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Author(s)	Lin, Chun; Fugetsu, Bunshi; Su, Youbo et al.
Citation	Journal of Hazardous Materials, 170(2-3), 578-583 https://doi.org/10.1016/j.jhazmat.2009.05.025
Issue Date	2009-10-30
Doc URL	https://hdl.handle.net/2115/39947
Type	journal article
File Information	JHM170-2-3_p578-583.pdf

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Studies on toxicity of multi-walled carbon nanotubes on *arabidopsis* T87 suspension cells

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ABSTRACT

The possible toxicities of agglomerates of multi-walled carbon nanotubes (MWCNTs) toward plant cells were evaluated. *Arabidopsis* T87 suspension cells were used as the model cells and decreases in values of cell dry weights, cell viabilities, cell chlorophyll contents, and superoxide dismutase (SOD) activities were seen for the cells cultured in media containing the agglomerates of MWCNTs, indicating the agglomerates of MWCNTs are toxic to the *Arabidopsis* T87 suspension cells. Moreover, the toxicities increased sharply as the diameters of the agglomerates of the MWCNTs became smaller. A concept based on the hypersensitive response is suggested to explain the possible toxic mechanism induced by the MWCNTs.

Keywords:
Multi-walled carbon nanotubes
White asbestos
Arabidopsis T87 suspension cells
Cell dry weights
Cell viabilities
Cell chlorophyll contents
Superoxide dismutase

1. **Introduction**

Carbon nanotubes (CNTs) are the key nano-materials epitomizing the field of nanotechnology. Production lines, especially the lines for multi-walled CNTs (MWCNTs) appeared worldwide (Belgium, Germany, Japan, China, etc.,); and some of these lines are capable for producing industrial quantities. Due to their fibrous shapes, CNTs have been compared to asbestos, raising great concerns that the widespread industrialization of such kind of the engineered nano-materials and/or their subsequent products may lead to negative impacts to environmental surroundings and the biological systems [1-4]. Early studies *in vivo* have shown that MWCNTs of the straight/rigid types introduced into the abdominal cavity of mice showed asbestos-like pathogenic behaviors [5], warning further research and careful attentions on their potential toxicities. Publications regarding the toxicities of CNTs toward animal cells and/or tissues have been increasing rapidly; however, there have been serious lacks on data regarding the possible toxicities of CNTs to plants [6, 7].

In this study we chose *Arabidopsis* T87 suspension cells, one of the well-characterized model plant cells, for evaluating the possible toxicity of CNTs toward the plant cells. To avoid any possible side-effects caused by dispersants and/or the chemically derivative functional groups, CNTs used throughout this study were the entire pristine tubes; the sizes of the agglomerates of the tubes were regulated simply by using ultra-sonication. Decreases in values of the cell dry weights, cell viabilities, cell chlorophyll contents, and superoxide dismutase activities were seen for the cells cultured in media containing the agglomerates of MWCNTs, indicating MWCNTs are toxic to the *Arabidopsis* T87 suspension cells even in the agglomerated forms.

2. Materials and methods

2.1. MWCNTs

Powders of MWCNTs were the CVD (chemical vapor deposition) products purchased from Nanocyl (Sambreville, Inc, Belgium). Two degrees of the agglomerates, one is the as-received products used without any further treatment (denoted as *loose-agglomerates*, in this paper) and the other, denoted as *fine-agglomerates*, which is obtained by dispersing the *loose-agglomerates* into agglomerates of smaller sizes in deionized water using ultra-sonication（in an ice/water bath for total 6 hours of ultra-sonication) without adding any kind of dispersants, were used throughout this study. The *loose-agglomerates* have the sizes up to few hundred μm; while the *fine-agglomerates* up to few ten μm, as were established from the light microscopic imagines (Figure 1). Other information regarding the CNTs is summarized in Table 1.

Our studies were also conducted using chrysotile (white asbestos, denoted as *white*-ASB, in this paper, obtained from Kanto Chemicals) as the reference material, for comparison.

