Preparation of biocompatible multi-walled carbon nanotubes as potential tracers for sentinel lymph nodes

Junjun Li, a Feng Yang, b Guiquan Guo, a Dong Yang, a Jiang Long, b Deliang Fu, b Jennifer Lu c and Changchun Wang a *

Abstract

Carbon nanotubes (CNTs) are capable of traversing cellular membranes by endocytosis and are therefore promising materials for use in imaging and drug delivery. Unfortunately, pristine CNTs are practically insoluble and tend to accumulate inside cells, organs and tissues. To overcome the poor dispersibility and toxicity of pristine CNTs, hydrophilic functionalization of CNTs has been intensively investigated. Water-soluble multi-walled carbon nanotubes (MWCNTs) were prepared by in situ polymerization of acrylic acid in a poor solvent for poly(acrylic acid) (PAA). The solvent type influenced the grafted density and chain length of PAA. MWCNTs with a high grafted density of PAA (22 wt%) could be well dispersed in water, NaCl aqueous solution (0.9 wt%) and cell culture media. The in vitro cytotoxicity of these MWCNTs for endothelial cells is reasonably low even at high concentration of PAA-g-MWCNT (70 μg mL −1 ). The experimental results show that the biocompatibility of these MWCNTs is sufficient for biological applications. PAA-g-MWCNTs were successfully utilized for lymph node tracing. Experimental results suggest that PAA-g-MWCNTs have potential to be used as a vital staining dye, which may simplify the identification of lymph nodes during surgery. © 2009 Society of Chemical Industry

Keywords: water-soluble multi-walled carbon nanotubes; poly(acrylic acid) (PAA); in situ polymerization; lymph node tracer

INTRODUCTION

Carbon nanotubes (CNTs) have attracted considerable attention due to their unique mechanical, optical and electronic properties. 1 – 5 Recently, CNTs have been explored for use in the fields of biomaterials and tissue engineering. 6 – 9 with several groups determining that CNTs are capable of traversing cellular membranes, 10 – 12 making them ideal candidates for use in imaging and drug delivery. 12 – 14 However, pristine CNTs are practically insoluble and tend to accumulate inside cells, organs and tissues, and therefore have an impact on health. 15 To overcome the poor dispersibility and cytotoxicity of pristine CNTs, hydrophilic functionalization of CNTs has been intensively investigated. 16 – 19

Acid oxidation treatment, as a conventional method to shorten and introduce hydrophilic groups (–COOH) to CNTs, 20 – 21 could greatly improve the dispersibility of CNTs in water. However, aggregation easily takes place when the ion concentration exceeds the critical coagulation concentration (CCC). The CCC of Na + for acid oxidation-treated CNTs (AO-CNTs) is about 37 mmol L −1 , 22 far lower than the ionic concentration of Na + in body fluids. Therefore, AO-CNTs cannot be well dispersed in physiological media. Many researchers have indicated that the dispersibility and cellular toxicity of carbon-based nanomaterials are greatly dependent on their surface chemistry. 23 – 26 Their biocompatibility can be obviously improved with surface modification using hydrophilic molecules.

Modification of CNTs with organic molecules, including noncovalent and covalent functionalization, has been developed for improving the solubility of pristine CNTs. 8 Through covalent functionalization, a wide range of molecules, including hydrophilic and hydrophobic ones, can be readily attached to CNTs. For example, Dyke and Tour 27 reported a method in a solvent-free system, from which could obtain CNTs with a high degree of functionalization. To date, many functionalization methods for CNTs have been developed and many hydrophilic polymer chains have also been attached to CNTs. 28 – 32

Recently, we established a new method to prepare poly(methyl methacrylate)-functionalized single-walled CNTs via in situ polymerization using a system in which the solvent is a good solvent for monomer but a poor solvent for polymer. 33 Exploiting this approach, we herein report a method to prepare water-soluble multi-walled carbon nanotubes (MWCNTs). The functionalization process can be carried out at low acrylic acid/MWCNT feed ratio (2.5:1 by weight) and the amount of poly(acrylic acid) (PAA) in
PAA-grafted MWCNTs (PAA-g-MWCNTs) can be well controlled through utilizing different solvents. This method is capable of producing MWCNTs with sufficiently long PAA chains and high graft density. We demonstrate that PAA-g-MWCNTs with acceptably low cytotoxicity can be used as a vital staining dye to visualize lymph nodes. Therefore, this could allow surgeons to locate blackened lymph nodes during surgery and enables pathologists to look for the nodes in fatty tissue.

