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# Biological effects of agglomerated multi-walled carbon nanotubes

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ABSTRACT

The physicochemical properties of nanomaterials play crucial roles in determining their biological effects. Agglomeration of nanomaterials in various systems is a common phenomenon, however, how agglomeration affects the biological consequence of nanomaterials has not been well investigated because of its complexity. Herein, we prepared variable sized agglomerates of oxidized multi-walled carbon nanotubes (O-MWCNTs) by using $Ca^{2+}$ and studied their cellular uptake and cytotoxicity in HeLa cells. We found the altered property of O-MWCNTs agglomerates could be controlled and adjusted by the amount of $Ca^{2+}$. Agglomeration remarkably facilitated the cellular uptake of O-MWCNTs at the initial contact stage, due to the easy contact of agglomerates with cells. But agglomeration did not induce evident cytotoxicity when the concentration of O-MWCNTs was less than 150 μg/mL. That was assayed by cell proliferation, membrane integrity, apoptosis and ROS generation. This study suggests us that the biological behaviors of nanomaterials could be altered by their states of agglomeration.



## 1. Introduction

Along with the development of nanotechnology, more and more nanomaterials (NMs) possessing fancy properties have been produced and applied in diverse fields. They have brought great benefits to humans. In the meantime, the increasing annual production and wide applications mean the great possibility that NMs would enter into the human life and environment via different pathways [1–3]. Several hundreds of personal care commercial products contain NMs [4]. The existence of many kinds of NMs in the environment, including water and soil, has been reported [5,6]. People worry seriously about the potential harm of NMs to the health and environment. At present, a large amount of research data on the toxicity and risk assessment of NMs have been published, reporting various toxic effects at different tiers [7–9].

It is well accepted that the properties of NMs play important roles in their toxicity [10,11]. How the properties, including chemical composition, size, shape, surface charge and groups, affect the bioeffects of NMs, have been extensively studied. But, the bioeffects of agglomeration of NMs have not been studied extensively. There are only few studies in this aspect on carbon nanotubes (CNTs) [12,13]. Muller et al. reported that dispersed multi-walled CNTs (MWCNTs) (small aggregates) were more biopersistent in the lungs than intact MWCNTs (big aggregates), thus inducing more serious inflammatory and fibrotic reactions [14]. Wick et al. demonstrated that the toxic potential of single-walled CNTs (SWCNTs) was related to their degree of dispersion [15]. Although agglomerated and dispersed MWCNTs had no different effects on cellular viability and membrane integrity, better dispersed MWCNTs and SWCNTs were observed to increase transforming growth factor β1 (TGF-β1) and fibroblast proliferation [16–19]. The agglomeration appears in almost all contexts for CNTs, including various biosystems and the real environment. Actually, it usually closely related with other properties of NMs, such as surface charges and functionalized groups. Therefore, to understand intensively how agglomeration influences the bioeffects of CNTs is necessary.

In the previous published papers, dispersed CNTs were usually prepared by suspending CNTs with proteins, surfactants and functionalization [20] or even grinding [14]. These treatments may change the properties of CNTs, such as surface charge and groups, and even length. Thus, it is difficult to attribute the obtained bioeffects to agglomeration per se. In our previous studies and literature, we found that metal ions could induce agglomeration of O-MWCNTs and MWCNTs [21–24]. Herein, we prepared agglomerates of oxidized MWCNTs (O-MWCNTs) by using $Ca^{2+}$, and studied the cellular uptake and toxicity of O-MWCNTs agglomerates in HeLa cells. The well-known derivative of MWCNTs, O-MWCNTs, were selected as the model because they are widely used in various fields directly and usually are the first step of the functionalization of MWCNTs. We found that agglomeration of O-MWCNTs

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facilitated significantly the cellular uptake of O-MWCNTs, but did not induce marked cytotoxicity.

## 2. Materials and methods

### 2.1. Preparation of O-MWCNTs and characterization of O-MWCNTs agglomerates

Raw MWCNTs (diameter: 10–20 nm, purity >95%), produced by chemical vapor deposition, were purchased from Nanoharbor Co. (Shenzhen, China). O-MWCNTs were prepared by sonicating MWCNTs in $H_2SO_4$/$HNO_3$ mixture following the literature [21,25].

O-MWCNTs agglomerates were prepared by adding $Ca^{2+}$ (calcium chloride, analytical grade, Sinopharm Group Chemical Reagent Co., Ltd., Shanghai, China) directly into O-MWCNTs water suspensions. The concentration of both O-MWCNTs and $Ca^{2+}$ is tenfold of the final tested concentration. Fifteen minutes later, the mixture was diluted to one tenth with water or cell culture medium for the following experiments. The final tested concentrations in the culture medium are 80, 110, 120, 130 and 150 μM for $Ca^{2+}$, and 50, 100 and 150 μg/mL for O-MWCNTs. Considering the toxicology studies should performed under realistic conditions, we chose a suitable concentration range (50, 100, 150 μg/mL) based on studies reported by other researchers. Correspondingly, the concentrations of $Ca^{2+}$ were chosen to make O-MWCNTs at certain concentration keep different agglomeration states, from less agglomerated to serious agglomerated.

O-MWCNTs and their agglomerates were characterized by transmission electron microscopy (TEM, JEM-200CX, JEOL, Tokyo, Japan) for the morphology and size of O-MWCNTs, dynamic light scattering technique (DLS, Nano ZS90, Malvern, UK) for the hydrodynamic size and zeta potential of O-MWCNTs in aqueous suspensions, and confocal laser scanning microscope (FV1000, Olympus, Japan) for the real morphology situation.

