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CRGO/alginate microbeads: an enzyme immobilization system and its potential application for continuous enzymatic reaction†

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In this work, novel hybrid microbeads composed of chemically reduced graphene oxide (CRGO) and alginate was fabricated, which could encapsulate enzymes by a simple non-covalent adsorption-entrapment method. Compared with alginate gel beads, the intervention of CRGO in alginate gel enhanced its mechanical strength, effectively prevented the leakage of enzyme, and greatly enhanced the stability and environmental tolerance. Compared with free enzymes or those on single carrier, the enzyme encapsulated in these hybrid microbeads can retain its optimum activity within a broad range (Temperature 45~60°C, pH 4~6). Additionally, the microbeads can be easily recycled by simple filtration and filled into a column to achieve a continuous fixed-bed enzyme catalytic reaction.

1 Introduction

Enzymes are nature's sustainable catalysts with highly controlled regio-, stereo- and substrate-specificity,[1,2] thus play indispensable roles in living systems and have been widely used in numerous fields, particularly in the pharmaceutical sciences, food sciences and chemical industries.[3,4] Despite all these advantages, their industrial applications are often hampered by lacks of long-term operational stability, highly environmental sensitivity and the difficulties in recovery and recycling. To overcome these drawbacks, immobilization of the enzyme on a suitable supporting medium is an effective method.[3,5-8] A broad variety of enzyme immobilization protocols have been applied on polymers or inorganic matrixes, involving physical or chemical methods.[5,9-14] Non-covalent bonding is the simplest immobilization method, which can keep the enzymatic activity in a better state,[15-17] without seriously losing the activity compared with chemical methods. However, the fatal disadvantage for the physical binding is the weak bonding force between the enzyme and the carrier, which always leads to enzyme leakage, especially under rigorous conditions.[8] Additionally, the enzymes always lost their activities quickly if not under the optimal conditions due to their environmental sensitivity. In fact, it is hard to simultaneously improve the stability and the activity of immobilized enzymes in one system due to the inherent characters. So, to best balance the stability and the activity, it is highly desirable to find a way to effectively control the interaction between the enzymes and the carrier.

Nanobiocatalysis, in which enzymes are immobilized on/in nanostructured materials, has gathered growing attention due to its excellent performance in stabilizing/enhancing the enzymatic activity.[18-24] Among them, graphene oxide (GO) and chemically reduced graphene oxide (CRGO), single-layered and 2D structure, have drawn great attentions of researchers. CRGO was also reported to be a star material for electron transfer[25, 26] and structure enhancement.[27, 28] Furthermore, graphene or its derivatives may have more potential applications in the field of biocatalysis. Recently, Zhang and coworkers[22] immobilized horseradish peroxidase and lysozyme on GO. They found that the interactions of substrate-enzymes were determined by the surface charges of the specified enzymes and the substrate. Huang *et. al.*[23] prepared a graphene oxide/hemoglobin (GO/Hb) composite hydrogel, which could catalyze a peroxidatic reaction in organic solvents with high yields, exceptional activity and stability. Zhang and coworkers [24] immobilized enzymes on CRGO through hydrophobic interaction. The CRGO–enzyme conjugates exhibit higher enzyme loading, better stability, and higher activity compared with GO–enzyme conjugate. In our previous work,[29] we have also fabricated a biocatalytic system by co-immobilizing multi-enzyme on CRGO, and found they exhibited higher activity and better stability compared with free enzyme by controlling the interaction between the enzyme and CRGO. However, the loss of stability and activity of enzyme still happened to some extent, due to the shortcoming of the weak interaction.[29] Therefore, it is urgent to find a simple and effective method to overcome these disadvantages.

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Due to the unique properties of hybrid materials combining with advantages of organic and inorganic components, such hybrid composite materials have been widely applied for the encapsulation of enzymes.[30-37] It is well known that alginate is a basically linear copolymer composed of α-L-guluronic acid (G) and β-D-mannuronic acid (M), which has been found to be the top candidates for immobilization and encapsulation technologies because of its excellent biocompatibility, processability and gel porosity.[38,39] Alginate microbeads, prepared in the presence of cationic cross-linkers such as calcium chloride, have been used for a long time to construct an immobilized enzyme reactor system;[40-44] however, low mechanical strength, high leakage of biomolecules and serious swelling greatly hinder its diverse applications. On the other hand, GO has been attracting tremendous attention as nanofiller for enhancing the mechanical performance of hydrogel.[22-24] Therefore, to fabricate a novel composite integrating the advantages of alginate and CRGO for the enzyme immobilization and hydrogel reinforcement may be an exciting and promising strategy. Based on this consideration, in this work, we reported a novel and effective approach to construct enzymatic microbeads (CRGO-GOD@Alg) by a simple adsorption-entrapment method, with CRGO and calcium alginate as scaffolds, and glucose oxidase (GOD) as a model enzyme.

