INTRODUCTION

Carbon nanotubes (CNTs) are endowed with intriguing physicochemical and biological properties. Their biological properties have received a great deal of attention in the medical field since its discovery. Previous studies have shown that CNTs, including single-walled CNTs and multi-walled carbon nanotubes (MWCNTs) are not only used for targeted delivery of drugs to specific tissue or organs, but also for killing cancer cells by the production of heat by near-infrared laser radiation. Moreover, CNTs are ideal reinforcement biomaterials for bone tissue engineering because they possess remarkable mechanical and structural properties. Furthermore, their capability of accelerating osteogenic differentiation of stem cells was in line with our unpublished findings. In addition to in vivo experiments, Siqueira et al. conducted in vivo studies and found poly D,L-lactic acid, superhydrophilic acid CNTs induced bone regeneration. Therefore, CNTs may be a promising osteo-inducing agent for regenerative medicine.

Bone-marrow derived stem cells (BMSCs) are considered multipotent progenitors, and can differentiate into adipocytes, osteoblasts, and neural cells. It has been previously reported that BMSCs increase the survival rate of transplanted adipocytes, suppress metastatic tumor growth, and improve bone formation. Janssens et al. conducted the first clinical trial on intracoronary artery transfer of BMSCs to ST-segment elevation myocardial infarction patients and found that BMSCs have a better effect on myocardial remodeling and regional systolic function recovery when compared to the placebo. Gutierrez-Fernandez et al. demonstrated that intravenous injection of BMSCs after a stroke had a positive effect on functional recovery and brain repair in Sprague-Dawley (SD) rats. Moreover, co-culturing of BMSCs and CNTs promoted directed differentiation of BMSCs, indicating that the combination of BMSCs and CNTs may be a promising strategy for clinical use. Moreover, the interaction and mutual promotion between stem cells and CNTs increased the focus of clinical studies on cytotherapy and bioengineering.

Because of van der Waals forces, CNTs can easily aggregate in water or organic solvents, and the poor dispersion of CNTs may cause toxicity. The use of CNTs has been reported to influence hemotoxicity, cytotoxicity to stem cells, inflammation, fibrosis, genotoxicity, and immunotoxicity. The effect of CNTs on cells is largely dependent on its colloid stability, composition, and size. Miscellaneous approaches have been adopted to change their physicochemical properties. Presently, functionalization is the most renowned and acknowledged approach.

In the rapid advancing field of nanomedicine, various functionalized CNTs have been studied, and the biocompatibility of which has been optimized. For example, acid oxidation is a classic strategy of CNTs’ functionalization because it sets the foundation for -OH and -COOH formation and facilitates subsequent addition of chemical surfactants. Polyethylene glycols (PEG)-MWCNTs exhibit extraordinary pharmacokinetic...
Materials and Methods

Materials

Pristine MWCNTs (diameter: 10–20 nm, length: 10–20 μm, purity ≥ 95%) were bought from Chengdu Organic Chemicals, Chinese Academy of Sciences (Chengdu, China). PEG, 4-dimethylaminopyridine (DMAP), 4-dichloromethane (CH₂Cl₂), HA, and nitric acid were bought from Kelong Chemical Reagent (Chengdu, China). Reactive oxygen species (ROS) assay kit, lactate dehydrogenase (LDH) assay kit and live-dead cell assay kit were purchased from Sigma (St. Louis, MO, USA). Comet assay kit was purchased from Trevigen (Gaithersburg, MD, USA). Fetal bovine serum (FBS), Alpha Minimum Essential Medium (α-MEM culture medium), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were purchased from Gibco (Gaithersburg, MD, USA).