The flow cytometer (BD FACS Calibur, BD Biosciences, USA) was used to confirm the size of O-MWCNTs agglomerates in cell culture medium. The data were analyzed using Cell Quest Pro software. Forward scatter (FSC) and side scatter (SSC) optical signals, which represent size and granularity of the particles, respectively, were acquired in linear scale using the primary data acquisition mode. Change of the suspension state of O-MWCNTs agglomerates modifies the quantum of laser light diffracted or scattered, thus causing altered population distribution in FSC-SSC planes.

### 2.2. Cell culture

HeLa cell (human epithelial cervical cancer) was obtained from the Cell Bank of Chinese Academy of Sciences in Shanghai, China. Cells were grown in DMEM medium (Zhejiang Tianhang Bio-Engineering Co., Ltd., China) with 10% (v/v) fetal bovine serum (Evergreen Bio-Engineering Materials Co., Ltd., Hangzhou, China) in a humidified atmosphere with 5% $CO_2$ and 95% air at 37 °C.

### 2.3. Cell proliferation and cell adhesion assay

HeLa cells were plated in 12-well plates ($5 \times 10^4$ cells per well). After 24 h, the cells were treated with culture medium containing O-MWCNTs agglomerates for another 24 h. Six parallel wells were adopted for each sample. After that, cells of three wells were collected and counted by Muse Cell Analyer (Merck Millipore, Darmstadt, Germany) for the cell proliferation assay. Cells in each well were counted three times. The other three parallel wells were used for the cell adhesion assay. Cells were washed three times with phosphate-buffered saline buffer (PBS). Cells in PBS were collected in a separate tube and counted; the adherent cells were trypsinized, collected and counted. The cell adhesion ability is represented with the ratio of the adherent cells to the total cells.

### 2.4. Cell viability and cell membrane integrity assay

The cell viability was evaluated by a WST-8 cell counting kit (CCK-8; Dojindo Molecular Technologies Inc., Kumamoto, Japan) and the cell membrane integrity was assessed by lactate dehydrogenase (LDH) test-kit (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega Corp., USA). Cells were plated in 96-well plates ($5 \times 10^3$ cells per well) and incubated for 24 h. Then, cells were cultured in culture medium containing O-MWCNTs agglomerates for another 24 h. After that, the culture medium was collected for LDH measurement by centrifugation (4500$g$ ×10 min) and cells for the viability assay, following the procedures reported previously [26,27]. Briefly, the cells were washed with D-Hanks and then incubated in CCK-8 solution (200 μL, contained 10% CCK-8) for 1 h at 37 °C. The optical density (OD) of each well at 450 nm was recorded on a Microplate Reader (Thermo, Varioskan Flash, USA). The cell viability (% of control) is expressed as the percentage of ($OD_{test}$-$OD_{blank}$)/($OD_{control}$-$OD_{blank}$), where $OD_{test}$ is OD of cells exposed to O-MWCNTs, $OD_{control}$ is OD of the control and $OD_{blank}$ is OD of the well without cells. For the LDH assay, the positive control was prepared by adding 10 μl lysis solution to the control cells at 45 min prior to centrifugation (4500$g$ ×10 min). Fifty microliter culture medium obtained above was assayed following the instruction of the kit. The OD of each sample at 490 nm was recorded on the Microplate Reader. LDH release (% of positive control) is presented as the percentage of ($OD_{test}$-$OD_{blank}$)/($OD_{positive}$-$OD_{blank}$), where $OD_{test}$ is OD of control cells or cells exposed to O-MWCNTs, $OD_{positive}$ is OD of positive control cells and $OD_{blank}$ is OD of well without cells.

### 2.5. Live/dead cell assay

The cell viability was quantified using the live/dead kit (Invitrogen, L-3224, USA). A mix of calcein AM and ethidium homodimer-1 was used to differentiate live cells (green. Ex 495 nm; Em517 nm) from dead cells (red. Ex 528 nm; Em 617 nm).

Cells were plated in 96-well plates and incubated for 24 h. Then the cell culture medium was replaced with culture medium containing O-MWCNTs agglomerates and incubated for another 24 h. After that, the above dye mix dissolved in PBS was added to cells and incubated for 30 min. Finally, cells were washed and detected under the confocal (F1000, Olympus, Japan).

### 2.6. Apoptosis assay

Apoptosis kit (FITC Annexin V Apoptosis Detection Kit I, BD Biosciences, USA) was employed to detect apoptotic and necrotic cells. Cells were plated in 6-well plates ($1 \times 10^5$ cells per well) and incubated for 24 h. Then the cell culture medium were replaced with culture medium containing O-MWCNTs agglomerates and incubated for another 24 h. The positive control was prepared by culturing the control cells in medium containing 200 mM $H_2O_2$ for 30 min. Finally cells were collected and stained with FITC-conjugated Annexin V (Annexin V-FITC) and propidium iodide (PI) following strictly the manual of the kit as reported [26,27]. The stained cells diluted by the binding buffer were analyzed by the flow cytometry. The cells were set as positive depending on the fluorescence intensity of Annexin V-FITC or PI. The early apoptotic cells were identified as Annexin V-$FITC^+$ and $PI^-$. The late apoptotic and necrotic cells were identified as Annexin V-$FITC^+$ and $PI^+$.