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Polymer coating on carbon nanotubes into Durobeads is a novel strategy for human environmental safety

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ABSTRACT

Carbon nanotubes (CNTs) have attracted much business interest in industrial applications due to their high electrical and heat conductivities while being both durable and versatile. However, multiwall CNTs (MWCNTs) of ~50 nm diameter (NT50) have been shown to cause mesothelioma in rodents after direct exposure to mesothelial cells, and thus were classified as a Group 2B carcinogen to humans, which requires considerable regulations for use. In contrast, tangled MWCNTs of ~15 nm diameter (NTtngl) are not carcinogenic to rats, indicating that the physical dimension linked with mesothelial cellular uptake is an important factor for human environmental risk. In the present study, hypothesizing that dustability is another distinct risk factor, for the first time, we evaluated the toxicity of CNT granules (Durobeads) that were generated with a polymer coating to mesothelial cells. Polymer coating induced prominent agglomeration and significantly suppressed the dustability of CNTs in a dose-dependent manner, with a 10% polymer coating resulting in 730 times less dustability. These CNT granules revealed significantly lower mesothelial uptake and cytotoxicity in comparison to NT50 in *in vitro* assays. Similarly, in *in vivo* analyses, CNT granules induced limited peritoneal inflammation 4 weeks after intraperitoneal injection, whereas NT50 caused severe fibrosing inflammation. Previously, we demonstrated that the severity of inflammation by intraperitoneal injection in the subacute studies are in agreement with the mesothelial carcinogenicity by CNTs. Therefore, we suggest that adding a polymer coating to CNTs provides another smart strategy for the safe use of CNTs.

Keywords: carbon nanotube, mesothelioma, polymer coating

INTRODUCTION

Carbon nanotubes (CNTs) have attracted much attention in engineering and materials science since their discovery in 1991[1)] due to their high electrical and heat conductivities while being both durable and versatile.[2)] The global market of CNT was predicted to expand exponentially. However, the market expansion has so far been delayed. One of the reasons that limited its industrial use was the concern for human safety after extended exposure. Unfortunately, the

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physical dimensions of CNTs are extremely similar to that of asbestos fibers regarding size and aspect ratio,[3-4] which have caused and are still causing serious social problems by the induction of mesothelioma/lung cancer over the course of many clinical trials.[5]

Indeed, multiwall CNTs (MWCNTs) of ~50 nm diameter (NT50) caused mesothelioma in $p53^{+/-}$ mice[6] and in wild-type rats[7] with intraperitoneal injections. It is important to note that the genetic alterations caused by MWCNTs observed in rat mesothelioma were very similar to those caused by asbestos fibers.[7-9] For example, homozygous deletion of the *p16*^{INK4A} tumor suppressor gene was induced in most of the cases.[7] We also found that local excess iron is largely responsible for the pathogenesis of mesothelioma and that CNTs have a high affinity for histones, hemoglobin and transferrin.[10] Another important point is the diameter of MWCNTs, which determined whether the MWCNTs would be phagocytosed by mesothelial cells.[7,10] This process appears indispensable in CNT-induced mesothelial carcinogenesis in that MWCNTs of ~15 nm diameter (NTtngl; tangled) are neither phagocytosed by mesothelial cells nor cause diffuse fibrosing peritonitis[7,10] or mesothelioma.[11]

For decreasing the human environmental risk in using this multifunctional nanomaterial, it would be important to consider each step of human exposure; 1) presence of airborne fibrous particles, 2) inhalation to alveoli, 3) activation of macrophage killers after phagocytosis, 4) durability of the needle-like structure with piercing of the visceral pleura into the pleural cavity and 5) stacking in the lymphatic system present on the parietal pleura.[12-14] Here, we focus on a novel strategy to agglomerate CNTs with a polymer coating to inhibit the processes described above. Many of the risk factors for respiratory diseases depend on dustability, as represented by tobacco smoke, bacteria, virus, asbestos and coal dust. Indeed, size enlargement can suppress dustability.[15] Polymers have been used for the reinforcement of CNTs as a polymer-nanotube composite[16-17] and can coat CNT surfaces to enable agglomeration. However, little is known about the effect of polymer-coated CNTs on mesothelial cells. In this study, we evaluated the biological effects of polymer coating on CNTs in mesothelial cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Two MWCNTs, NT50 as a positive control (positive carcinogenicity to mesothelial cells) and NTtngl as a negative control (negative carcinogenicity), were obtained from Showa Denko (Tokyo, Japan) as described.[7] K-Nanos 100P (pristine power-type by KUMHO) and K-Nanos 100T (granulation-type by KUMHO) were obtained from KUMHO PETROCHEMICAL (Seoul, Korea). Male Fischer-344 rats (6-week-old) were obtained from Chubu Kagaku Shizai (Nagoya, Japan). The animal experimental committee of Nagoya University Graduate School of Medicine approved these animal experiments. The Met5A mesothelial cell lines were obtained from the American Type Culture Collection (Manassas, VA).

