Preparation of a stable aqueous suspension of reduced graphene oxide by a green method for applications in biomaterials

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Abstract

A green approach for the preparation of a stable reduced graphene oxide (RGO) suspension from graphene oxide (GO) has been developed. This method uses L-serine (L-Ser) as the reductant and yellow dextrin (YD) as the stabilizing agent. X-ray photoelectron spectroscopy, UV-vis spectroscopy, X-ray diffraction and thermogravimetric analyses showed that L-Ser can efficiently reduce GO at a comparatively low temperature, and that the YD adsorbed onto the RGO facilitating the formation of a stable RGO aqueous suspension. Since L-Ser and YD are natural environmentally friendly materials, this approach provides a green method to produce stable RGO from GO on a large scale. Sodium salicylate (SS) which has an aromatic structure was loaded onto the RGO through π - π interactions and a maximum loading capacity of 44.6 mg/g was obtained. The release of the loaded SS can be controlled by adjusting the solution pH, and a 74.8% release was reached after 70 h at pH 7.4. The

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release profile of SS could be further controlled by incorporating it into RGO

Dispersed carboxylated chitosan films.

1 Introduction

Graphene, a single layer of a two-dimensional graphite carbon lattice, has stimulated great interest due to its extraordinary electronic, thermal and mechanical properties [1-6]. However to reach its full potential for use in practical applications, graphene needs to be produced on a large-scale. During the past several years, many approaches have been developed for the preparation of graphene, including micromechanical methods [7], epitaxial growth [8], the ultrasonic exfoliation of graphite [9], chemical vapor deposition (CVD) [10], and the chemical reduction of graphene oxide (GO). The micromechanical cleavage method involves the repeated exfoliation of natural graphite and is not practical for large-scale production since it produces graphene sheets with a very low yield [11]. CVD is the most widely used method to prepare graphene, however the electronic properties of the graphene are greatly influenced by the substrate used.

The chemical reduction of GO is considered to be the most promising route to produce graphene on a large scale [12]. The chemical reduction of GO is generally achieved by using the reducing agents like hydrazine [13], NaBH_4 [14], hydroquinone [15], ethylenediamine [16]. However, these reducing agents are either toxic or explosive. Recently, several environmentally friendly reducing agents, such as bovine serum albumin [17], reducing sugars [18], green tea [19], and wild carrot root [20] have been developed for GO reduction. Another problem that needs to be considered

is the stability of the reduced graphene oxide (RGO) suspension (or solution) because RGO easily aggregates and then precipitates from the suspension [21]. Therefore, an effective, low-cost, and green method to synthesize dispersible RGO is highly desirable.

Graphene-based materials are important for academic studies and technological applications in biomedical engineering [22, 23]. GO nanosheets chemically modified with polyethylene glycol (PEG) have been shown to have increased stability in physiological solutions and have better *in vivo* safety for the delivery of anticancer chemicals [24, 25]. Controlled drug delivery systems using graphene and graphene composites have also been developed [26-28]. For example, a pH-sensitive poly(acrylic acid)-grafted graphene oxide nanocarrier was synthesized by *in situ* atomic transfer radical polymerization to allow the oral delivery of hydrophilic macromolecular proteins in their active forms to specific cells or organs [29].

In this paper, an easy and environmentally friendly approach for the reduction of GO is reported. L-Serine (L-Ser) was utilized as the reductant and yellow dextrin (YD) as the dispersant. L-Ser is a non-toxic, biocompatible and environment friendly chemical. The amine group of L-Ser can reduce GO under mild conditions. YD is a starch degradation products and it is widely used in the pharmaceutical, food, and paper industries as an adhesive and stabilizing agent for emulsions. Sodium salicylate (SS) was used as a model drug and the release performance of SS loaded DRGO (RGO prepared in the presence of YD) and SS loaded DRGO/chitosan films were evaluated.

2 Experimental

2.1 Materials

Graphite was obtained from Qingdao Graphite Factory. Potassium permanganate, concentrated sulfuric acid, hydrogen peroxide (30%), hydrochloric acid, L-Ser, YD and all other reagents were purchased from Tianjin Chemical Reagent Co. All chemicals were analytical grade and used as received.

