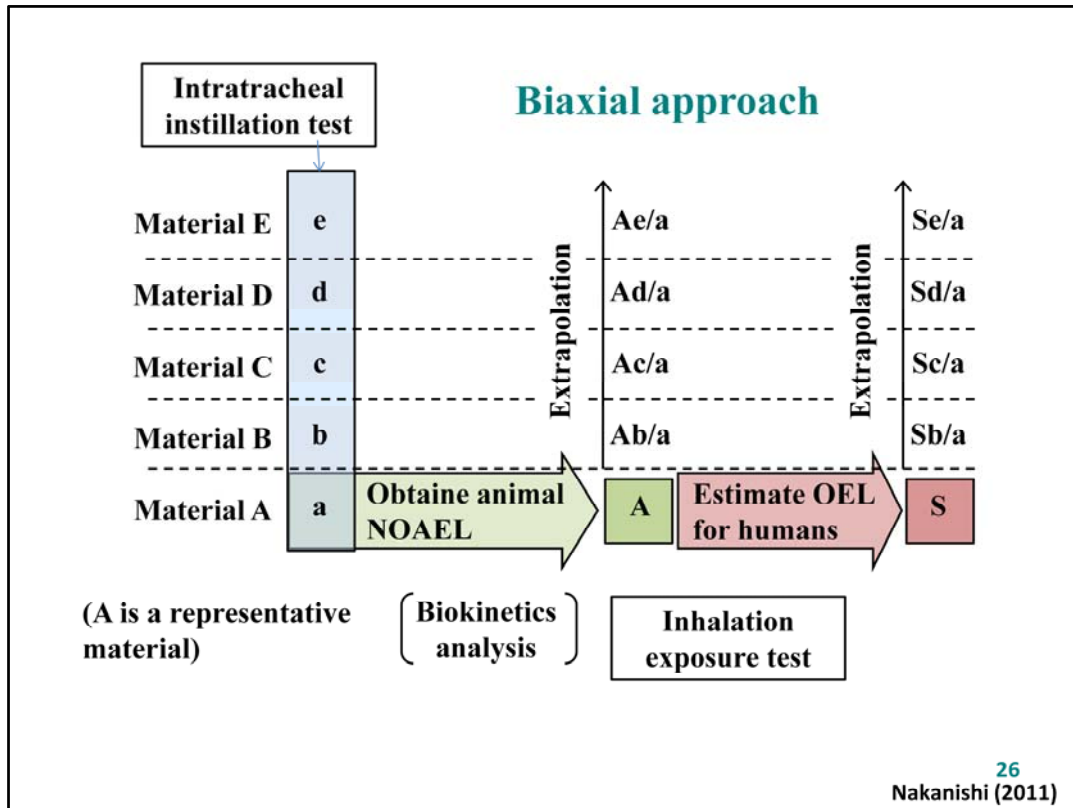
4. Critical Factors in Human Health Risks

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Let me move to the next topics. The subject is what is the critical factors in human health risks.

We determined rat NOAEL and OEL for three materials. However, those values are obtained for the representative one, for instance MWCNT(N) or SWCNT(A) .

What happens with other multi-walled CNTs or how about other single-walled CNT materials? I think you would have these questions in your minds. Now, in order to answer these questions, we have employed biaxial approach.



This chart explains Biaxial approach; We will conduct various testing for representative material, for instance, MWCNT(N). Therefore, we will have an animal NOAEL.

After that, we would estimate the human OEL. These results are plotted on the horizontal line. Now in the same MWCNT group, there are different materials, and for them, we measure the relative inflammation reaction size or scale, using simple tests and we obtained the ratio. These results are plotted on the vertical axis. Then, we'll again extrapolate and try to come up with a general picture.

We call this concept biaxial approach.

In reality, what are the simple tests that we have conducted? We have used intratracheal instillation tests.

What to Choose as the Vertical Metric?

The rate of increase of BALF neutrophil count at 1-month after instillation, compared with that of Min-U-Sil#5 as positive control.

→ **BALF inflammatory response indicator**

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In this case, 1 month later after the instillation, we had this BALF, the lavage fluid was used and looked at the increased rate of the neutrophils.

Neutrophil is something that you know leukocytes are almost equivalent to neutrophils.

Increase rate comparison of BALF neutrophil counts from the IT of SWCNT and DWCNT in rats							
Material	Manufacturing method	Specific surface area [m ² /g]	Particle diameter in liquid (width X length)	Animal species	Increase rate [%] ^a	Increase rate ratio [-] ^b	
Kobayashi <i>et al.</i> (2011) NEDO project	SWCNT (A)	CVD	1064	8.2 X 230 nm Dispersed	Male SD rats	269	1
	SWCNT (C)	HiPco	1000	Dispersed		120	0.45
	DWCNT (T1)	CVD	440	Dispersed	Male SD rats	58	0.22
NEDO project	DWCNT (T2)	CVD (with surface modifications)	310	Dispersed		39	0.14

a: $\frac{\text{CNT administered group BALF neutrophil count} - \text{negative control group BALF neutrophil count}}{\text{Positive control group BALF neutrophil count} - \text{negative control group BALF neutrophil count}} \times 100(\%)$
b: $\frac{\text{Increase rate of BALF neutrophil count of each CNT administered group}}{\text{Increase rate of BALF neutrophil count of CNT (A) administered group}}$
*BALF neutrophil count 1 month after intratracheal instillation of 1 mg/kg body weight of CNT
** BALF neutrophil count 1 month after administration of 5 mg/kg body weight of Min-U-Sil5 (crystalline silica)

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Nakanishi (2011)

As a result of that you saw this, SWCNT(A) is representative of SWCNT.. Then, the inhalation test was conducted,.

Then, the increased ratio of neutrophil was 269, a different single-walled one was 120 and double-walled nanotube 58 and 39 double wall. If the representative one is set at 1, then the ratio would be 0.45 and 0.22 and 0.14 respectively.

If you use the representative material as the standard for OEL, then you can see the numbers, 0.45, 0.22, 0.14, are small enough therefore, the OEL thus determined safe enough also for others..

