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Lactobionic acid and carboxymethyl chitosan functionalized graphene oxide nanocomposites as targeted anticancer drug delivery systems

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ABSTRACT

In this work, we report a targeted drug delivery system built by functionalizing graphene oxide (GO) with carboxymethyl chitosan (CMC), fluorescein isothiocyanate and lactobionic acid (LA). Analogous systems without LA were prepared as controls. Doxorubicin (DOX) was loaded onto the composites through adsorption. The release behavior from both the LA-functionalized and the LA-free material is markedly pH sensitive. The modified GOs have high biocompatibility with the liver cancer cell line SMMC-7721, but can induce cell death after 24 h incubation if loaded with DOX. Tests with shorter (2 h) incubation times were undertaken to investigate the selectivity of the GO composites: under these conditions, neither DOX-loaded system was found to be toxic to the non-cancerous L929 cell line, but the LA-containing composite showed the ability to selectively induce cell death in cancerous (SMMC-7721) cells while the LA-free analogue was inactive here also. These findings show that the modified GO materials are strong potential candidates for targeted anticancer drug delivery systems.

1. Introduction

A potent drug delivery system (DDS) must both achieve targeted delivery and a controlled rate of drug release. Such a system not only improves therapeutic efficacy, but also minimizes associated side effects. A vast number of DDSs have been developed (Arora, Al, Ahuja, Khar, & Baboota, 2005; Allen & Cullis, 2013; Bae & Park, 2011; Bamrungsap et al., 2012; Bruschi, 2015; Debbage, 2009; Gong, Chen, Zheng, Wang, & Wang, 2012); in the last 10 years or so, carbon based nanostructures – most notably carbon nanotubes (CNTs) – have attracted particular attention in this regard (Bianco, Kostarelos, & Prato, 2005; Faria et al., 2014; Feazell, Nakayama-Ratchford, Dai, & Lippard, 2007; Liu, Chen et al., 2008; Liu, Robinson, Sun, & Dai et al., 2008; Meng, Zhang, Lu, Fei, & Dyson, 2012; Zhang, Zhang, & Zhang, 2011; Zhu et al., 2014). Graphene oxide (GO) has been less explored than CNTs but offers a number of potential advantages, including an ultrahigh surface area for physical adsorption of a drug (mainly through π-π stacking) (Liu, Chen et al., 2008; Liu, Robinson et al., 2008; Sun et al., 2008) and abundant oxygen-containing functional groups (carboxyl groups, hydroxyl groups and epoxy groups), which make it dispersible in aqueous media and impart it with the ability to be further modified with functional molecules (Bao et al., 2011; Ma et al., 2012).

The first report of GO used as a DDS came from Sun et al. in 2008 (Sun et al., 2008). These authors synthesized nanoscale GO sheets, developed functionalization chemistry to permit GO to remain soluble in physiological media, and also proved that doxorubicin, a potent anti-cancer drug, could be adsorbed to the sheets and delivered to cells in vitro. At around the same time, Yang and co-workers independently reported the adsorption and release of doxorubicin using GO (Yang et al., 2008). Since then, there has been an explosion of interest in using GO for drug delivery purposes. The key features of GO causing it to attract this attention are its effective transportation capability, high levels of cellular uptake, and lack of obvious toxicity (Lv et al., 2012; Zhang et al., 2015). These properties have caused GO to be investigated in particular for the targeted cellular delivery of anticancer drugs (Long et al., 2013; Tao et al., 2012; Zhang, Xia, Zhao, Liu, & Zhang, 2010).

In order to maximize its functionality as a biomaterial, GO is often modified in order to prevent aggregation and ensure compatibility with biological tissue. Polyethylene glycol (PEG) has been used to this end (Ma et al., 2012; Sun et al., 2008; Wen et al., 2012), but another option is the naturally occurring chitosan material. This has been widely used in the biomaterial field due to its biodegradability and non-toxicity (Agnihotri, Mallikarjuna, &

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Aminabhavi, 2004; Jayakumar, Prabaharan, Kumar, Nair, & Tamura, 2011; Shan et al., 2010; Roosen, Spooren, & Binnemans, 2014), but suffers from poor water solubility (Zheng et al., 2011). To overcome this problem, chitosan can be derivatized to give carboxymethyl chitosan (CMC), a material which has shown excellent biocompatibility (Luo, Teng, & Wang, 2012; Vaghani, Patel, & Satish, 2012). CMC, which contains a number of amino groups, can be used to modify GO to improve its dispersibility in water and physiological fluids through covalent functionalization (Zheng et al., 2011). Very recently, Yang et al. (2016) have employed CMC-modified GO to prepare doxorubicin-loaded hyaluronic acid-functionalized systems for targeted delivery to cancer cells. The materials produced had excellent dispersibility and biocompatibility, and displayed pH-sensitive drug release: release at the cancer microenvironment pH of 5.8 was faster and reached a higher percentage of the drug loading than at the general physiological pH of 7.4. Further, the composites were taken up effectively by cancerous HeLa cells, but not by non-cancerous L929 cells, and the latter were largely unaffected by being incubated with the GO composites while the HeLa cells were much reduced in their viability (Yang et al., 2016).