2.2 Preparation of GO suspension

GO was synthesized from purified natural graphite by a modified Hummer's method (details are given in the SI) [30]. Briefly, concentrated H_2SO_4 was added to a 250-mL flask filled with graphite. Next NaNO_3 was added and then solid KMnO_4 was gradually added with stirring while the temperature of the mixture was kept below 20 °C. The temperature was then increased to 30 °C and excess distilled water was added to the mixture and the temperature was then increased to 80 °C. Finally, 30% H_2O_2 was added until the color of mixture changed to brilliant yellow. The mixture was filtered and the filter cake was washed several times with 5% aqueous HCl to remove the metal ions. The filter cake was then washed with distilled water to remove the acid. The resulting filter cake was dried in air and then re-dispersed into water with ultrasonic treatment to give suspended GO sheets.

2.3 Preparation of stable RGO suspensions using L-Ser as the reducing agent and YD as the stabilizing agent

First, solutions of L-Ser (3 mg mL^{-1}), YD (1 mg mL^{-1}) and GO (1 mg mL^{-1}) were prepared. Then equal volumes of the solutions were ultrasonically (150 W) mixed in a

flask for 10 min to obtain a mixed aqueous solution. The pH of the solution was then adjusted to the desired pH by adding dilute HCl or NaOH. The GO was then allowed to be reduced at 90 °C for at least 10 h to form DRGO. The extent of the reduction was monitored with a UV-Vis spectrophotometer (TU-1901, Matsushita Electric Japan). All the measurements were repeated three times, and the average results are reported.

The DRGO product was centrifuged and rinsed several times with distilled water to remove the excess L-Ser and YD, and then it was either re-dispersed in water to obtain a DRGO suspension or it was dried for 2 days in a vacuum at 40 °C to obtain DRGO powder. Similarly, GO was reduced in the absent of YD to give RGO

2.4 Characterization

X-Ray diffraction (XRD) analysis

The X-ray diffraction measurements of the graphite, GO and RGO samples were performed using a BDX3300 diffractometer (45 kV, 30 mA) with Cu Ka radiation ($\lambda = 1.54178 \text{ \AA}$). The relative intensities were recorded in the scattering range of 10° - 30° (2θ) with a step of 4° min^{-1} .

Thermogravimetric analysis (TGA)

The GO, RGO and DRGO samples were dried for 2 days in a vacuum at 40 °C before thermogravimetric analysis. TGA was performed on a Rigaku-TD-TDA analyzer at a heating rate of $10^\circ \text{ C min}^{-1}$. The analysis was performed on approximately 5 mg of the sample in an aluminum crucible from room temperature to 600 °C, under a flow of nitrogen.

Raman spectroscopy analysis

Raman measurements were performed from 500 to 2000 cm^{-1} at room temperature using a Raman spectrometer (Renishaw, UK) with a Nd:YAG laser excitation source (532 nm).

X-Ray photoelectron spectroscopy (XPS) analysis

The elemental composition of the samples were measured using X-ray photoelectron spectroscopy (PHI1600 ESCA System, PERKIN ELMER, USA) with Al K α radiation ($h\nu = 1486.6$ eV) and the XPS spectra were fitted using the XPS peak 4.1 software, in which a Shirley background was used to perform curve fitting and to calculate the atomic concentrations.

2.5 Preparation of SS loaded DRGO (S-DRGO) and DRGO/Chi (S-DGChi) films

S-DRGO was prepared by simply mixing DRGO and different concentrations of SS. Excess SS was removed by filtration and by thoroughly washing with distilled water. The amount of SS on S-DRGO was measured using a UV-Vis spectrophotometer at 242 nm.

Carboxylated Chitosan, DRGO, SS and glutaraldehyde solutions were mixed to obtain a homogeneous solution, and then the solution was poured onto a stainless steel plate and allowed to dry at 25 °C to obtain an S-DGChi film.

