Introducing a highly dispersed reduced graphene oxide nano-biohybrid employing chitosan/hydroxyethyl cellulose for controlled drug delivery

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Abstract

In this research, an attempt was made to stabilize reduced graphene oxide (rGO) in all pH ranges, incorporating both chitosan (CS) and hydroxyethyl cellulose (HEC) to make a proper drug carrier with suitable stability and drug release behavior. The stability of rGO-CS-HEC nanohybrid was assessed using field emission scanning electron microscopy (FE-SEM), ultraviolet-visible spectroscopy (UV-VIS) and Zeta potential measurements. Results depicted that the novel synthesized nanohybrid was stable in all pH ranges, due to the utilization of HEC, while without incorporation of this material, the rGO-CS nanohybrid aggregated at neutral and alkaline media, due to the ionic nature of chitosan. In addition, drug loading and release behaviour of folic acid (FA), as a model drug, was investigated to assess the role of chitosan on the release behavior of FA from the rGO-CS-HEC nanohybrid in comparison with rGO-HEC and rGO-CS nanohybrids. It was proved that the resultant nanohybrid could release nearly 27% more FA than the rGO-HEC nanohybrid and only 9% lower than the rGO-CS nanohybrid during 120 hours. Moreover, the biocompatibility of the resultant nanohybrid was also checked to introduce the novel rGO-CS-HEC nanohybrid as a suitable candidate for drug delivery application.

Keywords: Nano-biohybrid, Reduced graphene oxide, Chitosan, Hydroxyethyl cellulose, Stabilization, Drug delivery application

Chemical compounds studied in this article:

Chitosan (PubChem CID: 71853); Hydroxyethyl Cellulose (PubChem CID: 24846132); Graphene (PubChem CID: 5462310); Folic Acid (PubChem CID: 6037)

1. Introduction

Graphene oxide with its two-dimensional structure has shown potential applications in the field of targeted drug delivery (Mo et al., 2015; Shi et al., 2013; Wang et al., 2013; Wei et al., 2016; Xie et al., 2016; Yang et al., 2016; Zheng et al., 2015). This nanomaterial with its planar structure is capable of immobilizing different drug molecules, hydrophilic and hydrophobic, by non-covalent bonding or π - π interaction with high loading capacity, and it can respond to different stimuli for drug release (Hu et al., 2012; Rana et al., 2011; Yang et al., 2008; Zhang et al., 2011). However, the aggregation of GO in electrolyte solutions, such as blood media, restricts its effectiveness for drug delivery application (Hong et al., 2011; Wang and Hu, 2013). Therefore, stabilization of GO suspension in electrolyte media is the most important challenge for any biological application of this injectable drug carrier.

In this regard, functionalization of GO with different water soluble and biocompatible polymers has been practiced to stabilize GO in electrolyte solutions for various biological applications (Bao et al., 2011; Hu et al., 2012; Lee et al., 2011; Liu et al., 2008; Rana et al., 2011). For instance, chitosan (CS) as an ionic biopolymer, has been widely used for stabilization of GO in acidic media to form an effective pH-responsive drug carrier for cancer therapy (Ardeshirzadeh et al., 2015; Duran et al., 2015; Justin and Chen, 2014; Park et al., 2010). However, in contrary to high release rate and pH-responsivity of GO-CS nanohybrid, it starts to aggregate in neutral and alkaline solutions (Fang et al., 2010; Liu et al., 2012), such as blood media and normal cells, due to the deprotonation of chitosan's amino groups (Afshari et al., 2015). This aggregation severely affects the utility of the nanohybrid as a drug carrier, because the nanohybrid must be stable when it passes through the blood media to show the maximum effectiveness and evade the reticuloendothelial system (RES). Therefore, the stabilization of GO-CS nanohybrid in all pH ranges is crucial for its application as a drug carrier for cancer therapy, although in many researches, less attention has been paid to this important point, and the stability of the nanohybrid has been assessed only in acidic media, unlike the importance of colloidal stability in all pH ranges for drug delivery systems.

