Materials Science and Engineering C 75 (2017) 198–206

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Heparin modified graphene oxide for pH-sensitive sustained release of doxorubicin hydrochloride

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ARTICLE INFO

Article history:
Received 21 September 2016
Received in revised form 30 December 2016
Accepted 13 February 2017
Available online 15 February 2017

Keywords:
Graphene oxide
Heparin
pH-sensitive
Drug carrier
Cytotoxicity

ABSTRACT

A novel nanocarrier of heparin (Hep) modified graphene oxide (GO) was fabricated via a linker (adipic dihydrazide) and used as a pH-sensitive drug delivery system for controlling the release of anticancer drug doxorubicin (DOX) for anti-tumor therapy. The finally obtained nanocarrier was GO-ADH-Hep with better stability, blood compatibility and biocompatibility confirmed by the hemolytic test and *in vitro* cytotoxicity study. Its safety issue was greatly improved via Hep modification. The amount of DOX loaded onto GO-ADH-Hep was significantly high and dependent on pH value. The release rate of DOX from GO- ADH-Hep/DOX was pH-sensitive and much-slower than that of free DOX solution suggesting the sustained drug-release capacity of this prepared nanocomplexes. In addition, the results of cytotoxicity study illustrated that this fabricated nanocomplexes displayed effective cytotoxicity to MCF-7 and HepG2 cells. What's more, the results of the *in vivo* pharmacokinetic study was also indicated that the GO-ADH-Hep/DOX nanocomplexes could significantly prolong the retention time of DOX *in vivo* and this was consistent with the *in vitro* drug release performance. And finally, according to the biodistribution study, DOX delivered by GO-ADH-Hep could reduce cardiotoxicity deriving from DOX solution and also decrease the pulmonary toxicity deriving from unmodified GO. Based on the *in vitro* and *in vivo* investigations, the fabricated GO-ADH-Hep could be a promising candidate as an ideal nano-carrier for drug delivery and anti-cancer therapy.

1. Introduction

Cancer was traditionally treated by chemotherapy with limited efficacy and severe side effects due to the poor solubility, non-targeting ability and fast clearance of chemical drugs. To solve these issues, the construction of drug delivery system (DDS) is of vital importance [1,2].

Graphene oxide (GO), with excellent physico-chemical properties, was studied by an increasing number of people. Subsequently, various graphene-based nanomaterials were unceasingly emerging by utilizing non-covalent and covalent modifications of GO and used for cancer therapy [3], tissue engineering [4,5], photothermal therapy (PTT) [6–8], photodynamic therapy (PDT) [9–11] and so on. Compared to other nanoparticles, such as superparamagnetic iron oxide and superparamagnetic gadolinium ferrite nanoparticles [12–14], GO-based nanomaterials had a higher drug loading efficiency. However, the toxicity of GO-based derivatives wasn't completely settled. It was hoped that the toxicity was settled by modification methods. GO was stable in water for a long time but aggregated quickly in physiological environment. In order to improve its stability in physiological environment, modifications were critical.

Heparin (Hep) is one of glycosaminoglycans that naturally covers the surface of all eukaryotic cells and is reported to act as anticoagulant, preventing the formation of clots and extension of existing clots within the blood [15]. A large amount of research groups indicated that heparin-grafted materials exhibited excellent biocompatibility with reduced activation of complements, blood cells adhesion and coagulation formation [16–21].

Doxorubicin (DOX) is one of the widely used chemotherapeutic agents in clinical application currently due to its excellent anti-tumor efficiency against various solid tumors [22]. So, DOX was selected as a model drug. However, clinical application of DOX has been severely limited because the DOX is distributed throughout the body and can be harmful to healthy cells, such as the dose-dependent cardiotoxicity, myelosuppression, nephrotoxicity, and multidrug resistance [23,24]. It has been well documented that a targeted DDS is of vital importance to solve the above talked limitations. So a suitable drug delivery carrier was urgent to be constructed for DOX release.