2.2. Cell cultivations

Arabidopsis thaliana (Columbia ecotype) T87 suspension cells were obtained from RIKEN BioResource Center (Tsukuba, Japan) and were cultured at 22℃ under continuous illumination white fluorescent lamps (100 $\mu E \cdot s^{-1}m^{-2}$) with shaking at 120 rpm. Cells were subcultured every 5 days by adding 10 ml of the cell suspension to a 300-ml flask containing 100 ml of JPL medium [8]. Experiments were carried out in the exponential phase of growth

at about 72 hours of cell cultivation. The CNTs were added to the medium containing the suspension cells to the final required concentrations. For concentration-dependent experiments, the final CNTs concentrations were 10 $mg \cdot l^{-1}$, 60 $mg \cdot l^{-1}$, 100 $mg \cdot l^{-1}$ and 600 $mg \cdot l^{-1}$ respectively. For cell viability experiments, cells were exposed to 10 $mg \cdot l^{-1}$ of CNTs or 10 $mg \cdot l^{-1}$ of while asbestos for 2, 3, 4, 5, 6, 7 days, respectively. Each experiment was repeated independently for three times. In figures, data are presented as average values of three measurements.

2.3. Cell dry weight measurement

Cells (4 ml of the cell suspensions for each sample) were collected by using vacuum filtration on a 0.2 μm pore size polycarbonate membrane filter (Toyo Roshi Kaisha, Ltd. USA) fitted in a funnel and the cells were washed three times with PBS. Cell dry weight was measured after drying the cells in a vacuum dryer for 72 hours until a constant weight was recorded. Morphological changes of the treated cells were observed by using light microscopy and recorded by using a digital camera (IX-FLA, Olympus, Japan).

2.4. Viability assay

The triphenyl-tetrazolium chlorine (TTC) reduction assay [9] was used. Briefly, cells (2 ml of the cell suspensions for each sample) were incubated in 4 ml of TTC solution (23.9 μM TTC in 0.05 M potassium phosphate buffer, pH 7.5) for 24 h at 22℃ in the dark. The samples were then centrifuged for 5 min at 500 rpm, TTC solution was discarded and cells were subsequently incubated in 5 ml of 98 % ethanol for 15 h at 55℃. After centrifugation

for 5 min at 4000 rpm, the absorbance of supernatant was read at 485 nm wave length (V-530 UV/UISNIR Spectrophotometer, Jasco, Japan).

2.5. Superoxide dismutase (SOD) activity assay

SOD activity was determined by the photochemical method [10]. One unit of the SOD activity was defined as the amount of enzyme required to cause a 50 % inhibition of the rate of nitroblue tetrazolium (NBT) reduction at 560 nm. The sample preparation protocols are as follows: washing the cells by using PBS buffer; cells (1 ml of the cell suspensions for each sample) were transferred into a prechilled mortar and pestle in an ice bath and thoroughly ground after added homogenizing glass beads and the grinding medium 1.5 ml PBS buffer (pH 7.5). The homogenate was centrifuged for 30 min at 8500 rpm at 4℃ in a refrigerated centrifuge. The supernatant was used for protein contents and enzyme activity analysis. Protein contents determination was followed by the methods by using bovine serum albumin as standard described by Bradford [11].

2.6. Chlorophyll content assay

Cells (2 ml of the cell suspensions for each sample) were collected by centrifugation at 1500 rpm for 5 min in the centrifuge. Chlorophylls were extracted with 80 % acetone aqueous solution by keeping the vials in the dark at 22℃ for 12 h. Determination of chlorophylls was done on a spectrophotometer (V-530 UV/UISNIR Spectrophotometer, Jasco, Japan). The absorbance of chlorophyll a and b was recorded for each sample at 647 nm and 665 nm simultaneously and chlorophyll content was calculated as described by Arnon and Bruinsma

[12]. In order to prevent the decomposition of pigments all operations were performed under subdued light. All measurements were in three replicates.

3. Results and discussion

Cellular morphologies were observed using light microscopy. The morphologies for cells cultured under the regular cultivation; the media containing the *loose-agglomerates*, the *fine-agglomerates* of MWCNTs, and the *white-ASB* are shown Figure 2 a, b, c, and d, respectively. As can be seen from these observations, *arabidopsis* T87 suspension cells cultured in media containing the *loose-agglomerates* and the *fine-agglomerates* of MWCNTs were identical to that observed under the regular cultivation, indicating both the *loose-agglomerates* and the *fine-agglomerates* of the MWCNTs had no ability to physically injure the *Arabidopsis* T87 suspension cells into fragments, as compared to chrysotile, the white-asbestos (*white*-ASB). In other words, large populations of the fragmented cells those corresponding to protoplasm (this representing the irreversible cell injuries and cell death) were observed *only* for cells treated with *white*-ASB.