2.6 Measurement of SS release from the S-DRGO and S-DGChi films

A weighed S-DRGO or S-DGChi film (0.2 g) was placed into 20 mL phosphate-buffered saline (PBS, pH = 7.4) solution or a simulated stomach fluid (SSF, pH = 1.4) at 37 °C. The concentrations of SS in the media were measured using a

UV-Vis spectrophotometer. All the measurements were repeated three times, and the average results were used to calculate the release amounts.

3 Results and discussion

3.1 Reduction of GO

The GO used for reduction was prepared from natural graphite by a modified Hummer's method. An aqueous suspension of GO prepared via ultrasonication was stable for several months with no precipitation. The GO nanosheets were also dispersible in N, N-dimethyl formamide and dimethyl sulfoxide, implying that the GO nanosheets are both hydrophilic and thin enough to be suspended in numerous polar solvents [31]. Stable GO aqueous suspensions are crucial for preparing stable RGO suspensions.

When an aqueous solution of L-Ser was added to the GO suspension, the color of the GO suspension gradually changed from yellow to black during the reaction (Fig. 1a), suggesting that the GO was reduced by the L-Ser. The reduction of GO was monitored with UV-Vis spectroscopy, as shown in Fig. 1b. The maximum absorption peak (λ_{max}) of the GO solution is at 231 nm, but it gradually red shifted to 268 nm with a significant increase in intensity after 10 h of reaction. This indicates that the GO was reduced and that the aromatic structure and electronic conjugation of GO were restored [32]. For longer reaction times, no further changes were observed in the absorption intensity or in λ_{max} . The RGO solution prepared with L-Ser had a longer λ_{max} than those of RGOs reduced by other reducing agents such as zinc powder (265 nm) [33], urea (267 nm) [34], and formamidinesulfinic acid (264 nm) [35]. Therefore,

L-Ser is a natural, nontoxic and efficient reducing agent for GO reduction. However, the L-Ser-prepared RGO suspension is unstable and easily precipitates (Fig. 1a (B)).

When YD was added to the GO suspension before the reduction, the formed DRGO formed a stable suspension in aqueous solution as shown in Fig. 1a (C). So YD is a good stabilizing agent for RGO. The biocompatible and non-hazardous features of L-Ser and YD make them a good combination for the preparation of bulk quantities of DRGO which is dispersible in aqueous solutions.

Figure 1. Optical photos of aqueous suspensions of GO (A), RGO (B) and DRGO (C) (a); and UV-Vis absorption spectra of the DRGO suspension at different reaction times (b)

The effect of several factors such as reduction temperature, pH and the ratio of GO/L-Ser on the GO reduction were investigated. The effect of reduction temperature is shown in Fig. 2a. The reduction extent of GO increased as the temperature was increased. After 10 h at 90 °C, the $\lambda_{\max}$ was 268 nm and further increasing the temperature did not result in any further shifts in $\lambda_{\max}$. So, 90 °C was selected as the optimal reaction temperature for GO reduction.

Next, the amount of L-Ser needed to reduce GO was investigated and the results

are shown in Fig. 2b. When the GO/L-Ser ratio was 1:3 or 1:4, the $\lambda_{\max}$ shifted to 268 nm; but when the GO/L-Ser ratio was 1:2, the $\lambda_{\max}$ only shifted to 263 nm. So the optimal amount of L-Ser required for the reduction of GO is GO/L-Ser = 1:3.

GO reduction was also carried out at different pH values with the other conditions fixed and these results are shown in Fig. 2c. The pH of the solution was adjusted by adding 0.5 M HCl or 0.5 M NaOH to the GO suspensions. Under acidic conditions (pH = 3 or 5), the reduction of GO was limited as indicated by the small shift in the absorption peak from 231 to 240 or 245 nm, respectively. At pH 9, the absorption peak shifted to 268 nm. Further increasing the pH did not cause a change in $\lambda_{\max}$, so pH 9 was chosen as the optimal value.

Figure 2. Effects of reaction temperature (a), GO/L-Ser ratio (b), and pH (c) on

the reduction of GO.