Increase rate comparison of BALF neutrophil counts from the intratracheal instillation of MWCNT in rats

Material	Manufacturing method	Specific surface area [m ² /g]	Particle diameter in liquid (diameter X length)	Animal species	Increase rate [%] ^a	
Morimoto <i>et al.</i> (2011b) NEDO project	MWCNT (N)	CVD	69	48 nm X 1–10 μm	Male Wistar rats	-
Kobayashi <i>et al.</i> (2010) NEDO project	MWCNT (M)	CVD	37	60 nm X 1–20 μm	Male SD rats	6
NEDO project	MWCNT (S)	CVD	13		Male SD rats	5

* BALF neutrophil count 1 month after intratracheal instillation of 1 mg/kg body weight of CNT

** BALF neutrophil count 1 month after intratracheal instillation of 5 mg/kg body weight of Min-U-Sil5

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Nakanishi (2011)

We have done similar comparisons in the different types of multi-walled nanotubes.

Increase rate of BALF neutrophil counts observed in intratracheal instillation tests of SW/DW/MWCNT on rats

Material	SWCNT (A)	SWCNT (C)	DWCNT (T1)	DWCNT (T2)	MWCNT (N)	MWCNT (M)	MWCNT (S)
Increase rate [%] ^a	269	120	58	39	-	6	5
Increase rate ratio [-] ^b	1	0.45	0.22	0.14	-	0.02	0.02

a:
$$\frac{\text{BALF neutrophil count of CNT administered group}^* - \text{BALF neutrophil count of negative control group}}{\text{BALF neutrophil count of positive control group}^{**} - \text{BALF neutrophil count of negative control group}} \times 100$$
 (%)

b:
$$\frac{\text{Increase rate of BALF neutrophil count of each CNT administered group}}{\text{Increase rate of BALF neutrophil count of SWCNT (A) administered group}}$$

*: BALF neutrophil count at 1 month after intratracheal instillation of 1 mg/kg body weight of CNT

** : BALF neutrophil count at 1 month after administration of 5 mg/kg body weight of Min-U-Sil5 (crystalline silica)

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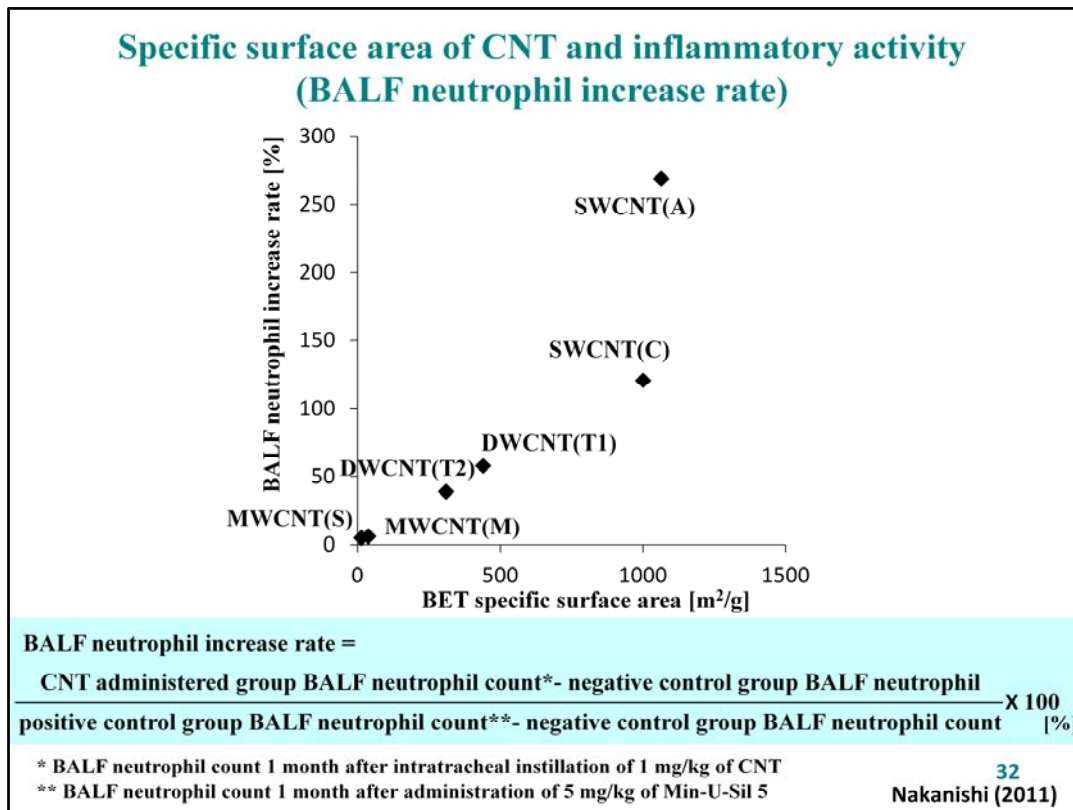
This table is summary of the relative inflammatory response for single-, double- and multi-walled carbon nanotube.

Thus, we can grasp the whole picture of inflammatory response due to CNT, based on which we can know the position of a CNT in terms of toxicity.