These CRGO-GOD@Alg beads offer several attractive advantages: (i) no covalent bond was introduced during the synthesis procedure, so the activity of enzyme can be well preserved; (ii) the dual interaction utilized of hydrophobic interactions and encapsulation can effectively prevent the enzyme leakage; (iii) the intervention of CRGO in alginate gel enhanced its mechanical strength and increased the number of channels in the gel beads which can facilitate the diffusion of substrates and products. More importantly, compared with the mono-carrier immobilized enzyme, the CRGO-GOD@Alg hybrid system clearly exhibited higher stability and better environmental tolerance, which is crucial for the industrial application of immobilized enzyme. Moreover, the immobilized enzyme microbeads can be filled into a fixed bed reactor to achieve a high-efficiency continuous enzyme reaction, which will undoubtedly expand the industrial applications of the immobilized enzyme.

2 Experimental

2.1 Chemicals.

GOD from Aspergillus niger was obtained from TCI Shanghai. Graphite was provided by Sigma-Aldrich. Sodium alginate and calcium chloride ($CaCl_2$) were purchased from Sinopharm Chemical Reagent Co., Ltd.. All other reagents used in the experiment were of analytical grade and used without further purification.

2.2 Preparation of CRGO.

Firstly, GO was prepared from natural graphite flakes using a modified Hummers method,[45, 46] and the detailed procedure was detailed described in our previous work.[29] Then L-ascorbic acid (L-AA) was used to reduce GO into CRGO.[47] Briefly, a 0.5 mg/mL GO dispersion in deionized (DI) water was exfoliated into sheets by ultrasonication (37 Hz) for 2 h, obtaining a GO colloidal solution. Then 0.5 g of L-AA was added into 100 mL of the GO colloidal solution under vigorous stirring. After two hours reduction at room temperature, the product was centrifuged for 30 min (12000 rpm), and rinsed and centrifuged alternately three times with DI water to remove the excess L-AA.

2.3 Enzyme Loading on CRGO (CRGO-GOD)

0.05 g of GOD was dissolved in 90 mL acetate buffer (pH 5, 0.1 M), and then 10 mL of CRGO dispersion (10 mg/mL) was added. The mixture was incubated for 4 h in a 4°C refrigerator with occasional shaking, followed by being centrifuged for 8 min at 8000 rpm. The solid collected by centrifugation was rinsed and centrifuged alternately with DI water for three times to remove the nonspecifically adsorbed enzymes. The supernatant was collected to estimate the amount of enzyme loading using Lowry's method,[48] which was determined by subtracting the amount of enzyme in the supernatant from the original solution.

2.4 Preparation of the GOD@Alg and CRGO-GOD@Alg Gel Beads

For the preparation of GOD@Alg gel beads, 0.3 g of sodium alginate was dissolved in 10 mL of DI water to form a colloidal solution, followed by 10 mL of GOD solution (1.7 mg/mL) was added and blended. Then the mixture was pumped drop by drop into an aqueous coagulation bath of $CaCl_2$ (2.0 M, 100 mL) by a syringe pump at a flow rate of 3 mL/min at room temperature. The beads were stirred in the $CaCl_2$ bath for 15 min, followed by being washed with DI water for 3 times to remove the redundant $CaCl_2$.

For the preparation of CRGO-GOD@Alg beads, the aforementioned enzyme-loaded CRGO was adequately dispersed in 10 mL DI water; 0.3 g of sodium alginate was dissolved in 10 mL DI water; then the two mixture was well blended, followed by being pumped dropwise into an aqueous coagulation bath of $CaCl_2$ (2.0 M, 100 mL) by a syringe pump at a flow rate of 3 mL/min at room temperature. The beads were stirred in the $CaCl_2$ bath for 15 min, followed by being washed with DI water for 3 times to remove the redundant $CaCl_2$.

2.5 GOD Activity Evaluation

The activities of free and immobilized enzymes were determined by measuring the amount of hydrogen peroxide (H_2O_2) formed with β-D-glucose as a substrate, using a UV/Vis adsorption method provided by Megazyme.[49,50] All the activities mentioned in this paper were relative activity. When investigating the effects of temperature and pH on the activities of free and immobilized GOD, the highest activity of each enzyme under its optimal conditions was taken as a reference, that is, 100%. The activity comparison of the free and immobilized enzyme was carried out at the optimum conditions of themselves, and the activity of free GOD was set to 100%. In the reusability experiments, the first-run activity of each immobilized enzyme was taken as 100%.

In this paper, all results were repeated for three times, and the error bar added in the curve was according to the calculated standard deviation.