**EXPERIMENTAL**

**Materials**

Pristine MWCNTs, synthesized by the chemical vapor deposition method (purity > 95%), were purchased from Shenzhen Nanotech Port Co. Ltd. 2,2'-Azobisisobutyronitrile (AIBN), acrylic acid (AA), toluene, tetrahydrofuran (THF) and acetone were all purchased from Shanghai Chemical Reagent Corporation. AIBN was recrystallized from ethanol. AA was redistilled prior to use. All other chemicals were used as received.

**Animals**

Male Sprague-Dawley (SD) rats (Shanghai SLAC Laboratory Animal Co. Ltd), 4 to 5 weeks old, were housed in sterilized cages and fed autoclaved food and water *ad libitum*. All animal handling procedures were approved by the institutional animal care committee. All guidelines met the ethical standards required by law and also complied with the guidelines for use of experimental animals in China.

**Synthesis of PAA-g-MWCNTs**

In a typical reaction, 0.25 g of AA was added dropwise into a solution containing 100 mL of acetone and 0.1 g of MWCNTs. After 10 min sonication (59 kHz), the solution was purged with dry nitrogen for 30 min to remove oxygen, followed by addition of 0.05 g of AIBN as free radical initiator. The reaction was kept at 55 °C for 4 h. PAA-g-MWCNTs were carefully washed with water and centrifuged alternately five times to remove non-covalently attached PAA. The PAA-g-MWCNTs were dried in a vacuum oven at 40 °C for 16 h.

**Characterization of PAA-g-MWCNTs**

TGA measurements were carried out using a Perkin Elmer Pyris-1 series thermal analysis system under nitrogen atmosphere at a scan rate of 10 °C min⁻¹. Gel permeation chromatography (GPC) measurements were carried out using an HP series 1100 chromatograph equipped with TSK columns and RI/UV dual-mode detectors. NaNO₃ (0.10 mol L⁻¹) was used as the mobile phase and the elution rate was 0.5 mL min⁻¹. Poly(ethylene oxide)/poly(ethylene glycol) standards were used for molecular weight and molecular weight distribution analysis.

High-resolution transmission electron microscopy (HR-TEM) measurements were carried out using a JEM 2010 analytical electron microscope with a field emission source. The accelerating voltage was 200 kV. Samples for HR-TEM measurements were prepared by casting an aqueous solution of pristine MWCNTs or functionalized MWCNTs on a carbon-coated copper grid.

¹H NMR analysis was carried out using a Philips DMX500 spectrometer with D₂O as solvent. UV-visible spectra were recorded with a Lambda 35 spectrophotometer using a quartz cuvette with 1 cm optical path length. Deionized water was used as reference.

**In vitro cytotoxicity**

PAA-g-MWCNT aqueous solution was kept at 120 °C for 20 min in an autoclave for sterilization. Endothelial cells in a 96-well plate (10⁴ cells per well) after 24 h incubation were treated with increasing concentrations of PAA-g-MWCNTs (1–100 µg mL⁻¹) for 24 h at 37 °C and 5% CO₂. An amount of 10 mL of 5 g L⁻¹ MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue tetrazolium bromide (M5655, Sigma) was added to the cells in every well and incubated for 4 h. Culture media were discarded followed by addition of 0.2 mL of dimethylsulfoxide and vibration for 10 min. The absorbance (OD) was measured at 570 nm using a microplate reader. The cell growth inhibitory rate was calculated using

\[
\left( \frac{\text{OD of control group} - \text{OD of experimental group}}{\text{OD of blank group}} \right) \times 100\%
\]

**Lymph tracing experiments**

PAA-g-MWCNTs were dispersed in deionized water under sonication (59 kHz) for 5 min. The solution was then centrifuged at 5000 rpm for 10 min. The supernatant was centrifuged again at 8000 rpm for 10 min. The collected sediments, PAA-g-MWCNTs, were redispersed in deionized water (50 mg mL⁻¹), and then 100 µL (5 mg of MWCNTs) of solution was subcutaneously injected into a rat left rear foot pad. After 24 h, the popliteal lymph nodes of rats with or without injection of MWCNTs were dissected and examined using optical microscopy.