We Found Something Even More Remarkable

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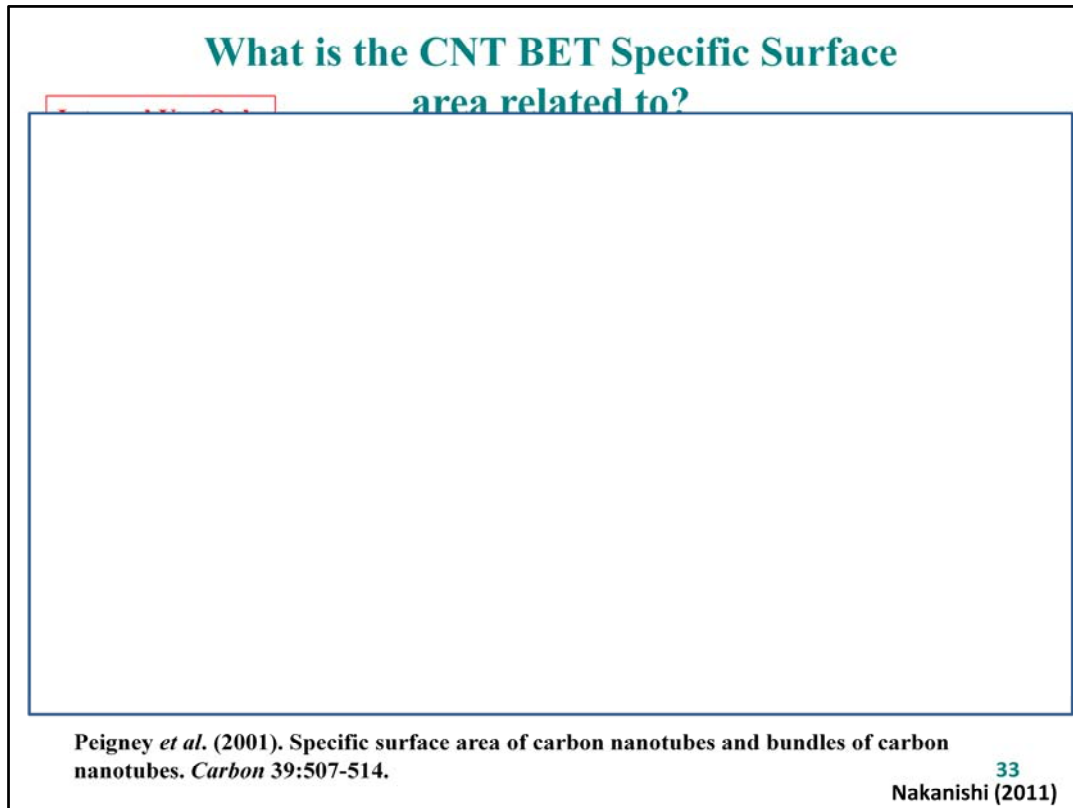
As we were undertaking that effort, we actually found out something that was really remarkable.



This is it.

The horizontal axis is the BET-specific surface area. The vertical axis is the BALF neutrophil increase rate. This is the relative indicator of inflammatory response. We can find that inflammatory response indicator is strongly related to the BET specific surface area.

They are not in linear relationship, but still there are some overlaps here. (The BET-specific surface area is where you have nitrogen gas, all the surface areas where the gas can come inside.)



This chart is from Peigney's paper. Vertical axis means BET specific surface area and horizontal one is CNT tube diameter. This chart indicates us that BET specific surface area is determined by the nature of CNT primary particle and the most outer wall is important in inflammatory response.

The BET specific surface area is very high for single-walled carbonnanotube. The greater the number of walls, the BET decreases.

Peigny's chart is obtained by theoretical consideration, However, it fits to the real CNT.

**How Different between the Mono-
dispersed and the Agglomerated in Terms
of Toxicity?**

**- Comparison between NEDO, Baytube
and Nanocyl -**

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These slide illustrates our sample.

SEM Image of Airborne Nanocyl(7000)

Lan Ma-Hock, et al. (2009) "Inhalation Toxicity of Multiwall Carbon Nanotubes in Rats Exposed for 3 Months" TOXICOLOGICAL SCIENCES, 112(2), 468–481. ³⁵

This is SEM image of Nanocyl 7000 particles collected from the test atmosphere chamber after dust generation.

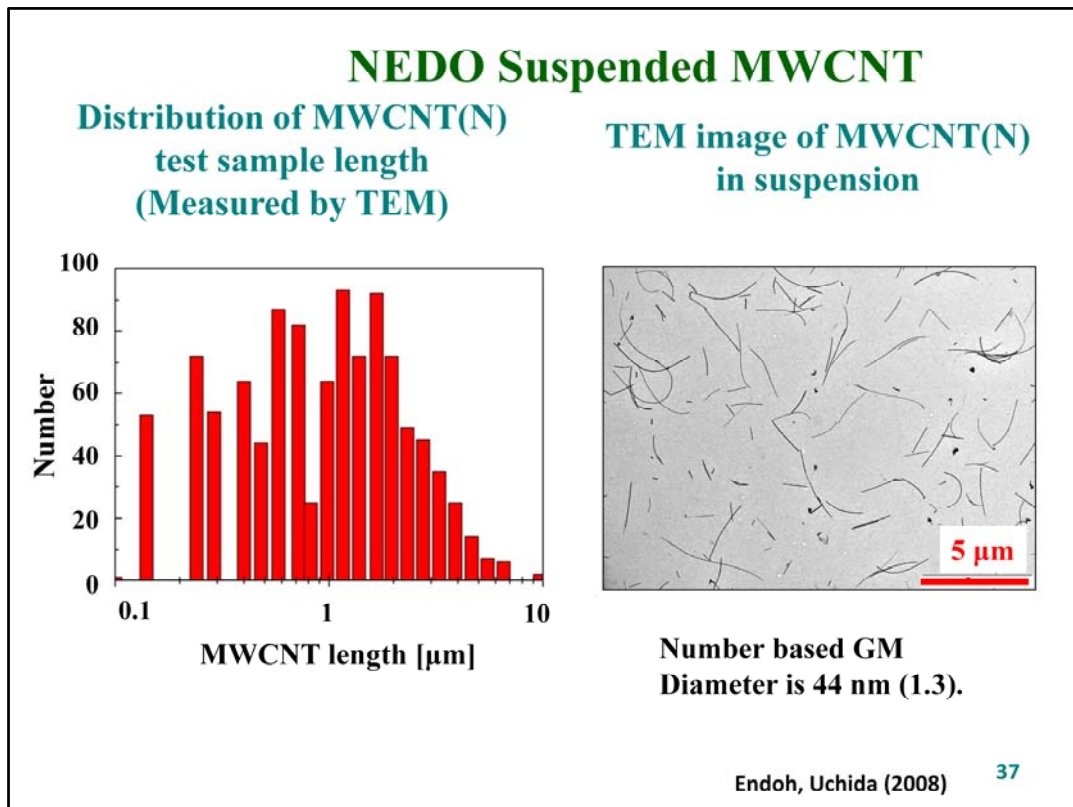
Scanning electron microscopy images of particles collected from the test atmosphere after dust generation, showing the wool-like clumps of agglomerated MWCNT with “hairy” surface at high magnification.

Comparison of SEM Images of Bulk, Micronized and Dispersed Baytube

Jürgen Pauluhn (2010) "Subchronic 13-Week Inhalation Exposure of Rats to Multiwalled Carbon Nanotubes: Toxic Effects Are Determined by Density of Agglomerate Structures, Not Fibrillar Structures" TOXICOLOGICAL SCIENCES, 113(1), 226–242.