Synthesizing CNT granules (Durobeads) with a polymer coating

K-Nanos 100P (45 g) of 7–23 nm diameter (Table 1) and pure water (2.5 L) were mixed using a homogenizer at 6,000 rpm to disperse the CNTs. The dispersion was transferred to a container equipped with a screw mixer (1,000 rpm), to which 360 ml of toluene containing olefin-based dissolved polymer with a melting temperature of 103 ºC was added to obtain spherical granules of 0.5–2.0 mm in diameter. The final preparation was obtained by filtering the solution through a 60–mesh metal screen and then drying for 7 h in a vacuum oven set at 70 ºC (Fig. 1A and B). Granule formation (granulation) is possible with 0% polymer in this synthetic system (Fig. 1C). CNT granules were sonicated prior to use for *in vitro* and *in vivo*

Table 1 Characteristics of K-Nanos 100P

Item	**K-Nanos 100P**
Diameter	7–23 nm
Bundle length	26 nm (average)
Bulk density	0.015 g /ml
Carbon purity	~ 95%
Polymer %	**Bulk density (g/ml)**
K-Nanos 100P	0.015
0%	0.095
6%	0.126
10%	0.148
20%	0.148

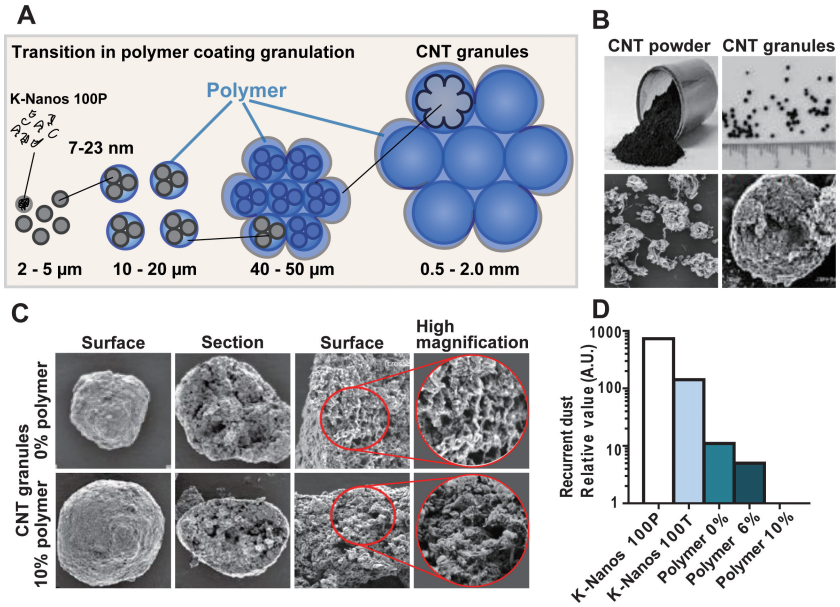

Fig. 1 Carbon nanotube (CNT) granules generated by polymer coating reduce the dustability
(A) Procedure to generate CNT granules with polymer coating. (B) Metamorphosis of K-nanos 100P from CNT powder to granules. Lower panels, scanning electronmicroscopy (SEM). (C) SEM images in CNT granules with or without polymer coating. (D) Dustability of each CNT evaluated by recurrent dust.

experiments, as described previously.[7,18)]

Scanning electron microscopy

CNT granules, named Durobeads, were observed with a scanning electron microscope (SU3500, Hitachi High Technologies; Tokyo) with magnifications ranging from 18–720x.

