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ABSTRACT

To enhance graphene stability, drug loading capacity and biocompatibility, β-cyclodextrin (β-CD) was grafted onto graphene oxide (GO) using L-phenylalanine (Phe) as a linker. The doxorubicin (DOX) loading efficiency and capacity of GO-Phe-CD were 78.7% and 85.2%, respectively. The cone shaped cavity of CD acts as a host for DOX loading through inclusion complex formation. The GO-Phe-CD nanocarrier showed higher release ratio of DOX in acidic milieu of cancer cells. In addition, general cytotoxicity of the nanocarriers was examined by MTT assay and trypan blue dye exclusion in MCF-7 cell lines. It was established that the MTT assay was not an appropriate technique for predicting the cytotoxicity of graphene based nanocarriers due to the spontaneous formation of MTT formazan by these materials; leading to a false high biocompatibility. According to the trypan blue experiment, the GO-Phe-CD had significant cytocompatibility, and the DOX-loaded GO-Phe-CD had outstanding killing capability to MCF-7 cells.

1. Introduction

Nowadays, drug nanocarriers can be considered as a bridge that links nanotechnology and drug delivery systems, which are of particular importance in clinical therapy of cancer. Among different kinds of nanocarriers, such as liposomes (Golkar, Samani, & Tamaddon, 2016), polymers (Wakaskar, 2018), nanoparticles (Karimi et al., 2017) and carbon based nanomaterials (Farvadi, Tamaddon, Sobhani, & Abolmaali, 2017), graphene derivatives have attracted much attention due to their desirable two-dimensional nanostructure containing a single layer of carbon atoms arranged in a honeycomb structure. High thermal and electrical conductivity, low cost, high strength and surface area, lack of band gap, the number of layers and lower toxicity are the unique properties that makes graphene different from other nanocarriers (Feng & Liu, 2011; Kim, Shen, Miao, Lee, & Oh, 2016; Mutthooamy, Bai, & Manickam, 2014; Zuchowska, Chudy, Dybko, & Brzozka, 2017).

The most prominent derivative of graphene is graphene oxide (GO), which consists of a wide range of oxygenated functional groups (hydroxyl, epoxy and carboxyl) on its basal plane and edges. Taking advantage of good π-π stacking and hydrophobic interactions, GO is an appropriate nanocarrier for carrying water insoluble drugs, such as antitumor ones (Duran et al., 2015; Wojtoniszak et al., 2013). However, the stability and dispersion of GO in biological solutions, such as culture medium and serum are low, leading to aggregation, that limits its applications in biomedical diagnosis and treatment (Dong et al., 2012; Hong, Compton, An, Eryazici, & Nguyen, 2012; Liu, Liu et al., 2008). Recently, efforts have been taken to improve GO solubility, stability and biocompatibility in physiological media by functionalization, especially for its usage in drug delivery system. Poly(ethylene glycol) (PEG) functionalization of GO was reported as a very useful method that effectively improves its biological dispersion and biocompatibility, which is widely used for loading and delivery of water insoluble drugs (Deb & Vimala, 2018; Liu, Robinson, Sun, & Dai, 2008). Hyaluronic acid (HA) (Yin et al., 2017), chitosan (Kumar et al., 2011; Wang et al., 2013), Pluronic F127 (Hu, Yu, Li, Zhao, & Dong, 2011), dextran (Zhang, Yang, Feng, & Liu, 2011), poly(ethyleneimine) (PEI) (Yan et al., 2013) and poly (amidoamide) (PAMAM) (Makharza et al.,...
2013) were reported for GO functionalization in designing new drug delivery systems. Nevertheless, relative low drug loading capacity and difficult procedures have limited further use of these systems.

An alternative approach for increasing drug loading is GO functionalization with cyclodextrin (CD), a biocompatible and water-soluble oligosaccharide. CDs comprise of six, seven, and eight glucopyranose units (named α-, β-, and γ-CD, respectively). The special structure of CDs (hydrophilic external surface and hydrophobic internal cavity), enables them to bind with several molecules (like hydrophobic drugs) into their cavity to form host-guest inclusion complexes (Abdolmaleki, Mallakpour, & Borandeh, 2015; Einashaf et al., 2018; Monteil, Lecouvey, Landy, Ruellan, & Mallard, 2017). CDs can absorb many compounds into their cavity through different kinds of intermolecular interactions, including van der Waals force, hydrophobic interaction, electrostatic affinity, dipole-dipole interaction, and hydrogen bond interaction (Chen, Diao, & Zhang, 2006; Liu, Robinson et al., 2008; Vogt & Strohmeier, 2012). Therefore, functionalization of GO with CD not only increase the drug loading capacity, but it also improves the solubility, stability and biocompatibility of GO.

Thus, in this study, we propose a new GO based drug delivery system using β-CD moieties as modifier and L-phenylalanine (Phe) as a linker. The prepared GO-Phe-CD nanocarrier was structurally and morphologically characterized by Fourier transform infrared spectroscopy (FT-IR), Raman, thermogravimetric analysis (TGA), dynamic light scattering (DLS), zeta-potential, field emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM) techniques. Doxorubicin (DOX) was used as chemotherapeutic drug model to examine the drug-loading and releasing properties while the effect of pH was also investigated. It is expected that GO functionalization with β-CD molecules will increase the loading capacity of DOX and the controlled release of the drug. In addition, the cell cytotoxicity of GO, GO-Phe and GO-Phe-CD nanocarriers and cell killing capability with these pH-responsive DOX-loaded drug delivery systems were also carried out via MTT assay and trypan blue dye exclusion in MCF-7 cell lines. The obtained data were compared and discussed.