Preparation of functionalized MWCNTs

1. AO-MWCNTs

Fifty milligram MWCNTs was resuspended in 70 mL 36.5 wt% hydrochloric acid solution and stirred for 2 h, following dilution and filtration. Next, the mixture was washed with deionized water and dried overnight under vacuum at 4°C. The mixture was refluxed for 4 h in 65 wt% hydrochloric acid solution and stirred for 2 min at 1,500 rpm. Cells were resuspended in 10 mL of 75 μm sieve and centrifuged for 4 min at 1,500 rpm. Cells were resuspended in 10 mL of α-MEM culture medium and 2×10⁶ BMSCs were seeded in 90 mm culture dishes in 90% α-MEM culture medium, containing 10% heat-inactivated FBS. Culture medium was changed every 24 to 48 h and cells were passaged every 5–7 days and expanded to the third passage for the following experiments.

2. PEG-MWCNTs

Two hundred fifty milligram PEG was dissolved in dichloromethane (CH₂Cl₂) solution, 50 mg AO-MWCNTs were added to the PEG/CH₂Cl₂ solution and allowed to react for 12 h at 25°C under catalytic reaction conditions of DMAP and dicyclohexylcarbodiimide. Next, the mixture was centrifuged at 15,000 rpm and the sediment was filtrated through a 0.22 μm cellulose membrane, and washed with deionized water. The mixture was dialyzed for 3 days to generate a mixture with a molecular weight between 8,000–12,000. The mixture was filtrated and dried for 24 h in vacuum chamber at 60°C.

3. HA-MWCNTs

AO-MWCNTs were added in sodium dodecyl sulfate solution and incubated in an ultrasonic bath for 2 h. After adding the biomineralization solution containing Ca(NO₃)₂·4H₂O, the mixed solution was adjusted to pH = 10. Then, the solution was incubated in an ultrasonic bath for 30 min. Next, (NH₄)₂HPO₄ solution (pH = 10) was added drop-wise to the above mixture (The ratio of Ca and P in mole was 1.667). After vigorously stirring and standing for 2 days, the mixture was repeatedly washed and filtrated until pH of the sediment was 7. After drying in vacuum chamber at 70°C, HA-MWCNTs were obtained.

Characterization of functionalized MWCNTs

Characterization of functionalized MWCNTs was assessed by scanning electron microscope (SEM, FEI, Philips, Eindhoven, the Netherlands). Dispersion was determined by general observation after 3 days of standing and the absorbance of supernatants of the suspension was measured. Static contact angles of functionalized MWCNTs powder were measured by a contact angle goniometer (Beijing HAKE, Beijing, China).

Preparation of BMSCs

Sprague Dawley rats (female, four weeks old, weighing: 100–150 g) were purchased from the Laboratory Animal center, Chinese Academy of Sciences (Beijing, China). Animal experiments were approved by the Animal Experimental Ethics Committee of Peking Union Medical College (Beijing, China) and were performed under the guidelines for care and use of laboratory animals as published by the National Institutes of Health (NIH). In brief, rats were euthanized by cervical dislocation, and the femur and tibia were harvested and sterilized with 70% alcohol for 20–30 min. After dissection of the soft tissue and osteoepiphysis, bone marrow was flushed out by phosphate-buffered solution (PBS). Bone marrow was filtered through a 75 μm sieve and centrifuged for 4 min at 1,500 rpm. Cells were resuspended in 10 mL of α-MEM culture medium and 2×10⁶ BMSCs were seeded in 90 mm culture dishes in 90% α-MEM culture medium, containing 10% heat-inactivated FBS. Culture medium was changed every 24 to 48 h and cells were passaged every 5–7 days and expanded to the third passage for the following experiments.

Cell experiments

1. MTT assay

Raw-MWCNTs, AO-MWCNTs, PEG-MWCNTs, and HA-MWCNTs were dissolved in α-MEM, and HA-MWCNTs were added to the MTT reagent (5 mg/mL) and cultured at 37°C and 5% CO₂ at day 1, 3 and 5, viability of the cells was determined. A total of 20 μL MTT reagent (5 mg/mL) was added to each well and incubated at 37°C for 4 h. The reagent was removed and 150 μL DMSO was added. After
shaking for 10 min. The absorbance was read at 490 nm using a spectrophotometer. Cell suspension to which no MWCNTs were added was used as a control.