Cell dry weight was measured during the courses of the suspension cultivations. As can be seen from the growth curves (Figure 3; note that the measurement was starting from the 2 days of cultivation) both the *loose-agglomerates* and the *fine-agglomerates* of MWCNTs have shown abilities to inhibit cell growth with the ability for the *fine-agglomerates* were much larger than that for the *loose-agglomerates*. In fact, the *fine-agglomerates* of MWCNTs have shown the inhibiting effects which were even stronger than that of the *white*-ASB.

Cell viabilities were estimated using the triphenyl-tetrazolium chlorine (TTC) reduction assay method during the courses of cell cultivation (Figure 4). Again, for cells cultured in media containing the *fine-agglomerates* of MWCNTs (10 $mg \cdot l^{-1}$), the optical density and thereby the viability of the cells was always the lowest value among the data obtained for the cells cultured in media containing the *loose-agglomerates* of MWCNTs and the *white*-ABS. These data again, highlight the increase in toxicity when the size of the agglomerates of MWCNTs became smaller.

Does-dependence was evaluated by verifying the ultimate amount of MWCNTs in culturing media from 10 to 600 $mg \cdot l^{-1}$ (10, 60, 100, and 600 $mg \cdot l^{-1}$). The ability of MWCNTs to inhibit the cell viabilities was found to behave in a concentration-dependent manner (Figure 5). This was truth for both the *loose-agglomerates* and the *fine-agglomerates* of MWCNTs. However, the linearity for these curves was rather poor; this can be attributed to the changes in situations of agglomeration of MWCNTs. As the concentrations of MWCNTs in the culturing media are changed for 60 times, the situations of agglomeration of MWCNTs in cell culture media will be largely changed. This hence changes the size, specific surface areas, and also surface properties of MWCNTs. In other words, the concentration-dependent manner observed in this study may attribute to the difference in agglomeration situations.

Superoxide dismutase (SOD) activity assay (Figure 6) gave a smallest value for cells expose to the *fine-agglomerates* of MWCNTs; while the values were almost identical for the cells exposed to *loose-agglomerates* and *white*-ASB. SOD activities were significantly decreased for cell exposed to the *fine-agglomerates* of MWCNTs; this again indicates a fact

that the certain toxicity of the agglomerates of MWCNTs is dependent strongly on the size of the agglomerates.

Contents of chlorophylls were measured; Figure 7 shows the experimental results. The cells exposed to the *fine-agglomerates* of MWCNTs gave the smallest values, followed by the cells exposed to the *loose-agglomerates* of MWCNTs. Cells exposed to *white-ASB* gave an value identical to that of the cells cultured under the regular cultivation. The decrease in contents of chlorophylls highlights a fact that the agglomerates of MWCNTs are capable of inhibiting chlorophyll synthesis.

The similarity in morphologies to asbestos has evoked scientists to concern about the possible analogous mechanism in toxicity for the carbon nanotubes. In previous study [5], the MWCNTs of the long, rigid types were found to behave in an asbestos-like pathogenic manner but did not for the short, tangled types. The MWCNTs used in our study are the short, tangled types and our experimental data indicate that MWCNTs, even for the short, tangled types are toxic to the *Arabidopsis* T87 suspension cells. Moreover, their toxicity increased sharply as the diameters of the agglomerates decreased.

Foreign metals (i.e, residual metals serviced as catalysts for synthesizing of the carbon nanotubes) and the physical piercing abilities have been considered to be the key parameters for causing the toxicities for the animal cells/tissues by CNTs. The residual metals induce oxidative stress [13], resulting in membrane damages for the mammalian cells [14-16]. On the other hand, due to their cylindrical shapes, CNTs were found to be able of penetrating

into the cells through cell membranes [17]. Carbon nanotubes, once inserted into the cells, can contact with cytoplasm or even contact with the nucleus [18].

The influence of metallic impurity in CNTs of their toxic responses, however, remains as debate so far, this is mostly because no reliable quantification method of analyzing the metallic impurities. This difficulty was also encountered in our studies; as can be seen from the analytical data on metallic impurity measurements (Table 1), differences with meaningful values were observed for the metallic contents for the *loose-agglomerates* and the *fine-agglomerates* of MWCNTs, although they have exactly the same lot numbers. Recently, Ge and co-workers have established a standard method for quantification of the metallic impurities in CNTs [19]. This method will help end the long-time debate on this issue and in our further works, the metallic impurities will be measured using this standard method.