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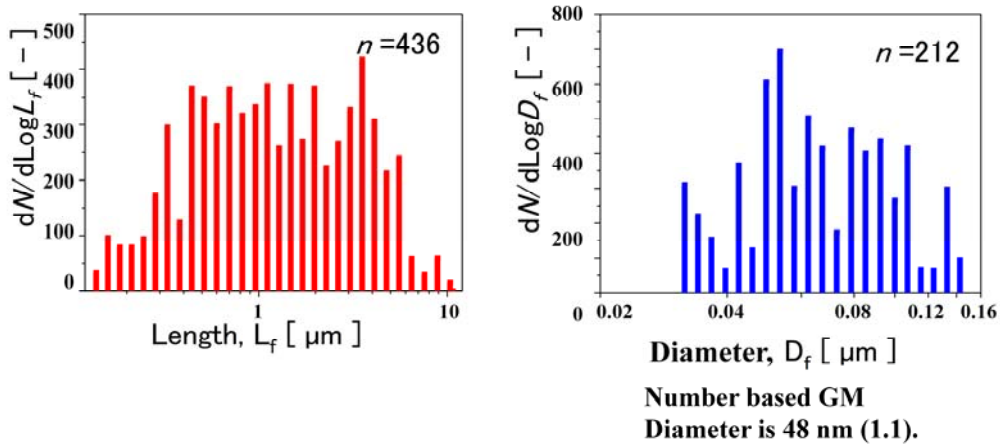
This is SEM image of dispersed test particles collected before the exposure chamber (lower and right panel).



These are the length distribution of MWCNT(N) suspended in the aqueous solution and the right panel is their TEM image. This suspended solution was used for IT test and raw material to generate aerosol in inhalation exposure chamber.

NEDO Airborne MWCNT

Distribution of the length and width of independent MWCNT(N) aerosol particles



Shimada(2011)

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These are the distribution of the length and tube diameter of independent MWCNT(N) in aerosol particles.

As you know from these illustrations, the status of test particles of Baytube and Nanocyl is very different from ours. Like I said a little while ago, our test particles are almost mono-dispersed and the tube diameter of MWCNT is nanoscale.

NOAEL and the MWCMT tube diameter

	MWCNT(N)	Baytube	Nanocyl
Tube diameter of primary particles (nm)	44 (1.3)	10 - 15	5 - 15
BET specific surface area(m²/g)	69	217	250 - 300
NOAEL (mg/m³)	0.185	0.10	0.05
NOAEL × BET	13.1	21.7	13.8
UF	2	1	2

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Nakanishi (2011)

I made up this table by extracting the critical figures. Tube diameter BET surface area and NOAEL. It is interesting to see the relationship between NOAEL and BET specific surface area. The greater the BET surface area, the smaller the NOAEL.

If NOAEL is multiplied by BET, the product is 13.1,21.7 and 13.8, these are very close each other. If we remove the uncertainty factor, the three product values are completely identical.

5. Tackling the Carcinogenicity Issues

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So far we have been talking about inflammability, but let's look at carcinogenicity.

Tackling the Carcinogenicity Issues

1. Issues related to PSLT:

- 1) Observation up to 2 years after administration in the IT test
- 2) Diagnosis of precancerous lesions in the IT test
- 3) Genotoxicity test
- 4) Considerations from mechanism

2. Issues related to fibers:

- 5) In addition to above 1)2)3), IT test using long fibers
- 6) Monitoring of airborne particle at the workplace

IT: Intratracheal instillation
OEL: Occupational Exposure limit

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Nakanishi (2011)

For this topic, PSLT or Poorly Soluble Low Toxicity particles are involved. Titanium oxide is representative of this group. It might cause toxicity or cancer by persistent retention.

Also, there is another cancer problem coming from the fiber form. How should we deal with that?

Regarding this issue, we implemented the subjects written in this slide. Today, I will talk about the subjects written in red.

Considerations from Mechanism - Tumor Due to PSLT -

- ◆ **Carcinogenicity due to a long-term, high-dosage exposure of PSLT is caused by persistent inflammation. Borm et al. (2004) concluded that the dosage that triggers lung cancer is about ten times that triggers neutrophil inflammation.**
- ◆ **Positive reactions in the genotoxicity tests are reported , though on rare occasions, and we also examined such problems. We concluded that this phenomenon is explained by the secondary genotoxicity.**
- ◆ **From this standpoint, NOAEL whose endpoint is persistent inflammation can be considered to satisfy the NOAEL for carcinogenicity.**
- ◆ **However, this conclusion should be suitably reviewed from the preventive viewpoint. From the context, it is appropriate to have set the period-limited OEL.**

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Nakanishi (2011)

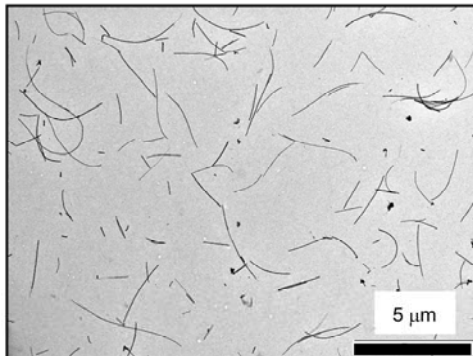
Generally speaking, it may be accepted by almost all experts that.

Let's begin from the PSLT carcinogenicity mechanism.

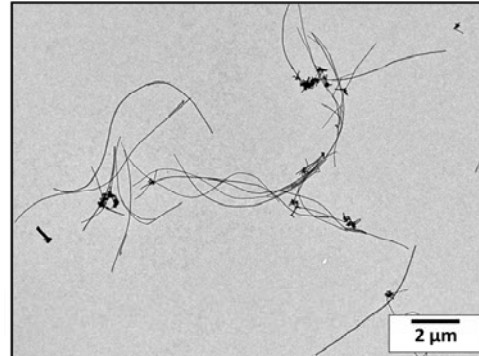
IT tests to compare the shorter MWCNT with longer one

TEM image of MWCNT(N) in suspension

(Conventional fiber)



(Long fiber)



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Endoh, Uchida(2008)

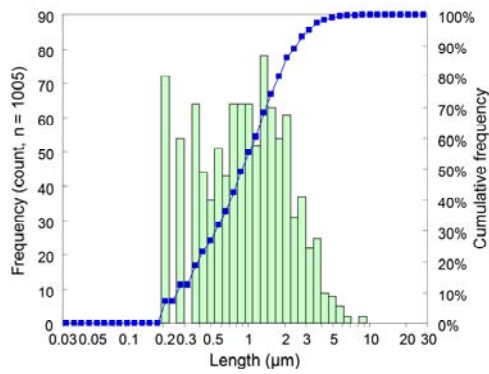
Let me move to the problem regarding the cancer due to long fiber.

