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Research Article

Manufacturing Strategy for Multiwalled Carbon Nanotubes as a Biocompatible and Innovative Material

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We investigated the relationship between differences in multiwalled carbon nanotubes (MWCNTs) and the biological responses they elicit in order to develop biocompatible MWCNTs. We exposed human bronchial epithelial (BEAS-2B) cells to two sizes and six grades of MWCNTs and measured the resulting cell viability, total reactive oxygen and/or nitrogen species (tROS/RNS) production, and cytokine secretion. Although differences in cellular tROS production were associated with differences in grades of MWCNTs, the graphitization temperature of MWCNTs apparently did not influence tROS production. However, cell viability was affected by MWCNT graphitization temperature and diameter. Moreover, cytokine secretion was apparently affected by treatment temperature, but not MWCNT diameter. We concluded that the highest temperature resulted in the most biocompatibility because impurities and carbon defects were removed from the MWCNTs. However, other mechanisms are possible. Therefore, it is important to optimize each type of MWCNT by monitoring biological responses that type elicits during the manufacturing stage for applications involving biology and medicine.

1. Introduction

Due to their unique properties, MWCNTs have potential applications in a wide variety of industries including biomedical fields [1]. Many uses for MWCNTs have been proposed including biosensors, drug and vaccine delivery vehicles, and novel biomaterials [2]. However, before such biomaterials can be incorporated into new and existing biomedical devices, the biocompatibility of MWCNTs must be thoroughly investigated because Takagi et al. and Poland et al. reported that mice injected intraperitoneally with MWCNTs exhibited toxicological responses similar to those seen in mice exposed to asbestos [3, 4]. Although some investigators have investigated the safety of inhalation or intratracheal administration of MWCNTs *in vivo*, a clear conclusion cannot be drawn from the results of these experiments [5–9]. Similarly, results from *in vitro* studies do not provide a consistent picture of the safety of MWCNTs; some studies indicate that MWCNTs cause cytotoxicity and cytokine production [10–12], but others indicate that MWCNTs did not cause any significant biological responses [13, 14].

It is a crucial to determine whether MWCNTs cause inflammation when used as a biomaterial. Oxidant stress is thought to be a likely cause of some possible MWCNT-mediated biological responses. Therefore, oxidative stress, as a cause of inflammation, attracts attention, and the transition metal catalyst residues CNTs might be major

Table 1: Basic properties of MWCNTs.

MWCNT type	MWCNT-150						Testing method
abbreviation name	NT15	NT15+Fe	NT15-30	NT15-26	NT15-22	NT15-13	
Graphitization temperature (°C)	3000[b]	3000[b]	3000[c]	2600[c]	2200[c]	—	
Additional treatment	—	Fe_2O_3[d]	—	—	—	—	
Diameter (nm)	150						FE-SEM
Length (μm)	7–10						FE-SEM
Iron content (ppm)	34	730	<20	<20	60	13000	ICP-MS
R value (I_d/I)[a]	0.3	0.3	0.2	0.5	0.9	1.4	Raman spectroscopy (785 nm)
MWCNT type	MWCNT-80						Testing method
abbreviation name	NT08	NT08+Fe	NT08-30	NT08-26	NT08-22	NT08-13	
Graphitization temperature (°C)	3000[b]	3000[b]	3000[c]	2600[c]	2200[c]	—	
Additional treatment	—	Fe_2O_3[d]	—	—	—	—	
Diameter (nm)	80						FE-SEM
Length (μm)	7–10						FE-SEM
Iron content (ppm)	1700	2200	<20	<20	360	21000	ICP-MS
R value (I_d/I)[a]	n/a						Raman spectroscopy (785 nm)

[a] R refers to the intensity of D band over the intensity of G band.
[b] MWCNTs were heated at Showa denko.
[c] MWCNTs were heated in our laboratory.
[d] NT15 and NT08 were added Fe_2O_3 (1000 : 1), mixed, and sonicated in 0.02% triton-X100 solution. Then, they were filtered and dried at 120°C.
n/a = not available.

cause of oxidative stress [15, 16]. It is known that the impurities in MWCNTs before graphitization (We named the MWCNTs "As-grown.") can be removed by thermal treatment for graphitization, and MWCNTs produced for commercial uses are additionally heat treated. In fact, we reported that the heat-treated MWCNTs did not represent cell proliferation inhibition although As-grown MWCNTs indicated cytotoxicity on U937 human monoblastic leukemia cells [17]. However, U937 cells do not endocytose MWCNTs, and the results were different for a human bronchial epithelial cell line (BEAS-2B cells), which endocytoses heat-treated MWCNTs [18]. BEAS-2B cells are of bronchial epithelium cell origin and are susceptible to cytotoxicity.

