Assembly of Graphene Oxide–Enzyme Conjugates through Hydrophobic Interaction

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***B**iochemical and biomedical applications of graphene oxide (GO) critically rely on the interaction of biomolecules with it. It has been previously reported that the biological activity of the GO–enzyme conjugate decreases due to electrostatic interaction between the enzymes and GO. Herein, the immobilization of horseradish peroxidase (HRP) and oxalate oxidase (OxOx) on chemically reduced graphene oxide (CRGO) are reported. The enzymes can be adsorbed onto CRGO directly with a tenfold higher enzyme loading than that on GO, and maximum enzyme loadings reach 1.3 and 12 mg mg^{-1} for HRP and OxOx, respectively. Significantly, the more CRGO is reduced, the higher the enzyme loading. The CRGO–HRP conjugates also exhibit higher enzyme activity and stability than GO–HRP. Excellent properties of the CRGO–enzyme conjugates are attributed to hydrophobic interaction between the enzymes and the CRGO. The hydrophobic interaction mode of the CRGO–enzyme conjugates can be applied to other hydrophobic proteins, and thus could dramatically improve the performance of immobilized proteins. The results indicate that CRGO is a potential substrate for efficient enzyme immobilization, and is an ideal candidate as a macromolecule carrier and biosensor.*

1. Introduction

Graphene oxide (GO) has rapidly become one of the most widely studied materials due to its unique structural features and extraordinary chemical, electrical, and mechanical properties, and has found potential applications in many fields.[1] Recently, due to its inertia and low toxicity under physiological conditions, the application of GO has been extending to biological systems.[2,3] For example, polyethylene glycol (PEG)-modified GO has been used as a carrier for water-insoluble cancer drugs.[4] GO has also shown promise in detection of biomolecules,[5] in situ molecular probing in living cells,[5b] immobilization of biomolecules,[5c,6] as DNA manipulating agent etc.[5d] Immobilization of enzymes on GO has been pursued by our group and others recently,[5c,6,7] motivated by the prospects of using GOs as new types of biosensors and new high enzyme loading materials.

Generally, enzymes can be immobilized on GO through covalent bonding by a crosslinker or using the functional groups on the GO surface, and/or noncovalent binding through weak interactions. We have previously shown that a GO sheet having a large specific surface area and bonded functional groups is an ideal substrate for enzyme immobilization.[6] The atomically flat surface of GO enabled us to observe the immobilized enzyme in the native state directly by using atomic force microscopy (AFM). We demonstrated that enzyme immobilization on GO sheets could take place

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readily without using any crosslinking reagents or additional surface modification. The binding of the enzyme molecules to GO is mainly through electrostatic interaction.[6] However, electrostatic interaction as the driving force for enzyme binding to GO severely affected the activity of the enzyme. The enzyme loading on GO, though higher than that on many classical materials, is still relatively low for practical applications. In addition, the stability of the GO-immobilized enzyme is also not good enough for practical use.

The interaction between enzymes and GO materials is a critical factor for enzyme loading, enzyme activity, and stability. Chemically reduced graphene oxide (CRGO) is possibly a promising candidate to be used in enzyme immobilization to improve the performance of the immobilized enzyme. Due to the lack of surface functional groups, CRGO may impart less perturbation to the enzymes. It is thus imperative to investigate the interaction between enzyme and CRGO that has different extents of reduction to improve the enzyme loading, activity, and stability. Recently, Lee et al. showed that the activity of heparin was improved by coupling CRGO with the protein.[8] Herein, we systematically investigate enzyme immobilization on CRGOs that have different reduction extents. We show that compared to GO, the enzyme loading and relative activity are much higher when using CRGO as an immobilization substrate. The adsorption of enzyme on CRGO was through hydrophobic interaction. The higher enzyme loading and the higher activity of CRGO-immobilized enzymes indicate potential applications in molecular carriers and detection.

