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# Hyaluronic acid-modified multiwalled carbon nanotubes for targeted delivery of doxorubicin into cancer cells

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ABSTRACT

Development of novel drug carriers for targeted cancer therapy with high efficiency and specificity is of paramount importance and has been one of the major topics in current nanomedicine. Here we report a general approach to using multifunctional multiwalled carbon nanotubes (MWCNTs) as a platform to encapsulate an anticancer drug doxorubicin (DOX) for targeted cancer therapy. In this approach, polyethyleneimine (PEI)-modified MWCNTs were covalently conjugated with fluorescein isothiocyanate (FI) and hyaluronic acid (HA). The formed MWCNT/PEI–FI–HA conjugates were characterized via different techniques and were used as a new carrier system to encapsulate the anticancer drug doxorubicin for targeted delivery to cancer cells overexpressing CD44 receptors. We show that the formed MWCNT/PEI–FI–HA/DOX complexes with a drug loading percentage of 72% are water soluble and stable. In vitro release studies show that the drug release rate under an acidic condition (pH 5.8, tumor cell microenvironment) is higher than that under physiological condition (pH 7.4). Cell viability assay demonstrates that the carrier material has good biocompatibility in the tested concentration range, and the MWCNT/PEI–FI–HA/DOX complexes can specifically target cancer cells overexpressing CD44 receptors and exert growth inhibition effect to the cancer cells. The developed HA-modified MWCNTs hold a great promise to be used as an efficient anticancer drug carrier for tumor-targeted chemotherapy.



## 1. Introduction

Although chemotherapy has been one of the most important methods to treat cancer, many anticancer drugs, like doxorubicin (DOX), not only kill the cancerous tissues, but also lead to adverse side-effect on normal tissues.[1,2] In addition, many cancer drugs lack water solubility, significantly limiting their bioavailability. Therefore, it is essential to develop a drug delivery carrier with targeting specificity,[3–5] good biocompatibility,[6–8] sufficient stability,[9–11] and long circulation time.[12,13] Currently, a wide range of nanocarrier systems, including but not limited to liposomes,[14] nanoparticles,[15,16] nanogels,[17] polymer micelles,[18,19] and dendrimers[20] have been developed to both improve the water solubility of the anticancer drug and achieve the specific targeting drug delivery.

Carbon nanotubes (CNTs)[21–23] have gained tremendous attention as a promising nanocarrier owing to their distinct characteristics, such as ultrahigh surface area, high drug-loading capability, effective transportation capability, and enhanced cellular uptake.[24–26] For biomedical applications, the poor aqueous dispersibility and high aggregation tendency of pristine CNTs can be resolved by appropriate surface functionalization via either noncovalent or covalent modification strategies,[27,28] rendering the CNTs water soluble and highly biocompatible.[29,30] In our previous work, we have shown that polyethyleneimine (PEI) covalently linked onto the surface of acid-treated multiwalled carbon nanotubes (MWCNTs) can be further modified with different surface functional groups for biomedical applications.[30,31] Our results reveal that the water-dispersibility and biocompatibility of PEI-modified MWCNTs can be significantly improved after further modification of the PEI amines with acetyl groups or polyethylene glycol moieties.

For targeted drug delivery applications, the MWCNTs can be modified with amine-terminated poly(amidoamine) dendrimers pre-modified with fluorescein isothiocyanate (FI) and folic acid (FA).[32,33] The developed multifunctional MWCNT-based delivery system can achieve a high drug payload of anticancer drug doxorubicin (DOX) with a pH-responsive release property showing fast DOX release under acidic environment and slow release at a physiological pH condition. Importantly, the complexes were able to target cancer cells overexpressing high-affinity FA receptors and effectively inhibit the growth of the cancer cells. These prior successes lead us to hypothesize that the MWCNTs may be covalently modified with other targeting ligands for targeted cancer therapy applications.

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Hyaluronic acid (HA) is a linear polysaccharide consisting of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine.[34,35] HA has unique and excellent physicochemical properties, such as biodegradability, biocompatibility, and nonimmunogenicity.[36,37] Besides, it is also known that HA has a strong affinity to bind cell-specific surface markers such as cluster determinant 44 (CD44) receptors,[38,39] which are overexpressed on the surface of several different cancer cells.[39] Such interesting selectivity of HA to CD44 receptor-overexpressing cancer cells has shown a great application potential in cancer diagnosis and therapy.[40–44]

In this present study, we synthesized and characterized multifunctional MWCNTs modified with FI and HA (MWCNT/PEI–FI–HA) as a new anticancer drug carrier for targeted delivery of DOX to cancer cells overexpressing CD44 receptors. Acid-treated MWCNTs were first covalently modified with PEI, followed by sequential modification with FI and HA (Fig. 1). The formed MWCNT/PEI–FI–HA conjugates were characterized using different methods. The loading of DOX onto the multifunctional MWCNTs and the release of DOX from the MWCNTs were monitored via UV–vis spectrometry. The targeted therapeutic efficacy of MWCNT/PEI–FI–HA/DOX complexes in vitro was investigated in detail.