We implemented the intratracheal instillation test using long fibers and not so long fibers and compared them.

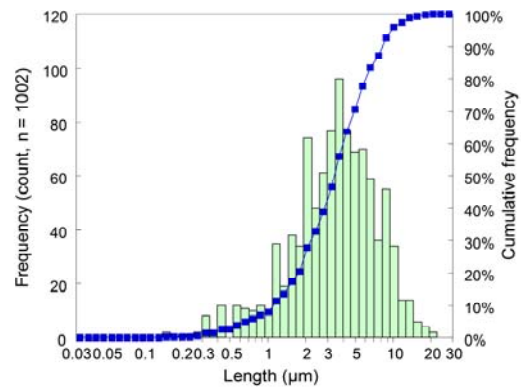
These are TEM image of MWCNT(N)in suspension. The left panel is conventional, whereas the right panel is longer fibers.

Fiber Length Distribution in Dispersion Liquid (MWCNT (N))

Conventional



Long fiber



The extent of inflammatory response is equal for the both.

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Next is fiber length distribution. The conventional ones are less than 10 microns or so. The longer one can go up to 20 microns. This is in numbers.

We did IT test using two fiber and compared them. We obtained the results up to one year after administration. There is no difference between the two for now.

CNT and Mesothelioma

The following papers back up the argument regarding the similarity between CNT and asbestos in terms of carcinogenicity: Takagi et al.(2008), Poland et al.(2008), Sakamoto et al., Donaldson(2010)(hypothesis):

However, it really boils down to two points:

- 1) When reached the mesothelium, CNT would have a high potential to cause mesothelioma, as asbestos.
- 2) Long, straight (needle-like) fibers remain in the pleura and generate tumors, but tangles or short fibers will be cleared.

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Nakanishi (2011)

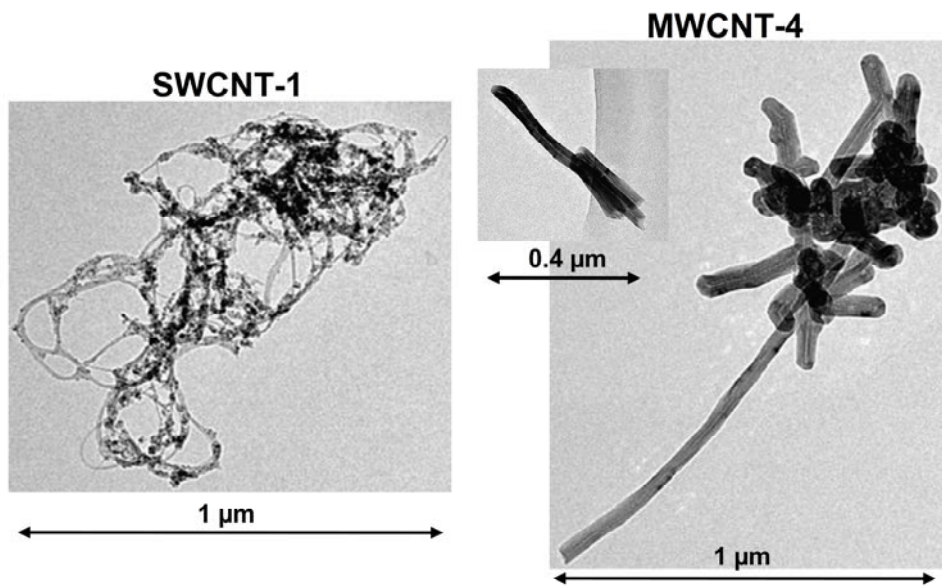
Next is the problem of CNT and Mesothelioma. In Japan, the public has a strong concern over the cancer problem, especially; they say strongly that CNT might be like asbestos and it might be as dangerous as asbestos.

However, I think recently it really boils down to two points: one is the notion that when CNT reached the mesothelium, it would have a high potential to cause mesothelioma as asbestos.

The second notation stated by Donaldson et al. which is stating long, straight, needle-like fibers remain in the pleura and generate tumors, but tangled or short fibers will be cleared. Still, we believe that the points at issue are very much boiled down to or narrowed down to these two points.

From this situation, the information of size and shape of airborne CNT is important.

Image from transmission electron microscope (TEM) of airborne CNT particle from Dustiness Test (NEDO Project)

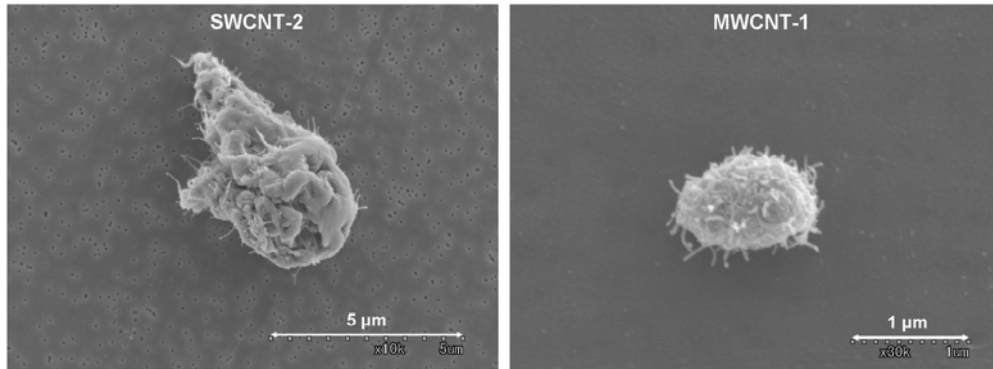


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Ogura (2011)

This is the TEM Image of airborne CNT particle from dustability Test.

A simulated process involving falling, collisions as well as aerosolization by the stream of air.