**RESULTS AND DISCUSSION**

Before use in biomedical applications, MWCNTs must be modified with hydrophilic molecules. In the study reported here, the surface of the MWCNTs was modified with PAA through precipitation polymerization. During the polymerization, polymer radicals are formed and become less solvent favorable. As the polymer radical chains reach a critical length, they are expelled from the poor solvent and are enthalpically favored to adsorb onto the surface of MWCNTs. The polymer radical chains still keep active with living radicals at the ends of the chains, some of the polymer radical chains will react with the MWCNTs and form covalent bonds.

When toluene is used as solvent for the surface modification of MWCNTs, the amount of grafted PAA is low (about 5 wt%); the main reason may be that the critical chain length of PAA in toluene is short. Although toluene, THF and acetone are all poor solvents for PAA, their solubility parameters are 8.9, 9.5 and 9.8 (cal cm⁻³)¹/², respectively, and therefore the critical chain length of PAA in these solvents differs. In order to compare the polymer critical chain length and graft density, functionalized MWCNTs in different solvents were studied. In each reaction system, the PAA-g-MWCNTs and associated PAA homopolymer were isolated from the solution, and the PAA homopolymer was analyzed using GPC. The results are shown in Fig. 1(A). It is found that the critical chain length of PAA is proportional to the solubility parameters (solvent polarity). Therefore, the amount of PAA grafted onto MWCNTs will increase with the solvent polarity, which is confirmed from the TGA results shown in Fig. 1(B). The amount of grafted PAA increases with the increase of solvent polarity: 5, 8 and 15 wt%. The samples of PAA-g-MWCNTs functionalized in THF and acetone have two main weight-loss regions as indicated in Fig. 1(B). The first weight-loss region (200–270 °C) may be assigned to the decomposition of carboxyl groups of PAA, and the significant weight reduction in the second region (270–500 °C) is possibly
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Figure 1. (A) GPC plots of PAA homopolymers and (B) TGA curves of PAA-g-MWCNTs and PAA-m-MWCNTs (The sample was prepared in: (a) toluene, (b) THF and (c) acetone).

Figure 2. TEM images of (A) pristine MWCNTs and PAA-g-MWCNTs prepared in different solvents: (B) toluene, (C) THF and (D) acetone.

due to the decomposition of the polymer backbone.\(^{31,34}\) As a controlled experiment, we prepared a mixture of free PAA and MWCNTs. The detailed procedure was as follows. Pristine MWCNTs (10 mg) were first dispersed in 5 mL of water containing 3.0 g of PAA homopolymer under bath sonication (59 kHz) for 10 min, and then 20 mL of acetone was added dropwise into the solution in order to induce precipitation. The precipitated material is MWCNTs non-covalently wrapped with PAA (PAA-m-MWCNTs). Two weight-loss regions are also found for the PAA-m-MWCNT sample. However, the first decomposition step is lower by about 100 °C than that of PAA-g-MWCNTs. A similar phenomenon has been reported before.\(^{31,35}\) For the sample of PAA-g-MWCNTs prepared in toluene, THF and acetone, respectively. This set of data suggests that the water solubility and the amount of grafted PAA are highest for PAA-g-MWCNTs prepared in acetone, which is in line with the TGA and TEM characterization results. Therefore, MWCNTs functionalized in acetone were selected for the subsequent lymph tracing experiments.

\(^1\)H NMR was further used to study the covalent grafting of PAA onto the MWCNTs. In this case, PAA-g-MWCNTs prepared in acetone were used. The \(^1\)H NMR spectra of PAA-g-MWCNTs
and PAA-m-MWCNTs are shown in Fig. 3. The signal peaks of PAA-g-MWCNTs are broader than those of PAA-m-MWCNTs due to slow tumbling of the grafted polymer brush in solution, indicating that PAA is covalently grafted onto the surface of MWCNTs after in situ grafting polymerization. The inherent peaks of PAA such as those of methylene (–CH₂–) and tertiary carbon hydrogen (–CH–) on the backbones of polymer chains clearly appear in the spectra at 1.16–2.05 ppm and 2.15–2.85 ppm. Moreover, PAA-m-MWCNTs in general precipitate within 10 h in water. In contrast, PAA-g-MWCNTs disperse well in water, and no obvious precipitation is found even after one month.

The dispersibility and toxicity of CNTs are usually dependent on the combination of the grafted polymer chain length and degree of sidewall functionalization. In order to obtain PAA-g-MWCNTs with a high degree of functionalization, the PAA-g-MWCNTs functionalized in acetone were screened out using different centrifugation speeds (3000, 5000, 8000 rpm) for 10 min. The amount of grafted PAA is 2 wt% for 3000 rpm, 10 wt% for 5000 rpm and 22 wt% for 8000 rpm.