For the plant cells, as the *Arabidopsis* T87 suspension cells, used in this study, we believe "hypersensitive response" is the key mechanism responsible for causing toxicities by CNTs. Unlike the animal cell cultures, for the plant cell cultures, they have high tendencies to form clumps, which are the small communities (multicellular clumps) made up by variable numbers of cells. These clumps arise as a result of the failure of new cells to separate after the division or from the adherence of free cells among themselves. In our cases, the clumps contained up to 20 cells, and reached up to few hundred μm in diameter. The cells aggregated into clumps led to form "gorges" with variable sizes in depth/width amount the cell populations. CNTs existed as the *loose-agglomerates* have less propensity to entry into the gorges because of their larger sizes. However, for the *fine-agglomerates*, they can

distribute into most kind of these gorges because their size is smaller. Data on measuring the particle size distribution of the fine-agglomerates of MWCNTs using dynamic light scattering are shown in Figure 8, the MWCNTs present as tube bundles with the size ranging from 400 nm to 6 μm. The tube bundles, especially the bundles having the sub-μm sizes, in the biological environments, in this study, the JPL media containing the *Arabidopsis* T87 suspension cells, can be dispersed further into smaller or even into the individual tubes, namely the smallest units of the agglomerates of the CNTs, through the so-called "bio-distribution" [20-24]. The individual tubes together with the agglomerates of smaller sizes, after distributed into the gorges, can be retained in an irreversible manner by the cell walls harbor proteins and polysaccharides through interactions and/or wrappings. CNTs once accumulated to a sufficient amount on the clumps can induce hypersensitive response because the multicellular organisms (namely, the clumps) misunderstand these CNTs as viral, or bacterial, or fungal elicitors. Hypersensitive response is one of the most important strategies developed by plants to defend themselves against pathogens involve restriction of the pathogen to its site of penetration [25, 26]. The defense responses cascaded (the decrease in superoxide dismutase activities is a good sign), once the hypersensitive response occurred, which in itself, is sufficient to stop microbial pathogens from completing their life cycle.

Plant cells have cell walls with cellulose as the major wall polymer. This cellulose-based cell wall is robust yet tougher; this makes the CNTs difficult to penetrate into the plant cells. In fact, in our studies, CNTs were not observed insides of the cells using TEM (data not shown).

Proteins and polysaccharides are deposited in cell walls and together provide the wall with its properties to regulate the cell development [27]. CNTs once accumulated on the cell walls will be having a plenty of chances to interact with the proteins and/or polysaccharides. Due to their nano-sized diameter and the micro-sized length, CNTs can mimic as either a viral, or bacterial, or fungal elicitor. These bio-mimetic elicitors induce the hypersensitive signals; cells die in either the apoptotic manner or the necrotic manner. Experimental data on the decrease in cell dry weights, cell viabilities, cell chlorophyll contents, and superoxide dismutase (SOD) activities obtained in this study highlight the cell death, which can be attributed to the hypersensitive response.

4. Conclusion

The results obtained in this study gave experimental evidence for a conclusion that MWCNTs are toxic to *Arabidopsis* T87 suspension cells. The certain toxicities were found to be dependent strongly on the size of the agglomerates. Agglomerates of smaller size induce stronger toxicity than the agglomerates of larger size. The agglomerates of smaller size (including the individual tubes) of CNTs can mimic either as a viral, or bacterial, or fungal elicitor; this induces hypersensitive signals. At sufficiently high does the induced hypersensitive signals can force the defense responses cascaded, and as a result, cells go to die. Studies are ongoing focusing on recognizing of the “CNT mimic elicitors” by using resistance (R) genes as the bio-indicators.

Acknowledgment

We thank Riken BioResource Center for providing *Arabidopsis* T87 cells.

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Table1. Data on quantitative analysis of the selected elements (wt %) using ICP-MS together with the diameter, length and the purities for the CNTs used in this study.