2. Live-dead cell assay
The third passage of BMSCs was co-cultured with functionalized MWCNTs (10 μg/mL) for 72 h. Fluorescent staining of viable and dead cells and calculation of the ratio of dead to live cells was performed using a fluorescence microscope according to the manufacturer’s guidelines provided with the assay kit.

3. ROS assay
ROS harms the cellular biomedical metabolism and reduces viability. Therefore, ROS are regarded as a critical factor for cytotoxicity. ROS levels are to some extent paralleled to the cytotoxicity. Dichloro dihydrogen fluorescein-acetoacetic ester (DCFH-DA) can be oxidized to fluorescent Dichloro dihydrogen fluorescein (DCF), and the fluorescence intensity reflects that of the ROS level. The third passage of BMSCs were seeded into 12 well plates in a density of 1×10^4 to 1×10^5 cells/well. BMSCs were co-cultured with functionalized MWCNTs (10 μg/mL) for 12 h, harvested using trypsin and resuspended. Flow cytometry analysis was performed at 488 nm.

4. LDH release assay
LDH is a stable protein that is contained in the cytoplasm and can be released when the cell membrane is damaged. LDH catalyzes lactic acid to pyruvate, which reacts with tetrazolium blue to form a purple, crystallized substance. Therefore, the absorbance of the suspension reflects the degree of cell membrane damage. In brief, the third passage of BMSCs was seeded into 12 well plates at a density of 1×10^4 to 1×10^5 cells/well. Cells were co-cultured with functionalized MWCNTs (10 μg/mL) for 12 h. Absorbance was read at a wavelength of 340 nm by enzyme linked immunosorbent assay.

5. Comet assay
The comet assay is a popular technique for evaluation of deoxyribonucleic acid (DNA) damage/repair, biomonitoring and genotoxicity testing[26]. When damaged, the structure of the lysed DNA structure migrates under electrophoresis and the fluorescence intensity reflects the extent of DNA damage. In brief, the third passage of BMSCs was seeded into 12-well plates at a density of 1×10^4 to 1×10^5 cells/well. Cells were co-cultured with functionalized MWCNTs (10 μg/mL) for 72 h, harvested using trypsin, and prepared into a single cell suspension. Subsequently, 1 mL of mono-suspension of 1×10^4 to 1×10^5 cells/mL was added to 50 μL H_2O_2 at a concentration of 1×10^{-3} mol/L. The cell suspension was incubated for 30 min at 37°C after which 30 μL of the mono-suspension was mounted on a microscope slide with 50 μL of low-melting-point agarose into a thin layer. The agarose was gelled at 4°C for 10 min. Slides were immersed in lysis solution for 2 h at 4°C and electrophoresis was performed for 20 min at 200 mA. Then, slides were stained with acridine orange.

Statistical analysis
Experiments were conducted in triplicate and the results were expressed as the mean±standard deviation (SD). Differences among samples were tested by one-way ANOVA test. *p<0.05 was considered statistically significant.

RESULTS
Characterization of functionalized MWCNTs
1. Characterization
SEM images of pristine and functionalized MWCNTs are presented in Fig. 1. The SEM images showed that after acid oxidation and purification, the MWCNTs were purified and the difference in diameter was reduced. The surface of HA-MWCNTs was smooth and regular. Among the three functional groups, HA significantly changed the morphology of MWCNTs. Moreover, the other three MWCNTs formed rod-like structures. The diameter of HA-MWCNTs was largely reduced and HA-MWCNTs exhibited a less rough surface. HA was evenly distributed onto the surface of MWCNTs, although in certain spots, HA was clustered. HA formation on MWCNTs was confirmed by transmission electron microscope and X-ray diffraction (data not shown).

![Fig 1](image_url) Scanning electron microscopy (SEM) of pristine MWCNTs (a), AO-MWCNTs (b), PEG MWCNTs (c), HA-MWCNTs (d).