In our previous study (Mianehrow et al., 2015), hydroxyethyl cellulose (HEC), a water soluble, non-ionic derivative of cellulose, was utilized as GO stabilizer for drug delivery application and the resultant nanohybrid presented high and long term stability in different electrolyte media, independent of the ionic nature of the solution. However, there was a conflict between stabilizing

effect of HEC and its drug release capability. The more HEC content in GO-HEC nanohybrid led to the longer stability of the nanohybrid, and less drug release because of the high interaction between HEC and folic acid (FA) drug, which dominates the drug release efficiency of such nanohybrid and makes the resultant nanohybrid undesirable for drug delivery application.

In the present study, stabilization of GO by incorporation of CS and HEC is assessed for the first time to exploit the advantages of both materials at the same time (Fig.1). HEC is used for stabilization of GO in all pH ranges and CS is utilized to get more drug release efficiency. Then, the dual functionalized GO is reduced to enhance its drug loading capacity through π - π interaction with drug molecules and at the end, loading and release behaviour of folic acid, as a model drug, from rGO-CS-HEC nanohybrid is investigated to check the effect of chitosan on the release rate of the drug from the novel nanocarrier, especially in comparison with rGO-HEC nanohybrid.

2. Experimental

2.1. Materials

Graphite flake with average lateral size of 20-100 micron, potassium permanganate, nitric acid 67%, sulfuric acid 98%, hydrochloric acid 37%, hydrogen peroxide 30% were purchased from Merck chemicals Co. Folic acid with molecular weight of 441.39 g/mol, L-ascorbic acid with molecular weight of 176.12 g/mol and low molecular weight chitosan (50,000-190,000 Da) were purchased from Sigma Aldrich Co. Hydroxyethyl cellulose with molecular weight of 300,000 g/mol and 1.5 (MS) was purchased from Kelong Chemical Co. (China), and all materials were used as received.

2.2. Synthesis of GO

GO was synthesized from natural graphite via preoxidation/thermal expansion of graphite followed by modified Hummer's method (Hummers Jr and Offeman, 1958; McAllister et al., 2007), following the same procedure as our previous work (Mianehrow et al., 2015). At first step, 10 g of natural graphite flake were mixed with 3:1 volume ratio of H_2SO_4 : HNO_3 for 24 h at room temperature. Then 400 ml of water was added to the resultant solution for quenching the reaction. The resultant product was then filtered and washed out with water and dried in vacuum. In the

second step, 2 g of the product was mixed with 200 ml of H_2SO_4 and 6 g potassium permanganate for 24 h resulting in a thick paste. Then, 600 ml of distilled water was added to the paste, and the reaction was terminated by addition of aqueous solution of H_2O_2 (30 wt. %), resulting in a yellow brown mixture. Then the mixture was centrifuged and washed out three times with 10% HCl solution and then three times with distilled water. The procedure was followed by sonication for 30 minutes to produce GO stable aqueous dispersion. At the end of this process, the mixture was centrifuged for 15 min at 4500 rpm in order to remove un-exfoliated graphite oxide particles. Synthesized GO full characterization could be found in the supporting information.

2.3. Preparation of rGO-CS-HEC, rGO-CS and rGO-HEC nanohybrids

Briefly, 1 ml of as-prepared GO dispersion (5.4 mg/ml) was added to 20 ml of acetic acid (0.2 M). It was first mixed with CS powder following by 15 minutes sonication, and then mixed with HEC powder by another 15 minutes sonication to produce a crystal clear solution of GO:CS:HEC with optimized mass ratio of 1:3:3. It is necessary to mention that we were aware that at least 1:6 mass ratio of rGO to HEC is required to get a stable suspension of rGO-HEC nanohybrid based on our previous work (Mianehrow et al., 2015) . Therefore, here we kept the ratio of rGO-polymer constant and changed the CS/HEC ratio. However, when the ratio of CS was more than HEC, spontaneous aggregation of the nanohybrid happened, which shows the dominating effect of CS over HEC in those ratios. In addition, in the case of higher HEC ratios, less drug release was expected. Therefore mass ratio of 1:3:3 was obtained as an optimum ratio. Then 25 mg of L-Ascorbic acid was added to the resultant nanohybrid and the solutions were kept at 70 °C for 1 hour for partial reduction of the functionalized GO. In the next step, the reduced nanohybrid was dialyzed against deionized water for 3 days for removal of L-ascorbic acid from the suspension. The same route was applied for preparation of rGO-CS and rGO-HEC nanohybrids with mass ratio of 1:6 (to have the same rGO: polymer ratio) for stability and drug release comparison.