	CNTs [a)]	CNTs [b)]
Average diameter*	9.5 nm	9.5 nm
Average length*	1.5 μm	N.A.
Carbon purity	90%	N.A.
Specific surface area	250-300 m^2/g	N.A.
B	0.439 %	0.483 %
Na	0.58 %	0.605 %
Mg	0.023 %	0.0114 %
K	0.1179 %	0.1198 %
Ca	0.268 %	0.094 %
Mn	0.0005 %	0.003 %
Al	3.64 %	3.3 %
As	0.002 %	0.007 %
Fe	0.208 %	0.188 %
Cu	0.001 %	0.0003 %
Zn	0.03 %	0.0036 %
Sr	0.004 %	0.0023 %
Mo	0.00003 %	0.00006 %
Cd	0.0011 %	0.00009 %
Pb	0.0004 %	0.0003 %
Ni	0.011 %	0.001 %
Total 16 elements	**5.326 %**	**4.819 %**

a) The CNTs were used as their received forms, namely the *loose-agglomerates.*

b): The *loose-agglomerates* after being converted into aggregates of smaller size, namely the *fine-agglomerates,* using ultrasonic waves.

N.A. = not analyzed.

* For the individual tubes.

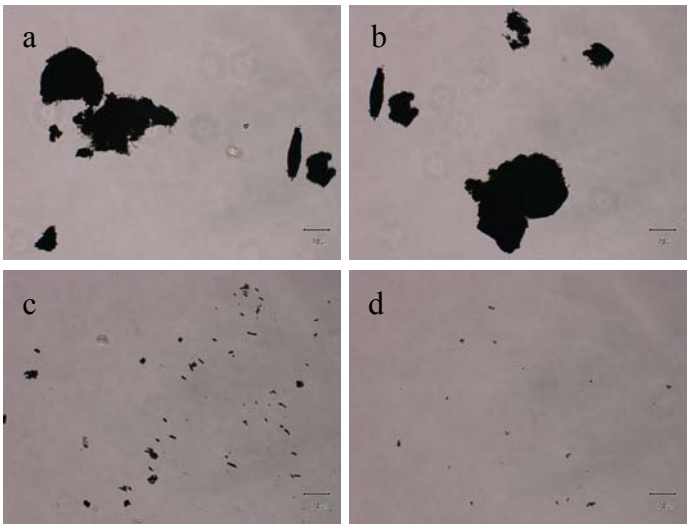

Figure 1 Light microscopic view of the *loose-agglomerates* (a, b) and the *fine-agglomerates* (c, d) of the CNTs. Aqueous suspensions containing the carbon nanotubes were mounted on glasses. Scale bars are 70 micrometers.