Evaluation of respirable particles (recurrent dust)

Measurements were performed based on recommendations by the Japan Industrial Safety & Health Association. A dust regenerator for sediment dust, Model SKY-2 (Shibata Scientific Technology; Saitama, Japan), was used. CNTs were introduced from the bottom in an air current at 10 L/ml and the scattered amount was calculated, based on the weight of the CNTs adsorbed on the filter paper set at the outlet. Five different samples were evaluated: 0%, 6%, and 10% polymer-coated K-Nanos 100P granules, as well as K-Nanos 100P alone and K-Nanos 100T alone.

Analysis based on cultured cells

The Met5A human mesothelial cell line, immortalized with SV40, was used. M199 containing 10% fetal bovine serum (#S1780, Biowest; Nuaillé, France) and 1% antibiotic-antimyotic (#15240-062, GIBCO; Grand Island, NY) was used in a humidified incubator with 5% CO_2 at 37 °C. Cells were used within two months after thawing. For cell death assay, we plated cells at a density of 1×10^4 cells/cm^2 48 h prior to the addition of each material in 96-well plates. The cell death detection assay (LDH assay kit, Sigma Aldrich; St. Louis, MO) was performed 48 h after addition of material and analyzed with a plate reader (Power Scan 4, DS Pharma Biomedical; Osaka, Japan). In the evaluation of cellular uptake, cells were observed 48 h after exposure of CNTs by phase contrast microscopy (BZ-9000, Keyence; Osaka, Japan).

Analysis based on an in vivo subacute study using rats

To observe the subacute reactions *in vivo*, male rats were injected with 10 mg of CNTs of each type intraperitoneally, by the same methods as described in our previous study.[18] Rats were placed under close observation and euthanized 4 weeks after the injection. Complete autopsies were performed and samples were fixed in 10% phosphate-buffered formaldehyde for routine paraffin-embedded sections with hematoxylin and eosin staining, as well as Masson trichrome staining for staining collagen fibers. We used three rats in each group (0%, 6%, 10% and 20% polymer-coated K-nanos 100P, NT50 and NTtngl).

Statistics

All experiments were performed in triplicate. Data are expressed as the means ± SEM. Significance was evaluated by using a one-way ANOVA with a post hoc Tukey test or Student's *t*-test, using Prism 5 software (GraphPad; La Jolla, CA).

RESULTS

Reduction in the dustability of CNTs by polymer coating

CNTs were modified with a polymer coating for the possible reduction of dustability (Fig. 1A). The diameter of the original K-Nanos 100P ranged from 7–23 nm and polymer coating significantly increased bulk density (Table 1). Fibrous K-Nanos 100Ps were agglomerated to spherical CNT granules with a diameter of 1.0–2.0 mm. After this modification, we found that fibrous components were mostly embedded on the surface whereas the original K-Nanos 100Ps were mostly tangled in shape (Fig. 1B and 1C). As expected, the dustability of CNT granules was significantly decreased in a polymer concentration-dependent manner (Fig. 1D). Respirable amounts of polymer-coated CNT (10%), as an index of dustability, were >730 times lower in comparison with the original CNTs (Fig. 1D and Table 2).

Table 2 Amounts of respirable particles (dustability)

Subject of comparison		Respirable amounts (mg)	Comparison of dustability	
			10% polymer as 1	K-Nanos100P as 1
K-Nanos 100P		9.053	730	1
K-Nanos 100T		1.756	142	1/5.06
CNT granules (polymer %)	0%	0.134	10.8	1/67.6
	6%	0.059	4.76	1/153
	10%	0.0124	1	1/730

K-Nanos 100P: pristine powder type of K-Nanos100
K-Nanos 100T: granulation type of K-Nanos100 (KUMHO)