2.4. FA loaded nanohybrid and in-vitro release analysis

Loading of FA was performed by mixing of 10 ml of FA (with 80 ppm concentration at pH of 9) with 15 mg of as-prepared nanohybrid overnight in a dark room. The mixture was then centrifuged

at 6000 rpm for 1 hr to remove the unloaded drug from the mixture. The drug loading efficiency of the nanocarrier was measured by UV-VIS spectrophotometer and was calculated as follows:

Drug loading efficiency (%) = $100 \times [\text{Total concentration of FA} - \text{Concentration of FA in the supernatant}] / \text{Total concentration of FA}$

The release behaviour of the loaded nanohybrid was monitored in phosphate buffer solution (PBS) with adjusted pH at 5.3. Briefly, 15 mg of the nanohybrid including 5 ml of the buffer was sealed in a dialysis tube with 12 kDa pore size. The dialysis tube was then immersed in 25 ml of the same saline buffer and placed in a shaking bed with a rotational speed of 150 rpm. 3 ml of release media was withdrawn every hour for the first 10 h and thereafter every 24 h until 120h. Released amount of folic acid in the samples was measured by UV-VIS spectrometer and each measurement has been done for three times.

2.5. Cytotoxicity of the synthesized nanohybrid

In vitro cytotoxicity of rGO-CS-HEC nanohybrid at different concentrations of 10, 20, 50 µg/ml was evaluated by MTT assay. Briefly, human fibroblast (HDF) cells (obtained from National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran) were seeded into 96-well plates (10,000 cells/well) using 100 µL RPMI medium and incubated in a humidified 5% CO₂ balanced air incubator at 37°C for 24 h. Then, except from control wells, the medium in remaining wells was replaced with 100 µL of culture medium containing rGO-CS-HEC nanohybrid with different concentrations, respectively. After incubated at 37°C for 24 h, the medium in each well was replaced with 100 µL of MTT solution with 0.5 mg/ml concentration. After continuous incubation for 4 h, the optical density (OD) of each sample was measured at wavelength of 545nm using an Elisa plate reader (STAT FAX 2100, USA). Triplicate samples were analyzed for each experiment.

2.6. Characterization

The chemical structure of the nanohybrid was assessed by Bomem Fourier transform infrared (FT-IR) spectrometer and its morphology was studied using Hittachi S4160 field emission scanning electron microscope (FE-SEM). X-ray diffraction (XRD) analysis has been done by using an Inel

Equinox 3000 diffractometer to study the interlayer spacing of rGO in the nanohybrid. For stability assessments, Analytic Jena (Specord 210) UV-VIS spectrophotometer and *ZetaPlus* Zeta potential analyzer; Brookhaven Instruments were employed. Fluorescence spectrophotometer, Varian Inc. was utilized for investigation of the molecular interaction between folic acid and the nanohybrid. The size of the nanohybrid particles were measured by particle size analyzer, VASCO-2, Cordouan Technologies, France.

