

Preparation and Characterization of Disulfide Functionalized Multi-Walled Carbon Nanotubes for Biomedical Applications

Ke Long Huang (Corresponding author)

Institute of Functional Materials and Chemistry, College of Chemistry and Chemical Engineering
Central South University, Changsha, Hunan 410083, China
Tel: 86-731-887-9850 E-mail: huangkelong@yahoo.com.cn

Ling Hu

Institute of Functional Materials and Chemistry, College of Chemistry and Chemical Engineering
Central South University, Changsha, Hunan 410083, China
E-mail: huling19821216@163.com

Su Qin Liu

Institute of Functional Materials and Chemistry, College of Chemistry and Chemical Engineering
Central South University, Changsha, Hunan 410083, China

Abstract

To interact with different biologic systems for biomedical applications, chemical modification of carbon nanotubes is always a key step. Disulfide is sensitive to the reductive intracellular environment, and such stimulus-responsive covalent bonds were used to modify carbon nanotubes. After deprotection of N-tert-butoxycarbonyl (Boc) groups of the N-(tert-butyloxycarbonyl) cystamine modified multi-walled carbon nanotubes (MWNTs), positively charged ammonium (NH_3^+) functionalized MWNTs (MWNTs-S-S-NH_3^+) with disulfide linkages were obtained. Their surface functional groups and changes of morphologies were characterized by infrared (IR) spectroscopy and transmission electron microscope (TEM), respectively. The weight percentage of the immobilized disulfide was estimated by thermogravimetric analysis (TGA). And their cytotoxicity in vitro on cultured human nasopharyngeal SUNE1 cells was evaluated. The biocompatibility of MWNTs was improved compared to that of MWNTs without functional groups.

Keywords: Multi-walled carbon nanotubes, Disulfide, Functionalization, In vitro cytotoxicity tests

1. Introduction

Recently, carbon nanotubes (CNTs) have attracted considerable attention in biomedical applications. CNTs can act as carriers to deliver a variety of biological and bioactive components such as proteins, peptides, DNAs/RNAs, drugs and other biological molecules into cells (Wong *et al.*, 2005, p.6021; Bianco *et al.*, 2005, p.571; Pantarotto *et al.*, 2004, p.5242; Wu *et al.*, 2005, p.6358). To get a kind of well physiologically acceptable material, CNTs should be functionalized firstly. Amines can be further modified with a wide range of biological molecules and therapeutic agents. For example, NH_3^+ -functionalized CNTs (CNTs-NH_3^+) is such a useful material, which can conjugate biomolecules and deliver them into cells. Georgakilas and Prato (Georgakilas *et al.*, 2002, p.3050; Prato *et al.*, 2008, p.60) have reported that amphotericin B, anticancer agent methotrexate, immunogenic peptides have been carried successfully into cells by CNTs-NH_3^+ .

Disulfide linkage is stable under extracellular physiological conditions but sensitive to the reductive intracellular environment. Introducing disulfide linkages to CNTs could facilitate releasing the targets from modified CNTs. The concentration of intracellular glutathione is adequate to break the disulfide linkages, while glutathione outside the cells has no such ability due to the relative low concentration (Piest *et al.*, 2008, p.308; Lin *et al.*, 2006, p.130). You (You *et al.*, 2007, p.16161) has decorated MWNTs with small molecules of pyridyl-ended disulfide and conjugated bovine serum albumin (BSA) onto the material through a disulfide-exchange reaction, and the BSA could be smartly released from MWNTs in the presence of glutathione in vitro.

However, there is little information available of functionalizing MWNTs in literature for the purpose of conjugating biomolecules onto CNTs conveniently as well as releasing these molecules smartly. The aim of the work described herein was to functionalize MWNTs, and then to assess the characteristics of toxicity of MWNTs functionalized with disulfide. By this procedure, we can obtain the disulfide functionalities decorated MWNTs with ammonium terminal to endue MWNTs with more convenience for transporting targets and releasing these molecules. The chemical reactions for functionalization of MWNTs are illustrated in Figure 1.

2. Experimental

2.1 Reagents and Measurements

All solvents and reagents were purchased from commercially available sources and used without further purification unless otherwise stated. The MWNTs (98%, 40-60 nm outside diameters, 5-15 μm lengths) were purchased from Shenzhen Nanotech Port Co., Ltd.

Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet 6700 IR spectroscopy. Transmission electron microscopy (TEM) images were acquired on a JEOL JEM-1230 electron microscopy at an accelerating voltage of 100 kV. Thermal gravimetric analysis (TGA) was carried out on a Diamond TG-DTA 6300 instrument in flowing Argon at a heating rate of 10 $^{\circ}\text{C}/\text{min}$ from room temperature to 600 $^{\circ}\text{C}$.

2.2 Preparation of functionalized MWNTs

The N-(tert-butyloxycarbonyl) cystamine, containing disulfide linkage, was synthesized via a procedure reported by Niu (Niu *et al.*, 2008, p.11988).

Oxidized MWNTs (MWNTs-COOH) were obtained by refluxing MWNTs and concentrated nitric acid for 28h. The as-prepared MWNTs-COOH (100mg) were suspended in 15 mL of DMF solution by sonicating the mixture for 30min, then EDC (4.5 mmol) and NHS (4.5 mmol) were added to the above suspension and mixed sufficiently. After stirring the mixture for 24 h, N-(tert-butyloxycarbonyl) cystamine (1.14 g, 4.5 mmol) was added. Then, the reaction was allowed to stir for another 36 h under Ar gas protection, following by centrifuging, sonication and repeated washing. The products of N-(tert-butyloxycarbonyl) cystamine functionalized MWNTs (MWNTs-S-S-NHBoc) were obtained by drying under vacuum. The Boc protecting groups of the MWNTs-S-S-NHBoc are cleaved by treatment with trifluoroacetic acid in dichloromethane for 2 h at the room temperature under Ar gas protection. After centrifuging and washing, the disulfide functionalized MWNTs (MWNTs-S-S-NH₃⁺) were obtained.

2.3 Toxicity test in vitro

We studied the toxicity effects of MWNT-S-S-NH₃⁺ on human nasopharyngeal SUNE1 cells by MTT assay which was used to determine cell survival in the past few years. SUNE1 cells were incubated with either MWNT-S-S-NH₃⁺ or pristine MWNTs. As a control, cells were exposed to physiological saline. The cells were grown in RPMI-1640 cell culture media at 37 $^{\circ}\text{C}$ in 5 % CO₂ humidified incubator and treated for 24 h, 48 h or 72 h with doses of MWNT-S-S-NH₃⁺ increasing from 6.25 to 200 $\mu\text{g}/\text{mL}$. The dose of pristine MWNTs used was 50 $\mu\text{g}/\text{mL}$.

3. Results and discussion

3.1 IR spectroscopy

IR spectra of the oxidized MWNTs and positively charged MWNTs are shown in Figure 2. The presence of the added functional groups onto MWNTs is verified. In the spectrum of MWNTs-S-S-NH₃⁺ (Figure 2(b)), the characteristic C-H stretching vibration (2923 cm^{-1}), as well as amide I band C=O stretching and amide II band N-H bending vibration (1624 and 1540 cm^{-1} , respectively) are observed. These peaks confirm that disulfide were connected to MWNTs successfully.

3.2 TEM analysis

Morphology and nano-structures of pristine MWNTs, MWNTs-S-S-NH₃⁺ are observed by TEM which are shown in Figure 3, TEM observations give definitive proof of the presence of MWNTs-S-S-NH₃⁺ in the solution. The MWNTs cut by concentrated nitric acid appear in bundles. After functionalization, the MWNTs-S-S-NH₃⁺ are shorter and thinner than pristine MWNTs, with diameters of 20-40 nm and lengths of 400-600 nm.

3.3 TGA analysis

Thermogravimetric analysis (TGA) measurements of the MWNTs-S-S-NH₃⁺ material supplies the quantitative evaluation of the disulfide grafted onto MWNTs (Figure 4). The weight loss of all samples below 200 $^{\circ}\text{C}$ is mainly attributed to release of water molecules and organic molecules adsorbed by samples. Oxidized MWNTs

sample (Figure 4(a)) displays a small quantity of weight loss in the region 200-500 °C. A weight loss, corresponding to 21% for MWNTs-S-S-NH₃⁺ sample is observed in the region 200-500 °C (Figure 4(b)), which is attributed to phrolysis of the disulfide connected with the carboxyl groups of MWNTs. The thermal analysis indicate the surface of MWNTs are grafted with a large number of disulfide.