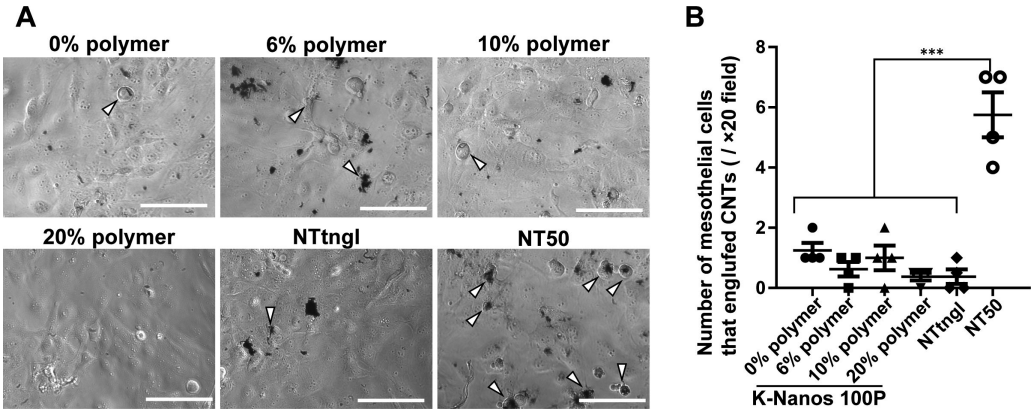

Fig. 2 Polymer coating reduces mesothelial uptake of CNTs
(A) Met5A mesothelial cells phagocytizing MWCNTs. K-Nanos 100P of 0%, 6%, 10% and 20% polymer fraction, NT50 (carcinogenic to mesothelial cells) and NTtngl (non-carcinogenic). Arrowheads indicate mesothelial uptake of CNTs. Scale bar = 100 μm. (B) Quantification of Fig. 2A. Cells with CNT uptake was counted per field at a magnification of ×20 objective lens (means ± SD, N = 4; ***$p < 0.001$).

Polymer coating inhibited mesothelial cellular uptake of CNTs

To study the effect of polymer coating on the mesothelial cellular uptake of CNTs, we observed mesothelial cells after exposure either to the original or the polymer-coated CNTs with phase contrast microscopy (Fig. 2A). NT50 was efficiently phagocytosed by mesothelial cells whereas polymer-coated CNTs were mainly observed in the extracellular space, similar to NTtngl (Fig. 2A and 2B). Thus, polymer coating was shown to inhibit the mesothelial uptake of CNTs.

Polymer coating reduced CNT-induced necrosis of mesothelial cells

To study the effect of polymer coating on the cytotoxicity of CNTs, we performed a cell death detection assay (LDH assay). The necrotic fraction of mesothelial cells was significantly lower with polymer coating in comparison to pristine NT50 (Fig. 3).

Polymer-coated CNTs induced a low level of localized inflammation after intraperitoneal injection

To study the effect of polymer coating of CNTs on inflammogenicity, we used a rat model of intraperitoneal injection, which allows direct contact of each CNT with peritoneal mesothelial

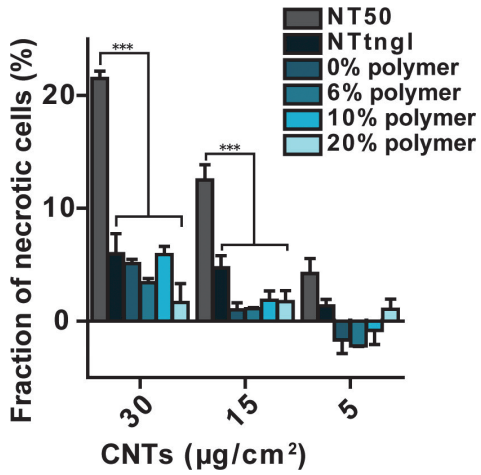

Fig. 3 Low incidence of mesothelial cell necrosis after exposure to polymer coated CNTs seen by LDH assay Necrosis assay for Met5A mesothelial cells after 48 h incubation with each CNT at 5, 15 and 30 μg/cm^2 (means ± SD, N = 3; ***$p < 0.001$).

