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Research paper

Preparation and characterization of an amylase-triggered dextrin-linked graphene oxide anticancer drug nanocarrier and its vascular permeability

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ABSTRACT

We synthesized a dextrin (DEX)-conjugated graphene oxide (GO) nanocarrier (GO_{100}-DEX) as a potential drug delivery system to respond to a tumor-associated stimulus, α-amylase, that has high permeability through the fenestrated endothelial barrier to the tumor site. At acidic pH and in the presence of α-amylase to simulate tumor conditions, GO_{100}-DEX released a 1.5-fold higher amount of doxorubicin (DOX) than of GO_{100}. Under the same conditions, the cytotoxic effects of GO_{100}-DEX/DOX were 2-fold greater than those of free DOX and 2.9-fold greater than those of GO_{100}/DOX. Employing an *in vitro* biomimetic microfluidic blood vessel model lined with human umbilical vein endothelial cells, we evaluated the tumor vasculature endothelial permeation of GO_{100}-DEX and GO_{100} using dextrans of 10 and 70 kDa for comparison and as standards to validate the microfluidic blood vessel model. The results showed that the permeabilities of GO_{100}-DEX and GO_{100} were 4.3- and 4.9-fold greater than that of 70 kDa dextran and 2.7- and 3.1-fold higher than that of 10 kDa dextran, thus demonstrating the good permeability of the GO-based nanocarrier through the fenestrated endothelial barrier.

1. Introduction

Nano-sized drug delivery carriers, including polymers, micelles and liposomes, have been extensively explored as nanocarriers to improve the selectivity and targeting of small drug molecules for tumor tissues over healthy tissues (Blum et al., 2015), and some have advanced into clinical stages (Pillai, 2014). Recently, a carbon-based nanomaterial, graphene oxide (GO), has sparked growing interest in the biomedical field owing to its two-dimensional structure, which provides an extremely large surface area (2600 m^2/g) and a high drug loading capacity, which is usually above 100 wt% (Siriviriyanun et al., 2015) and is far greater than the loading values of most nanomaterials (Kim et al., 2010; Sun et al., 2008a). GO contains hydrophobic graphenic domains for interacting with water-insoluble drug molecules and hydrophilic edges anchored with carboxyl groups, which give a wide range of chemical functionalization opportunities and good water dispersibility (Kiew et al., 2016).

To improve tumor targeting and selectivity and to reduce the premature leakage of the drug from the nanocarrier, GO-based nanocarriers have been studied, with the release of their drug cargos being activated by stimuli such as near-infrared light, pH and electricity (He et al., 2014; Kurapati and Raichur, 2013; Weaver et al., 2014). In this study, we seek to develop a GO-based nanocarrier that selectively unloads drugs to tumors with high expression levels of α-amylase (Casadei Gardini et al., 2016; Kawakita et al., 2012; Minami et al., 2014; Shingu et al., 2013; Yanagitani et al., 2007). According to Takeuchi et al. (1981) ovary and lung adenocarcinoma and thymoma show 6.8–15 U/g α-amylase activity, whereas normal tissues have less than 1 U/g of tissue (Lenler-Petersen et al., 1994; Takeuchi et al., 1981). The reason for the overexpression of α-amylase by these tumors remains elusive, but it is conceivable that such overexpression might play a role in the acquisition of nutrients and energy for rapidly growing cancer cells. α-amylase catalyzes polysaccharides into smaller disaccharides optimally at pH 6.7–7.0. Thus, it is expected to function in tumors because the extracellular pH of tumor tissue is between pH 5.8 and 7.6 (Tannock, 1998).