3.4 In vitro toxicity

Insoluble and pristine CNTs have induced cell death in vitro (Cui *et al.*, 2005, p.73; Bottini *et al.*, 2006, p.121; Kiura *et al.*, 2005, p.359). To assess the biological properties of the functionallized MWNTs-S-S-NH₃⁺, we study their toxicity effects on SUNE1 cells. The dates were taken for 24, 48 and 72 h time points. As can be seen in Figure 5, the percentages of cell death are all less 25 % when cells were treated with MWNTs-S-S-NH₃⁺, showing no significant loss of cell viability compared to untreated cells. According to the 72 h time point dates, more than 50 % of the cells died in the presence of pristine MWNTs, whereas the vast majority of cells remained alive upon treatment with MWNTs-S-S-NH₃⁺. However, an expected dose-response relationship is observed. At higher concentrations of MWNTs-S-S-NH₃⁺, regardless of 24, 48 or 72 h points dates, MWNTs-S-S-NH₃⁺ have continuous reinforcing effects on the cells. The initial studies indicate functionalization of MWNTs reduces toxicity and improves biocompatibility of cells in vitro. These results agree well with previous observations (Sayes *et al.*, 2006, p.135; Lacerda *et al.*, 2006, p.1460).

4. Conclusions

We have prepared positively charged MWNTs by attaching disulfide to the nanotubes followed cleavage of the Boc protecting groups. The method is effective for functionalization. Infrared spectroscopy characterization, electron microscope measurements and thermal gravimetric analysis reveal that disulfide linkages are introduced onto the surface of MWNTs via amide bonds. The toxicity studies show MWNTs-S-S-NH₃⁺ material has only very limited impact upon cell proliferation and cell viability, indicating that functionalization of nanotubes dramatically improves the toxicity profile of this nanomaterials.

Acknowledgements

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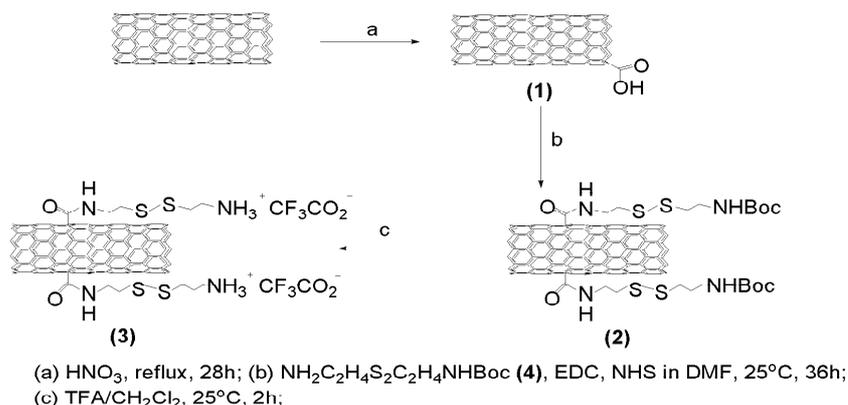


Figure 1. Preparation of disulfide functionalized MWNTs and positively ammonium functionalized MWNTs-S-S- NH_3^+