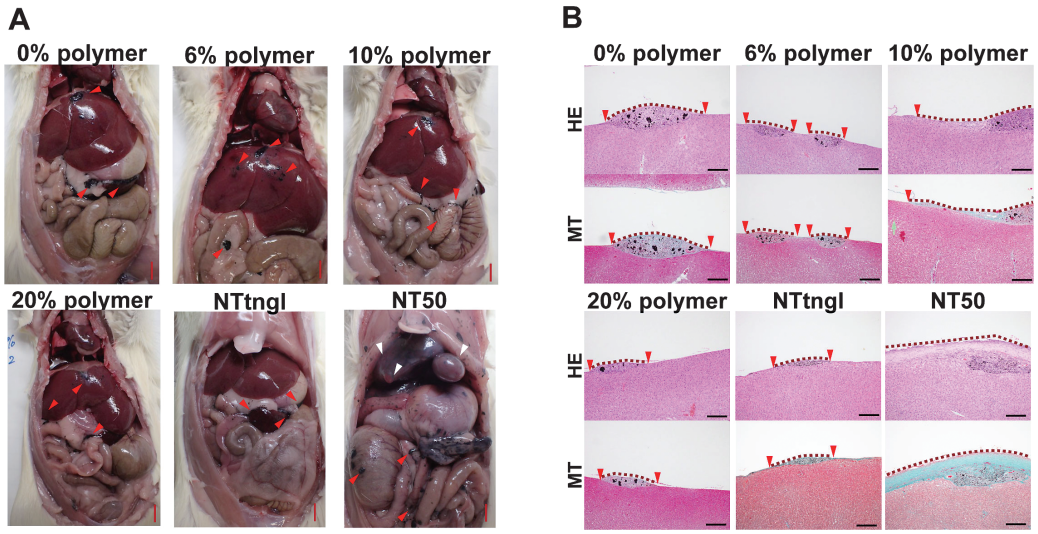

Fig. 4 Subacute *in vivo* evaluation by intraperitoneal injection into rats reveals low and local inflammogenicity of polymer-coated CNTs
(A) Peritoneal overview on dissection 4 weeks after intraperitoneal injection of CNTs. Scattered CNTs with granuloma formation (red arrowheads). Only NT50 shows diffuse severe fibrosis with deformed liver (dull hepatic edge, white arrowheads). Scale = 1 cm. (B) Microscopic images of the hepatic mesothelium show foreign body granuloma formation and different degree of fibrosis. Arrowheads indicate local margin of fibrosis with dotted line as fibrotic areas. Only NT50 shows diffuse severe fibrosis. HE, hematoxylin & eosin; MT, Masson trichrome (collagen fibers as green). Scale = 200 μm.

cells. NT50 induced severe fibrosing inflammation in the entire peritoneal cavity, thus changing the liver to a dull-edged round shape (Fig. 4A). Fibrosis was evident with NT50 injection on the surface of all the intraperitoneal organs, revealing diffuse fibrosis recognized with Masson trichrome staining (Fig. 4B). In contrast, polymer-coated CNTs caused only local mild inflam-

mation surrounding the CNTs (Fig. 4B).

Indeed, fibrosis of the polymer-coated CNT groups was confined to the areas of CNT exposure. However, peritoneal fibrosis in the NT50 group was diffuse on the whole peritoneal surface.

DISCUSSION

This is the first report on the biological effects of polymer coating on MWCNTs in mammals. Our method of polymer coating on the surface of MWCNTs provided agglomeration, leading to the formation of CNT granules on the order of millimeters. This is an ~100-fold difference in size, which significantly decreased the dustability of the CNTs. This clearly indicates that this novel method can decrease the human environmental risk of CNTs at the entrance of the respiratory system because there are lower chances for such a large granule to reach pulmonary alveoli even if some of them are degraded by cracking.

CNT granules were used in the *in vitro* and *in vivo* experiments after sonication. This was performed exactly the same way as described previously,[7,18] considering that some of the CNT granules may be decomposed *in vivo* by phagocytic cells. With this experimental condition, MeT5A mesothelial cells revealed low phagocytic activity for polymer-coated CNTs and for NTtngl (noncarcinogenic CNT). The result was the same for the evaluation of mesothelial cell death, which was evaluated by LDH release. There was no significant trend for the dependence on the amounts of polymer used. An evaluation using intraperitoneal injection in rats confirmed the results. Only local inflammation was observed for all the groups of polymer-coated CNTs and for NTtngl. These data suggest that the agglomeration process itself is important to decrease the environmental risks.

We propose, based on the current results, that dustability should be included as an independent risk factor for CNTs. Polymer coating methods significantly decreased dustability of CNTs. Regarding the safety of CNTs against mesothelial carcinogenesis, the total risk may be considered as a multiplication of exposure level (inhaled CNTs to alveoli) and mesothelial cellular toxicity. Of note, unless a CNT is phagocytosed by mesothelial cells, it is neither cytotoxic nor carcinogenic.