In order to visualize the structural changes that occurred with reduction, XRD patterns of pristine graphite, GO and RGO were measured and they are given in Fig. 3a. The graphite has a strong diffraction peak at about 26.52° which represents an interlayer distance of 0.34 nm. However, this peak disappears in GO and a new weak diffraction peak at 11.4° which represents an interlayer space (d-spacing) of 0.78 nm is observed. This large interlayer distance is due to the formation of hydroxyl, epoxy and carboxyl groups which accommodate the intercalation of water molecules and increase the distance between the layers. However, this peak disappears in the XRD diagram of RGO, which indicates the successful reduction of GO by L-Ser.

The thermal stability of graphite, GO, RGO and DRGO (RGO with YD) was measured using TGA in a N_2 atmosphere and the results are shown in Fig. 3b. Graphite does not show any mass loss when heated from room temperature to $600^\circ C$. GO shows a 21% mass loss below $200^\circ C$, which is the result of the evaporation of adsorbed water and the removal of some functional groups. There is also a rapid mass loss (20%) from 200 to $250^\circ C$ which is due to the removal of the oxygen-containing functional groups. A gradual mass loss above $250^\circ C$ is due to the further removal of functional groups.

The mass losses of RGO are obviously smaller than those of GO, especially the mass loss between 200 to $250^\circ C$. This indicates a decrease in the amount of oxygen-containing functional groups due to the reduction of GO. The DRGO has

larger mass losses than those of RGO. The sudden mass loss at about 300 °C is caused by the decomposition of YD [36]. This phenomenon indicates that YD has been adsorbed on the RGO nanosheets and this may be the reason why DRGO dispersed so well in water.

Raman spectroscopy was also used to evaluate the L-Ser-reduced GO. Raman spectroscopy is often used to detect defect structures and doping levels. The Raman spectrum of reduced GO has two characteristic peaks, the G band at around 1580 cm^{-1} and the D band at around 1350 cm^{-1} . The G band is due to the in-plane sp^2 carbon-carbon bond stretching motions (symmetric E_{2g} mode) and the D band arises from the A_{1g} mode, which is induced by disorder and the edges. Changes in the relative intensities of the D and G bands (D/G) indicate changes in the electronic conjugation state of GO [37].

As shown in Fig. 3c, the D/G ratio increased after reduction. Since the Raman D/G intensity ratio is inversely proportional to the average size of the sp^2 domains, the increase in the D/G intensity ratio suggests that smaller in-plane sp^2 domains were formed during the reduction of GO [37]. This also indicates effective reduction of GO by L-Ser.

Figure 3. XRD diagrams of graphite, GO and RGO (a); TGA curves of graphite, GO, RGO and DRGO (RGO with YD) (b), and Raman spectra of GO and RGO (c)

XPS analysis was used to evaluate the degree of reduction and to determine the composition of the prepared RGO (Fig. 4). In the C1s spectrum of GO, the binding energy at 284.47 eV can be attributed to the C–C, C=C, and C–H bonds. The deconvoluted peaks centered at 286.45 and 288.1 eV are assigned to the C–O of the epoxy and/or alkoxy groups and the C=O groups, respectively [38]. Upon reduction with L-Ser, there is a slight shift of the peak positions along with a large decrease in the peak intensities which can be attributed to the removal of the oxygen moieties. In addition, the peak at 284.4 eV is more intense than that peak in GO, suggesting a restoration of the sp^2 network [39].

The C/O ratio is often used as a convenient measure of the degree of oxidation in GO samples [39]. The C/O ratio of GO is about 3.1 and that for RGO prepared with L-Ser is 7.9. This is higher than those of RGO reduced by $NaBH_4$ (4.78), chitosan (6.9), p-phenylene diamine (7.4), and pyrrole (7.7) and it indicates that many of the oxygen-containing functional groups were removed after the reduction. This again

demonstrates that GO was effectively reduced by L-Ser. The C/O ratio of DRGO is 5.4 which is lower than that for RGO, because YD contains more O atoms compared with RGO.

