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A graphene oxide/hemoglobin composite hydrogel for enzymatic catalysis in organic solvents†

Cancan Huang, Hua Bai, Chun Li and Gaoquan Shi*

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A graphene oxide/hemoglobin (GO/Hb) composite hydrogel was prepared for catalyzing a peroxidatic reaction in organic solvents with high yields, exceptional activity and stability.

Enzymes have been widely used as catalysts for organic reactions in organic solvents.[1] However, these enzymatically catalyzed reactions usually suffer from some inherent shortcomings, such as poor solubility of reactants and products in organic media, difficulty in recovering the enzymes from reaction systems and unwanted side reactions.[2] Moreover, the catalytic activities of enzymes in organic solvents are strongly weakened by several factors including unfavourable substrate desolvation, suboptimal pH and reduced conformational mobility.[3] To improve the activities of enzymes, several strategies have been implemented.[1,3,4] Among them, the immobilization of enzyme in an aqueous microenvironment was tested to be effective for retaining its activity in organic media. For this purpose, chemical or physical hydrogels were chosen as the supports of enzymes to provide the enzymes with an aqueous microenvironment.[4–7] Interestingly, it was found that the enzymes bound in these hydrogels showed even higher catalytic activities in organic media than those in water.[6] However, the preparation of these hydrogels usually involves chemical reactions (for chemical hydrogels) or heating treatments (for physical hydrogels). These processes may damage the structures of enzymes and decrease their activities. Therefore, a mild, convenient and cheap method for immobilizing enzymes is required. Recently, our group found that graphene oxide sheets (GO, a precursor of chemically converted graphene) could be easily assembled into a hydrogel by just shaking the mixed dispersion of GO and a crosslinker under ambient condition.[8] The GO based hydrogels were formed by various supramolecular interactions including hydrogen bonding, coordination, electrostatic interaction and π–π stacking.[8,9] Therefore, it is expected that biomacromolecules such as DNA and proteins can act as physical crosslinkers of GO sheets to form multifunctional composite hydrogels.[9] In this communication, we prepared a GO/hemoglobin (Hb) composite supramolecular hydrogel by directly mixing the dispersions of both components and explored its application as a catalyst for a peroxidatic reaction in organic solvents. The activity and stability of the enzyme-containing hydrogel were tested to be much higher than those of Hb itself in organic solvents.

GO was prepared by a modified Hummers' method (ESI†),[10] and the single-layered GO sheets have topographic thicknesses of 0.6–0.8 nm and lateral dimensions of 2–5 μm (Fig. S1, ESI†). To prepare a GO/Hb composite hydrogel, 4 mg of Hb was dissolved into 0.1 mL deionized water, in which 0.8 mL of GO solution (9 mg mL^{-1}) was added. The blended solution was shaken violently for about 15 s to form a hydrogel (Fig. 1A). The water content of this gel was calculated to be 98.5%.

The rheological properties of the composite hydrogel were studied by small-deformation oscillatory tests (Fig. 1B). The results revealed that the storage or elastic modulus (G′) of the hydrogel is about 3 times its loss or viscous modulus (G″) over the entire tested range of angular frequencies (1–100 rad s^{-1}). This phenomenon implies that elastic response is predominant and the hydrogel has a permanent network.[8] The G′ and G″ of the hydrogel are slightly sensitive to angular frequency and do not cross over each other in the tested frequency range,

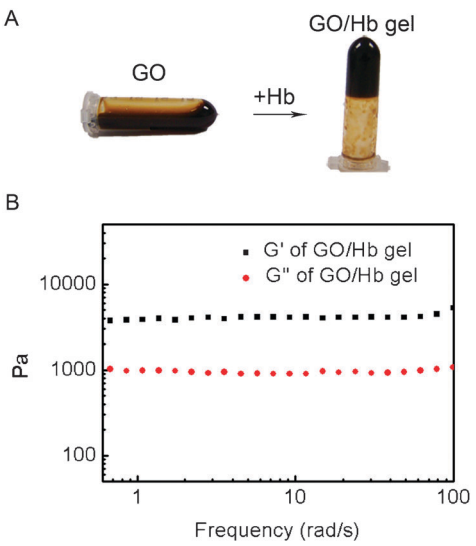

Fig. 1 The preparation of GO/Hb composite hydrogel (A) and its rheological behaviour (B).

Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology, Department of Chemistry, Tsinghua University, Beijing 100084, People's Republic of China.
E-mail: gshi@tsinghua.edu.cn

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which is a characteristic of gels with a high degree of non-covalent cross-linking. The G′ of the gel at 10 rad s^{-1} was measured to be about 4.1 kPa and this value is much higher than that of the GO solution with a concentration of 9 mg mL^{-1} (Fig. S2, ESI†). The viscosity of the GO/Hb composite hydrogel is also much higher than that of the GO solution at a given shear rate (Fig. S3, ESI†). The SEM images of both lyophilized GO solution and GO/Hb hydrogel exhibit a 3D network with pore sizes of several micrometres, indicating that the addition of Hb did not change the morphology of GO sheets (Fig. 2). The formation of GO/Hb hydrogel can be ascribed to the strong electrostatic interaction between its both components. The pH of the GO aqueous solution was measured to be 1.87, and this value is much lower than the isoelectric point of Hb (pH $\approx$ 6).[5,11] Therefore, there is an electrostatic attraction between positively charged Hb molecules and negatively charged GO sheets, which is responsible for the formation of the composite hydrogel. Hb in the composite hydrogel acted as a crosslinking reagent of the GO 3D network. This is different from immobilization of enzyme in other conventional hydrogels, in which enzyme is not an essential component. Since the electrostatic attraction between Hb and GO is a weak intermolecular interaction, the structure of Hb has not been changed significantly in the hydrogel. As a result, the hydrogel kept the catalytic activity of the enzyme.

To test the catalytic activity of the composite hydrogel, the oxidation of pyrogallol by H_2O_2 (Fig. 3A) was chosen as a model reaction. We evaluated the catalytic performance of the GO/Hb composite hydrogel by measuring the reaction rate, which can be calculated from the absorbance changes of the product, purpurogallin, at 425 nm (Fig. S4, ESI†).[12] Since purpurogallin is liposoluble, we chose several hydrophobic solvents including methylene dichloride, toluene, and chloroform as the reaction media. Taking the reaction in methylene dichloride as an example, one can see that the GO/Hb composite gel exhibits higher stability than those of pure Hb and the GO in methylene dichloride (Fig. 3B). The absorbance at 425 nm in the system catalyzed by GO/Hb gel increased continually in the first 1.5 h, indicating that the gel has high catalytic activity in this period (Fig. 3B). However, the activity of the GO/Hb gel gradually decreased during the successive reaction process, showing that the product and surrounding residues inhibited the catalytic activity of the hydrogel (Fig. S5, ESI†).[16] For the system with free Hb molecules, the enzyme lost its activity after 30 min and the product concentration reached a plateau. Pure GO showed a stable but very low activity in the whole experiment period. The high stability of the composite hydrogel provided an aqueous microenvironment for protecting

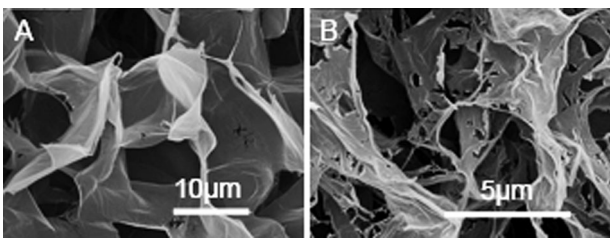

Fig. 2 SEM images of freeze dried GO solution (9 mg mL^{-1}) (A) and GO/Hb composite hydrogel (B).

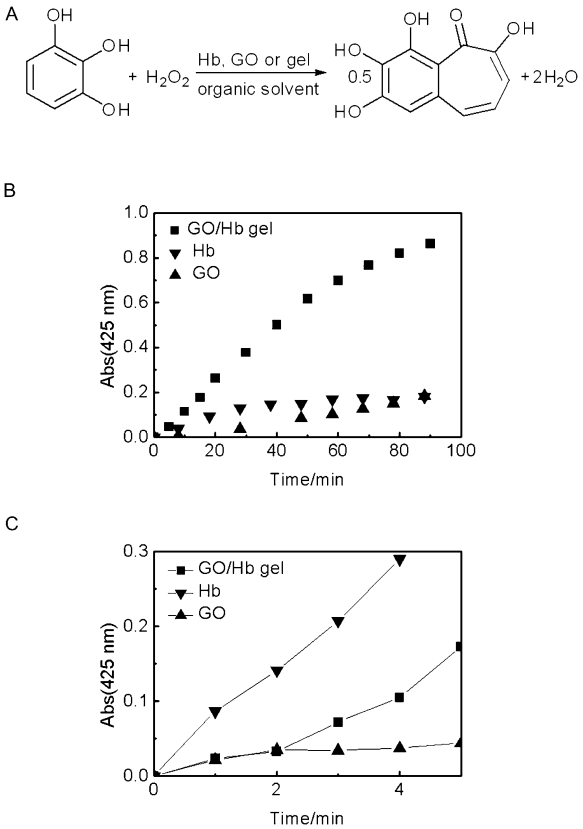

Fig. 3 (A) Scheme of the peroxidatic reaction; (B) the extended 90 min reaction courses in methylene dichloride with different catalysts; (C) the reaction courses for the first 5 min in methylene dichloride with different catalysts.