In conclusion, there are three major merits of Durobeads over pristine CNTs in industry. Durobeads are safer to humans due to its much lower dustability and mesothelial cytotoxicity. Automation in the manufacturing process may be possible with higher liquidity of Durobeads. Furthermore, lower bulk density of Durobeads than pristine CNTs would decrease logistics cost.

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CONFLICT OF INTEREST

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REFERENCES

1) Iijima S. Helical Microtubules of Graphitic Carbon. *Nature*, 1991; 354: 56–58.
2) Endo M, Strano MS, Ajayan PM. Potential applications of carbon nanotubes. In: Carbon nanotubes, edited by Jorio A., Dresselhaus G., Dresselhaus M.S. pp. 13–62, 2008, Springer, Berlin, Heidelberg, Berlin.
3) Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WA, Seaton A, *et al.* Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study [In Process Citation]. *Nat Nanotechnol*, 2008; 3: 423–428.
4) Nagai H, Toyokuni S. Differences and similarities between carbon nanotubes and asbestos fibers during mesothelila carcinogenesis. *Cancer Sci*, 2012; 103: 1378–1390.
5) Robinson B, Lake R. Advances in malignant mesothelioma. *N Engl J Med*, 2005; 353: 1591–1603.
6) Takagi A, Hirose A, Nishimura T, Fukumori N, Ogata A, Ohashi N, *et al.* Induction of mesothelioma in p53+/– mouse by intraperitoneal application of multi-wall carbon nanotube. *J Toxicol Sci*, 2008; 33: 105–116.
7) Nagai H, Okazaki Y, Chew SH, Misawa N, Yamashita Y, Akatsuka S, Ishihara T, *et al.* Diameter of multi-walled carbon nanotubes is a critical factor in mesothelial injury and subsequent carcinogenesis. *Proc Natl Acad Sci USA*, 2011; 108: E1330–1338.
8) Nagai H, Toyokuni S. Biopersistent fiber-induced inflammation and carcinogenesis: lessons learned from asbestos toward safety of fibrous nanomaterials. *Arch Biochem Biophys*, 2010; 502: 1–7.
9) Jiang L, Akatsuka S, Nagai H, Chew SH, Ohara H, Okazaki Y, *et al.* Iron overload signature in chrysotile-induced malignant mesothelioma. *J Pathol*, 2012; 228: 366–377.
10) Wang Y, Okazaki Y, Shi L, Kohda H, Tanaka M, Taki K, *et al.* Role of hemoglobin and transferrin in multi-wall carbon nanotube-induced mesothelial injury and carcinogenesis. *Cancer Sci*, 2016; 107: 250–257.
11) Nagai H, Okazaki Y, Chew SH, Misawa N, Miyata Y, Shinohara H, *et al.* Intraperitoneal administration of tangled multiwalled carbon nanotubes of 15 nm in diameter does not induce mesothelial carcinogenesis in rats. *Pathol Int*, 2013; 63: 457–462.
12) Toyokuni S. Mechanisms of asbestos-induced carcinogenesis. *Nagoya J Med Sci*, 2009; 71: 1–10.
13) Toyokuni S. Genotoxicity and carcinogenicity risk of carbon nanotubes. *Adv Drug Deliv Rev*, 2013; 65: 2098–2110.
14) Chew SH, Toyokuni S. Malignant mesothelioma as an oxidative stress-induced cancer: An update. *Free Radic Biol Med*, 2015; 86: 166–178.
15) Brown JS, Gordon T, Price O, Asgharian B. Thoracic and respirable particle definitions for human health risk assessment. *Part Fibre Toxicol*, 2013; 10: 12.
16) Moniruzzaman M, Winey KI. Polymer nanocomposites containing carbon nanotubes. *Macromolecules*, 2006; 39: 5194–5205.
17) Coleman JN, Khan U, Blau WJ, Gun'ko YK. Small but strong: a review of the mechanical properties of carbon nanotube–polymer composites. *Carbon*, 2006; 44: 1624–1652.
18) Toyokuni S, Jiang LI, Kitaura R, Shinohara H. Minimal inflammogenicity of pristine single-wall carbon nanotubes. *Nagoya J Med Sci*, 2015; 77: 195–202.