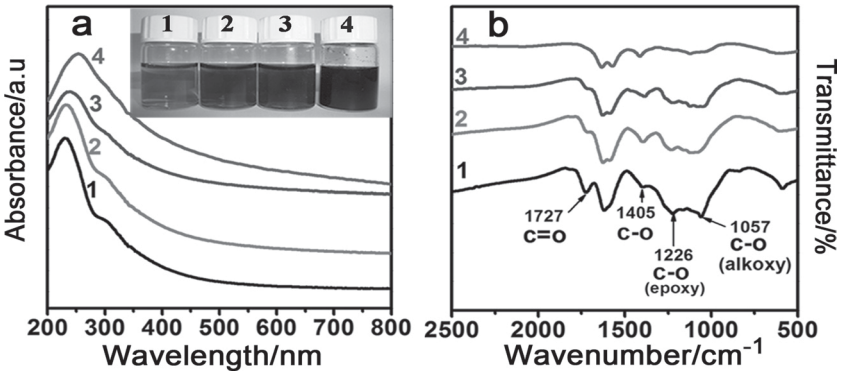

Figure 1. UV/vis spectra (a), photographs (a, inset) and FTIR spectra (b) of the GO (1) and GO reduced with L-AA for 2 (2), 4 (3), and 12 h (4).

2. Results and Discussion

2.1. Preparation and Characterization of the CRGO

CRGO was prepared by reducing GO sheets with L-ascorbic acid (L-AA) as previously described.[9] The reduction degree of the CRGO was controlled by the reaction time: the longer the reaction time, the greater the reduction extent. As shown in the inset of **Figure 1**a, the color of the CRGO changed from yellowish brown to black with increase in the reduction extent. The UV/Vis absorption peak at 230 nm for GO also red-shifted gradually to 233, 240, and 255 nm for CRGO-2, CRGO-4, and CRGO-12, respectively (Figure 1a; CRGO-2, CRGO-4, and CRGO-12 represent GOs that were reduced with L-AA for 2, 4, and 12 h, respectively). The absorption intensity of the entire spectrum of the CRGO, especially in the region above 300 nm, increased dramatically, thus suggesting that the GO might be reduced. The reduction process was also monitored using FTIR spectroscopy. Figure 1b shows that the intensity of the FTIR peaks of hydroxyl, epoxide, and carboxylic groups decreased with increase in the reduction time, thereby revealing the removal of the oxygen-containing groups on the GO. The morphology of the GO was maintained after reduction, as evidenced by transmission electron microscopy (TEM) and AFM images of CRGO (data not shown).

The zeta potentials of the GO and CRGO-12 solutions at pH 2–12.5 were also compared to examine the reduction state of the CRGO (Figure S1, Supporting Information).[10] Zeta potentials directly indicated that the electrostatic interaction between CRGO sheets is weaker than that between GO sheets. The lower absolute zeta potential value of CRGO-12 also suggested that the degree of Coulomb repulsion of CRGO was weaker. The zeta potential results confirmed the reduction of GO, which is consistent with the FTIR results.

2.2. Enzyme Immobilization and Mechanism

Previously, we have shown that horseradish peroxidase (HRP) can be immobilized on GO directly without any pretreatment, and the maximum loading is about 0.1 mg mg^{-1}.[6] We also found that the enzyme loading on GO is governed by the electrostatic interaction between the GO and HRP. According to that result, when CRGO was used in enzyme immobilization, we would expect a lower enzyme loading. However, the opposite result was observed using the same immobilization procedure. As shown in **Figure 2**a, the maximum HRP loadings on CRGO-2, CRGO-4, and CRGO-12 were 0.2, 0.7, and 1.3 mg mg^{-1}, respectively, which are much higher than that on the GO.[6] More importantly, the enzyme loadings on CRGOs are closely related to their reduction extents: the more GO is reduced, the higher is the enzyme loading. A similar result was obtained using oxalate oxidase (OxOx; Figure 2b). The maximum loading for OxOx was increased from 1.6 mg mg^{-1} for GO to 12 mg mg^{-1} for CRGO. Such enzyme loading on CRGO is more than 60-fold higher than that using many other classical materials.[11] The high loadings of OxOx should be related to its structural and surface properties, as detailed below.

