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A comparative study on non-covalent functionalization of carbon nanotubes by chitosan and its derivatives for delivery of doxorubicin

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ABSTRACT

Three targeting drug delivery systems were formulated by functionalization of single-walled carbon nanotubes using chitosan and its derivatives (Palmitoyl Chitosan and Carboxymethyl Chitosan) for delivery of doxorubicin, an anti-cancer drug. Loading efficiency was higher than 75% for all carriers. The systems were stable under neutral pH, while effectively released drug at reduced pH. The drug loading efficiency and the release rate were revealed to be dependent on the type of applied polymer and could be adjusted to a desired rate by changing the hydrophobic/hydrophilic substitution degree. Folic acid was attached and cytotoxicity of system was compared with free drug.

1. Introduction

Cancer, known as one of the top three killers in the world, acutely threatens human's health and causes many problems for families and societies. A variety of cancer treatments were applied to date, mostly include surgery, chemotherapy, radiotherapy, thermotherapy, gene therapy and immunotherapy [1]. The effectiveness of chemotherapy is limited because almost all anticancer drugs cause severe side effects resulted from their toxicities, low selectivity and low aqueous solubility [2]. Because of which, they present small therapeutic indices, limited half time circulation, and fast clearance by the reticuloendothelial system [3,4].

A smart and efficient drug delivery system (DDS) should be biocompatible, biodegradable, non-toxic, and present targeting delivery capacity with high drug-loading capability and a convenient controlled release profile. In recent decades, a number of nanoscale drug delivery carriers such as liposomes [5,6], peptides [7,8], lipids [9,10], polymeric nanoparticles [11,12], inorganic nanoparticles [13] and nanotubes [14–16] have been introduced. Among them, the single-walled carbon nanotubes (SWNT) have appeared to be encouraging candidates for anti-cancer drug delivery systems, since they provide potential advantages such as have a higher surface area (1300 m^2/gr) allowing for higher drug loading and possibility for accompanying additional therapeutic ligands through surface functionalization [17]. In addition, their inherent stability and architectural flexibility lead to prolonged circulation time; hence improving bioavailability of the drug molecule [18,19].

However, DDSs based on SWNTs have still critical challenges. SWNTs are potentially toxic and extremely hydrophobic. Furthermore, they tend to form bundles that disperse poorly in aqueous solutions. These drawbacks may be conquered by functionalization of carbon nanotubes by covalent/non-covalent interactions. Surfactants [20], peptides and polymers such as poly ethylene glycol [21,22], amphiphilic phospholipids (PL-PEG) [23], poly acrylic acid and polysaccharides [24] have been used to modify SWNTs *via* non-covalent interactions in order to improve their compatibility and cellular uptake, to decrease their hydrophobicity, and to make them non-toxic[25,26].

Chitosan (CS) is a non-toxic, biodegradable, biocompatible and cationic natural polymer that can enhance water solubility and biological compatibility of carbon nanotubes [27]. Chitosan has been used for modification of carbon nanotubes by some researchers [18,28]; but existence of amine groups in its structure endow positive potential surface to the carrier which can restrict cationic drug adsorption such as doxorubicin due to the repulsive forces. Therefore, drug loading efficiency will decrease in drug delivery systems involving chitosan. *O*-carboxymethylchitosan (CMCS) is one of hydroplilic derivatives of chitosan that shows good biocompatibility and antibacterial activity [29]. CMCS acts as a week poly anion in solution, thus regarded as an excellent candidate for

Abbreviations: FA, Folic Acid; CMCS, O-carboxymethylchitosan; CS, Chitosan; DDS, Drug Delivery System; DOX, Doxorubicin; NPCS, N -palmitoyl chitosan; Ox-SWNTs, Oxidized Single Walled Carbon Nanotubes; PBS, Phosphate Buffer Saline; SWNTs, Single Walled Carbon Nanotubes.

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interaction with cationic drugs such as doxorubicin. Hydrophobically modified chitosan (*N* -palmitoyl chitosan, NPCS) is another chitosan derivative that has amphiphilic structure and presents proper drug release properties [30,31]. Moreover, changing the hydrophobic/hydrophilic substitution degree ratios of applied polymer can optimize the ability of DDs to release drug in a controlled manner and desired rate.

Doxorubicin (Supplementary Fig. S1,a) is a member of the anthracycline class of chemotherapeutic agents that is widely applied in many cancer treatments such as breast, ovarian, prostate, bladder and lymph nodes tumors [32]. However, doxorubicin has a high-level toxicity such as gastrointestinal toxicity and cardio toxicity [33]. Doxorubicin (DOX) loading on carbon nanotubes not only could decrease the drug toxicity but also increase its therapeutic indices. Folic acid (FA) (Supplementary Fig. S1, b) has a high affinity to the folate receptor protein, which is mostly over expressed on the surface of many human cancer cells and has been widely used for targeting cancer cells [34–36].

In this study, for the first time, to the best of our knowledge, palmitoyl chitosan and carboxymethyl chitosan have been applied to functionalization of carbon nanotubes and their performances were compared with chitosan. Then, doxorubicin was loaded on the modified SWNTs, and finally, folic acid was attached to the carrier surface by covalent interaction to improve the carrier targeting capabilities. The release profile of studied delivery systems were obtained at pH 7.4 (serum pH) and pH 5.5 (tumor pH) and the therapeutic efficacy of the drug delivery system was determined by *in vitro* cell viability assays.