## 2. Experimental

### 2.1. Materials

MWCNTs (diameter = 30–70 nm, length = 100 nm–2 μm) were synthesized and characterized in a previous report.[45] HA (*Mw* = 31,200) was obtained from Zhenjiang Dong Yuan Biotechnology Corporation (Zhenjiang, China). Hyperbranched PEI (*Mw* ≈ 25,000) and all other chemicals and solvents were purchased from Aldrich (St. Louis, Missouri) and used as received. HeLa cells (a human cervical carcinoma cell line) and L929 cells (a mouse fibroblast cell line) were from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). RPMI 1640 medium, DMEM medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Regenerated cellulose dialysis membranes with molecular weight cut-off (MWCO) at 14,000 and 50,000 were acquired from Fisher (Pittsburgh, PA). Water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with resistivity higher than 18 M Ω cm.

### 2.2. Synthesis of MWCNT/PEI–FI–HA conjugates

The modification of MWCNTs with PEI to form MWCNT/PEI was adapted from our previous report.[30] Briefly, acid-treated MWCNTs with carboxyl residues (120.0 mg) dispersed in DMSO (50 mL) were activated with EDC (120.0 mg) and NHS (62.0 mg) co-dissolved into DMSO (5 mL) under vigorous magnetic stirring. The reaction was continued for 3 h to activate the carboxyl residues of MWCNTs, followed by the addition of PEI solution (120 mg in 12 mL DMSO). The reaction mixture was sonicated for 2 d to obtain MWCNT/PEI conjugates. Finally, the DMSO and the excess of reactants and byproduct were removed from the reaction mixture by extensive dialysis against phosphate buffered saline (PBS) solution (3 times, 2 L) and water (5 times, 2 L) using a dialysis membrane with MWCO of 50,000 for 3 d. The MWCNT/PEI conjugates were obtained following lyophilization.

Multifunctional MWCNTs (MWCNT/PEI–FI–HA) were synthesized by conjugating FI and HA onto the surface of MWCNT/PEI. Firstly, MWCNT/PEI (140.0 mg) was dispersed into DMSO (50 mL). Then, FI (4.85 mg) dissolved into DMSO (4 mL) was added into the DMSO solution of MWCNTs under vigorous magnetic stirring. The reaction was continued for 12 h to get the raw product of MWCNT/PEI–FI. Then, HA (120.0 mg) dissolved in DMSO (10 mL) was activated by EDC (120.0 mg in 5.0 mL DMSO) for 3 h and was added dropwise into the DMSO solution of MWCNT/PEI–FI under vigorous magnetic stirring. The reaction was continued for 48 h to obtain the raw product of MWCNT/PEI–FI–HA conjugates. Then, the MWCNT/PEI–FI–HA conjugates were purified and lyophilized according to the procedure used for the preparation of MWCNT/PEI conjugates.

### 2.3. Characterization techniques

$^1$H NMR spectra of functionalized MWCNTs were recorded using a Bruker DRX 400 nuclear magnetic resonance spectrometer. Samples were dispersed in $D_2O$ before measurements. UV–Vis spectra were collected on a PerkinElmer Lambda 25 UV–Vis spectrophotometer. Samples were dissolved in water before the experiments. Thermogravimetric analysis (TGA) was performed using a TG 209 F1 (NETZSCH Instruments Co., Ltd, Germany) thermogravimetric analyzer with a heating rate of 20 °C/min and a temperature range of 30–900 °C in air. Zeta potential measurements were carried out using a Zetasizer Nano ZS system (Malvern, UK) equipped with a standard 633 nm laser. The morphology of the MWCNTs was observed by transmission electron microscopy (TEM) using a JEOL 2010F analytical electron microscope (JEOL, Japan) operating at 200 kV. A 5 μL aqueous solution of samples (3 mg/mL) was dropped onto a carbon-coated copper grid and air dried before TEM analysis.

### 2.4. Loading of DOX onto MWCNT/PEI–FI–HA conjugates

The loading of DOX onto the multifunctional MWCNTs was carried out under the optimal conditions as described in our previous work.[33] Briefly, MWCNT/PEI–FI–HA dispersed in water (2 mg/mL, 5 mL) was mixed with an aqueous DOX solution (0.8 mg/mL, 5 mL) under magnetic stirring. The mixture was adjusted to have a pH value of 8.0. The mixture was stirred for 48 h at room temperature in the dark. The formed MWCNT/PEI–FI–HA/DOX complexes were purified by repeated centrifugation (15,000 rpm, 5 min) and redispersion in water having a pH value of 8.0 until the supernatant became colorless. The DOX payload was calculated according to the method described in our previous work.[33] The final purified MWCNT/DOX complexes were lyophilized and stored at −20 °C in the dark before use.

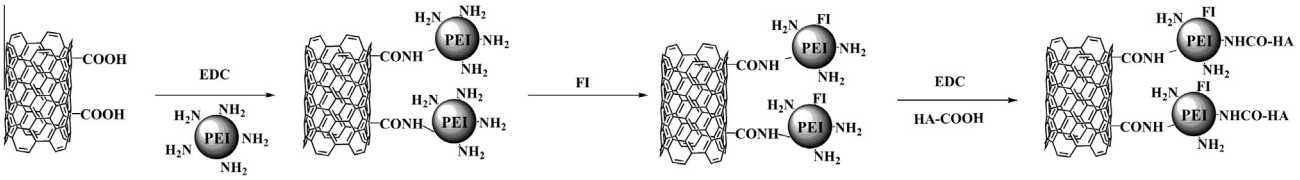


**Figure 1.** Schematic illustration of the synthesis of multifunctional MWCNTs.