The interaction between CRGO and the enzymes is apparently unlikely to be electrostatic interaction, because the numbers of surface functional groups on CRGO are much less than for GO as shown in the FTIR spectra, and also

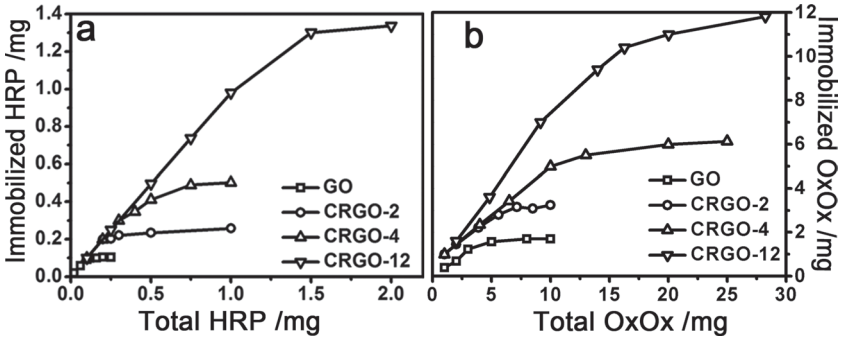

Figure 2. HRP (a) and OxOx (b) loadings on GO and CRGO as a function of the total amount of enzyme. GO and CRGO weights are all 1 mg.

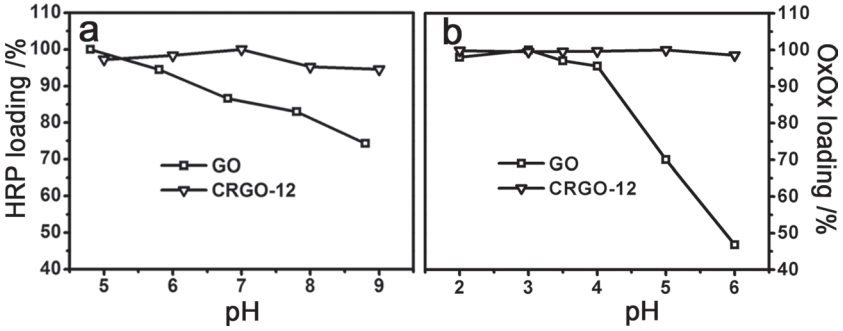

Figure 3. Effect of pH on the enzyme loading on GO and CRGO-12; HRP (a) and OxOx (b).

evidenced by zeta potential measurements. To explore the interaction between CRGO and the enzymes, the effects of pH on the enzyme loading on GO and CRGO with different reduction extents were examined. **Figure 3** shows the HRP and OxOx loadings on GO and CRGO-12 under different pH conditions. The enzyme loadings were barely affected by pH in the case of CRGO-12, whereas the enzyme loadings dramatically dropped in the case of GO when the pH was above the enzymes' pI values (7.2 for HRP, 4.2 for OxOx). The fact that enzyme loading on CRGO-12 is insensitive to pH confirmed that electrostatic interaction is not involved in the enzyme binding to the CRGO, as we assumed.

To further understand the interaction between enzyme and CRGO, the surface property of the CRGO was evaluated. As shown in **Figure 4**, the contact angle of water on the GO is much smaller than that of the CRGO, and also the water contact angles on the CRGOs increased from 46.8° to 80.7° with increasing reduction extent. The results suggest that the hydrophobicity of the CRGO surface is proportional to its reduction extent. Apparently, such a hydrophobic surface of CRGO was the result of the removal of functional groups on the GO surface by chemical reduction. The surface of the highly reduced CRGO sheets should be similar to the surface of graphene, which is hydrophobic.[12] Our previously reported results also indicated that the CRGO with a higher reduction extent exhibited a higher electrical conductivity.[13] The facts that the enzyme loading is insensitive to pH and that CRGO has a hydrophobic surface suggested that the adsorption of the enzymes on CRGO should be governed by a hydrophobic interaction instead of electrostatic interaction as on GO.[14]