In this study, we examined cytotoxicity, oxidative stress, and inflammation, as for index of biological responses, using two sizes of MWCNTs with varying iron concentration in BEAS-2B cells to clarify more important factor during the manufacturing stage to improve the biocompatibility.

2. Materials and Methods

2.1. Carbon Nanotubes. We used commercial MWCNT materials, vapor-grown carbon fiber (VGCF, Showa Denko, Tokyo, Japan) and vapor-grown carbon fiber-S (VGCF-S, Showa Denko, Tokyo, Japan), that were manufactured by a chemical vapor deposition method [19], and As-grown MWCNTs before the graphitization were also provided by Showa Denko. Information on each type of MWCNT, including the abbreviated name (e.g., NT15, NT08), additional treatments in our laboratory, and properties, is listed in Table 1. MWCNTs were sterilized in an autoclave at 121°C for 15 min. MWCNTs were vortexed for 1 min in PBS (−) containing 0.1% gelatin (Nacalai Tesque, Kyoto, Japan) and sonicated with a water-bath sonicator for 30 min. Dispersed

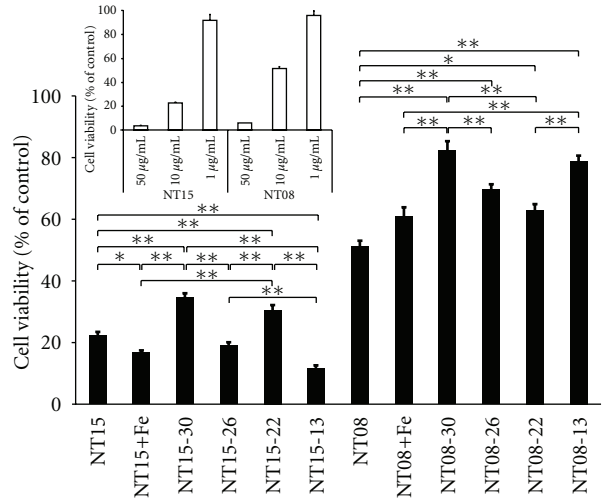

Figure 1: The viability of BEAS-2B cells treated with different MWCNTs. The cells were exposed to MWCNTs for 24 h. The small graph shows the dose effects of NT15 and NT08 on the cell viability. The large graph shows the cell viability associated with 10 μg/mL treatment of each MWCNT in the MWCNT-150 and MWCNT-80 series. (Mean ± SE, $n = 6$, $^*P < 0.05$, $^{**}P < 0.01$.)

MWCNTs suspended in the PBS-gelatin dispersant were added to cell culture medium at 1/100 volume in each of the following experiments.

2.2. Cell Culture. The human bronchial epithelial cell line, BEAS-2B, was purchased from the American Type Culture Collection (Manassas, Va, USA). BEAS-2B cells were cultured in Ham's nutrient mixture F-12 with 10% fetal bovine serum at 37°C in a 5% CO_2 humidified incubator and passaged