3. Results and discussion

3.1.Characterization of rGO-CS-HEC nanohybrid

The FT-IR spectra of GO, CS, HEC and rGO-CS-HEC are presented in Fig. 2a. As-prepared GO shows characteristic FTIR peaks at 3438 cm^{-1} , 1716 cm^{-1} , 1618 cm^{-1} , 1180 cm^{-1} and 1049 cm^{-1} which are typical indication of vibration of O-H , C=O groups, C=C bonds, C-O-C and C-O groups. The FT-IR spectra of CS shows a broad -OH stretch absorption band at 3469 cm^{-1} and the aliphatic C-H stretch between at 2877 cm^{-1} . As the O-H stretch band and N-H stretch band are aligned, it appears as a broad band. Other major absorption bands between 1243 and 1097 cm^{-1} represent the free primary amino group (-NH₂) at the C2 position. The peak at 1656 cm^{-1} corresponds to the acetylated amino group of chitin, which indicates that the sample was not fully deacetylated. The peak at 1382 cm^{-1} identifies the C-O stretch of primary alcoholic group (-CH₂-OH). In FT-IR spectrum of HEC, the bands due to ether C-O-C stretch at 1149 - 1051 cm^{-1} appear as a strong peak. The peak at 1149 cm^{-1} corresponds to the secondary alcohol groups of HEC. In the rGO-CS-HEC spectrum, characteristic peaks of GO, CS and HEC are presented, which confirms the presence of two biopolymers on the surface of GO and the small shift of some peaks depicts the electrostatic interactions between the GO and incorporated biopolymers.

XRD patterns of GO, HEC, CS and the rGO-CS-HEC nanohybrid are investigated as well. As it is depicted in Fig.2b, HEC shows a sharp peak at $2\theta = 20.8^\circ$ which is characteristic peak of its cellulosic backbone (Chen et al., 2010) and a slight shoulder at $2\theta = 40^\circ$ which shows the presence of microcrystalline parts in its structure. Chitosan also shows a sharp peak at $2\theta = 19.4^\circ$ related to its backbone and two small peaks at $2\theta = 10^\circ$ and 14° and a broad shoulder around $2\theta = 40^\circ$ which

shows the presence of different crystalline types in the structure of chitosan. As-prepared GO also shows a sharp peak at $2\theta = 11^\circ$ which corresponds to the presence of oxygen-containing functional groups between GO layers. It is expected that after the reduction of functional groups of GO, the interlayer distance between GO sheets become significantly shorter, corresponding to a sharp peak at higher degrees. However, rGO-CS-HEC nanohybrid only shows the characteristics peaks of chitosan and HEC and no sign of graphene stacking is observed, which confirms the role of these biopolymers on stabilization of rGO. Moreover, the shoulder observed in case of HEC and CS is less pronounced in the pattern of the resultant nanohybrid, which could be due to the disruption of microcrystalline parts of HEC and CS, suggesting stretching of their chains in the presence of GO.

FE-SEM images of GO and rGO-CS-HEC were used to study the structure and morphology of the synthesized nanohybrid and provide more details about the surface of the nanohybrid (Fig.2c). The SEM image of GO clearly deduce the ultrathin and homogeneous layered structure. After incorporation of CS and HEC to the surface of GO, SEM image shows that the structure of the GO were constantly remained. Also surface changes of GO was clearly observed which confirms the great affinity and interactions between GO surface and two mentioned biopolymers.

The DLS size measurement employing Pade Laplace method showed that the resultant nanohybrid had a particle size range from 33.85 ± 1.64 up to 478.57 ± 22.50 nm ($n=3$). It worth noting that about 97% of particles mostly included an average size of 478.57 ± 23.50 nm and the rest had an average size of 33.85 ± 1.44 nm.

3.2. Stability assessments

Stability assessment of rGO-CS and rGO-CS-HEC in acidic, neutral and alkaline solutions is presented in Fig.3a. As expected, rGO-CS nanohybrid shows stability only in acidic media and start to aggregate in neutral and alkaline solutions, because of the deprotonation of chitosan. In contrast, rGO-CS-HEC nanohybrid seems completely stable in all pH ranges, which confirms that the steric stabilization strength of non-ionic HEC is able to stabilize rGO-CS nanohybrid independent of the acidity of the solution.

UV-VIS absorption spectra of rGO-CS-HEC suspension at different concentrations in alkaline media also correlation between the absorbance peaks at 272 nm and concentrations are presented in Fig.3b. According to the Beer–Lambert law, linear correlation between UV-VIS absorbance and the concentration of a suspension at different concentrations is a proof of its stability (Yang et al., 2010), and as it is depicted in the inset of Fig.3b, rGO-CE-HEC suspension shows the same correlation, which confirms its colloidal stability in alkaline media (the same results were obtained for the nanohybrid in acidic and neutral media (See the supporting information)).