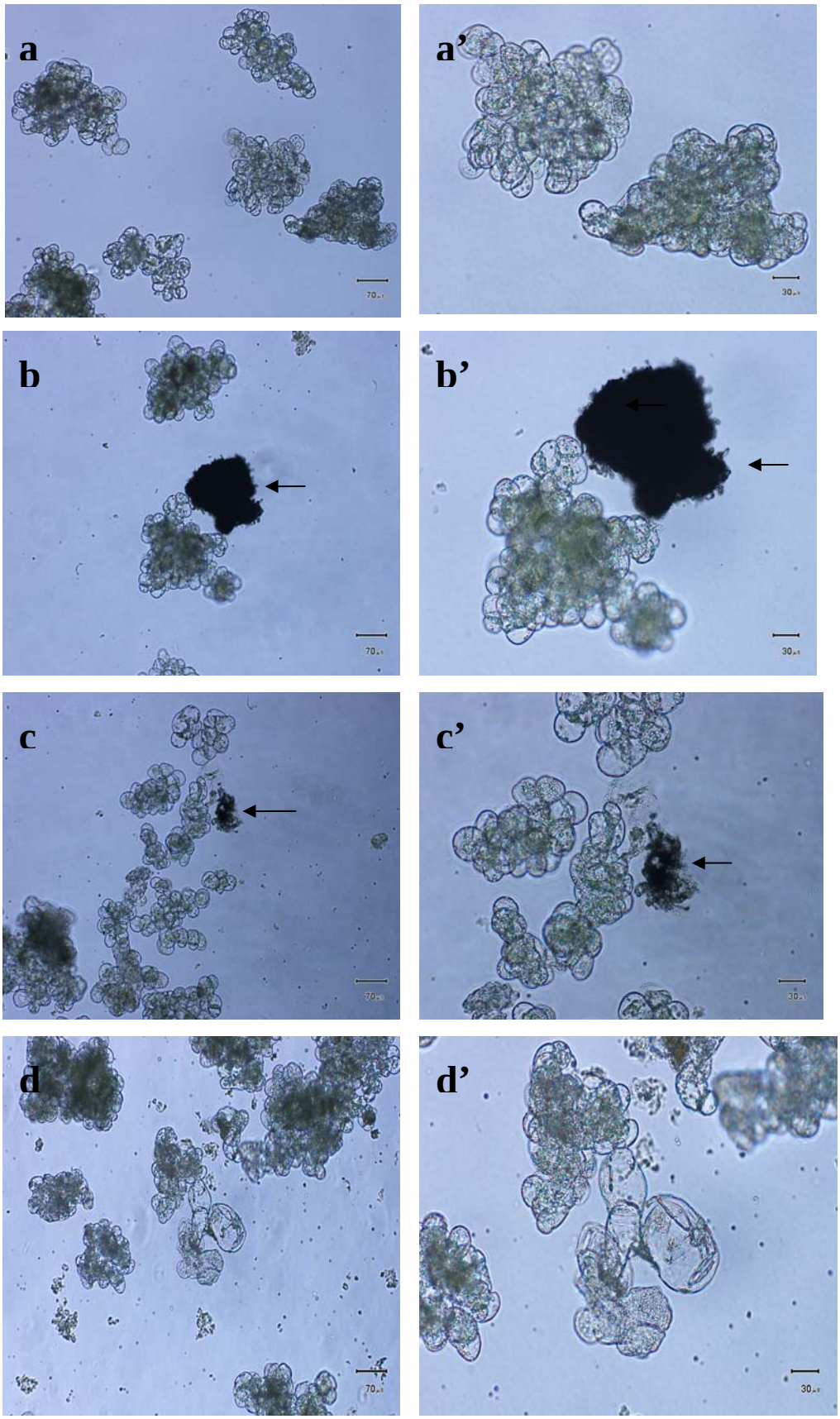

Figure 2 Light micrographs of *Arabidopsis* T87 suspension cells exposed to 10 mg·l^{-1} of CNTs and 10 mg·l^{-1} of white-asbestos for 3 days: (a, a') control cells; (b, b') cells exposed to

loose-agglomerates, arrows point agglomerates of CNTs; (c, c') cells exposed to *fine-agglomerates*, arrows point to the aggregates of the CNTs; (d, d') cells exposed to *white*-asbestos. Scale bars are 70 micrometers (a, b, c, and d) and 30 micrometers (a', b', c', and d'), respectively.

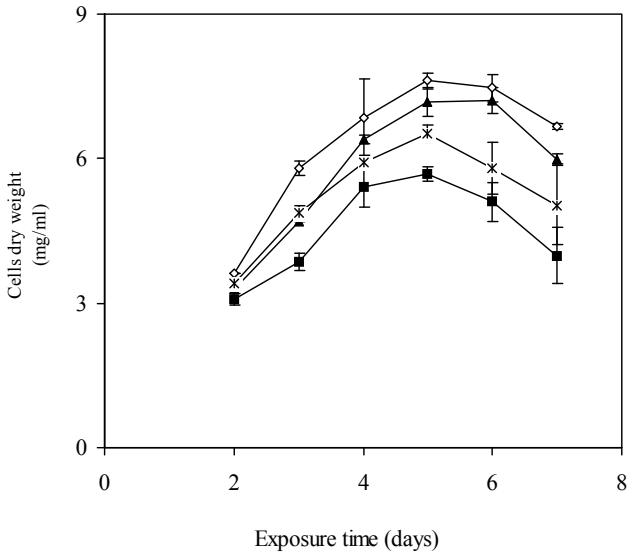

Figure 3 Representative growth curves for *Arabidopsis* T87 suspension cells for the control cells (◇) and that of the cells exposed to 10 mg·l^{-1} of *loose-agglomerates* (▲) and 10 mg·l^{-1} of the *fine-agglomerates* (■) of MWCNTs, and 10 mg·l^{-1} of white-asbestos (×). Cells were incubated at a constant temperature 22 ℃ in a growth room. Results are the means± standard error of the mean (S.E.M.; vertical bars); n=3.