In our study, a novel covalently modified GO nanocarrier was reported *via* a linker–adipic dihydrazide (ADH). Through the formation of amido bond, GO-ADH-Hep was fabricated. And then, its hemolytic characteristic and drug loading performance were studied. The amount of

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http://dx.doi.org/10.1016/j.msec.2017.02.048
0928-4931/

DOX loaded onto GO-ADH-Hep was significantly high (more than 60%) and dependent on pH value. The release rate was significantly slow with pH- sensitivity simultaneously. In addition, the results of *in vitro* cytotoxicity study illustrated this fabricated nanocomplexes exhibited effective cytotoxicity to MCF-7 and HepG2 cells. What's more, the *in vivo* pharmacokinetic study of GO-ADH-Hep/DOX nanocomplexes was also carried out in this paper and suggesting that the GO-ADH-Hep/DOX nanocomplexes could significantly prolong the retention time of DOX *in vivo*. Finally, based on the result of its biodistribution study, we can say this prepared nanocarrier-GO-ADH-Hep was beneficial to reduce cardiotoxicity deriving from DOX solution and also decrease the pulmonary toxicity from unmodified GO. So, according to the *in vitro* and *in vivo* investigations, the present work paved the way for expanding the application of graphene-based derivatives in biomedicine.

2. Experimental section

2.1. Materials

Nano graphite powder (99%, $D_{50} < 400$ nm,Lot: A1402053) was supplied from Aladdin Industrial Co., Ltd.; Concentrated sulfuric acid (H_2SO_4), potassium permanganate ($KMnO_4$), 30% hydrogen peroxide (H_2O_2), HCl and all other chemicals were purchased from Tianjin Chemical Reagent Co., Ltd. and of analytical reagent grade. Deionized (DI) water was used throughout the whole experiment. Heparin (~1.5 kDa) was supplied in our own laboratory. Mica was obtained from Beijing Zhongjingkeyi Technology Co., Ltd. and was cut into 9.9 mm of the diameter as substrate for the AFM film transfer. Dialysis tube was obtained from Beijing Solarbio Science & Technology Co., Ltd.

2.2. Preparation of GO

The GO nanosheets were prepared *via* a modified Hummers method [25]. In this method, 3 g of natural graphite powder was added into a solution consisting of 12 mL of concentrated sulfuric acid (97%), 2.5 g of potassium persulfate, and 2.5 g of phosphorus pentoxide at 80 °C. The mixture was kept at 80 °C for 6 h. Then, the mixture was diluted with 0.5 L deionized water and left to cool down to room temperature (23 °C) overnight. The mixture was filtered with a 0.2 μm nylon filter *via* vacuum filtration and washed with deionized water to remove the residual acid. The product was dried at room temperature overnight. This preoxidized graphite was then subjected to oxidation by Hummer's method. The preoxidized graphite powder (1 g) was placed in concentrated H_2SO_4 (23 mL) at 0 °C. $KMnO_4$ (3 g) was added gradually with stirring while keeping the temperature of the mixture below 20 °C. The mixture was then stirred at 35 °C for 2 h, followed by the addition of distilled water (46 mL), and stirring was continued for 15 min at ~100 °C. Distilled water (140 mL) and 30% H_2O_2 (2 mL) were then added to terminate the reaction. Subsequently, the color of the mixture changed to bright yellow. The mixture was centrifuged (13,000 r/min) and washed with 10% HCl solution to remove residual metal ions. The precipitate was then washed with distilled water and centrifuged (13,000 r/min) repeatedly until the solution became neutral. To exfoliate the oxidized graphite, the product was treated with an ultrasonic probe and then dialysis against water to remove the residual ions.

2.3. Synthesis of GO-COOH

Firstly, 200 mL GO (1 mg/mL) was treated with an ultrasonic probe for 0.5 h to fully exfoliate it. Then NaOH (20 g) and $ClCH_2COONa$ (20 g) were added to the GO suspension, followed by 2 h of bath sonication for converting the OH groups into COOH groups. GO-COOH, the resulting product, was neutralized with concentrated hydrochloric acid and purified by repeated rinsing and centrifugation until the product was well dispersed in deionized water. Then, the GO-COOH suspension was dialyzed against distilled water for more than 48 h to remove any ions.