2. Materials and methods

2.1. MATERIALS

Single-walled carbon nanotubes (SWNTs) were purchased from Neutrino Co (Tehran, Iran). (Purity >99%, length >5 μm, diameter 1–2 nm, surface area >380 m^2/g). The sample was cut and purified *via* oxidative acid treatment using the procedure documented in the literature [37]. Briefly, SWNTs were purified by reflux in a mixture of concentrated nitric and sulfuric acid (2: 3 (v/v)) at 50 °C for 24 h. The resultant oxidized SWNTs (ox-SWNT) were washed by ultra-distillated water followed by centrifugation (13000 rpm, 15 min) for several times to obtain neutral pH. Finally, samples were dried at 60 °C over night, and then characterized by AFM and FTIR spectroscopy.

Low molecular weight chitosan (~50 kDa) with 75–85% degree of deacetylation, folic acid, ethanol (≥ 99.5%), *N, N′*-dicyclohexyl carbodiimide (DCC), dimethylsulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Monochloroacetic acid and *N*-Succinimidyl palmitate were obtained from Sigma-Aldrich (UK). Doxorubicin hydrochloride from Sterling Biotech Ltd., India was obtained from Red Cross, Iran.

Dialysis tubing's membrane (12 kDa) was purchased from Spectrum laboratories. Fetal bovine serum (FBS), RPMI 1640 and penicillin/streptomycin were obtained from Gibco Inc. Human cervical cancer HeLa cells were purchased from the Pasteur Institute of Iran, Tehran, Iran. Analytical reagent grade salts and chemicals were used without further purification. HPLC grade (> 99%) organic solvents were used in this work.

Atomic force microscopy (AFM) measurements were performed using a PicoPlus instrument (Agilent Technologies, Chandler, AZ, USA,), and were used for size measuring of oxidized nanotubes. High-resolution electron microscopy (HR-TEM) was conducted on a CM 30 (Philips) operates at 200KV. UV–visible spectroscopy (UV-2501, Shimadzu, Japan) was used for doxorubicin concentration measurement, and Fourier transform infrared (FTIR) (Perkin–Elmer, Spectrum65) was used for characterization of functional groups. Infrared (IR) spectra of the samples were scanned in the range from 400 to 4000 cm^{-1} and obtained at a resolution of 4 cm^{-1}. Zeta potentials were measured on a zeta potential analyzer (Zetasizer Nano ZS (UK), Malvern) used to characterize the polymer-wrapped carbon nanotubes.

2.2. POLYMER WRAPPING CARBON NANOTUBES

In this study, three kinds of biopolymers: CS (Supplementary Fig. S2,a), CMCS(Supplementary Fig. S2,b) and NPCS(Supplementary Fig. S2,c) were used to modify ox-SWNT through surface wrapping. First *O*-carboxymethyl chitosan (CMCS) was synthesized based on Poon and colleagues method [38] with the same modification and *N*-palmitoyl chitosan (NPCS) was synthesized based on Chiu and colleague's method [39]. Then, briefly, ox-SWNT (50 mg) and biopolymer (100 mg) were suspended in PBS (pH 7.4, 100 ml); and sonicated for 1 h. The mixture was stirred at room temperature for 16 h. The polymer-wrapped ox-SWNTs were filtered and rinsed with distilled water to remove the unbound polymers; then, the solid was freeze dried. The products were named according to the corresponding polymer as CS-SWNT, CMCS-SWNT, and NPCS-SWNT, respectively.

2.3. FOLATE TARGETING

Folic acid (FA) as a targeting agent can selectively bind to folate receptors on various cancer cells [40]. To this end, Folic acid was conjugated to carrier to make it targetable to cancer cells. Folic acid (6 mg) was stirred with a mixture of 10 mg DDC and 10 ml DMSO for 1 h at room temperature. Then, NPCS-SWNTs (3 mg) were added to the mixture and stirred for 16 h at room temperature in darkness. The unreacted folic acid was removed by filtration and washing with distilled water. Finally, the resultant solid was freeze dried and named as FA-NPCS-SWNT.

2.4. DRUG LOADING

Doxorubicin loading onto carriers (CS-SWNT, CMCS-SWNT and NPCS-SWNT) was conducted by mixing 10 mg doxorubicin hydrochloride with the carrier (4 mg) and submerge both components in 50 ml PBS (pH 8) followed by stirring for 16 h at 4 °C in darkness. Doxorubicin degradation is significantly enhanced by light and temperature; also, its activity is lost at pH > 8 [41]. Therefore, drug loading experiments should be operated at low temperatures, PBS (pH 8) and in darkness to maintain drug activity.

The suspension was centrifuged for 30 min and 13000 rpm, filtrated, and washed thoroughly with PBS (over 10 times) until the filtrate became free of reddish color (corresponding to free DOX). Finally, the products (denoted as DOX/ox-SWNTs, DOX/CS-SWNTs, DOX/CMCS-SWNTs and DOX/NPCS-SWNTs) were collected and freeze dried.

The amount of unbound doxorubicin in the filtrate was measured by UV–vis absorption spectroscopy at 485 nm (the characteristic absorbance of DOX) with respect to the calibration curve accomplished under the same conditions. The spectroscopy results were used to calculate drug loading efficiency and drug loaded/carrier (gr/gr) ratio according to the following equation:

$$\text{Drug loading efficiency}(\%) = 100 \times \frac{w_{\text{feed DOX}} - w_{\text{free DOX}}}{w_{\text{feed DOX}}} \quad (1)$$

$$\text{Drug loaded/carrier(gr/gr)ratio} = \frac{w_{\text{feed DOX}} - w_{\text{free DOX}}}{w_{\text{SWNTs}}} \quad (2)$$

where $W_{\text{feed DOX}}$, $W_{\text{free DOX}}$, and W_{SWNTs} are the total amount of DOX, the amount of unbound DOX in the filtrate, and the amount of carrier, respectively.