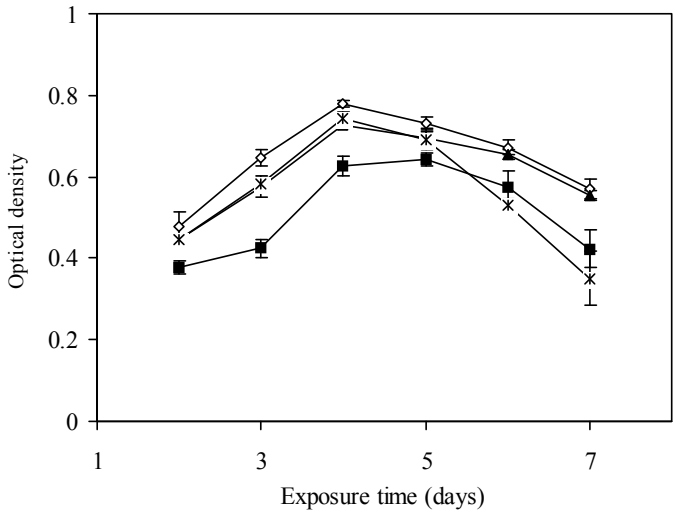

Figure 4　TTC assay in *Arabidopsis* T87 suspension cells of the control cells (◇) and that of the cells after the exposure to 10 mg·l^{-1} of *loose-agglomerates* (▲) and 10 mg·l^{-1} of the *fine-agglomerates* (■) of MWCNTs, and 10 mg·l^{-1} of white-asbestos (×) in the time course period. Cells were incubated at a constant temperature 22 ℃ in a growth room. Results are the means±S.E.M.(bars); n=3.

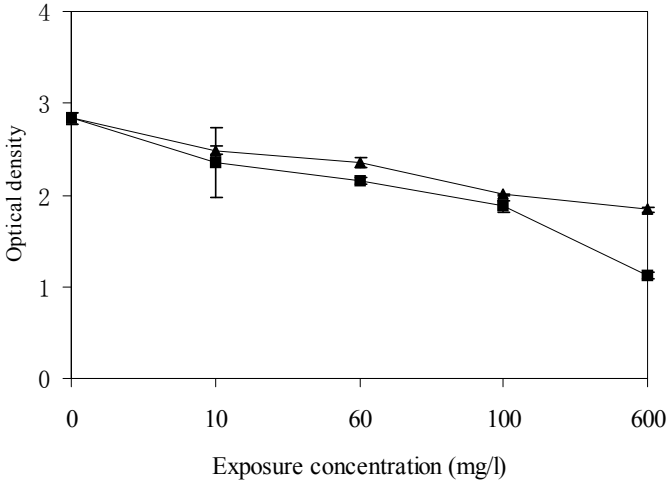

Figure 5　Concentration dependence of the viability of *Arabidopsis* T87 cells upon exposure to the *loose-agglomerates* (▲) and *fine-agglomerates* (■) of multi-walled CNTs. Cells were treated with 10 mg·l^{-1}, 60 mg·l^{-1}, 100 mg·l^{-1} and 600 mg·l^{-1} of the

loose-agglomerates and the *fine-agglomerates* of tMW CNTs for 48 hours, respectively. Cells were incubated at a constant temperature 22 ℃ in a growth room. Results are the means±S.E.M.(bars); n=3.

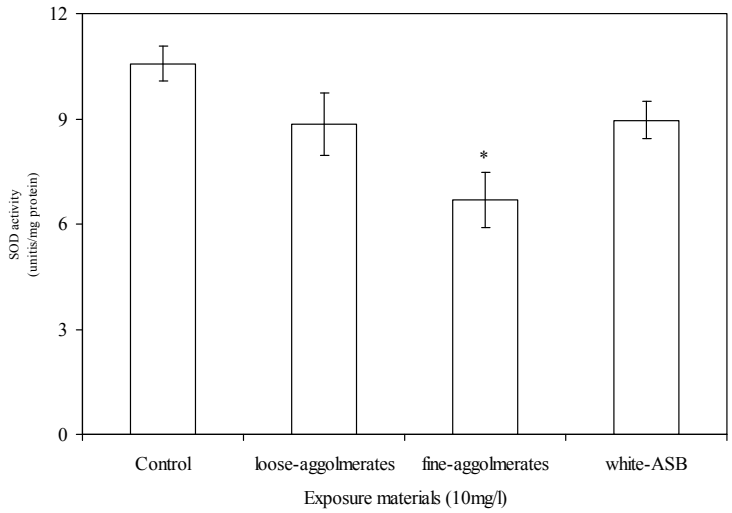

Figure 6　Total cellular SOD activities in control and treated cells in response to the CNTs (10 mg·l^{-1}) and the white-asbestos (10 mg·l^{-1}) exposure for 30 min. Cells were incubated at a constant temperature 22 ℃ in a growth room. Results are the means ±S.E.M. from three experiments (n=3).