To confirm this assumption, the effect of the ionic strength of the immobilization buffer on the enzyme loading was studied. As shown in **Figure 5**a, when the phosphate buffer concentration was increased from 10 to 100 mM, the enzyme loading was improved about 1.4-fold. Similarly, a high salt concentration could also improve the enzyme loading on CRGO, as shown in Figure 5b. The results indicate that a higher ionic strength is favorable for the interaction between enzyme and CRGO. This phenomenon is similar to the principle of the hydrophobic separation resin that is used for protein separation in biological science, in which proteins are bound to the hydrophobic separation resin with a higher salt concentration buffer, and proteins are released by washing the resin with a lower salt concentration buffer (Figure S2).[15] The results convinced us that hydrophobic interaction is the driving force for enzyme immobilization on the CRGO. The hydrophobic interaction between enzyme and CRGO allows us to control the enzyme state as bound or free simply by tuning the ionic strength of the buffer.

However, the two enzymes used in this work are both soluble proteins, so there should not be large hydrophobic areas on their surfaces, because most hydrophobic residues are located in the interior of the soluble enzyme.[16] The high loadings of the enzymes on the hydrophobic CRGO suggested that a structural conformation change may occur to these enzymes to break the balance between the interior hydrophobic interaction and electrostatic interaction on the periphery of the enzyme molecule for its immobilization. **Figure 6**a shows the circular dichroism (CD) spectra of free and immobilized HRP with the same concentration. Apparently, the secondary structure of the HRP that immobilized on CRGO is partially lost. The most dramatic change occurred to the enzyme immobilized on the highly reduced CRGO-12. Figure 6b shows that the most severe conformation change resulted in the lowest activity as expected. The conformation changes of the enzymes were

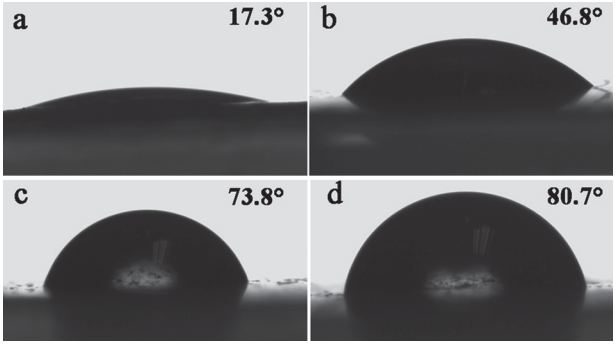

Figure 4. Water contact angles of GO and CRGO thin films; a) GO, b) CRGO-2, c) CRGO-4, and d) CRGO-12.

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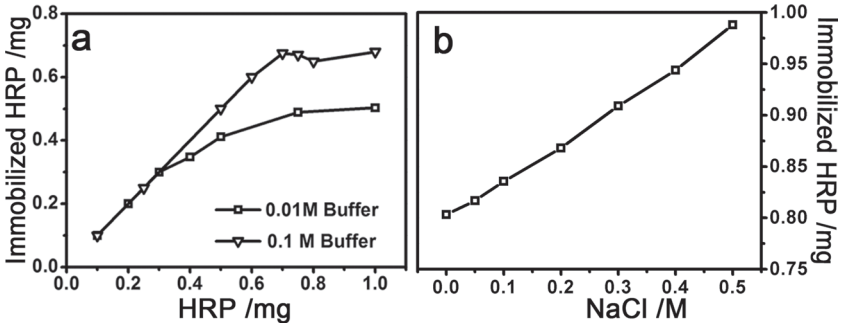

Figure 5. Effect of concentrations of the buffer (a) and NaCl (b) on the HRP loading on CRGO-4.