**Image from scanning electron microscope (SEM) of airborne
CNT particle from Dustiness Test (NEDO Project)**



Size and Shape of Airborne CNT Particles

- ◆ **The Electron microscope observations indicated that many of the CNT particles emitted in the air were submicron to micron in size.**
- ◆ **SWCNT, DWCNT, or thin MWCNT did not take the form of single, independent fibers but were present as wool-like clumps that were intricately interwoven.**
- ◆ **Particles in single straight fibers or in straight bundles of several fibers, though not so long, were observed among thick MWCNT or CNF.**

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Ogura (2011)

In this context, Dr. Ogura's investigation is very useful and interesting. He investigated the size and shape of airborne CNT particles at workplace and in the dustability test apparatus.

Individual Management - Long Fiber Issue

- ◆ **It is difficult to conduct the inhalation exposure testing of long-fibrous CNTs. It is, therefore, hard to prove experimentally that they will or will not cause mesothelioma.**
- ◆ **In addition, it would also be difficult to prove experimentally that they will not reach the pleura.**
- ◆ **Taking this into account, it would be rather appropriate, for the time being, to see if there are any long, straight, needle-like fibers, and if there are, an individual response considering a greater than normal safety factor would be required.**

6. Exposure Assessment

Hazard Ratio of Worker:							
No.	Material	Working scale	Process	Total dust or Inhalable dust level [$\mu\text{g}/\text{m}^3$]	Estimated respirable exposure level of worker ^(A) [$\mu\text{g}/\text{m}^3$]	OEL(B) [$\mu\text{g}/\text{m}^3$]	Hazard ratio(A/B)
Site 1	SWCNT (unpurified): Laser ablation or HiPco	Laboratory & plant	Collection, cleaning	0.7–52.73 (Estimated CNT conc. based on catalytic metal)	0.35–26.4	30	0.012–0.88
Site 2	MWCNT: CVD, Approx. 50 nm dia., approx. 1.5 μm long	Laboratory	Blending (opening blender)	332	166	80	2.1
			Weighing, CNT solution spraying	113–193	57–95	80	0.71–1.2
Site 3	MWCNT: CVD	Plant	Synthesis, recovery, weighing, packaging, dispersion	31–286	16–143	80	0.19–1.8
Site 4	MWCNT: CVD	Plant	Bagging (manual)	63 (ECI)	31	80	0.39
Site 5	CNF: approx. 100 nm dia., 500 mg/batch	Laboratory	Wet saw cutting of CNF composite	1,094 (TC)	547	80	6.8
			Weighing, mixing	64–221 (TC)	32–110	80	0.40–1.4
Site 6	CNF: CVD, 70–200 nm dia., 50–100 μm long, 10–20 kg/shift	Plant	Chemical treatment	31–248 (TC)	15.5–124	80	0.19–1.6
			Transferring (dumping), drying	1,839 (TC)	920	80	11
			Scooping (bagging)	1,729 (TC)	865	80	11
<p>•The hazard ratios for the exposure levels estimated from results of on-site investigations are about 0.1 to 10. •Processes in which high exposure is likely to occur are those for handling dry CNT powder including collecting, transferring, weighing, mixing, bagging, and maintenance.</p>							
EC: Elemental carbon concentration, TC: Total carbon concentration						51 Ogura (2011)	

This is a result of exposure level of worker and Hazard Ratio for health risk, which was obtained by processing the published and our monitored data. If you look at the Site 3, the respirable dust concentration is shown here.

Some of it is estimate, and 80 is the OEL value that we use for the multi-walled CNT, and the hazard quotient A/B is 0.19 to 1.8.

Generally, you say that if it is over 1, there is risk, but this is not the actual factory site because they actually opened up the equipment to see how much release there is. It does not mean that that site is really dangerous. This is how risk assessment can be done.

Ratio of Estimated Exposure Level of Worker to OEL

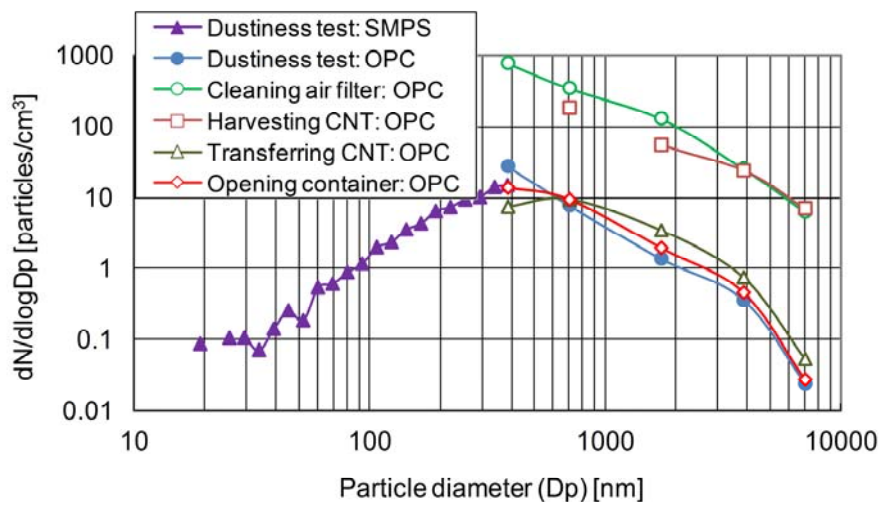
No	Material	Working scale	Process	Total dust or Inhalable dust level [$\mu\text{g}/\text{m}^3$]	Particle size category [μm]	Estimated respirable exposure level of worker A [$\mu\text{g}/\text{m}^3$]	OEL B [$\mu\text{g}/\text{m}^3$]	Hazard ratio A/B
site 3	MWCNT :CVD	Plant	Synthesis, recovery, weighing, packaging, dispersion	31–286*	0.01 < D_m < 0.1	0.00058 – 0.0054	31	0.000019 – 0.00017
					0.1 < D_m , D_a < 1	2.7 – 25	90	0.030 – 0.28
					1 < D_a < 4	13 – 118	71	0.18 – 1.7
					Total	16 – 143		0.21 – 1.9
*Cited from Lee et al.(2010)				EC: Elemental carbon concentration, TC: Total carbon concentration				

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Ogura (2011)

This table shows the data of Site 3.