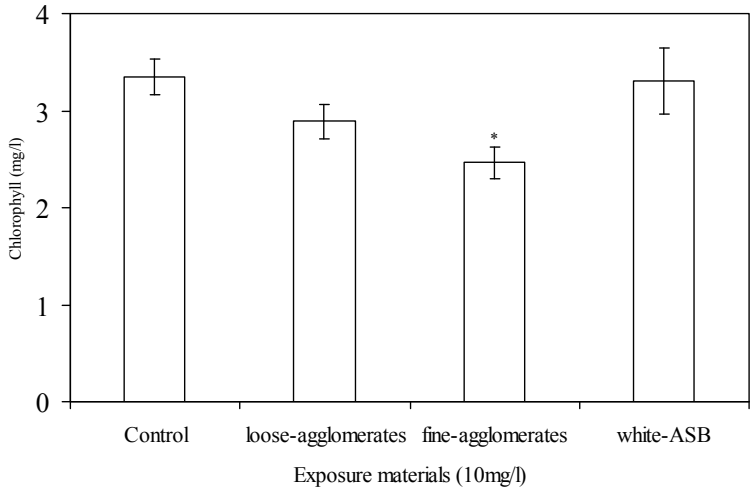

Figure 7 Chlorophyll contents in control cells and the treated cells in response to the MWCNTs (10 $mg \cdot l^{-1}$) and the white-asbestos (10 $mg \cdot l^{-1}$) exposure for 48 hours. Cells were incubated at a constant temperature 22 ℃ in a growth room. Results are the means±S.E.M. from three experiments (n=3).

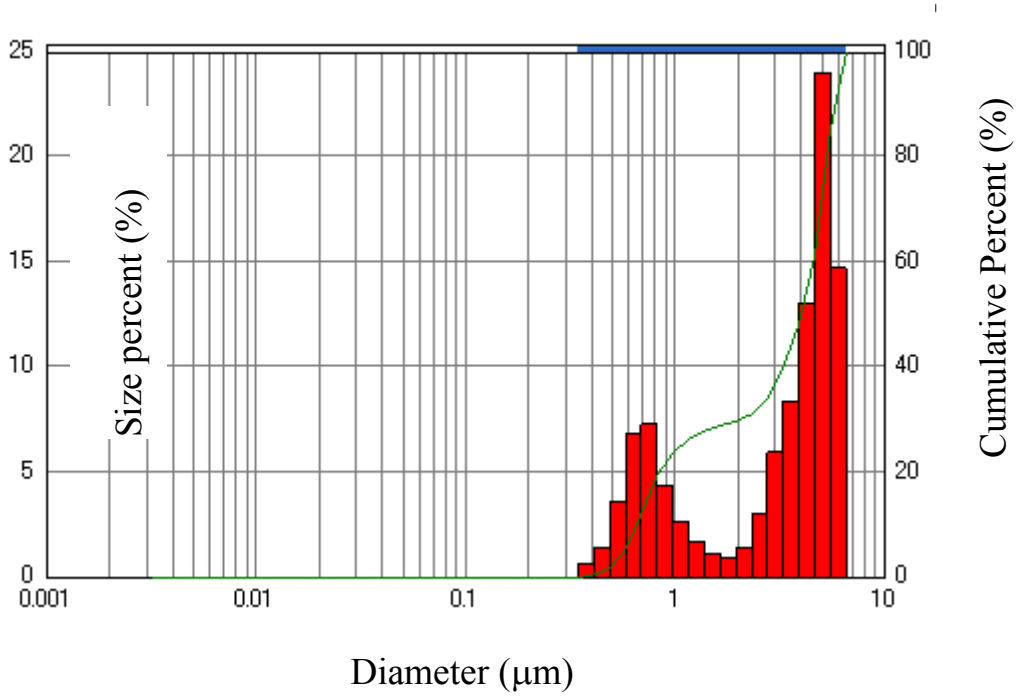

Figure 8 Dynamic light scattering size distributions of individual tubes and the agglomerates

of smaller sizes for the *fine-agglomerates* of MWCNTs.