One way to achieve targeted drug delivery to a given cellular population is to exploit particular biological signatures of the cell type of interest. The high levels of expression of asialoglycoprotein receptors (ASGPRs) on the surface of hepatocytes (Ashwell & Harford, 1982) have been widely utilized in this regard. ASGPRs bind galactose moieties and thus lactobionic acid (LA), a disaccharide comprising gluconic acid and galactose, can be employed to develop liver-targeted DDSs. This technology can be coupled with pH-sensitive components to yield a system which can both target liver cells and also ensure that drug release only occurs in the acidic microenvironment typical of cancerous cells (Zhang, Meng, Lu, Fei, & Dyson, 2009). LA has been used to functionalize a number of different carriers such as magnetite nanoparticles (Song et al., 2015) and laponite (a synthetic clay) (Chen et al., 2015) for targeted drug delivery, or dendrimer-entrapped gold nanoparticles for computed tomography imaging applications (Cao, Tao, Wen, Hou, & Shi, 2015; Cao, He et al., 2015). However, the possibility of using functionalized GO to target hepatocytes via this route has not been explored.

In this study, a drug delivery system based on graphene oxide, carboxymethyl chitosan and lactobionic acid was developed and loaded with the anti-cancer drug doxorubicin. We hypothesized that the GO-CMC materials (both LA functionalized and LA free) could be effectively loaded with DOX, and would give more rapid release at the lower pH typical of the cancer cell microenvironment than at the general physiological pH. We further anticipated that functionalization of the GO-CMC composites with LA would permit their selective uptake by cancerous cells with only minimal uptake by non-cancerous cells, and thus that the LA-containing materials would be able to act as precisely targeted anti-cancer drug delivery systems.

2. Experimental

2.1. Materials and methods

Chemicals were procured as follows: graphite (power, 99.95% metals basis, 500 mesh; Nanjing Xiaofeng Nanomaterials Co. Ltd); carboxymethyl chitosan (CMC; viscosity average molecular weight 8.6×10^4 Da and DS of 0.9; Shanghai Shifeng Biological Technology Co. Ltd); lactobionic acid (LA; purity > 98%; J&K scientific Co. Ltd); doxorubicin (DOX; purity > 98%; J&K Scientific Co. Ltd); fluorescein isothiocyanate (FITC; Sigma-Aldrich); *N*-hydroxysuccinimide (NHS; analytical grade; Sigma-Aldrich); 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (EDC; analytical grade; J&K Scientific Co. Ltd); acetic anhydride (analytical grade; Sinopharm Chemical Reagent Co. Ltd); and triethylamine (analytical grade; Sinopharm Chemical Reagent Co. Ltd). Phosphate buffered saline (PBS; pH = 7.4) and acetate buffers (pH = 5.8) were prepared in-house.

SMMC-7721 cells and L929 cells were provided by the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), phosphate buffered saline (PBS), RPMI 1640 and DMEM media were supplied by the Shanghai Pumai Biotechnology Co. Ltd. Dialysis membranes (molecular weight cut-offs 8,000–14,000 or 100,000 Da) were also sourced from the Shanghai Pumai Biotechnology Co. Ltd.

2.2. Synthesis and purification of GO-CMC

Graphene oxide was synthesized by using an improved Hummers' method (Hummers & Offeman, 1958). GO-CMC (graphene oxide-carboxymethyl chitosan) was subsequently prepared by first dispersing GO (100 mg) in 25 ml distilled water, then adding NHS (45 mg) and EDC (35 mg) to activate the GO and stirring for 3 h at room temperature. A solution of CMC (200 mg in 20 ml distilled water) was subsequently gradually added to the GO dispersion and the resultant mixture stirred for a further 24 h at room temperature. The reaction product was purified to remove residual GO, CMC and byproducts through dialysis (molecular weight cut-off: 100,000 Da). Dialysis was performed in PBS (2 l) for 1 day then in distilled water (2 l) for 3 days, with the dialysis medium being changed three times per day. GO-CMC was finally obtained from lyophilization of the dialyzed material.

2.3. Synthesis and purification of GO-CMC-FI-LA-Ac

The modification of GO-CMC with FITC and LA to form GO-CMC-FI-LA was undertaken by sequential conjugation. 1 mg FITC was added to an aqueous solution of GO-CMC (25 ml; 2 mg/ml) and stirred for 2 h to obtain GO-CMC-FI. Separately, LA (10 mg) was dissolved in 10 ml of PBS with 25 mg EDC and 20 mg NHS added. This solution was stirred for 3 h at room temperature.

Next, the LA solution was added drop-wise to the GO-CMC-FI solution, and the mixture stirred at room temperature for 24 h to yield GO-CMC-FI-LA. To eliminate any residual amino groups in CMC, 280 μl triethylamine and 160 μl acetic anhydride were added to the mixture and reaction continued for an additional 24 h. The GO-CMC-FI-LA-Ac product was collected following dialysis (molecular weight cut-off: 8000–14,000 Da) and lyophilization as described in Section 2.2 above.

2.4. Synthesis and purification of GO-CMC-FI-Ac

GO-CMC (50 mg) was dispersed in 25 ml distilled water, and 1 mg FITC added. The resultant solution was allowed to stir for 2 h, followed by acetylation for 24 h as described in Section 2.3. GO-CMC-FI-Ac was obtained after dialysis (molecular weight cut-off: 8000–14,000 Da) and lyophilization following the same procedures as described previously.

2.5. Characterization

^{1}H NMR spectra were obtained using a Bruker DRX 400 nuclear magnetic resonance spectrometer. Samples of GO, GO-CMC, LA, GO-CMC-FI-Ac, GO-CMC-FI-LA-Ac were dispersed in D_2O before spectra acquisition. Fourier transform infrared (FTIR) spectroscopy was carried out on a Nicolet-Nexus 670 spectrometer (Nicolet Instrument Corporation) over the range 4000–500 cm^{-1} and with a resolution of 2 cm^{-1}. Morphological examination of GO and the modified GO samples was performed using a JEOL 2010 F