After acid oxidation, MWCNTs were purified and the difference in diameter was reduced, the surface of HA-MWCNTs was smooth and regular. Among these three functional groups, HA significantly changed the morphology of MWCNTs, while the other three MWCNTs had rod-like structures. HA-MWCNTs exhibited delicate surface, and HA was evenly distributed onto the surface of MWCNTs.
2. Dispersion
After incubation in an ultrasonic bath for 6 h and standing for 3 days, sediments were observed in the pristine MWCNTs, whereas the functionalized MWCNTs suspension was evenly dispersed and stable at room temperature (Fig. 2). The absorbance at 600 nm from supernatants obtained from pristine MWCNTs was low, whereas the absorbance of functionalized MWCNTs was high (Fig. 3). Moreover, HA-MWCNTs showed the highest priority in static contact angle test and absorbance, indicating that HA-grafted MWCNTs significantly improved the dispersion of MWCNTs. Static contact angles are presented in Table 1. The data showed that the static contact angle of pristine MWCNTs (110±1.7) was the largest and that the static contact angle of functionalized MWCNTs was decreased.

**Morphology of BMSCs**
After 3 days of culturing, the morphology of BMSCs was evaluated using a phase contrast microscope (Fig. 4). BMSCs that were co-cultured with HA-MWCNTs and PEG-MWCNTs showed a dendritic-like morphology and proliferated well, whereas an apoptotic trend was seen in the AO-MWCNTs and Raw-MWCNTs groups.

**MTT assay**
Figure 5 demonstrated that, compared with the blank control, AO-MWCNTs and Raw-MWCNTs significantly suppressed the proliferation of BMSCs, whereas the influence of HA-MWCNTs and PEG-MWCNTs on the cells was trivial. Differences between the control group and HA-MWCNTs/PEG-MWCNTs were not statistically significant after 5 days of culture, whereas the difference between AO-MWCNTs/Raw-MWCNTs and control group were statistically significant.

**Live-dead cell assay**
Live cells showed a green fluorescence and adhered to the bottom of culture flask. In contrast, dead cells showed a red fluorescence. Figure 6 shows that compared with HA-MWCNTs and PEG-MWCNTs groups, the ratio of dead cells was higher in Raw-MWCNTs and AO-MWCNTs groups. Moreover, among all groups, the AO-MWCNTs group displayed the highest cytotoxicity to MSCs.

![Image of pristine MWCNTs and three functionalized MWCNTs](image)

**Table 1** Water contact angle of pristine MWCNTs and three functionalized MWCNTs

| Sample        | Water contact angle/deg±SD |
|---------------|---------------------------|
| Raw-MWCNTs    | 110±1.7                   |
| AO-MWCNTs     | 89.6±1.3                  |
| PEG-MWCNTs    | 81.5±1.4                  |
| HA-MWCNTs     | 75.8±1.5                  |
Fig. 4 Observation of BMSCs using phase contrast microscopy after 3 days of culture. 

a: pristine MWCNTs, b: AO-MWCNTs, c: PEG-MWCNTs, d: HA-MWCNTs, e: control group. HA-MWCNTs and PEG-MWCNTs co-cultivated BMSCs showed a dendritic morphology and a favorable trend of proliferation, while the AO-MWCNTs and Raw-MWCNTs groups showed an apoptotic trend.

Fig. 5 MTT assay.

Compared with the blank control, AO-MWCNTs and Raw-MWCNTs showed significant suppression on MSCs proliferation, while HA-MWCNTs and PEG-MWCNTs exhibited beneficial effects on cell proliferation. Differences between the control group and HA-MWCNTs/PEG-MWCNTs were not statistically significant after 5 days of culture, whereas the difference between AO-MWCNTs/Raw-MWCNTs and control group were statistically significant.