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Employing α-amylase as the tumor-associated stimuli, we conjugated GO (average diameter = 100 nm, GO_{100}) with dextrin (DEX), a long chain α-1,4-poly(glucose) polymer that is readily degraded by α-amylase, to yield GO_{100}-DEX as a stimulus-responsive nanocarrier. The clinical safety of DEX has been well documented. It has been used as a peritoneal dialysis solution, and it has been formulated with 5-fluorouracil for peritoneal administration in cancer treatment (Kerr et al., 1996). We hypothesized that the GO_{100}-DEX nanocarrier effectively cages its drug payload within its nanostructure *via* π-π interactions with the GO surface and physical trapping by the DEX chains. Upon the arrival of the drug-loaded GO_{100}-DEX at the tumor site, α-amylase in the tumor interstitium degrades the DEX coating of the GO_{100}-DEX, and the trapped drug molecules are released into the tumor microenvironment.

Similar to other nanocarriers, drug delivery by GO_{100}-DEX relies on the enhanced permeability and retention effect as a means to passively accumulate at the tumor site. Importantly, for accumulation to occur, the nanocarrier must first be able to permeate through the fenestrated vascular endothelial barrier. To study this, we employed a previously established microfluidic device lined with an endothelial cell monolayer that can approximate cancer endothelial permeability (Ho et al., 2017) to evaluate the permeability of GO_{100}-DEX through the endothelial barrier. Our findings suggest that permeability through the vascular endothelial barrier depends on both the size and the shape of the nanocarriers. The 100 nm GO_{100} permeated better than the 130 nm GO_{100}-DEX did, and the permeability of these non-spherical GO-based nanocarriers was at least 4.3-fold higher than that of the spherical 70 kDa dextran (Blanco et al., 2015).

This paper is the first study to explore the possibility of using DEX as a surface coating of a GO nanocarrier. DEX acts as the key component in fabricating the GO into a nanocarrier responding to the tumor-associated stimulus α-amylase. This is also the first study to investigate the permeability of GO-based nanocarriers through the vascular endothelial barrier by using a biomimetic microfluidic device to ensure the efficient targeting of this potential nanocarrier to the tumor site.

2. Materials and methods

2.1. Materials and chemicals

Aqueous dispersion of graphene oxide (GO) (4 mg/mL), doxorubicin (DOX), dextrin (DEX, corn, type 1), α-amylase (porcine pancreases, Type VI-B), *N*-(3-dimethylamiopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), fibrinogen, thrombin and fibronectin were purchased from Sigma-Aldrich, USA. Hydrochloric acid (37%) and sodium hydroxide were from Fisher Chemical, UK. Sodium bicarbonate ($NaHCO_3$) and acetonitrile (ACN) were supplied by Merck, Germany and ACROS, USA, respectively. Pullulan standards (MW, 6 & 12 kDa) for gel permeation chromatography (GPC) analysis were purchased from Fluka, Germany. Dialysis tubing (MWCO 3500 Da) were from Fisher Scientific, USA. Fetal bovine serum (FBS) and RPMI medium 1640 (1×) supplemented with L-glutamine were purchased from GIBCO, Brazil. Endothelial growth medium (EGM-2) and human umbilical vein endothelial cells (HUVECs) were supplied by Lonza, Switzerland. Oregon Green 488-tagged 10 kDa and Texas Red-tagged 70 kDa dextrans were purchased from Life Technologies, USA, and rhodamine and sulfo-*N*-hydroxysulfosuccinimide (NHS) were from Thermo Fisher Scientific, USA. 4T1 (murine breast carcinoma) cells were supplied by ATCC, USA. Sterilized ultrapure water with a resistivity of 18.2 MΩ.cm (ELGA, UK) was used for all synthesis, characterization and evaluation.

2.2. Preparation of GO_{100} nanocarrier

To prepare GO_{100} (100 nm), 5 μm GO sheets were fractured *via* ultrasonication for several hours using an ultrasonic processor equipped with a standard probe with a tip diameter of 19 mm (Sonics & Materials, VCX 400), with the amplitude intensity set at 25% and power at 40 W (Siriviriyanun et al., 2015). Throughout the ultrasonication process, the sample was kept in an ice-bath for heat dissipation. The sonicated GO was then filtered through a cellulose acetate membrane with a pore size of 0.2 μm to remove oversized GO sheets. A smaller pore size (*e.g.*, 0.1 μm) was not used due to clogging and excessive sample loss. The size of the filtered GO dispersion was determined using a Malvern Zetasizer (Nano ZSP, Worcestershire, UK). The ultrasonication and filtration processes were repeated until the size of the GO sheets was reduced to 100 nm.