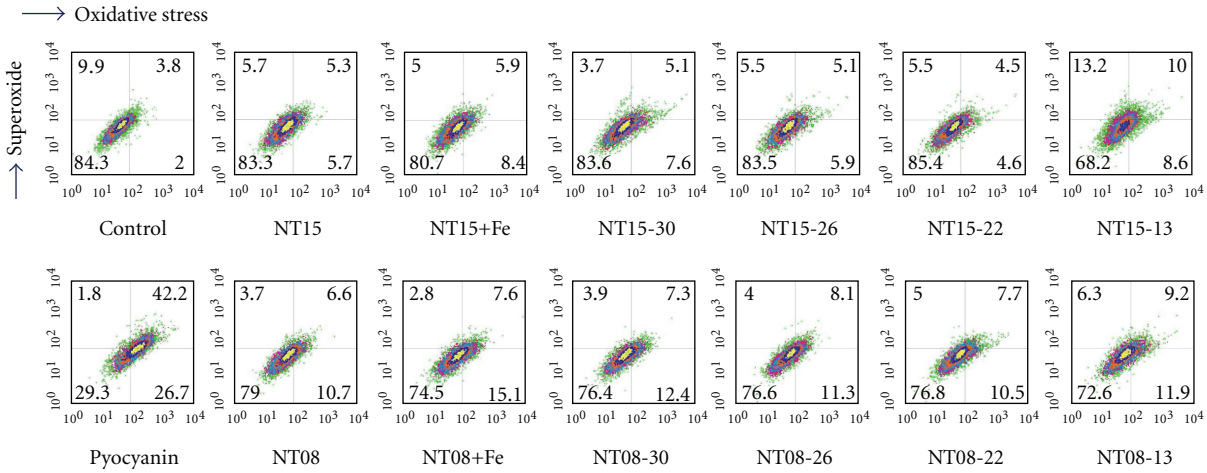

Figure 2: The tROS production in BEAS-2B cells treated with different MWCNTs. The cells were exposed to MWCNTs for 1 or 24 h, then stained with two color ROS detection reagents, and analyzed using FCM. Image shows the cell population of the fraction separated by oxidative stress and superoxide for 1 h (10,000 cells). The numbers in the image reflect the percentage of the cell population in each quadrant.

twice each week. For each study, the cells were seeded at a density of 5×10^5 cells/mL and adhered for 24 h.

2.3. Alamar Blue (AB) Assay. To assess the viability of cells exposed to different MWCNTs, we performed an Alamar blue assay (AlamarBlue cell viability reagent; Invitrogen, Carlsbad, Calif, USA), according to the manufacturer's instructions. Cells were plated in 96-well plates and incubated for 24 h at 37°C in the culture medium containing VGCF in dispersant or only dispersant control medium. Viable cells metabolized the dye, resulting in an increase of fluorescence following excitation/emission at 550/600 nm with a fluorescence multiplate reader (PowerScan 4, DS Pharma Biomedical, Osaka, Japan). Cytotoxic activity was calculated as follows: percent cytotoxicity = 100 × experimental value/control value. Test media were assayed six times for each treatment condition.

2.4. Total ROS/Superoxide Production. To determine total reactive oxygen and/or nitrogen species (tROS/RNS) production in the cells exposed to MWCNTs, we used a total ROS/superoxide detection kit (Enzo Life Sciences, Plymouth Meeting, Pa, USA). Cells were plated into 24-well plates and incubated for 24 h so cells could adhere to the substrate. Cells were then incubated for 1 or 24 h at 37°C in the presence or absence of MWCNTs. Pyocyanin (100 μM) was used as a reactive oxygen species (ROS) inducer. Following exposing to MWCNTs, the cells were treated with oxidative stress detection reagent (OSDR) and superoxide detection reagent (SDR) for 30 min. Cells were then washed once in 1 × wash buffer and harvested with trypsin-EDTA. Finally, the cells were suspended with 0.3 mL of 10% FBS in 1 × wash buffer and passed through nylon mesh. These cells were subjected to flow cytometry (FCM; FACSCalibur, Becton Dickinson, San Jose, Calif, USA) in the FL1 channel for OSDR signals and FL2 channel for SDR signals. The cells were separated into four fractions, and the fractions were named tROS (FL1 and FL2 positive), superoxide (FL1 negative and FL2 positive), peroxide (FL1 positive and FL2 negative), and negative (FL1 and FL2 negative).

2.5. Cytokine Measurement. Cytokines in the culture supernatant were measured by a BD cytometric bead array flex set assay (Human soluble protein master buffer kit & Human IL-6 and IL-8 flex sets; BD Biosciences, San Jose, Calif, USA), according to the manufacturer's protocol Briefly, BEAS-2B cells that had been cultured in 24-well plate for 24 h were exposed to 10 μg/mL of MWCNT in dispersant for 24 h, and the resulting supernatant was collected by centrifugation. Then cytokine capture beads (for IL-6 and IL-8) were mixed with supernatant samples or cytokine standards in FCM tubes. The mixtures were vortexed, and antibody for fluorescence detection was added to each tube. The samples were then incubated at room temperature for 2 h. Following incubation, beads were washed once by wash buffer and resuspended prior to reading with an FCM.

2.6. Statistical Analysis. Data are presented as mean ± SE. Statistical significant was determined by analysis of variance (ANOVA) followed by the Student's t-test to compare the controls with each sample, and the Tukey-Kramer method for comparisons between different types of MWCNTs. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cell Viability. Cell viability was measured using AB assay method. The viability value for each experimental sample was expressed as percentage of the control sample, which was designated as 100% viable (Figure 1). NT15 and NT08 which are commercial MWCNTs decreased the cell viability depending on the concentration. At a concentration of 10 μg/mL, all MWCNTs in the NT08 series were associated with a viability associated higher than that associated with MWCNTs in the NT15 series. Moreover, commercial MWCNTs (NT15 and NT08) were associated with lower

Table 2: Fractionation rate using two fluorescent reagents for tROS/RNS with FCM on BEAS-2B cells exposed to MWCNTs at 1 and 24 h.

