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Carboxymethyl chitosan-mediated synthesis of hyaluronic acid-targeted graphene oxide for cancer drug delivery

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ABSTRACT

In order to enhance the efficiency and specificity of anticancer drug delivery and realize intelligently controlled release, a new drug carrier was developed. Graphene oxide (GO) was first modified with carboxymethyl chitosan (CMC), followed by conjugation of hyaluronic acid (HA) and fluorescein isothiocyanate (FI). The resulting GO–CMC–FI–HA conjugate was characterized and used as a carrier to encapsulate the anticancer drug doxorubicin (DOX) to study *in vitro* release behavior. The drug loading capacity is as high as 95% and the drug release rate under tumor cell microenvironment of pH 5.8 is significantly higher than that under physiological conditions of pH 7.4. Cell uptake studies show that the GO–CMC–FI–HA/DOX complex can specifically target cancer cells, which are over-expressing CD_{44} receptors and effectively inhibit their growth. The above results suggest that the functionalized graphene-based material has potential applications for targeted delivery and controlled release of anticancer drugs.

1. Introduction

To circumvent the drawbacks associated with anticancer drugs in chemotherapy, many novel drug carriers have been reported in the biomedical field (Kim, Kabanov, & Bronich, 2009; Litzinger & Huang, 1992; Peer et al., 2007; Yih & Al-Fandi, 2006). Although such modified carriers have shown numerous advantages, such as drug solubilization and prolonged blood circulation (Moghimi, Hunter, & Murray, 2001; Nagahama, Mori, Ohya, & Ouchi, 2007; Yallapu, Jaggi, & Chauhan, 2011), their efficacy is largely constrained by their lack of ability for targeted delivery (Abeylath, Ganta, Iyer, & Amiji, 2011; Adair, Parette, Altınoglu, & Kester, 2010; Cho & Kwon, 2011) and controlled drug release (Salim, Minamikawa, Sugimura, & Hashim, 2014; Uhrich, Cannizzaro, Langer, & Shakesheff, 1999). Moreover, insufficient cell uptake further decreases the therapeutic efficacy (Yang et al., 2011) and nonspecific accumulation in normal tissues leads to serious side effects and thus limits their clinical use (Erttmann, Erb, Steinhoff, & Landbeck, 1988; Kalivendi et al., 2005).

Graphene oxide (GO) has drawn considerable attention as a potential drug carrier because of its 2D plate-like structure, which provides a large surface area on both sides of the sheet for the physical adsorption of nucleobases and aromatic compounds, mainly through π–π stacking (Wang et al., 2009; Yang et al., 2008). Moreover, any functional group attached to the GO such as epoxy, hydroxyl, or carboxylic acid moieties, facilitates easy modification with biomolecules that could improve the stability, solubility, biocompatibility and, more importantly, interactions with target molecules (Dreyer, Park, Bielawski, & Ruoff, 2010; Rao, Biswas, Subrahmanyam, & Govindaraj, 2009). In addition, its effective transportation capability (Sun, Liu, et al., 2008), enhanced cellular uptake (Kam, Liu, & Dai, 2006; Lu et al., 2010; Wang, Li, et al., 2010) and lack of obvious toxicity (Liu, Robinson, Sun, & Dai, 2008) make it a promising material for drug carrier substances (Yang et al., 2008).

Carboxymethyl chitosan (CMC) is a biocompatible and biodegradable derivative of chitosan, but compared with chitosan, CMC has better water solubility and can be applied in more biomedical systems (Dong et al., 2010; Wang, Chen, et al., 2010). Moreover, with a flexible molecular backbone, some free carboxylic groups and unsubstituted amino groups on the CMC molecular chains cross-linked with other residual groups can act as linkers between the graphene oxide sheets and bioactive molecules. Thus the CMC-functionalized graphene oxide (CMC–GO) composite can acquire good aqueous dispersibility which is desirable for biomedical applications (Bao et al., 2011; Yang, Cao, Li, Rana, & Zhu, 2013).

For targeted drug delivery applications, hyaluronic acid (HA) was chosen as a functional group as it has a strong binding affinity to cell-specific surface markers such as cluster determinant 44 (CD_{44}) receptors, which are over-expressed on the surface

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of several different cancer cells (Aruffo, Stamenkovic, Melnick, Underhill, & Seed, 1990; Li, Bae, & Na, 2010). Besides, as a linear polysaccharide consisting of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine (Bae, Yoon, & Park, 2006; Jiang et al., 2008), HA also has unique and excellent physicochemical properties, such as biodegradability, biocompatibility and non-immunogenicity (Lapčík, Lapcik, De Smedt, Demeester, & Chabrecek, 1998; Necas, Bartosikova, Brauner, & Kolar, 2008), which all confer excellent application potential in cancer diagnosis and therapy.

In this study, a new anticancer drug carrier system with the abilities of targeted delivery and controlled release was developed. GO was firstly prepared *via* an improved Hummers' method (Becerril et al., 2008) and then was modified with CMC, followed by sequential modification with a tracing ligand FI and a targeting ligand HA. The resulting GO–CMC–FI–HA conjugate was characterized by FTIR, TEM and Zeta potential measurements. DOX, as an anti-tumor drug, was then loaded onto the surface of this conjugate *via* π–π stacking, and *in vitro* release behavior at different pH conditions was monitored *via* UV–vis spectrometry. Cell culture experiments were conducted to evaluate the potential of the GO–CMC–FI–HA/DOX complex as a targeting delivery system that can transport DOX to tumor cells which are over-expressing CD_{44} receptors effectively.