2. Experimental

2.1. Materials

Graphite powder (particle size = 70 µm, purity = 99.999%) was purchased from Merck Chemical Company (Germany). β-Cyclodextrin (β-CD), L-phenylalanine (Phe), potassium permanganate (KMnO₄), 37% hydrochloric acid (HCl), sulfuric acid (H₂SO₄), 30% hydrogen peroxide (H₂O₂), sodium nitrate (NaNO₃), 1,1′-carbonyldiimidazole (CDI), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were all purchased from Sigma-Aldrich company and were used as received. MCF-7 cells were purchased from Pasteur Institute (Iran).

2.2. GO synthesis

Graphite was oxidized through Hummer’s method (Hummers & Offeman, 1958), which describes as follow. Graphite powder (0.5 g) was poured into cold solution of concentrated H₂SO₄ (12 mL) and NaN₃ (0.25 g). Then, KMnO₄ (1.5 g) was gradually added while stirring and cooling (the temperature of the mixture must have kept below 20 °C). The mixture was then stirred at 35 °C for 30 min and as the reaction progressed the color of the mixture turned to light brown. Then, distilled water (25 mL) was added and the temperature was raised to 98 °C for 1 h. The reaction was terminated by addition of distilled water followed by treated with 2 mL of H₂O₂ (30%) and the mixture changed into brilliant yellow color. The mixture was filtered and washed with distilled water and 10% HCl solution in order to remove metal ions. The obtained graphite oxide powder was dispersed in deionized water. The resulting yellow brownish suspension was centrifuged at 3000 rpm to eliminate unexfoliated graphitic plates. Finally, an aqueous suspension containing GO sheets was obtained through exfoliation of the filtered graphite oxide suspension through its sonication for 1 h. Finally, the resulting GO was then freeze dried and stored in vacuum for further use.

2.3. Phe functionalization of GO

Phe was attached to GO according to our previous report (Mallakpour, Abdolmaleki, & Borandeh, 2014). Briefly, GO (0.1 g) powder was dispersed in 10 mL distilled water and 10 mL solution of Phe (0.3 g) and an equimolar amount of NaOH was added. The mixture was stirred for 24 h at room temperature. The reaction was terminated by adding ethanol. The resulting precipitate was centrifuged, washed well with H₂O/EtOH mixture and finally freeze dried. In addition, to convert the carboxylate groups into carboxylic acid groups, the obtained solid was stirred and sonicated in 5% HCl solution and dried with freeze drier for 24 h.

2.4. GO-Phe-CD nanocomposite synthesis

At first 25 mg of GO-Phe was dispersed in 3 mL distilled water at room temperature. Then, CDI (25 mg) and β-CD (25 mg) were added to the suspension and the mixture was stirred for 2 h at room temperature and was sonicated for 1 h. Finally, the mixture was centrifuged at 14,000 rpm for 10 min and the precipitant was washed several times with distilled water and dried by freeze drier for 24 h.

2.5. Instrumentation

The FT-IR spectra of synthesized graphene based nanocarriers were obtained using FT-IR spectrometer (Vertex, Bruker, Germany) with a resolution of 4 cm⁻¹ in range of 400–4000 cm⁻¹ using KBr pellets of solid samples. Raman spectroscopy was recorded from 500 to 3500 cm⁻¹ on an Almega Thermo Nicolet Dispersive Raman Spectrometer using a Nd:YLF laser source operating at wavelength of 532 nm. The thermal stability of nanocarriers were measured using Perkin-Elmer TGA instrument under nitrogen atmosphere at the heating rate of 10 °C/min. The morphology of GO-Phe-CD was observed using FE-SEM (HITACHI S-4160, Japan) and TEM (Philips CM 120 operated (Netherlands) at voltage of 150 kV). Hydrodynamic size and surface charge of GO, GO-Phe and GO-Phe-CD were measured based on dynamic light scattering (DLS) method using Zetasizer 3000HSA (Malvern, UK).

2.6. DOX loading

GO, GO-Phe, GO-Phe-CD (0.25 mg) were dispersed in 0.5 mL of distilled water using ultrasound irradiation. Then, DOX solution at the concentration of 1 mg/mL was added to the mixture and the pH was set to 7.4. The mixture was shaken for 72 h at room temperature in the dark. The samples were ultra centrifuged at 14,000 rpm for 30 min. The DOX concentration in the supernatant solution was measured using a standard DOX concentration curve, generated with an UV–vis-NIR spectrophotometer at the wavelength of 480 nm from a series of DOX solutions with different concentrations. The DOX loading capacity and efficiency of different nanocarriers were calculated according to the following equations:

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\% \text{DOX Loading capacity (LC)} = \left( \frac{\text{mass of loaded DOX}}{\text{mass of nanocarrier}} \right) \times 100
\]

\[
\% \text{DOX Loading efficiency (LE)} = \left( \frac{\text{Initial DOX conc.} - \text{Supernatant DOX conc.}}{\text{Initial DOX conc.}} \right) \times 100
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