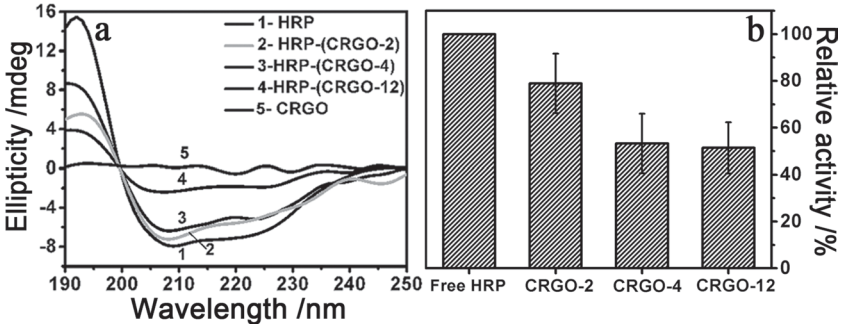

Figure 6. CD spectra (a) and relative activity (b) of the native HRP and HRP bound to CRGO. Immobilized HRP is 0.2 mg mg^{-1} CRGO.

also observed in AFM images of the immobilized enzymes. Figure S3 displays the AFM images of the immobilized HRP, and the heights of the HRP-loaded GO and CRGO at the maximum enzyme loading. The thicknesses of the enzyme-loaded CRGO are summarized in the table included in Figure S3. In addition, the higher the enzyme loading, the thicker is the CRGO and the lower the immobilized enzyme activity (Figure S4). This result also suggests that more than one layer of enzymes is possibly loaded on the highly reduced CRGO.[17] Nevertheless, the results further convinced us that hydrophobic interaction between CRGO and the enzyme is the driving force for enzyme immobilization.

2.3. Potential Applications

In the case of the immobilized OxOx, activity loss was not observed. On the contrary, the activity of the immobilized OxOx is even higher than that of free OxOx (Figure S5). OxOx comprises β-jellyroll monomers locked into a homohexamer (a trimer of dimers). Owing to the extensive surface burial and hydrophobic nature of the interactions between the monomers and dimers, OxOx has extreme resistance to proteases, heat, and dehydration.[18] That is possibly the reason why the conformation of OxOx was not affected by dehydration during the immobilization on hydrophobic CRGO, and nor was its activity. The immobilized OxOx maintained its 60% activity after more than ten times of usage, as shown in **Figure 7**a. While the reduction extent of CRGO increased, about 90% activity of the immobilized OxOx was maintained after ten cycles (Figure 7b and c). The immobilization of OxOx has been extensively explored for its critical application in clinical oxalate determination since 1980.[19,20] Many solid materials have been employed for this purpose, and the best enzyme loading so far discovered is 182 mg g^{-1} on alkylamine glass beads; however, the activity of the immobilized OxOx was only 5.17% that of the free OxOx.[11c] The high activity of the immobilized OxOx on CRGO can dramatically decrease the cost of using free OxOx in clinical oxalate detection and determination.[21] The higher enzyme loading, better stability, and high activity of the immobilized OxOx suggested that CRGO is a promising immobilization substrate for proteins and that structural alteration caused by dehydration is less prominent.[14b,18]

3. Conclusion

We have investigated enzyme immobilization on CRGO with different reduction extents using HRP and OxOx as model enzymes. Both enzymes can be loaded on CRGO directly and simply. Compared with GO–enzyme conjugate, the CRGO–enzyme conjugates exhibit higher enzyme loading, better stability, and higher activity. CRGO-immobilized OxOx displays higher activity than free OxOx, and it can be