Values of zeta potential for rGO-CS and rGO-CS-HEC are presented in Table 1. rGO-CS-HEC nanohybrid shows lower surface charges in comparison to rGO-CS at acidic media, which shows the covering effect of HEC. In addition, by increasing the pH of the solution, the deprotonation of chitosan is observed. However, the solutions still show high stability, which is directly because of the steric stabilizing strength of HEC, and that is why the stability of such nanohybrid is independent of the pH of the solution.

Chemical behaviour of rGO-CS and rGO-CS-HEC nanohybrid in different pHs are shown schematically in Fig.4. rGO-CS nanohybrid is stable in acidic media, because of the protonation of chitosan, which causes electrostatic repulsion between functionalized rGO sheets (Fang et al., 2010). By changing the acidity nature of the medium from acidic to neutral and alkaline, chitosan becomes deprotonated, and as a result, disappearance of electrostatic repulsion happens that leads to aggregation of rGO-CS nanohybrid. However, in case of rGO-CS-HEC suspension, the steric hindrance between HEC chains of different nanohybrid sheets, make it stable in all pH ranges, and at different ionic solutions.

3.3. Drug loading and release behaviour

As it is explained in stability assessments part, rGO-CS-HEC nanohybrid is highly stable in all pH ranges, due to the stabilizing effect of HEC, and this characteristic introduces this nanohybrid as a proper nanocarrier for any biological application. In the next step, for examining the role of chitosan on the drug release behavior of the nanocarrier, FA loading and release behavior is examined.

The interaction between the nanohybrid and FA drug is assessed by UV-VIS and fluorescence spectroscopy. In Fig.5a, UV-VIS spectra of the supernatant of FA loaded rGO-CS-HEC nanohybrid after centrifugation is compared to the UV-VIS spectra of pure FA solution before loading on the nanohybrid, and it is obvious that a significant amount of FA is loaded by nanohybrid, since its absorbance is noticeably decreased.

Fluorescence emission of FA before and after conjugation with rGO-CS-HEC nanohybrid, with the same concentrations of FA is depicted in Fig. 5b to assess the nature of interaction between the samples. The significant quenching of FA emission after conjugation with the nanohybrid, shows the efficient π - π interaction between FA and rGO-CS-HEC, and proves that even after presence of HEC and CS on the surface of GO, it still has a great surface area for making π - π interaction with FA, especially because of its partial reduction by L-ascorbic acid. This observation is confirmed by high calculated drug loading capacity of the nanohybrid which is $92\pm2\%$ ($p<0.05$, $n=3$), and it depends on hydrogen bonding also π - π interaction between rGO-CS-HEC and FA.

Fig.6 shows a schematic of drug loading on rGO-CS-HEC nanohybrid. It is well known that CS and HEC chains have the affinity to be conjugated to the carboxyl groups of GO on its edges. Therefore, both materials occupy some places in the edges of GO not its surface, and that is why a huge surface area of the nanohybrid would still be accessible for drug molecules to make π - π interaction. In addition, hydrogen bonding between FA and adsorbed CS and HEC could be also possible, which explains high drug loading capacity of the nanohybrid.

Release behaviour of FA from rGO-CS-HEC nanohybrid in PBS solution with pH of 5.3, (which has the same acidity as cancer cells(Du et al., 2011)), is presented in Fig.7a. As it is mentioned in our previous work (Mianehrow et al., 2015), FA release from the GO-HEC nanohybrid is quite low, which is due the low affinity of FA to acidic media and the strong interaction between FA and HEC. In addition, it was observed that by increasing the HEC amount in the nanohybrid, the FA release become significantly less (nearly 3% release after 72h for GO-HEC (1:25)), which shows the dominating role of HEC on release behaviour. This characteristic reduces the effectiveness of the nanohybrid as a drug carrier, even if it shows superb stability in different ionic media. Therefore, chitosan is utilized in this work to improve the release efficiency of the nanohybrid, because of its ionic nature. Results depict that rGO-CS-HEC nanohybrid releases more drug than the control sample of rGO-HEC nanohybrid. As it is observable in Fig 7a, rGO-

HEC sample releases up to 13% of FA during 120 hr, while rGO-CS-HEC nanohybrid releases 30% of FA during the same time, which is a significant increase in comparison to our control sample of rGO-HEC. Moreover, it is noticeable that the rGO-CS-HEC sample releases only 9% less FA than rGO-CS nanohybrid, which confirms that the resultant nanohybrid could show the high stability and more release rate at the same time.