(a) Fractionation rate (%) of 10,000 cells at 1 h (mean ± SE, $n = 3$)

Fraction	Control	NT15	NT15+Fe	NT15-30	NT15-26	NT15-22	NT15-13
tROS	3.8 ± 0.4	5.3 ± 0.7	5.9 ± 0.4	5.1 ± 0.6	5.1 ± 0.2	4.5 ± 0.3	10.0 ± 0.2
Superoxide	9.9 ± 0.2	5.7 ± 0.1	5.0 ± 0.6	3.7 ± 0.2	5.5 ± 0.5	5.5 ± 0.7	13.2 ± 2.2
Peroxide	2.0 ± 0.2	5.7 ± 1.2	8.4 ± 1.3	7.6 ± 1.4	5.9 ± 0.7	4.6 ± 0.9	8.6 ± 1.3
Negative	84.3 ± 0.3	83.3 ± 1.8	80.7 ± 1.2	83.6 ± 2.0	83.5 ± 0.3	85.4 ± 0.7	68.2 ± 1.0
Fraction	Pyocyanin	NT08	NT08+Fe	NT08-30	NT08-26	NT08-22	NT08-13
tROS	42.2 ± 2.1	6.6 ± 0.8	7.6 ± 0.6	7.3 ± 0.6	8.1 ± 0.3	7.7 ± 0.8	9.2 ± 0.4
Superoxide	1.8 ± 0.3	3.7 ± 0.5	2.8 ± 0.3	3.9 ± 1.0	4.0 ± 0.7	5.0 ± 0.8	6.3 ± 0.7
Peroxide	26.7 ± 1.5	10.7 ± 0.8	15.1 ± 1.6	12.4 ± 2.5	11.3 ± 1.6	10.5 ± 2.0	11.9 ± 1.4
Negative	29.3 ± 2.6	79.0 ± 1.3	74.5 ± 1.9	76.4 ± 1.9	76.6 ± 1.0	76.8 ± 2.0	72.6 ± 0.9

(b) Statistical significance compared between different types of MWCNTs by the Tukey-Kramer method (mean ± SE, $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$)

Fraction	MWCNT-150	MWCNT-80
tROS	Control versus NT15+Fe*, NT15-13** NT15-13 versus NT15**, NT15+Fe**, NT15-30**, NT15-26**, NT15-22**	Control versus NT08+Fe*, NT08-30*, NT08-26**, NT08-22*, NT08-13**
Superoxide	Conrol versus NT15**, NT15+Fe*, NT15-30**, NT15-26**, NT15-22* NT15-13 versus NT15**, NT15+Fe**, NT15-30**, NT15-26**, NT15-22**	Control versus NT08**, NT08+Fe**, NT08-30*, NT08-26*, NT08-22*, NT08-13*, NT08+Fe versus NT08-13*
Peroxide	Control versus NT15+Fe*, NT15-26*, NT15-13*	Conrol versus NT08**, NT08+Fe*, NT08-26*, NT08-13*
Negative	Control versus NT15-13* NT15-13 versus NT15**, NT15+Fe**, NT15-30**, NT15-26**, NT15-22**	Conrol versus NT08*, NT08+Fe*, NT08-26*, NT08-13**

(c) Fractionation rate (%) of 3,000 or fewer cells at 24 h (mean ± SE, $n = 3$)