2. Experimental

2.1. Materials

Graphite, with an average particle diameter of 30 nm, was purchased from Nanjing Xianfeng Nanomaterials Co. Ltd. (Nanjing, China). Carboxymethyl chitosan (CMC) and hyaluronic acid (HA) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and used without further purification. N-hydroxysuccinimide (NHS), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), doxorubicin (DOX), fluorescein isothiocyanate (FI) 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 obtained from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). DMEM medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology Co. Ltd. (Hangzhou, China). Dialysis membranes (diameter = 34 mm), which had molecular weight cut-offs of 8000–14,000 and 100,000 respectively were purchased from Shanghai Yuanye Biological Technology Co. Ltd. (Shanghai, China). All the other reagents were analytical grade.

2.2. Synthesis of the GO–CMC–FI–HA conjugate

Graphene oxide (GO) was prepared from purified natural graphite according to a modified Hummers' method (Becerril et al., 2008). In detail, graphite powder (1 g), $NaNO_3$ (0.5 g) and $KMnO_4$ (2 g) were vigorously stirred in concentrated H_2SO_4 (75 mL) at room temperature for 7 days. On completion of the reaction, 5% H_2SO_4 (200 mL) aqueous solution was added and the temperature was kept at 98 °C for 2 h. Then the temperature was reduced to 60 °C, H_2O_2 (30%, 6 mL) was added and the reaction was further stirred for 2 h. The above mixture was centrifuged and the lower layer was removed and sequentially washed with 5% H_2SO_4/0.5% H_2O_2 (15 times), 5% HCl solution (5 times), and then washed repeatedly with distilled water until the pH of the supernatant was neutral. Finally the material was dried with P_2O_5 in a desiccator to obtain a loose brown powder.

CMC, as a mediator, was firstly modified onto GO to form GO–CMC. A typical procedure was as follows: GO (50 mg) was first sonicated in water (40 mL) for 30 min to be dispersed sufficiently. EDC (50 mg) and NHS (25 mg) co-dissolved in water (10 mL) were added into the GO dispersion under magnetic stirring to activate the carboxyl residues of the GO. After activation for 3 h, CMC aqueous solution (50 mL, 1 mg/mL) was added drop wise, and the mixture was stirred at room temperature for a further 24 h, followed by extensive dialysis to remove the excess of reactants and byproducts. The procedure consisted of sequential dialysis 3 times with phosphate buffered saline (PBS) solution (2 L) and 6 times with water (2 L) using a dialysis membrane with MWCO of 100,000 for 3 d. Finally, GO–CMC conjugate was obtained following lyophilization.

The final material, GO–CMC–FI–HA was synthesized by sequentially conjugating FI and HA onto the surface of the intermediate product GO–CMC. Firstly, GO–CMC (50 mg) was dispersed sufficiently in 50 mL water by sonication, followed by the addition of a FI aqueous solution (5 mL, 1 mg/mL) under magnetic stirring. The reaction was continued for 4 h to get the raw product of GO–CMC–FI. At the same time, EDC (50 mg) and NHS (25 mg) was added to an HA aqueous solution (50 mL, 1 mg/mL) and stirred for 3 h to achieve activation. Then, the above GO–CMC–FI mixture was added drop wise into the activated HA aqueous solution, and the mixture was kept stirring at room temperature for 24 h. Finally, the GO–CMC–FI–HA conjugate was obtained after dialysis and lyophilization according to the procedure used for preparation of GO–CMC conjugate.

2.3. Characterization techniques

Fourier transform infrared (FTIR) analysis was carried out on a Nicolet-Nexus 670 spectrometer (Nicolet Instrument Corporation, Madison, WI, USA) over the range 4000–500 cm^{-1} and with a resolution of 2 cm^{-1}. Samples were prepared using the KBr disk method (2 mg sample in 200 mg KBr). Zeta potential was measured using a Zetasizer Nano ZS system (Malvern, UK) equipped with a standard 633 nm laser. Transmission electron microscopy (TEM, JEOL 2010F, Japan) operating at 200 kV. A was used to characterize the morphology of the GO–CMC–FI–HA conjugate. An aqueous solution of sample (3 mg/mL) was dropped onto a carbon-coated copper grid and air dried before TEM analysis.

2.4. Loading of DOX onto GO–CMC–FI–HA

GO–CMC–FI–HA (20 mg) dispersed in water (10 mL) was first sonicated with an aqueous solution of DOX (20 mL; 2 mg/mL) for a few minutes to mix sufficiently and then treated with sodium hydroxide to adjust to pH 9.0. The mixture was protected from light and was stirred for 48 h at room temperature, followed by extensive centrifugation (12,000 rpm, 30 min) to remove the unconnected DOX. The resulting precipitate was lyophilized to afford the GO–CMC–FI–HA/DOX drug complex. The extent of DOX loading onto GO–CMC–FI–HA/DOX was determined with a UV spectrophotometer by measuring the concentration of the loss of DOX in the upper layer using a standard DOX concentration curve generated at the wavelength of 481 nm from a series of DOX solutions with different concentrations.

2.5. Release of DOX from GO–CMC–FI–HA/DOX complex

The release behavior of DOX from GO–CMC–FI–HA/DOX was evaluated at two different pH values by dialysis. The GO–CMC–FI–HA/DOX complex (1.0 mg) dispersed in PBS (1.0 mL, pH 7.4) or acetate buffer (1.0 mL, pH 5.8) was placed in a dialysis bag, with MWCO of 14,000, which was then immersed in 50 mL