In this table, the particle was classified by particle size and the hazard ratio was calculated. If we take the assumption that the toxicity of the CNT is not so different by particle size, then the risk is caused by particles larger than 1 micron, 1 to 4 microns, those are the particles that you really have to control well; that is what this is indicating.

Comparison of SWCNT in on-site investigation and Dustiness test results: Number-based particle size distribution



See Ogura *et al.* (2011) for details on on-site investigation results. For on-site investigation results, background concentration has been deducted. The particle sizes are expressed in terms of electrical mobility diameter for the SMPS results and optical diameter for the OPC results. N is number concentration, Dp is particle diameter.

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Ogura (2011)

How did we get this particle concentration estimation by size? This is the curve that we assumed.

7. Final Proposal of OEL and Conclusive Remarks

Our Proposal regarding OEL (1)

	BET specific surface area (m ² /g)	Diameter (nm)	OEL (PL) (mg/m ³)	Manufacturer
MWCNT	69	44	0.08	N
SWCNT	1064	2.8	0.03	A

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Nakanishi (2011)

This is our proposal regarding OEL as indicated earlier. For multiwalled carbon nanotube, OEL is 0.08, and for a single wall, it's 0.03. let us look at the BET-specific surface area for the multi-walled CNT, it is 69.

That is related to the fact that the tube diameter is 44 nanometers.

Of course, the size of the primary particle will determine or the diameter will determine the specific surface area. Thinking this, there is one problem with this.

The Tube Diameter of MWCNT(N) is too Thick

- 1. We used MWCNT(N) as a representative of MWCNT, the tube diameter of which is 44nm thick, too thick to be a standard MWCNT.**
- 2. Taking this into account, it might be better to propose the OEL obtained for SWCNT, 0.03 as a common for all CNTs, rather than having a separate value as the OEL for MWCNT.**

Since the BET specific surface area of SWCNT(A) is over 1000 m²/g , nearly marginal for CNT, this proposal is reasonable.

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The carbon nanotubes that are to be manufactured will be much thinner.

In other words, this 44 nanometer diameter is too thin. This will be not conservative and should we use such a non-conservative figure was the question that we've had.

Our New Proposal regarding OEL (2)

	OEL mg/m ³	Manufacturer (model number)	Values of other institutions
TiO ₂	0.61	E. Degussa P25	0.30 (NIOSH)
C ₆₀	0.39	Company F	
CNT	0.03	SWCNT(A)	0.05 (Bayer) 0.0025 (BASF) 0.007(NIOSH)

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We revised our proposal as this. These are the values proposed by other institutions.

Furthermore, we examined the meaning of OEL and defined it more clearly.

Proposing a Period-limited OEL

- ◆ **Our tests are subchronic at best, while many toxicity tests are being conducted including long-term inhalation exposure tests around the world and in near future they will be available.**
- ◆ **Taking this into consideration, it is better to propose the period-limited OEL for exposure of around 15 years, which corresponds to subchronic, on the basis of the premise that the results will be reviewed within approximately 10 years.**
- ◆ **The period-limited OEL thus obtained is expressed as the OEL(PL), in which PL stands for period-limited.**
- ◆ **The proposal of such a period-limited criterion can be an application example of the adaptive management, which we believe to be essential for risk evaluation for new technology.**

Our Proposal regarding OEL(PL) (3)

	OEL (PL) mg/m ³	Manufacturer (model number)	Values of other institutions
TiO ₂	0.61	E. Degussa P25	0.30 (NIOSH)
C ₆₀	0.39	Company F	
CNT	0.03	SWCNT(A)	0.05 (Bayer) 0.0025 (BASF) 0.007(NIOSH)

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Nakanishi (2011)

This is a final result.

Remarks (1)

- 1. In order to derive rat NOAELs for CNT and fullerene, we conducted pulmonary toxicity tests using test samples most of whose secondary particles are nanoscale. Our TiO₂ NOAEL, on the other hand was obtained through compilation of published data.**
- 2. These rat NOAELs thus obtained to control the persistent inflammation are, in principle, also effective in preventing tumor.**
- 3. We have extrapolated human OELs from the rat NOAELs for three nanomaterial groups. Considering that the NOAELs are based on the subchronic test, we proposed the OEL to be period-limited, expressed as the OEL (PL). The proposal of such a period-limited criterion can be an application example of the adaptive management, which we believe to be essential for risk evaluation for new technology.**

Remarks (2)

- 4. Whether the test sample is nanoscale dispersed or agglomerated (like cotton candy) did not result in a critical difference, comparing the various toxicity tests.**
- 5. We have also discovered that the degree of CNT inflammatory response is strongly related to the BET specific surface area, and is determined by the primary particle size rather than the secondary particle size.**
- 6. While the BET specific surface area itself could hardly catalyze the biological reaction, we, nevertheless, consider it extremely useful for such purposes as estimating the strength of inflammatory response and classifying CNTs from the viewpoint of toxicity.**

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Nakanishi(2011)

Using the biaxial approach, we can select the representative one among the group materials.

Remarks (3)

- 7. The three test results on NOAEL of MWCNT that have been conducted so far including ours are consistent (if we take the BET specific surface area into account), which will allow us to conclude that they are globally conclusive. We, therefore, would encourage to use this NOAEL value though more tests with various BET surface areas must be conducted.**

- 8. We would like to touch on the hypothesis by Donaldson et al. that long and straight (needle-like) CNT fibers would cause tumor, especially mesothelioma. Although we were unable to determine experimentally the appropriateness of this hypothesis, we would recommend the business owners to first check the size and shape of airborne particles at their own workplaces. Should such particles be found, a response considering a greater than normal safety factor would be required.**

Remarks (4)

9. On a final note, in conducting toxicity assessment and/or risk assessment of new technology or novel material, we believe that a top priority should be given to such researches as to aim to quickly grasp the whole picture of toxicity and/or risk of target material rather than detailed and time-consuming ones. Going forward, we should discuss more about the methodology for risk evaluation of new technology or new materials, because the risk evaluation and risk management is essential for putting the innovative technology into market.

Thank you for your attention.