Hb from deactivation induced by an organic solvent. Moreover, the yield of the reaction catalyzed by the GO/Hb hydrogel was about 4 times that catalyzed by free Hb after reaction for 1.5 h. Thus, the catalytic activity of the hydrogel is much higher than that of pure Hb or GO in a long reaction time. However, in the first 5 min, the reaction rate of the system containing Hb is higher than that of the system with the GO/Hb hydrogel or GO (Fig. 3C). After the addition of the catalyst (*e.g.* GO/Hb hydrogel) into the reaction system, a microinterface between the water phase and organic solvent was formed. Since the substrate, pyrogallol, is hydrophilic and insoluble in organic solvents, its molecules were accumulated at the surfaces of the gel, and then crossed the interface to enter the gel for reaction. Therefore, this reactant diffusion process took time and limited the initial composite gel. However, the free Hb lost its activity in a short time because of the organic circumstance. In summary, free Hb molecules have higher initial catalytic activity but a shorter reaction rate. However, for the reaction catalyzed by free Hb, a small amount of Hb and substrate dissolved in H_2O_2 solution immediately for the reaction to occur. This is a relatively fast process. Thus, its initial reaction rate is higher than that catalyzed by the lifetime. On the other hand, the GO/Hb composite hydrogel has a lower apparent activity in the initial stage of the reaction, while it can maintain its activity for a much longer time, which results in a higher average activity in the whole reaction period. The interrelated catalytic performances of the GO/Hb hydrogel in toluene

Table 1 The apparent Michaelis–Menten constant (K_m) and maximum initial velocity (V_{max}) of a GO/Hb hydrogel catalyzed oxidation of pyrogallol in different organic solvents

Solvent	K_m/mM	V_{max}/min^{-1}
Methylene dichloride	0.1255	0.05016
Toluene	0.3697	0.03940
Chloroform	0.5049	0.04579

and chloroform are similar to that in methylene dichloride (Fig. S6, ESI†), indicating the applicability of the hydrogel in different solvents.

As shown in Fig. 3B, we also observed a low catalytic activity of pure GO, which is also reported by other researchers.[13] Therefore, the catalytic activity of GO/Hb hydrogel can be partly attributed to the synergetic effect of both components. Except the synergetic effect, several other factors also have contributions to the high activity of the GO/Hb hydrogel. First, hydrophilic pyrogallol molecules entered the aqueous microenvironment of the hydrogel to form a micro-reaction system.[6,14] Second, the GO/Hb gel has a 3D network constituted by 2D GO sheets, and the pore sizes of the network are as large as 5–10 μm (Fig. 2). Thus, the molecules of reactants or product could be easily diffused to or released from the reaction system. Third, pyrogallol is hydrophilic and purpurogallin is hydrophobic, which promoted the indiffusion of a reactant to the hydrogel and the outdiffusion of the reaction product. Moreover, the outdiffusion of the product reduced catalyst inhibition, improving the activity of GO/Hb hydrogel.

We also investigated the catalytic kinetics of the hydrogel in different organic solvents. It is assumed that the reaction occurred only in the gel, and the product diffused out the gel as soon as it was produced. The reaction rates in the first five minutes were measured to evaluate the activity of the catalyst. Two kinetic parameters, maximum initial velocity (V_{max}) and Michaelis–Menten constant (K_m), calculated from the Lineweaver–Burk plots[15] are list in Table 1 (see ESI†). According to this table, the V_{max} measured in methylene dichloride is higher than those observed in toluene and chloroform. However, the apparent K_m of the GO/Hb hydrogel in the first solvent is lower than those in the latter two media. These results indicate that methylene dichloride is the best among these three solvents for the reaction, most possibly due to its suitable polarity. It should be noted here that the reaction product can be collected and purified simply by decantation to remove the hydrogel. It was also found that the GO/Hb composite gel had an improved thermal stability. Hb usually should be stored in relatively low temperatures to keep its activity. In contrast, the GO/Hb composite gel can maintain its activity even after keeping it at room temperature for more than two weeks (Fig. S7, ESI†). Thus, the reaction system developed here has potential industrial application.

In summary, we have demonstrated that 2D GO sheets are able to form stable composite hydrogels with Hb. The hydrogels showed higher activity and stability than free Hb or GO when they were utilized for catalyzing oxidation of pyrogallol in organic solvents. The catalytic activity of the composite hydrogel could be preserved even after it was stored at room temperature for a long time. In the system, the hydrogels provide aqueous microenvironment to protect the enzyme from deactivation, and it acted as the transit station to attract the substrate and exclude the product. Considering the easy separation of the catalyst and the biocompatibility of GO, our composite hydrogel has great potential in industrial applications, such as organic synthesis and enzymatic biotransformations.

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