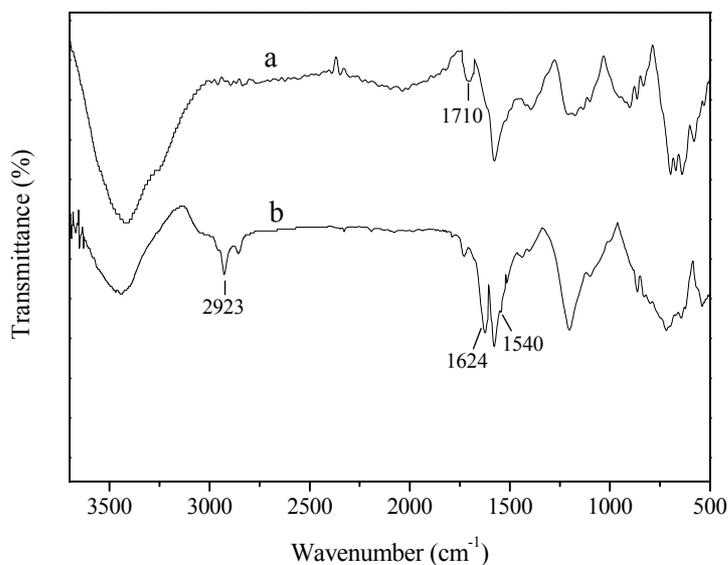


Figure 2. FT-IR spectra of (a) oxidized MWNTs and (b) MWNTs-S-S- NH_3^+

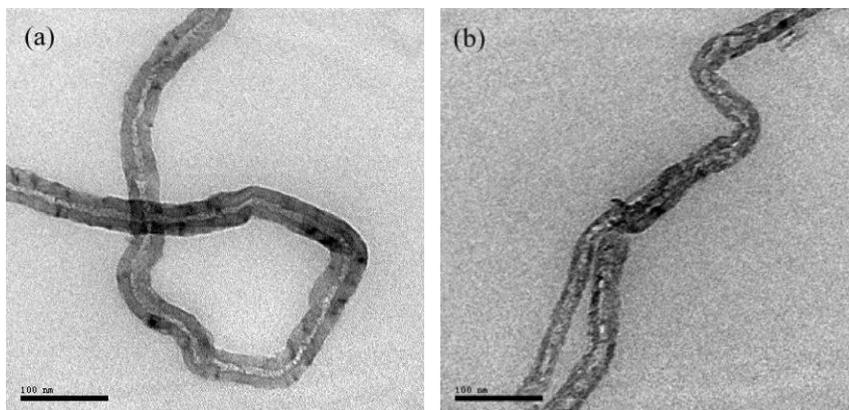


Figure 3. TEM images of (a) pristine MWNTs and (b) MWNTs-S-S-NH₃⁺. The scale toolbars represent 100 nm

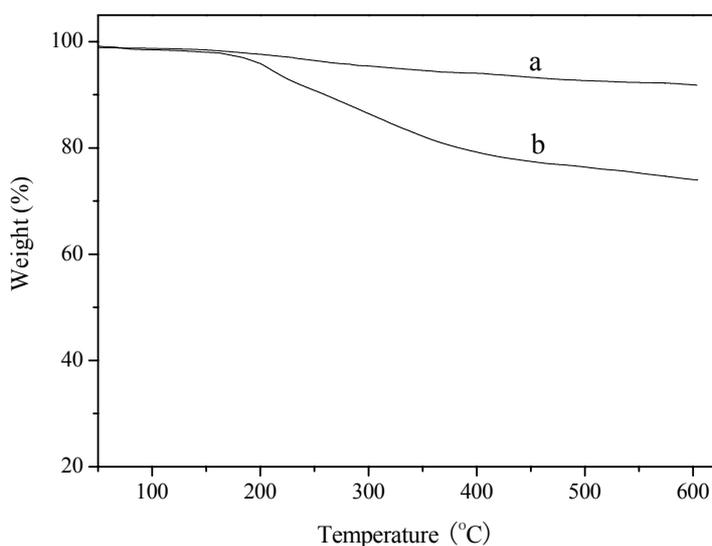


Figure 4. Thermal gravimetric analysis of (a) oxidized MWNTs and (b) MWNT-S-S-NH₃⁺

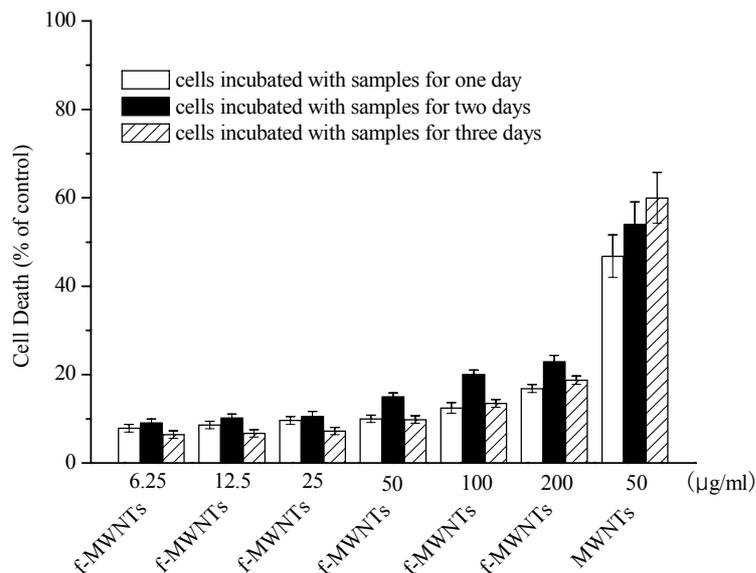


Figure 5. Cell death after treatment with MWNTs-S-S-NH₃⁺ (f-MWNTs) and pristine MWNTs for 24 h, 48 h or 72 h, respectively