Fraction	Control	NT15#	NT15+Fe#	NT15-30#	NT15-26	NT15-22	NT15-13#
tROS	3.4 ± 2.1	6.3 ± 0.6	7.1 ± 0.6	11.1 ± 1.8	7.0 ± 0.4	7.4 ± 0.9	18.9 ± 3.4
Superoxide	5.9 ± 0.3	17.9 ± 2.6	14.5 ± 1.0	19.8 ± 2.0	17.7 ± 3.2	17.6 ± 2.6	20.1 ± 1.7
Peroxide	2.0 ± 1.5	4.7 ± 0.2	7.3 ± 0.6	8.9 ± 0.9	5.8 ± 0.2	5.5 ± 1.0	8.9 ± 1.8
Negative	88.7 ± 3.5	71.1 ± 2.1	71.2 ± 1.6	60.2 ± 4.2	69.5 ± 3.1	69.5 ± 1.9	52.1 ± 3.8
Fraction	Pyocyanin	NT08	NT08+Fe	NT08-30	NT08-26	NT08-22	NT08-13
tROS	50.5 ± 2.3	9.9 ± 1.0	9.9 ± 2.3	9.6 ± 2.2	10.1 ± 1.8	9.9 ± 1.3	10.7 ± 1.5
Superoxide	20.8 ± 0.7	21.5 ± 1.9	17.6 ± 1.4	17.3 ± 1.6	25.3 ± 1.3	28.7 ± 2.5	41.7 ± 3.1
Peroxide	1.3 ± 0.1	4.2 ± 0.8	5.6 ± 1.3	4.3 ± 1.6	3.0 ± 0.6	2.5 ± 0.4	1.4 ± 0.4
Negative	27.4 ± 1.7	64.5 ± 0.3	66.9 ± 2.3	68.8 ± 2.2	61.6 ± 1.4	58.9 ± 0.9	46.2 ± 1.4

= less than 3,000 cells.

cell viability than the MWCNTs treated thermally in our laboratory for each series except the NT15-26. Based on these observations, MWCNT treatment temperature was apparently not directly related to cell viability, indicating that the impurities (i.e., mainly iron) and/or carbon defects were not the principal cause of cytotoxicity. Interestingly, NT15 MWCNTs were associated with higher viability than NT15+Fe and NT15-13, which both contain substantial amounts of iron, but NT08 MWCNTs were associated with lower cell viability than NT08+Fe and NT08-13.

3.2. tROS/RNS Production. Generally, cellular tROS/RNS are produced and eliminated rapidly. However, we assayed tROS/RNS at 1 h and at 24 h after exposing cells to MWCNTs because the cells internalized MWCNTs over time. Only 3,000 or fewer cells were assayed for the 24 h time point because the cells were injured, but we assayed 10,000 cells at the 1 h time point. Figure 2 and Tables 2(a) and 2(b) show each fraction of the population resulting from FCM analysis. At 1 hr, the fraction of tROS-positive cells was significantly higher in NT15+Fe and the MWCNT-80 series without NT-08 than in the control samples. However, the tROS-positive fractions were no higher in the experimental sample than in the pyocyanin sample, which was a positive control. At 1 hr, the superoxide-positive fraction was significantly lower in all experimental samples than in the control sample, except that the superoxide-positive fraction in NT15-13 sample was not significantly different from that in the control sample.

Although the peroxide-positive fraction of cells treated with some grades of MWCNTs was significantly higher than that of the control cells, the values were consistently less than half of the values associated with the pyocyanin samples. The alteration of MWCNT-80 series was larger than that of MWCNT-150 series in the peroxide-positive fractions. NT15-13 MWCNTs were significantly different from the MWCNT-150 series in the tROS, superoxide, and negative fractions, and there was a significant difference between only NT08+Fe and NT08-13 in the MWCNT-80 series for the superoxide fraction. The cell counts from the 24 h timepoint were too low to result in statistically significant differences; nevertheless, we present the results as reference data (Table 2(c)). At 24 hr, the tROS, superoxide, and peroxide fractions were larger in all MWCNT-treated cells than in control cells, and the tROS and superoxide fractions were larger in all 24 h samples than in the 1 h samples.

3.3. Cytokine Secretion. Recently, we reported that BEAS-2B cells exposed to MWCNTs secreted IL-6 and IL-8 [18]. All MWCNT treatments resulted in significant increased IL-6 and IL-8 secretions when compared to the control treatment, and all, except NT15-30, resulted in higher IL-8 secretion than did LPS, which was a positive control (Figure 3). There were statistically significant differences in the MWCNT-150 series though there were no significant differences in the MWCNT-80 series. However, secretion associated with NT15-30 and NT15-13 in the MWCNT-150 series and with NT08-30 and NT08-13 in MWCNT-80 tended to be lower than that with other MWCNTs in the respective series for both cytokines.