2.6 Mechanical Strength Test

200 grains of alginate and CRGO-alginate hybrid gel beads were added into 50 mL acetate buffer (pH 5, 0.1 M), respectively, which were vigorously stirred at the same conditions (1000 rpm). The mechanical strength of the alginate and CRGO-alginate hybrid gel beads was investigated by counting the number of fractured beads

at selected intervals of time.

2.7 Fixed Bed Reaction

The fixed bed equipment was composed of a temperature controller, a liquid chromatography pump and a reaction column. 1300 grains of CRGO-GOD@Alg beads were filled into the reaction column and some quartz wool was fixed on the both ends of the column in case its orifice was blocked. 0.5 mg/mL of glucose solution was prepared with pH 5 buffer solution which was pumped into the column from bottom to top with a flow velocity of 0.05 mL/min controlled by the performance liquid chromatography pump. The temperature of the column was controlled at 50°C. The reaction solution was collected at selected intervals of time and analyzed by high performance liquid chromatography (HPLC).

2.8 Characterizations

Fourier transform infrared (FTIR) spectra were recorded on a Nicolet 6700 FTIR Spectrometer (ThermoFisher) with a resolution of 4 cm^{-1} in 4000-600 cm^{-1} region, using the KBr pellet technique. The specimens were prepared by forming thin transparent KBr pellets containing the samples. UV/Vis spectra of samples were measured using a UV-250 spectrophotometer. Transmission electron microscopy (TEM) images were obtained on a Hitachi H-7650 transmission electron microscope at 100 kV. Samples were made by placing a drop of the analyte suspension onto a carbon-coated copper grid. The surface morphology of the samples was recorded on a scanning electron microscopy (SEM, Hitachi S-4800). Atomic force microscopy (AFM) was acquired on a MultiMode Nanoscope V scanning probe microscopy system (Agilent 5400 AFM) using tapping mode. To prepare the specimens, the aqueous dispersion was dropping on a freshly cleaved mica surface and dried with a flow of nitrogen.

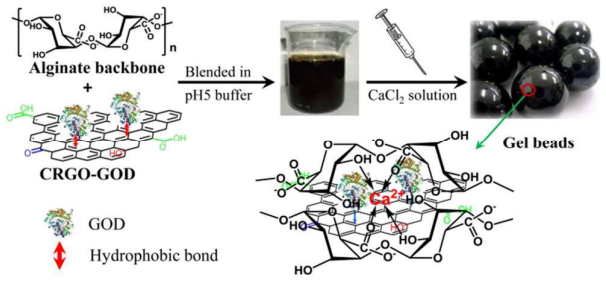
2.9 HPLC analysis

Glucose and gluconic acid analysis in the reaction solution was performed on a Agilent 1100 HPLC, which was equipped with a refractive index detector (RID) and a variable wavelength detector (VWD).[51] A BioRad Aminex HPX-87H column was used thermostated at 55°C with H_2SO_4 (0.05 M) as the eluent.

3 Results and discussion

3.1 Preparation and Characterization of CRGO and CRGO-GOD

The synthesis procedure of the microbeads is depicted in Scheme 1. GOD as a model of biocatalyst was first adsorbed onto CRGO with



Scheme 1 Schematic representation of the synthesis of CRGO-GOD@Alg beads.

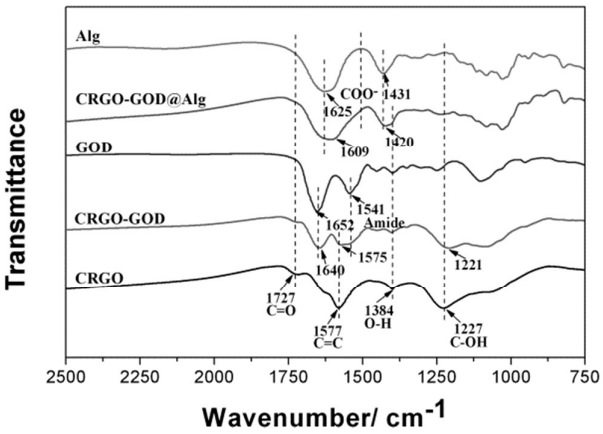


Fig. 1 FTIR spectra of the GO, CRGO, CRGO-GOD, GOD, CRGO-GOD@Alg and calcium alginate.

controlled hydrophobic association (CRGO-GOD); the CRGO-GOD hybrid nanocomposite was then mixed with sodium alginate in pH 5 buffer solution; finally, the mixture was dropped into calcium chloride solution to form the ionic cross-linked gel beads (CRGO-GOD@Alg) with a syringe pump.