Cytotoxicity of rGO-CS-HEC (with concentrations of 10, 20 and 50 $\mu\text{g/ml}$) exposed to human fibroblast (HDF) cells is investigated within 24 h to assess the biocompatibility of the proposed nanocarrier and to get comparable results according to the cytotoxicity results of pure graphene oxide exposed to the same cell line in the previous published researches (Wang et al., 2011) (Fig.7b). Results prove suitable biocompatibility of the nanohybrid within 24 h. Sample with 10 $\mu\text{g/ml}$ concentration shows almost no toxicity. Even the sample at 50 $\mu\text{g/ml}$ concentration, shows more than 80% of cell viability, which confirms the biocompatibility of the rGO-CS-HEC nanohybrid. Consequently, the introduced nanohybrid could be utilized as a proper drug carrier, because of its high stability in all pH ranges and in different ionic media, controllable drug release and biocompatibility.

Conclusions

With respect to the importance of the stability of a drug carrier in biological media, an attempt was made to introduce the novel nano-biohybrid based on reduced graphene oxide decorated with chitosan and hydroxyethyl cellulose that is highly stable in all pH ranges and ionic media. HEC was used as a non-ionic, soluble and biocompatible stabilizer for rGO, and CS was utilized to improve the low drug release characteristic of HEC contained nanohybrid. Results showed that steric hindrance between HEC chains can stabilize rGO in all pH ranges, and CS is able to enhance the drug release behaviour from the resultant nanocarrier significantly, due to its ionic nature. In addition, the nanohybrid showed high loading capacity, which represents the effect of hydrogen bonding and π - π interactions between rGO-CS-HEC and FA drug. Therefore, considering the before-mentioned results, it seems that rGO-CS-HEC nanohybrid can be introduced as a promising nanocarrier for drug delivery application, because of its stability, high loading capacity, controllable drug release and biocompatibility.

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Tables:

Table 1. Zeta potential values for rGO-CS and rGO-CS-HEC

	pH 2	pH 7	pH 9
rGO-CS-HEC	+ 8.5 mV	+ 4.3 mV	-18.2 mV
rGO-CS	+ 20.7 mV	aggregated	aggregated

Figure captions:

Figure 1. Schematic of rGO-CS-HEC nanohybrid synthesis

Figure 2. FT-IR spectra (a) and XRD patterns (b) of GO, CS, HEC and rGO-CS-HEC nanohybrid, FE-SEM images of GO and rGO-CS-HEC nanohybrid (c)

Figure 3. Digital images of rGO-CS and rGO-CS-HEC nanohybrid in three different pHs (a), UV-VIS spectra of rGO-CS-HEC suspension at different concentrations in alkaline media (Inset: correlation between the absorbance of the nanohybrid at 272nm and its concentration) (b)

Figure 4. Response of rGO-CS and rGO-CS-HEC nanohybrid toward neutral and alkaline media

Figure 5. UV-VIS spectra of supernatant solution of FA loaded nanohybrid after centrifugation and pure FA (a), Fluorescence spectra of FA and FA loaded nanohybrid (excitation wavelength 360 nm) (b)

Figure 6. Schematic of folic acid loading on rGO-CS-HEC nanohybrid

Figure 7. Release behavior of FA from rGO-CS-HEC, rGO-CS and rGO-HEC nanohybrids in PBS and pH=5.3 (a), MTT assay of rGO-CS-HEC nanohybrid at different concentrations for 24 hours (b) (The data points are average of three independent experiments. Error bars represent the range over which the values were observed)