Fig. 6 Live-dead cell assay of pristine MWCNTs (a), AO-MWCNTs (b), PEG-MWCNTs (c), HA-MWCNTs (d). Dead cells exhibit red fluorescence. Compared with HA-MWCNTs and PEG-MWCNTs groups, the ratio of dead cells was higher in Raw-MWCNTs and AO-MWCNTs groups. Among all the groups, AO-MWCNTs group displayed greatest cytotoxicity to MSCs.

**ROS assay**

Figure 7 shows the density of fluorescence of DCF, which reflects the amount of ROS. Compared with AO-MWCNTs/Raw-MWCNTs, the PEG-MWCNTs and HA-MWCNTs groups generated less ROS, suggesting that PEG-MWCNTs and HA-MWCNTs had a trivial influence on the cellular biochemical reactivity and exhibited a lower cytotoxicity.

**LDH release assay**

The amount of LDH in HA-MWCNTs and PEG-MWCNTs was reduced when compared to the AO-MWCNTs and Raw-MWCNTs group (Fig. 8). These data indicated that HA-MWCNTs and PEG-MWCNTs have a minor influence on the cellular membrane.

**Comet assay**

The comet assay (Fig. 9) demonstrated that the AO-MWCNTs group had an obvious comet tail and the intensity of tail was relative to the amount of DNA breaks. Compared with the AO-MWCNTs and Raw-MWCNTs groups, the HA-MWCNTs and PEG-MWCNTs groups displayed a regular and relatively round appearance after electrophoresis. These findings
DISCUSSION

Although CNTs have gained significant popularity in the field of medicine and bioengineering, its safety issue is currently still unresolved. The major barrier for the use of CNTs is their unfavorable biocompatibility. Aggregation of CNTs can generate toxicity to cells and organisms. For example, inhalation of a substantial amount of CNTs may cause pulmonary fibrosis\(^\text{28}\). Animal experiments have proven that intravenous or intraperitoneal injection of CNTs not only caused degradation of red blood cell, but also affected function of heart, brain, testis, etc.\(^\text{29–31}\). Therefore, understanding the mechanism of cytotoxicity is critical, both for the discovery of safe and effective drug carriers and for protecting the ecological environment\(^\text{32}\).

The toxicity of CNTs is determined by their physicochemical properties, which include diameter, length, surface area, purity, and solubility. Moreover, the contact time of cells and organisms with CNTs is also a principal factor\(^\text{30}\). Thus, several strategies have been performed to change the physicochemical property/dispersibility/toxicity, among which functionalization was the most promising and effective approach\(^\text{2}\). AO, as a classic strategy for the functionalization of CNTs, has become a basic step for further modification of CNTs. PEG-MWCNTs have been proven to be a safe and effective option as a drug carrier system\(^\text{34}\). HA-MWCNTs possess the mutual advantages of MWCNTs and HA and showed better mechanical properties and biocompatibility when compared with HA or MWCNTs alone\(^\text{27}\).

As a multipotent stem cell, promising applications of BMSCs in the field of regenerative medicine can be envisaged\(^\text{35}\). Deligianni et al.\(^\text{5}\) investigated the influence of pristine MWCNTs on human BMSCs and found that MWCNTs delayed the proliferation of BMSCs while increasing their differentiation towards bone. Moreover, Das et al.\(^\text{21}\) studied the reaction of canine BMSCs to COOH-functionalized CNTs and found that...
COOH-SWCNTs promoted neuronal and chondrogenic differentiation, whereas COOH-MWCNTs promoted neuronal and osteogenic differentiation. To use BMSCs and CNTs to the largest extent and ameliorate their disadvantages, studies have focused on the interaction between stem cells and CNTs.