According to our previous study, in order to obtain a better enzyme activity on the surface of GO, the GO must be chemically reduced in a controlled manner.[29] The preparation of GO was described in our previous work In detail .[29] The CRGO was prepared by reducing GO with L-AA for 2 hours at room temperature. The successful reduction was verified by FTIR and UV/Vis spectroscopy (Fig. S1 and S2, ESI†).The spectra of GO exhibits several characteristic IR features, such as the C=O stretching vibration peak at 1726 cm^{-1}, the vibration and deformation at 1404 cm^{-1}, the C–O (epoxy) stretching and deformation peaks at 1225 and 855 cm^{-1}, and the C–O (alkoxy) stretching peak at 1052 cm^{-1}, respectively. However, after reduction with L-AA, the peak at 1727, 1053 and 855 cm^{-1} greatly decayed, indicating the removal of the related oxygenated groups. Meanwhile, a strong C=C stretching peak at 1574 cm^{-1} coupled with the doublet peaks at 2923 and 2846 cm^{-1} (symmetric and antisymmetric stretching vibrations of $–CH_2$ group, respectively) appeared, suggesting a restoration of partial aromatic ring structure in GRGO. In the UV/Vis spectra, GO shows a main C=C plasmon peak at about 234 nm representing the π-π* transition of aromatic and a shoulder band at about 301 nm ascribed to n-π* transition of the carbonyl groups.[52,53] After reduction with L-AA for 2 hours, the peak at 234 nm red-shifted to 243 nm whilst the shoulder peak at around 301 nm was weakened, suggesting the reduction of GO and the probable restoration of aromatic structure. CRGO was reported to be a promising candidate for enzyme immobilization mainly through the hydrophobic interaction between the carrier and enzymes, due to the lack of some oxygen-containing groups.[24]

GOD was immobilized on CRGO in pH 5 buffer solution at 4°C for 4 h with occasional shaking. The CRGO-bounded GOD (CRGO-GOD) was centrifuged and rinsed three times to remove the nonspecific adsorbed enzymes. The amount of immobilized GOD on CRGO was determined to be 0.259 mg mg^{-1} using the Lowry method, evaluated by the change of enzyme concentration in the solution

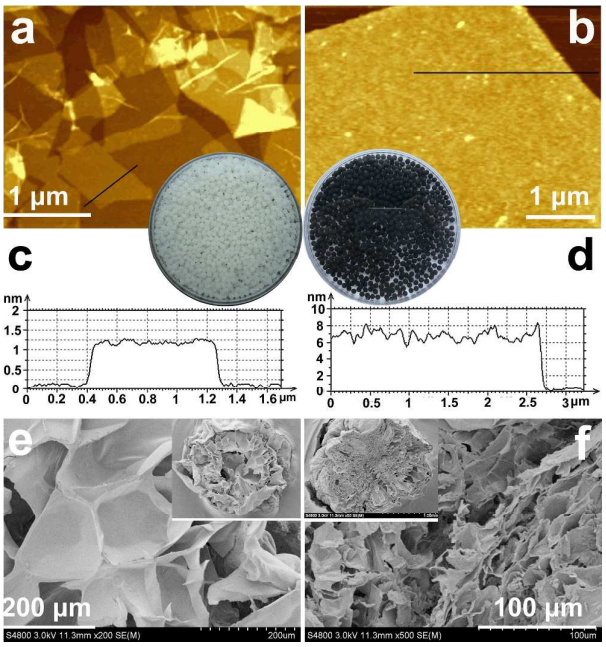


Fig. 2 Tapping mode AFM images of CRGO (a) and CRGO-GOD (b). The height profile of the AFM images of CRGO (c) and of CRGO-GOD (d). SEM images of the section GOD@Alg (e) and CRGO-GOD@Alg (f) beads dried in a freezer dryer.

before and after enzyme loading. To confirm the successful enzyme immobilization, AFM, TEM, FTIR and UV/Vis spectra were employed. As shown in the FTIR spectra in Fig. 1, CRGO gives several characteristic peaks corresponding to the oxygen containing groups because of the incomplete reduction, such as the C=O stretching vibration peak at 1727 cm^{-1}, the O-H deformations in the C-OH groups at 1384 cm^{-1} and the C-OH stretching vibration peak at 1227 cm^{-1},[25,53] as well as the skeletal vibration absorption of graphene at around 1570 cm^{-1}. GOD shows the characteristic peaks at around 1652 cm^{-1} corresponding to C=O stretching vibrations of Amide I band and around 1541 cm^{-1} attributing to N-H bending vibrations of Amide II band in the infrared spectra of the protein.[54] Compared to the spectrum of CRGO, CRGO-GOD retained the skeletal vibration and the oxygen-containing residue absorption peak of CRGO at about 1575 cm^{-1}, 1727 cm^{-1}, 1384 cm^{-1} and 1227 cm^{-1}, yet new band at about 1645 cm^{-1} emerged, which should attribute to the C=O stretching vibrations of Amide I band in GOD. Furthermore, the peak at 1575 cm^{-1} was obviously broadened and flattened until the N-H bending vibrations of protein at about 1541 cm^{-1}. The appearance of the new band at 1645 cm^{-1} and the changes of peak shape at 1575 cm^{-1} indicated the successful enzyme loading. The shift of Amide I band from 1650 to 1645 cm^{-1} and the broadened peak at the range of 1575-1541 cm^{-1} indicated the interaction between CRGO and enzyme. The UV/Vis spectrum further confirmed the successful combination of GOD and CRGO (Fig. S2, ESI†). Compared to CRGO, the spectrum of CRGO-GOD exhibits a new peak at around 226 nm, which is in accord with GOD peak at the same wavelength, indicating the successful loading of GOD on CRGO.