PAA-g-MWCNTs and AO-MWCNTs centrifuged out at 5000–8000 rpm were dispersed in NaCl aqueous solution (0.9 wt%). The concentration of all the solutions was 5 mg mL⁻¹ (because of their original absorbance curves being similar, we only give one curve for the original PAA-g-MWCNTs solution). These solutions were left undisturbed for 12 h at room temperature, and then the supernatants were collected for UV-visible absorbance measurements. As shown in Fig. 4, the absorbance of PAA-g-MWCNT supernatant centrifuged at 8000 rpm (Fig. 4, spectrum b) is almost identical to that of the initial solution (Fig. 4, spectrum a), no appreciable absorption decreases at 250 nm. This result indicates that the supernatant, prepared in acetone and centrifuged at 8000 rpm, can be well dispersed in NaCl aqueous solution. It can also disperse well in cell culture media.

Recently, it has been reported that the cytotoxicity of CNTs is related to the degree of sidewall hydrophilic functionalization, and most water-soluble CNTs display non-toxicity in vivo. We also studied the cytotoxicity of PAA-g-MWCNTs. Firstly, the sterilized PAA-g-MWCNTs and AO-MWCNTs centrifuged at 5000–8000 rpm were dispersed in cell culture media. A series of cell culture media containing endothelial cells was prepared with the concentration of functionalized MWCNTs ranging from 10 to 100 µg mL⁻¹. After culturing the cells for 24 h, the cytotoxicity of functionalized MWCNTs was evaluated using the MTT assay. The assay is based on the accumulation of dark-blue formazan.
crystals inside living cells (but not in dead cells) after their exposure to MTT. A linear relationship between the number of living cells and the optical density can be established. This experiment allows an accurate quantification of the number of living cells. Analysis results, shown in Fig. 5, indicate that AO-MWCNTs exhibit stronger cytotoxicity due to the lowest number of living cells. The PAA-g-MWCNTs are more biocompatible due to the high degree of sidewall functionalization. When the concentration of PAA-g-MWCNTs exceeds 70 µg mL⁻¹, the number of living cells decreases dramatically from approximately 100 to 85%. This finding indicates that the PAA-g-MWCNTs with a concentration lower than 70 µg mL⁻¹ are relatively safe for physiological applications such as drug delivery and medical diagnosis. Upon subcutaneous injection of PAA-g-MWCNTs to the left foot pad of rats, the regional skin is dyed black right away, as shown in Fig. 6(A). After 24 h, a biopsy result shown in Fig. 6(B) indicates that left popliteal lymph nodes are blacker than other regions. This result suggests that PAA-g-MWCNTs are readily taken up selectively by lymphatic vessels, and are delivered to and blacken the popliteal lymph nodes. The lymph nodes of rats injected with and without PAA-g-MWCNTs were necropsied and fixed with 10% formalin, to keep the formalin in place until paraffin embedding, sectioning and hematoxylin–eosin (H&E) staining had been carried out. Large collections of black particles are found in the popliteal lymph nodes of rats injected with PAA-g-MWCNTs (Fig. 6(D), shown by white arrows); however, no black particles are found in the popliteal lymph nodes of rats not injected with PAA-g-MWCNTs (Fig. 6(C)). At the same time, the biopsy experiments did not identify the presence of PAA-g-MWCNTs in the major internal organs such as liver, kidney and lung. This result means that the PAA-g-MWCNTs are preferentially absorbed by lymph vessels, and then blacken the lymph nodes.

CONCLUSIONS

MWCNTs can be effectively functionalized with PAA via in situ precipitation polymerization of acrylic acid. Employing solvents with different polarity can tune the chain length of PAA grafted onto the MWCNTs. MWCNTs with different degrees of sidewall functionalization can be separated by centrifugation, the maximum amount of PAA grafted on the surface of MWCNTs being about 22 wt%. The as-prepared PAA-g-MWCNTs can well disperse in water, NaCl aqueous solution (0.9 wt%) and cell cultures. MTT experimental results showed that the biocompatibility of these PAA-g-MWCNTs was sufficient for biological applications. For the first time, PAA-g-MWCNTs were successfully utilized for lymph node tracing. The results suggest that the PAA-g-MWCNTs have the potential to be used as a vital staining dye, which may simplify lymph node identification during surgery even when they are very small.

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REFERENCES