L-alanine was also used to reduce GO, but the extent of GO reduction was not as high as that with L-Ser (Table S1), though they all contain one $-\text{NH}_2$ group in a molecule. L-arginine and L-lysine were also tested for the reduction of GO and both were more efficient than L-Ser in reducing GO (Table S1). Both L-serine and L-alanine contain one $-\text{NH}_2$ group whereas L-arginine and L-lysine both contain more than one $-\text{NH}_2$ group (molecular structures are shown in SI). These results indicate that amino acids that contain more $-\text{NH}_2$ groups are more efficient in GO reduction.

Some small molecules and macromolecules containing amine groups have shown reduction ability towards GO. These include ethylenediamine [16], aniline [40], p-phenylene diamine [41], pyrrole [42], poly(ethylenimine) [43], polylysine [44], and amino terminated poly(amidoamine) (PAMAM) dendrimers [45]. The present work and these previous works prove that amino groups can reduce GO.

Figure 4. The C1s XPS spectra for GO (a), RGO (b) and DRGO (c)

3.2 Release profiles of S-DRGO and S-DGChi films

Graphene has a wide range of potential applications including electronic and optical devices, batteries, catalyst supports, composite materials and biomedical engineering. Drug delivery systems based on graphene and modified graphene have also attracted attention [46-49]. Aromatic drugs can be loaded onto the surfaces of carbon nanomaterials such as carbon nanotubes and graphene materials because of their noncovalent interaction including π - π stacking and hydrophobic interactions. GO or RGO is usually used instead of graphene because the latter is nondispersible in aqueous solution. However, RGO easily aggregates and precipitate in aqueous solution, so RGO often needs to be modified before it can be used in applications. Since, DRGO is dispersible in water, and considering the nontoxic properties of L-Ser and YD, DRGO was investigated as a drug delivery system.

SS has aromatic structure similar with RGO and may be loaded on the surface of RGO to form a delivery system (Figure S2). Sodium salicylate is a drug used to

mitigate pain. However, it has side effects when orally administered so other forms of the drug have been investigated. For instance, SS loaded hydrogels have been studied for external use [50, 51]. A pH-sensitive hydrogel [P(CE-co-DMAEMA-co-MEG)] was synthesized by free-radical crosslinking polymerization, and sodium salicylate was used as the model drug to study the controlled-release properties of the hydrogels. In vitro release studies showed that the release rate depended on swelling of the network as a function of the environmental pH and the DMAEMA content [52]. Sodium salicylate is water soluble. It also absorbs ultraviolet light, so it can be easily monitored with UV spectroscopy. Finally it has an aromatic structure similar to RGO (Fig. S2) so it can be loaded onto the surface of RGO to form a delivery system. Thus SS is a good candidate to load onto DRGO in chitosan to investigate its drug release. The sodium salicylate loaded chitosan film will be antibacterial (come from chitosan) and acesodyne.

The drug loading was conducted by simply physical mixing SS with DRGO. The unbound SS was removed by repeated rinsing. Figure 5 shows the effect of the initial SS concentration on the loading amount. When the concentration of SS was 0.01 mg/mL, the loading capacity of SS onto DRGO was only 6 mg/g. As the SS concentration increased, the loading capacity gradually increased until it reached a maximum value of 44.6 mg/g for a SS concentration of 0.12 mg/mL. Higher SS concentrations resulted in more loaded SS because of the load-release equilibrium. Under the same conditions, the maximum loading capacities of SS on RGO and YD were 32 and 13.8 mg/g, respectively. The loading capacity of DRGO is higher than

those of RGO and YD because the DRGO is well-dispersed in the aqueous solution so more RGO is accessible to the SS molecules.

Figure 5. Loading capacities of SS on S-DRGO for different SS initial concentrations

Figure 6a shows the release behavior of SS from S-DRGO over 70 h in buffers with pH values of 7.4 and 1.4. It was found that the release of SS from S-DRGO was pH-dependent. The release of SS was similar during the first 8 h at both pH values, however, at longer times, the release of SS at pH 7.4 was faster. After 70 h, 54.5% and 74.8% of the SS was released from S-DRGO at pH 1.4 and pH 7.4, respectively. In an acidic environment, RGO becomes more hydrophobic and tends to aggregate which hinders release [53].