Typical AFM images of CRGO and GOD-loaded CRGO are shown in Fig. 2a and b. As expected, before enzymes loading, the CRGO sheets exhibited a fairly smooth surface (Fig. 2a) while it became quite rough after enzymes loading due to the dense covering of enzyme molecules over all the surface of CRGO sheets. Hecht etc. reported the size of GOD was about 6 nm × 5 nm × 7.7 nm.[55] The height profiles of AFM images showed that the thickness of CRGO sheet was about 1 nm while that of CRGO-GOD sheet increased to around 6 nm (Fig. 2c and d), which confirmed that the CRGO sheet was fully covered by GOD with a single layer. The shorter height of the immobilized GOD molecules revealed that immobilization maybe induced some conformational changes of the enzyme molecules.[22] Moreover, TEM images also clearly exhibit that the GOD molecules covered densely over all the surfaces of CRGO sheets (Fig. S3b and c, ESI†).

3.2 Preparation and Characterization of CRGO-GOD@Alg and GOD@Alg

CRGO-GOD can be well dispersed in the sodium alginate solution forming a uniform solution. The mixture solution was then pumped into calcium chloride aqueous solution for ionically crosslinking to form CRGO-GOD@Alg beads. As a comparison, another kind of gel beads in absence of CRGO was made by direct encapsulation of GOD within sodium alginate beads (GOD@Alg). As shown in Fig. 2 (inset), all the beads have a uniform and smooth spherical shape with an average diameter of around 0.27 cm.

It is observed from AFM and TEM (Fig. 2a, 2b) that the enzyme has been loaded on the surface of CRGO due to the morphology change after the non-covalent interaction. Fig. S3c and d show the TEM images of CRGO-GOD and CRGO-GOD@Alg, respectively. After crosslinking by the sodium alginate, the surface of CRGO-GOD@Alg became rough and almost cannot be penetrated by the electron beam due to something thick covered on the surface. The SEM images of the GOD@Alg and CRGO-GOD@Alg after being dried in a freezer dryer were also investigated. As can be seen from the micrographs of the beads (Fig. S4a and b, ESI†), the surface of GOD@Alg beads displays a relatively homogeneous, smooth morphology (Fig. S4a, ESI†) while that of CRGO-GOD@Alg beads reveals a rather rough morphology with some wrinkle structures (Fig. S4b, ESI†), which might be attributed to the CRGO sheets embedded into alginate gel. Fig. 2e and f show the section micrographs of the two kinds of gel beads. As shown in Fig. 2e, there are some cellular cavities in the GOD@Alg dehydrated beads. In the case of the CRGO-GOD@Alg gel beads (Fig. 2f), more compact and smaller cavities are found, which might be caused by the layered structure of CRGO. This may be beneficial to protect the enzyme in the system. Even more important, as shown in Fig. S5 (ESI†), with the help of CRGO, the number of damaged CRGO-GOD@Alg beads was obviously reduced under the vigorous agitation, which indicated that the mechanical strength of CRGO-GOD@Alg gel beads was efficiently enhanced due to the addition of CRGO compared with that of GOD@Alg beads. The enhanced mechanical strength of the hybrid microbeads will no doubt facilitate the following treatment for the immobilized enzyme. Both TEM and SEM images of CRGO-GOD@Alg beads indicate the well and homogenous dispersion of the CRGO in the alginate matrix. On

the other hand, it is suggested that some interaction among the CRGO, enzyme and alginate interface may exist.