Supplemental Information

Outputs released to the Public

The following is available on-line:

http://www.aist-riss.jp/main/modules/product/nano_rad.html

1. Risk assessment documents for the three nanomaterial groups -
1) Titanium dioxide (TiO₂), 2) Fullerene (C₆₀), and
3) Carbon nanotubes (CNT)
2. Executive summary of the three risk assessment documents and
“Approaches”. (Japanese and English)
3. Methods of Preparation and Characterization of Manufactured
Nanomaterials for Toxicity Testing. (Japanese and English)
4. Guideline for Hazard Assessment Testing of Industrial Nanomaterials
in Animal Analysis by Respiratory Exposure. (Japanese and English)
5. Emission and Exposure Assessment of Manufactured Nanomaterials.
6. Others (9 booklets)

Summary of Genotoxicity Tests (1)

	<i>in vitro</i>		<i>in vivo</i>	
	Bacterial Reverse Mutation Test (with or without S9 mix)	Mammalian Chromosome Aberration Test (with or without S9 mix)	Mammalian Erythrocyte Micronucleus Test	Comet Assay following intratracheal instillation in rats (single or five-repeated dose)
SWCNT (A)	Negative	Negative	Negative	–
SWCNT (N)	Negative	Negative	Negative	Negative
MWCNT (N)	Negative	Weak positive on numerical chromosomal aberration (without S9 mix)	Negative	Negative
MWCNT (M)	Negative	Positive on numerical chromosomal aberration (with or without S9 mix)	Negative	–

Ema & Naya(2011) 66

Summary of Genotoxicity Tests (2)

	<i>in vitro</i>		<i>in vivo</i>	
	Bacterial Reverse Mutation Test (with or without S9 mix)	Mammalian Chromosome Aberration Test (with or without S9 mix)	Mammalian Erythrocyte Micronucleus Test	Comet Assay following intratracheal instillation in rats (single or five repeated dose)
Anatase TiO₂ (ST-01)	–	–	–	Negative
Fullerene C₆₀ (Nanom Purple SU)	Negative	Negative	Negative	Negative

Ema & Naya(2011) ⁶⁷

Publications Referred in this Presentation (1)

- Horie et al. (2010a) In vitro evaluation of cellular responses induced by stable fullerene C₆₀ medium dispersion. *Journal of Biochemistry*. 148(3):289-298.
- Horie et al. (2010b) Cellular responses by stable and uniform ultrafine titanium dioxide particles in culture-medium dispersions when secondary particle size was 100 nm or less. *Toxicology in Vitro*. 24:1629-1638.
- Kobayashi et al. (2009) Comparative pulmonary toxicity study of TiO₂ particles of three different primary sizes in rats. *Toxicology*. 264(1-2):110-118.
- Kobayashi et al. (2010) Biological response and morphological assessment of individually dispersed multi-wall carbon nanotubes in the lung after intratracheal instillation in rats. *Toxicology* 276:143-153.
- Kobayashi et al. (2011) Pulmonary and systemic responses of highly pure and well-dispersed single-wall carbon nanotubes after intratracheal instillation in rats. *Inhalation Toxicology*. 23(13):814-828.
- Morimoto et al. (2010) Inflammogenic effect of well-characterized fullerene in inhalation and intratracheal instillation studies. *Particle and Fibre Toxicology*. 7(1):4.
- Morioto et al. (2011a) Pulmonary toxicity of well-dispersed single-wall carbon nanotubes after inhalation. *Nanotoxicology*. Posted online on September 26, 2011. doi:10.3109/17435390.2011.620719.
- Morimoto et al. (2011b) Pulmonary toxicity of well-dispersed multi-wall carbon nanotubes following inhalation and intratracheal instillation. *Nanotoxicology*. Posted online on June 29, 2011. doi:10.3109/17435390.2011.594912.
- Oyabu et al. (2011) Biopersistence of inhaled MWCNT in rat lungs in a 4-week well-characterized exposure. *Inhalation Toxicology*. 23(13):784-791.
- Shimada et al. (2009) Development and Evaluation of an Aerosol Generation and Supplying System for Inhalation Experiments of Manufactured Nanoparticles. *Environmental Science and Technology*. 43:5529-5534.
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- Tamura et al. (2011) A determination method of pristine multiwall carbon nanotubes in rat lungs after intratracheal instillation exposure by combustive oxidation-nondispersive infrared analysis. *Talanta*. 84(3):802-808.

A complete list of publications and presentations are available at http://www.aist-riss.jp/projects/nedo-nanorisk/index_e.html.

Publications Referred in this Presentation (2)

- Ema et al. (2009) Genotoxicity evaluation of titanium dioxide nanoparticles. *Jpn J Environ Toxicol.* 12(2):71-84. (in Japanese)
- Ema et al. (2010a) Review of carcinogenicity of titanium dioxide. *Jpn J Environ Toxicol.* 13(1):15-26. (in Japanese)
- Ema et al. (2010b) Reproductive and developmental toxicity studies of manufactured nanomaterials. *Reprod Toxicol.* 30(3):343-352.
- Ema et al. (2010c) Tissue distribution and toxicity of titanium dioxide after various routes of exposure. *Jpn J Environ Toxicol.* 13(2):85-103. (in Japanese)
- Ema et al. (2011a) Genotoxicity evaluation of fullerenes and their derivatives. *Jpn J Environ Toxicol.* 14(1):69-80. (in Japanese)
- Ema et al. (2011b) Genotoxicity evaluation of carbon nanotubes. *Jpn J Environ Toxicol.* 14(1):81-97. (in Japanese)
- Ema et al. (2011c) Evaluation of dermal and eye irritation and skin sensitization due to carbon nanotubes. *Regul Toxicol Pharmacol.* 61(3):276-281.
- Ema et al. (submitted) Dermal and eye irritation and skin sensitization studies of fullerenes C₆₀ nanoparticles. *Drug Chem Toxicol.*
- Ema et al. (submitted) Genotoxicity evaluation of fullerene C₆₀ nanoparticles in a comet assay using lung cells of intratracheally instilled rats. *Regul Toxicol Pharmacol.*
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- Naya et al. (submitted) *In vivo* genotoxicity study of titanium dioxide nanoparticles using comet assay following intratracheal instillation in rats. *Regul Toxicol Pharmacol.*
- Obata and Honda (2011) Dynamic behavior of carbon nanotube and bio-/artificial surfactants complexes in an aqueous environment. *J.Phys.Chem.C* ,115,19659-19667

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