2.4. Synthesis of GO-ADH-Hep

Adipic dihydrazide (ADH), as a linker, was firstly conjugated to heparin (Hep) *via* the formation of amido bonds [26]. In brief, 0.20 g of Hep (MW 15 KDa) was dissolved in water to give a concentration of 4 mg/mL and agitated for 0.5– 1 h. 0.048 g EDCI and 0.035 g HOBt were added into the above system. Subsequently, the mixture was agitated for 2 h to active the —COOH. And then 1.2 g ADH was added under agitation. The reaction was carried out for 24 h. The resulting solution was dialyzed (MWCO 3500) exhaustively against distilled water. Finally, the as-made product Hep-ADH was lyophilized.

According to the previous study [27], 10 mL GO-COOH (1 mg/mL) was treated with an ultrasonic probe for 0.5 h to fully exfoliate it. Subsequently, 10 mg EDCI was added and the mixture was sonicated for 5 min and agitated for another 30 min. And then, 100 mg Hep-ADH was added into this reaction system. This mixing system was maintained for 24 h under agitation. After 24 h, this system was dialyzed (MWCO 100 kDa) against distilled water. Finally, the as-made product GO-ADH-Hep was lyophilized.

2.5. Characterization

UV spectroscopy was determined by a UV–visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China). The topography and thickness of GO or GO-ADH-Hep was characterized by atomic force microscope (AFM, MultiMode IIId, Veeco Instruments Inc., Bruker AXC Inc., Madison, Wisconsin, USA) in non-contact mode. The AFM sample was prepared by dropping 5– 10 μL GO or GO-ADH-Hep (~10 μg/mL) solution onto Si substrate covered with SiO_2, and left them at room temperature for 1 h to make the samples adhere to the substrate surface. The samples were then dried in oven, and observed under AFM. The images were flattened and plain fitted using NanoScope analysis software (version 1.7, Bruker AXC Inc., Madison, Wisconsin, USA). Raman spectroscopy was obtained using a Horiba Jobin Yvon LabRAM HR-800 Raman microscope ($\lambda = 532$ nm). Fourier transform infrared (FTIR) transmittance spectroscopy was done by Nicolet 6700 Fourier transform infrared spectrometer (Thermo Nicolet Corp., Madison, WI, USA) and collected in the region of 400– 4000 cm^{-1} by OMNIC software (Thermo Nicolet Corporation, Madison, WI, USA) to confirm the presence of functional groups. The intermediate product's structure was characterized by ^1H NMR spectroscopy (AvanceTM DPX-300, Bruker BioSpin GmbH, Rheinstetten, Germany) using D_2O as the solvent.

2.6. Hemolytic toxicity of GO-ADH-Hep

The hemolysis of the GO-ADH-Hep was evaluated using 2% erythrocytes suspension according to previous studies [28]. The fresh blood samples were centrifuged and resuspended in normal saline (0.9 wt% NaCl solutions) to get 2% red blood cells (RBCs). Then, 1.2 mL RBCs suspension was dispersed in 1.2 mL normal saline solution as a negative control and dispersed in 1.2 mL distilled water as a positive control, respectively. In experimental groups, 1.2 mL of the erythrocyte suspension was mixed with GO-ADH-Hep at different concentrations, with the total volume of 2.4 mL. All the mixed suspensions were incubated at 37 °C for 2 h. Thereafter, the above suspension was centrifuged at 3000 rpm for 10 min to remove intact erythrocyte. The supernatant was collected and analyzed for the released hemoglobin with a UV-2450 spectrophotometer (Shimadzu) at 540 nm. All the samples were performed in triplicate.

The degree of hemolysis was calculated by the following equation:

$$\text{Hemolysis}\ (\%) = \frac{A_{\text{sample}} - A_{\text{negative control}}}{A_{\text{positive control}} - A_{\text{negative control}}} \times 100\%$$