As shown in the FTIR spectrum of alginate (Fig. 1), the peaks at 1625 cm^{-1} and 1432 cm^{-1} correspond to the asymmetric and symmetric COO^- stretching vibration of the carboxylate salt group.[56] When entrapping CRGO-GOD into the alginate beads, the peaks at 1625 cm^{-1} and 1432 cm^{-1} obviously broadened and shifted to smaller wavelengths (from 1625 cm^{-1} to 1609 cm^{-1} and from 1431 cm^{-1} to 1420 cm^{-1}, respectively), which might be attributed to the interaction among the alginate ($-COO^-$), CRGO (the remaining hydroxyl after the incomplete reduction) and GOD ($-NH_2$) through intermolecular hydrogen bonds. The achieved interaction may induce good interfacial adhesion at the interface of CRGO-GOD /alginate and results in an enhanced mechanical response of CRGO-GOD@Alg gel beads compared with GOD@Alg beads. Also these interactions can probably improve the stability of the composite gel immobilized enzyme.

3.3 Activity Assay

The activities of immobilized GOD were examined (Fig. S6, ESI†). It showed that CRGO-GOD@Alg could retain about 92% of its original enzyme activity after the immobilization.

Generally speaking, enzyme is sensitive to external environment, the reaction conditions should be strictly controlled within a relatively small range to keep its optimum activity, which is also one of the headaches for the enzyme application. The stability of immobilized enzyme towards temperature and pH is of general interest, especially when entrapping an enzyme in beads or capsules, which has the potential to change the microenvironment (such as temperature and pH) surrounding enzyme molecules. Therefore, we examined the enzymatic activities of free and immobilized GOD by the enzymatic reaction of glucose oxidation under different pH and temperature conditions. The influence of temperature on the activities of free and immobilized GOD was shown in Fig. 3a. It was found that the optimum temperature for both free and CRGO-adsorbed GOD was 50°C, while that for alginate-encapsulated GOD shifted to 60°C. These results indicated that the enzyme absorbed on the surface of 2D CRGO sheets showed the similar behavior as the free one when the temperature changed. By contrast, the alginate can provide a buffer region around the enzyme molecules, which protected them from being destroyed under the higher temperature. It should be noticed that no matter the enzyme immobilized on the CRGO or into the alginate, the enzyme keeps its activity only in a narrow range of optimum temperature (Fig. 3a). However, when embedding CRGO-GOD into alginate gel, the enzyme could still retain its optimum enzymatic activity to a higher degree (more than 90%) over a broad temperature range of 45~60°C (Fig. 4a). That is to say, the temperature change has less impact on the enzymatic activity of CRGO-GOD@Alg, which is preferable in the practical application. However, for the free GOD and CRGO-GOD, when the temperature rises to 60°C, the enzyme activity decreases to 59.5% and 65.2% of their optimum activity, respectively. For the GOD@Alg, the enzyme activity is only 45.4% of its optimum activity at 45°C. Similar results were also obtained for the pH effect on the activities of free and immobilized enzyme. As shown in Fig. 4b, free GOD, CRGO-GOD and GOD@Alg exhibited maximum activity at pH 5.5, 5 and 4.5, respectively, while the optimum pH of CRGO-GOD@Alg was much broadened, over the pH range of 4~6. Moreover, its ability to resist over acid and alkali got greatly enhanced compared with free enzyme and the enzyme protected by a single carrier (CRGO or alginate). It is well known that the graphene nanosheet is an excellent candidate for carrier material. Due to its unique structure, the heat or gust molecule will be isolated very well.[57] Then, it is believed that the hybrid microbeads might not only increase the stability of enzyme structure, but also buffer the impact of external environment changes to avoid the structure change of enzyme under violent conditions. In the system of microbeads, the effect of temperature and pH changes will be reduced greatly, and also the alginate may act as a good buffer gel at the microscale due to its gel net. For the CRGO-GOD@Alg beads, GOD was first immobilized on CRGO sheets through hydrophobic interaction and then embedded into the alginate gel, which provided enzyme dual protection with non-covalent bond. The synergy of the dual immobilization for enzyme made it more capable of resistance to the external environment, regardless of temperature and pH conditions, which will facilitate the application of the enzyme.