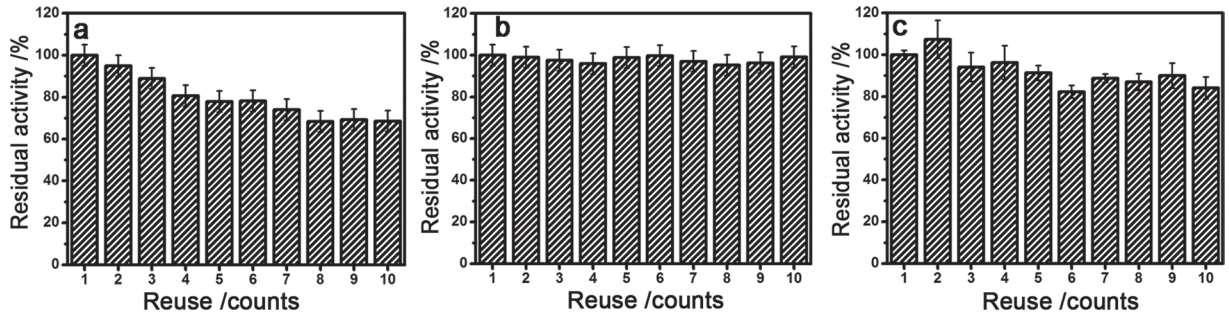

Figure 7. Reuse of the immobilized OxOx on GO (a), CRGO-4 (b), and CRGO-12 (c).

cycled ten times without losing activity. Studies of their surface properties and immobilization conditions indicate that the enzymes interact with CRGO through hydrophobic interaction. The hydrophobic interaction mode between enzyme and CRGO can be applied to other hydrophobic proteins, and thus could dramatically improve their performances after immobilization. The structural property of CRGO and the hydrophobic interaction with enzymes render it a potential enzyme-immobilizing substrate.

4. Experimental Section

Materials and General Methods: Horseradish peroxidase (HRP) was purchased from Majorbio BioTech. Oxalate oxidase (OxOx) was purified from barley roots using a previously published procedure.[22] L-Ascorbic acid (L-AA) was purchased from Sigma–Aldrich. Phenol (99%), H_2O_2 (30%), and analytical grade 4-aminoantipyrine (4-AAP) were purchased from Sinopharm Chemical Reagent Co.

Preparation and Characterization of GO and CRGO: GO sheets were prepared following the literature procedures.[9,23] CRGO was obtained by reducing GO with L-AA, which was developed by our group.[9] The degree of reduction was controlled by the reaction time. Briefly, the reduction reaction was performed by adding L-AA (200 mg) to an aqueous dispersion of the GO (2 mg mL^{-1}, 50 mL). After the reduction reaction was complete, the solid was isolated by filtration and washed with distilled water until the pH of the solution was 7.0. The reduction progress of GO was followed by UV/Vis spectroscopy (Shimadzu UV-2550, Japan). FTIR spectra of GO before and after being reduced with L-AA for 2, 4, and 12 h were recorded on an FTIR spectrometer (Bruker Co., Germany). Zeta potentials of GO, CRGO-4, and CRGO-12 as a function of pH were obtained on a Nicomp 380/ZLS instrument (PSS, USA). The contact angles measured with an optical contact angle measuring device (Dataphysics, Germany) were obtained for GO and CRGO films. AFM images of the immobilized HRP on GO and CRGO were acquired on a MultiMode Nanoscope V scanning probe microscope system (Veeco, USA) using the tapping mode. The samples for AFM were prepared by dropping the aqueous suspension (0.02 mg mL^{-1}) on a freshly cleaved mica surface. The CD spectra of free HRP and HRP bound to CRGO were acquired by CD spectroscopy (JASCO J-815, Japan) at 18 °C.

Immobilization of HRP and OxOx: HRP was dissolved in phosphate buffer (0.1 M) at pH 7. CRGO (0.1 mg mL^{-1}, 200 μL) was incubated in the HRP solution at 4 °C for 30 min. The mixture was then centrifuged at 15 000 rpm for 15 min, and the supernatant was collected to determine the enzyme loading. The solids were centrifuged and rinsed alternately three times with the buffer to remove nonspecifically adsorbed enzyme. The solid was stored at 4 °C for the activity assay. OxOx was immobilized using a similar procedure except that pH 3.8 succinate buffer (0.02 M) was used.

HRP and OxOx Activity Assay: HRP activity was determined by the 4-AAP method.[24] Phenol (0.6 mM) and H_2O_2 (1.57 mM) were used as substrates and 4-AAP (14 mM) as chromogen in phosphate buffer (0.1 M, 1 mL) at pH 7.0. The enzyme loading was calculated by taking away the amount of free enzyme in the supernatant from the total weight of added enzyme. The activity of the free and immobilized OxOx was determined using the literature procedure.[25]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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