2.3. Preparation of low molecular-weight dextrin

Low molecular weight DEX was prepared by a simple and facile method: filtration of the dextrin aqueous (8 mg/mL) solution through a series of cellulose acetate membranes with pore sizes of 0.2, 0.1 and 0.02 μm to gradually reduce the molecular weight. The molecular weight of the resultant DEX was characterized by gel permeation chromatography (GPC; Agilent GPC 1260 Infinity Multi-Detector Suite assembled with viscometer, refractive index, dual angle light scattering and various wavelength detectors, USA) equipped with 7.8 × 300 mm Ultrahydrogel™ Linear columns (Waters, Japan). Water was used as the eluent at a flow rate of 0.6 mL/min. Pullulans (MW, 6 and 12 kDa) were used as standard references because they have similar chemical structures and functional groups as DEX does.

2.4. Preparation of GO_{100}-DEX, GO_{100}/DOX and GO_{100}-DEX/DOX

Dextrin-conjugated graphene oxide (GO_{100}-DEX) was synthesized by a modified esterification process in water-containing solvent as shown in Fig. 1(a) (Wang et al., 2012), using EDC as the coupling agent to chemically conjugate the DEX to GO_{100}. The process began with 30 min of bath sonication of GO_{100} (400 μg/mL, 4 mL) and DEX (32 μg/mL, 4 mL) separately to ensure the even dispersion of the samples. The GO_{100} was vigorously stirred using a magnetic stirrer for 15 min before adding $NaHCO_3$ (0.4 g), ACN (200 μL, 0.2 M) and EDC (0.2 g). After another 15 min of stirring, DEX was added. The mixture was further stirred for 5.5 h at 0 °C to allow the esterification reaction to occur. At the end of the reaction, the mixture was centrifuged three times at 16000 × *g* with ultrapure water to remove the excess unreacted reagents. GO_{100}-DEX was collected and redispersed in ultrapure water, and the concentration was determined by the absorbance at 230 nm using a standard calibration curve plotted with absorbance of GO dispersions of known concentrations, 0–25 μg/mL. The presence of DEX did not affect the concentration determination of GO_{100}-DEX because its absorbance at this wavelength is negligible.

To load DOX onto GO_{100} and GO_{100}-DEX, GO_{100} (2 mL, 100 μg/mL) and GO_{100}-DEX (2 mL, equivalent to 100 μg/mL of GO) were separately mixed with an aqueous solution of DOX (2 mL, 200 μg/mL; Fig. 1(b)). Both single-sheet surfaces of the two-dimensional GO are accessible for drug interaction. Because a drug loading rate of 235 wt% has been reported for GO nanocarrier (Yang et al., 2008), 2 mL of DOX at a concentration of 200 μg/mL was loaded onto 2 mL of GO nanocarriers at a concentration of 100 μg/mL. The mixture was stirred for 24 h in the dark before being washed twice with ultrapure water by centrifugation at 16000 × *g* for 1 h to remove the unbound DOX. Finally, GO_{100}/DOX and GO_{100}-DEX/DOX dispersions were made by resuspension of the pellet.

The absorbance value of the unbound DOX remaining in the supernatant was measured using a UV–vis spectrophotometer at a wavelength of 480 nm. The DOX loading (% w/w) was calculated based on the formula below (Siriviriyanun et al., 2015):

$$\text{Drug loading (\%w/w)} = ((M_{DOX} - M_{DOX}^{*})/M_{GO}) \times 100$$

where M_{DOX} is the initial amount of DOX, M_{DOX}^{*} is the total amount of