Based on the above, we investigated and compared the toxicity of several promising functionalized MWCNTs in rat’s BMSCs. Our findings suggested that AO-MWCNTs caused the most severe cytotoxicity, which was in line with previously published results. Albini et al. found that acid-treated MWCNTs displayed a more severe cytotoxicity. Chatterjee et al. evaluated the toxicity of pristine MWCNTs and different surface-functionalized MWCNTs, including carboxylated (COOH), hydroxylated-oxygenation O(+), and amination (NH3), in human cell line (BEAS2B) and Caenorhabditis elegans, and found that COOH-MWCNTs was most toxic. Nonetheless, several studies presented opposite findings. For example, Porter et al. used the Raman technique to verify the existence of acidized functional groups outside the MWCNTs, however no significant effects on cell viability or structure were observed. These results indicated that physiological morphology and structure of CNTs and targeting cells were principle factors for toxicity. When observed by phase contrast analysis, BMSCs that were co-cultivated with HA-MWCNTs and PEG-MWCNTs demonstrated a dendritic-like morphology and favored proliferation, while the AO-MWCNTs and Raw-MWCNTs groups showed an apoptotic trend. These data demonstrated that HA-MWCNTs and PEG-MWCNTs have trivial cytotoxicity and favorable biocompatibility, and therefore may be used as promising drug carriers and biological scaffolds.

The mechanism of CNTs’ toxicity has not yet been identified. In our study, the ROS assay showed that MWCNTs-treated MSCs exhibited a higher fluorescence, which indicated an increase in oxidative stress. Moreover, the MTT assay showed that MWCNT-treated MSCs exhibited a higher absorbance, indicated damage of the mitochondriosome. The LDH release assay showed that MWCNT-treated MSCs displayed a higher fluorescence, indicating damage of cellular membranes. Obvious comet tails showed proof of DNA damage. The increase in ROS levels has been demonstrated in various cells types and animals treated with CNTs. Cui et al. and Reddy et al. reported that SWCNTs could decrease the proliferation of human embryonic HEK293 cells, reduce the expression of cytokines associated with cellular adhesive ability, such as laminin, fibronectin and focal adhesion kinase, and increase the level of ROS, cellular adhesive ability, such as laminin, fibronectin and other cytokines associated with proliferation of human embryonic HEK293 cells, reduce the expression of cytokines associated with cell apoptosis, cell-cycle arrest. Ghanbari et al. studied the toxicity of MWCNTs and detected the loss of mitochondrial activity, increased mitochondrial ROS formation, and mitochondrial membrane potential collapse before mitochondrial swelling. Furthermore, Visalli et al. observed the increase of ROS production in differentiated SH-SY5Y cells and found that ROS overproduction was dose and time-dependent and associated with mitochondrial impairment, DNA damage and decreased viability. In conclusion, mitochondriosome, membrane, DNA damage, and ROS overproduction may occur in MWCNT-treated BMSCs.

Many in vivo studies have evaluated its systemic toxicity to animals. Pulmonary inflammation and subsequent granuloma were observed pathologically after intratracheal instillation of SWCNTs, as SWCNTs that were deposited in the pulmonary surface, damaged alveolar cells and mesothelial cells, induced immunoreactivity, and generated pulmonary failure. Moreover, the observed toxicity was concentration dependent. SWCNTs activated heme oxygenase-1 and accelerated plague formation and atherosclerosis. Furthermore, CNTs can activate the immune system, raise antigen levels in blood serum, and disrupt immune function. Toxicity to other organs, such as the brain, liver and kidney, have also been reported.

CONCLUSION

While AO-MWCNTs and Raw-MWCNTs exhibited significant toxicity on BMSCs, PEG-MWCNTs and HA-MWCNTs showed favorable biocompatibility and beneficial effects on BMSCs. Therefore, PEG-MWCNTs and HA-MWCNTs may serve as promising biomaterials for future use. The order of toxicity was as follows: AO-MWCNTs>pristine MWCNTs>PEG-MWCNTs>HA-MWCNTs. The possible mechanism of MWCNTs’ toxicity may include cellular membrane damage, DNA damage, mitochondriosome damage, and an increase in oxidative stress. In conclusion, this study provided novel clues for the application of nanomaterials and understanding the mechanism of carbon nanotube-related toxicity. Additional experiments will be required for understanding the cytotoxic mechanism of action and the factors involved.

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