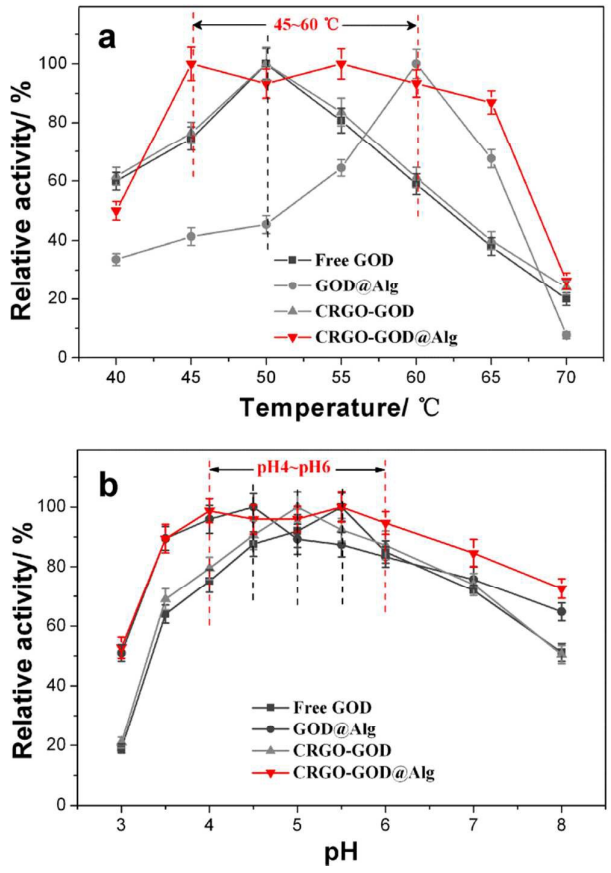


Fig. 3 Effect of temperature (a) and pH (b) on the activities of free GOD, GOD@Alg, CRGO-GOD and CRGO-GOD@Alg. The highest activity of each enzyme under its optimum temperature was set to 100%.

3.4 Reusability

The reusability of immobilized enzyme is always taken into account for industrial application with the purpose of cost reduction. Compared with other fine powder carriers (such as porous silica,

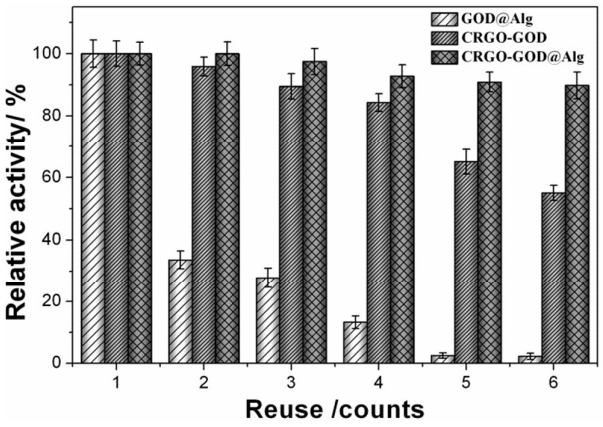


Fig. 4 Reusabilities of GOD@Alg, CRGO-GOD and CRGO-GOD@Alg in the enzymatic catalytic oxidation of glucose. The first run activity of each immobilized enzyme was taken as 100%.

active carbon, molecular sieve etc. powder), in our experiments, the enzyme immobilized in the microbeads can be easily recycled by simple filtration even without using centrifugation or filtration under vacuum. The reusability was evaluated by examining the remaining activity of immobilized GOD after each cycle, and the specific activity in the first run was set to 100%. Fig. 4 illustrated the recycling stability of the CRGO-GOD@Alg beads, with CRGO-GOD and GOD@Alg as comparison. It showed that the enzymatic activity of GOD@Alg decreased rapidly with each cycle and the remaining activity was only 8% after six cycles, due to the serious leakage of enzyme from alginate beads where only physical entanglement happened between alginate and GOD.[58] The mainly driving force between CRGO and GOD is hydrophobic interaction, which is much stronger than the simple physical entanglement, so the reusability of CRGO-GOD was obviously enhanced compared with that of GOD@Alg. However, the enzymatic activity of CRGO-GOD was still decreasing with each cycle, and only 60% of its initial activity was remaining after recycling six times. More remarkably, when embedding CRGO-GOD into alginate beads, the reusability was further improved. For CRGO-GOD@Alg beads, above 90% of its original activity was retained even after six batches, and the activity was almost balanced after the fourth cycle. Just as described above, the enzyme showed excellent stability when immobilized by CRGO and alginate. Based on structure analysis (FTIR), the synergistic effect among CRGO, GOD and alginate in CRGO-GOD@Alg beads might play an important role. In addition, the thermal stability of free and immobilized enzyme was also investigated at 50°C (Fig. S7, ESI†). It was found that CRGO-GOD@Alg maintained the highest enzymatic activity compared with CRGO-GOD and free one when incubated at 50°C for equal time, which indicated that the thermal stability of the CRGO-GOD@Alg was obviously enhanced compared with the other two samples. This may be another reason that the reusability of the CRGO-GOD@Alg microbeads was improved compared to the other two immobilized enzymes.