The surface charge before and after SS loading was determined by zeta potential measurements. The zeta potential changed from -20.3 to -26.1 mV after SS loading. The charge became more negative because SS has -COO- groups.

Similar release values have been reported for other graphene-based drug carriers: 71% of Rhodamine B was released from Fe_3O_4 /graphene nanosheets [54], and 70% of doxorubicin was released from a phospholipid monolayer membrane functionalized

graphene at pH 5 [55]. At pH 7.4, the SS released from YD and RGO were lower than the amount released from S-DRGO (Fig. 6b). These results demonstrate that DRGO is a good drug carrier.

Figure 6. Release of SS from S-DRGO at different pH values (a); release of SS from YD and RGO at pH = 7.4 (b)

Chi is a natural polycationic polysaccharide, with excellent biocompatibility, biodegradability and film-forming ability and it has broad applications in bio-medicine and biological materials. Films containing dispersed drugs are a kind of drug delivery system that has applications in wound dressings and tissue engineering. Carboxylated Chi and SS are both water-soluble and DRGO is dispersible in aqueous solutions, so it is easy to make SS dispersed S-DGChi films as a controlled drug release system. The SS loading capacity of the S-DGChi films was 58.86 mg/g.

The effects of the amount of crosslinking agent and DRGO on the SS release from the S-DGChi films were studied and the results are shown in Fig. 7. The release of SS from the S-DGChi films is rapid at the beginning of the experiment and then

levels off at longer times. The rapid release is due to the dissolution of the SS from the film surfaces, and the subsequent sustained-release process is due to the diffusion of SS molecules through the membranes into the solution. As shown in Fig. 7a (pH 7.4, 2% RGO) the amount of crosslinking agent influences the release profile. When the amount of crosslinking agent was 0.1% and 0.2%, the total release of SS was the same (82.4%), but the release rates were different. When the amount of crosslinking agent was 0.4%, the release rate slowed significantly, and the total release was about 75.3%. So the optimal amount of crosslinking agent is 0.1 or 0.2%, and the prepared S-DGChi films show total release of about 82.4%. This may be due to the hydrophilic property of Chi [53, 56].

The effect of RGO on the SS release from the S-DGChi films is shown in Fig. 7b (pH 7.4, crosslinking agent: 0.1 %). As the amount of RGO increased up to 2%, the total release of SS gradually increased. More RGO did not result in a larger release. RGO nanosheets may hinder the interactions between the Chi macromolecules which enhances the diffusion of SS and results in a high release rate [28]. A more complete release of a drug is desirable for more effective therapeutic treatments. Figure 7c shows the release profile of SS from S-DGChi films (crosslinking agent: 0.1 %, rGO: 2%) under acidic conditions (pH 1.4). More SS is released under acidic conditions than at pH 7.4. This is because Chi swells in acid media [53].

Figure 7. Effects of the crosslinking agent (a), DRGO content (b) and pH (c) on the release of SS from S-DGChi films

4. Conclusions

An environmentally benign method for reducing GO has been developed by using L-Ser as the reductant and YD as the dispersant. The L-Ser efficiently reduced GO and adsorbing YD onto the RGO allowed the formation of a stable DRGO aqueous dispersion. This should facilitate the large-scale production of RGO and increase its potential for applications, especially in biomaterials because L-Ser is nontoxic and YD is biodegradable. This is an improvement over previous chemical reductants which are often toxic and non-biodegradable dispersants. The highly dispersed DRGO showed excellent performance as a carrier for SS, with a maximum loading capacity of

44.6 mg/g. The S-DRGO exhibited pH-controlled drug release behavior. The release profile of SS can be further controlled by incorporating it in S-DGChi films. Therefore, the as-prepared nontoxic DRGO is a promising candidate for applications in drug delivery and other biomedical engineering areas.

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