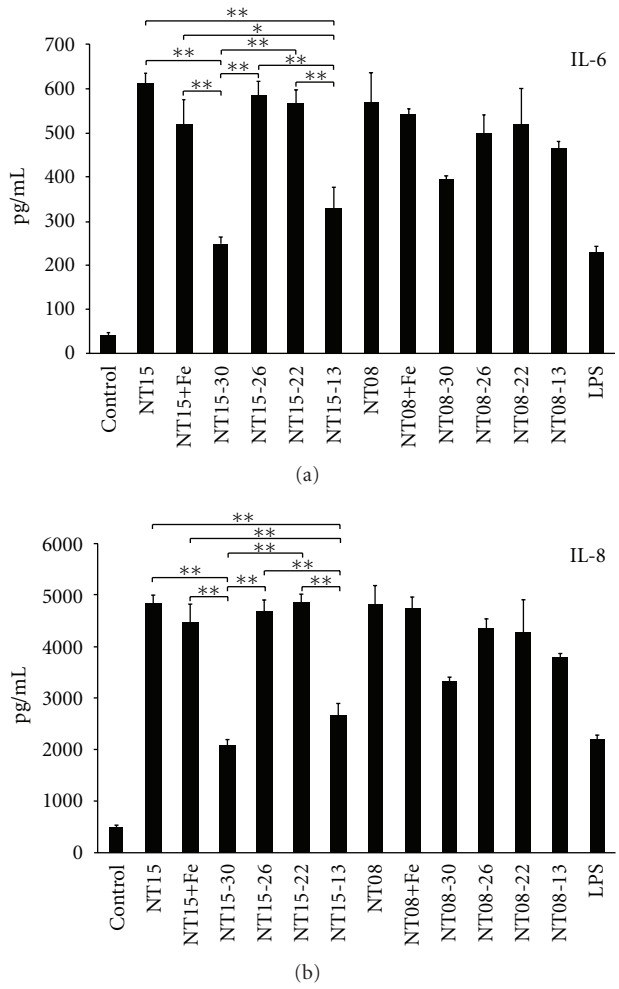

Figure 3: The cytokine secretion from BEAS-2B cells treated with different MWCNTs. The cells were exposed to MWCNTs for 24 h. The upper graph shows IL-6 secretion, and the lower graph shows IL-8 secretion from cells treated with 10 μg/mL of each MWCNT, the MWCNT-150 and MWCNT-80 series (mean ± SE, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$).

4. Discussion

Carbon nanotubes (CNTs) are expected to be useful for a wide variety of industrial applications, and postprocessing procedures will depend on the individual application. In the biomedical field, the research on drug delivery systems and diagnostic imaging that use CNTs is advanced [20, 21]. However, doubts about efficacy and safety of modified CNT remain [22], and the influence of different manufacturing processes for CNTs, as biomaterials, has not been examined thoroughly. In this study, we evaluated the influence of different graphitization temperatures on the MWCNTs of two different diameters.

The graphitization temperature has a crucial influence on the impurities content and crystalline of MWCNTs, and these factors are reportedly critical for the safety of CNTs [23–25]. However, our results did not indicate that carbon defects and impurities directly affected three biological responses, cell viability, tROS production, and cytokine secretion. Each biological response did not show mutual relativity and did not correlate to impurities or the defects, although both the MWCNT-150 and MWCNT-80 series had fewer defects and impurities depending on the treatment temperature. However, the diameter of MWCNTs did affect cell viability and tROS production. MWCNT-80 series gave rise to the seemingly contradictory results that relative cell viability was high while tROS production was also high. Therefore, cellular tROS production may have been critical to cytotoxicity. IL-6 and IL-8 secretion increased with all MWCNT exposure regardless of the MWCNT diameter, and secretion associated with NT15-30 and NT15-13 and NT08-30 and NT08-13, treatments tended to be lower than secretion associated with treatments involving MWCNT of the same diameter. However, the data from NT15-30 or NT08-30 treated with graphitization at 3000°C and NT-13 or NT08-13 without graphitization were not able to clarify commonalities in this research. Additional iron did not affect the biological responses of original MWCNTs except for the cell viability counts associated with the N15 and N15+Fe treatments. These results also indicated that the iron contained in the MWCNTs is not crucial because the cell viability of NT15-13, which included 13,000 ppm iron, was the lowest in the MWCNT-150 series, but NT08-13, which included 21,000 ppm iron, was associated with high

viability. Finally, the MWCNTs processed at the maximum temperature in the lab had the highest biocompatibility overall regardless of diameter.

In this study, we found that the graphitization temperature of MWCNTs in the manufacturing process affected biological response to the MWCNTs, but the biological responses did not have regularity and was affected by the diameter of the MWCNTs. In other words, we should investigate the condition that results in the lowest biological responses for each MWCNT in the manufacturing process before that MWCNT is used for applications in biology and medicine. It is our duty to optimize the biocompatibility of the nanomaterial itself in order to develop application for the nanomaterials.

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