3.5 Fixed Bed Reaction

Based on above experiments, the enzyme immobilized in the hybrid microbeads shows better capability for the environment tolerance and stability, and can be recycled easily by simple filtration. At the same time, with the introduction of CRGO, the physical strength of CRGO-GOD@Alg gel beads was effectively enhanced compared with that of GOD@Alg beads. So, it is desirable to achieve a continuous enzymatic reaction to avoid the cumbersome process for the catalyst recycle. Therefore, we design a continuous-flow fixed bed reactor filled with the CRGO-GOD@Alg beads to evaluate our hybrid system (Fig. 5, inset) in laboratory scale. For a fixed bed reaction, both the size and shape of the fixed bed reactor can affect the catalytic reaction. In this work, a specific column (Length/Diameter=4) was employed to be the container of the fixed bed. Based on the results and analysis above, the optimum reaction conditions of the CRGO-GOD@Alg beads were in the temperature range of 45~60°C and pH range of 4~6, respectively. So, the effect of the flow velocity and concentration of substrate on the glucose conversion was investigated on the conditions of pH 5 and 50°C (Fig. S8 and S9, ESI†). As shown in Fig. S8 and S9, the glucose conversion decreased as the flow velocity or the concentration of substrate increased. Fig. S8 showed that when the flow velocity increased from 0.05 mL/min to 0.5mL/min, the conversion of the glucose decreased from 82% to 12% after reaching equilibrium. That is because as the flow velocity getting fast, the residence time of the substrate in the reaction column became shorter, which allowed more substrate to leave the enzyme before reaction. Otherwise, the effect of the concentration of substrate on glucose conversion was also investigated in the concentration range of 0.5 mg/L to 1 mg/L at the conditions of 50°C and flow velocity on 0.05 mL/min (Fig.9, ESI†). It showed that when the substrate concentration rose from 0.5 mg/L to 1 mg/L, the glucose conversion declined from 82% to 24%. In the concentration range of 0.5 mg/L to 1 mg/L, the substrate cannot convert completely, which suggested that the amount of substrate was redundant. For a catalytic reaction, the turnover number is constant within a certain range. When increasing the substrate concentration, the ratio of reacted substrate and total substrate decreased, so the conversion decreased. Based on the data of Fig. S8 and S9, we investigated the stability and selectivity of the fixed bed reaction at 50°C with 0.5 mg/mL substrate and 0.05 mL/min flow velocity. As shown in Fig. 5, the conversion of the enzymatic reaction is above 80% to reach equilibrium after six hours, and the CRGO-GOD@Alg enzyme system can still preserve their activity even after 60 hours.

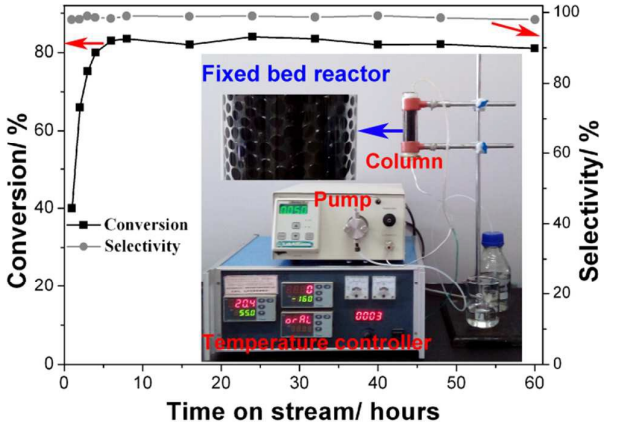


Fig. 5 The conversion and selectivity of fixed-bed reaction and the photograph of fixed-bed reactor (inset).

Furthermore, the process has a high selectivity for gluconic acid (more than 99% detected by HPLC), just like other biocatalyst system. The implementation of the continuous enzyme reaction will make it possible for the large-scale application of immobilized enzyme.

4 Conclusions

In summary, we have reported a novel and feasible method for enzyme immobilization in CRGO/alginate hybrid gel beads with non-covalent bond. The enzyme encapsulated in the hybrid microbeads showed excellent stability and environmental tolerance, and it could retain its optimum activity within a broad range (Temperature 45~60°C, pH 4~6). Additionally, the microbeads showed good mechanical strength, could be easily recycled by simple filtration and filled into a column to achieve a continuous fixed-bed enzymatic reaction, which rendered it a promising candidate for the large-scale application of immobilized enzyme. Further studies on the immobilization of various enzymes are under way in our laboratory.

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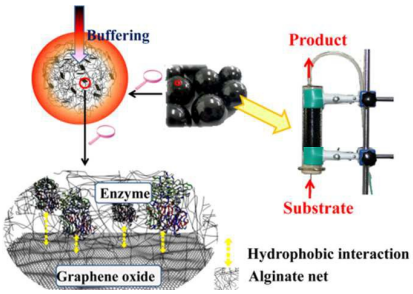
Table of Content



Hybrid bio-inorganic microbeads composed of CRGO-enzyme and alginate exhibited better stability and higher environmental tolerance, which can be used in a continuous fixed-bed enzymatic reaction.