

Molecular hydrogel-immobilized enzymes exhibit superactivity and high stability in organic solvents†

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This communication describes the use of a molecular hydrogel to immobilize enzymes for catalyzing reactions in an organic solvent to attain superactivity and exceptional stability.

Despite that enzymatic catalysis in organic solvents offers some technological advantages¹ (e.g., enhanced stability of enzymes, easy recovery of products, and novel biosynthesis) for a variety of applications and has already led to some successful commercial processes,^{1,2} enzymes, however, often display drastically lower activity in organic solvents than in water.³ It is believed that the water layer on the molecular surface of enzymes determines their activity in organic media,¹ and there are three major causes of the low activity—unfavorable substrate desolvation, suboptimal pH, and reduced conformational mobility.³ Among several known approaches⁴ to remedy these problems, it is quite effective to immobilize enzymes within an aqueous microenvironment in the organic solvent. For example, enzymes bound inside polymer hydrogels⁵ or organic plastics⁶ show enhanced activity and stability relative to native enzymes in organic media, and the enzymes within the water phase of reverse micelles^{7,8} exhibit near or even higher activity in organic media than that in water. These promising results led us to develop molecular hydrogels as new materials to immobilize enzymes for catalysis in organic media.

Although molecular hydrogels, formed by self-assembly of amphiphilic molecules, have served as scaffolds for tissue engineering, medium for screening inhibitors of enzymes, and biomaterials for wound healing,⁹ their application as matrices for enzymatic reactions in organic media has yet to be explored. In this work, we mixed enzymes into the molecular hydrogel formed by the self-assembly of two simple derivatives of amino acids (**1** and **2**) and carried out oxidation reactions in organic media (Fig. 1). We found that the enzymes enclosed in the molecular hydrogel exhibited higher activity (e.g., up to eight times) in the organic media (e.g., toluene) than that of unconfined enzymes in water. Moreover, the molecular hydrogel acts as a more effective carrier of enzymes in organic solvents than a polymeric hydrogel does, suggesting a unique role of the self-assembled nanofibers in molecular hydrogels for the observed superactivity and stability because covalently crosslinked random coils form the network in polymeric hydrogels and self-assembled nanofibers of amphiphilic molecules as the network in molecular hydrogels. This result is particularly significant because it implies that tailoring the

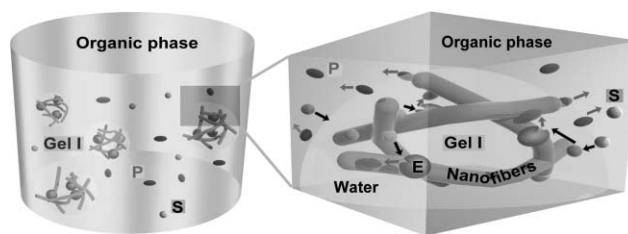
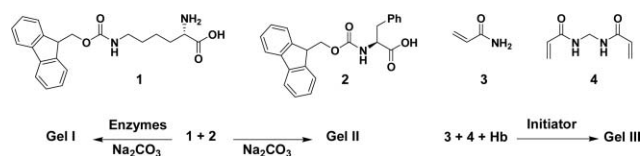


Fig. 1 Illustration of the molecular hydrogel-immobilized enzymes that catalyze a reaction in organic media (E: enzymes; S: substrates; P: products).

nanofibers *via* the control of the structure of hydrogelators may provide an optimal microenvironment for enzyme-catalyzed biotransformations.¹⁰

It is simple and straightforward to make a molecular hydrogel for confining an enzyme (Scheme 1). The mix of sodium carbonate (20 mg), Fmoc-L-lysine (**1**, 36 mg), and Fmoc-L-phenylalanine (**2**, 38 mg) into 0.9 mL water gives a suspension, which turns into a clear solution upon heating to about 333 K. The addition of 0.1 mL methemoglobin solution (Hb, 40 mg) into the solution at 308–313 K and subsequent cooling to room temperature affords gel I. A similar procedure allows the immobilization of other enzymes (e.g., horseradish peroxidase (HRP, 50 U), laccase from *trameters versicolor* (Laccase, 3.6 U), or alpha-chymotrypsin from bovine pancreas (Alpha-CT, 100 U)). Without the addition of enzymes, the same process produces gel II as a control. A crosslinked poly(acrylamide) hydrogel⁵ containing Hb serves as another control (gel III).

The rheological test (Fig. 2(a)) confirms the elastic nature of gels I and II.¹¹ The dynamic storage modulus of gel I is ten times lower than that of gel II, indicating that crosslinks in gel I exist at lower density than that in gel II and suggesting that the interaction of Hb with the nanofibers decreases the density of the crosslink. TEM images (Fig. 3(a), (b)) reveal that Hb molecules aggregate and shorten the nanofibers (~16 nm in diameter) made of the hydrogelators, thus reducing the density of crosslink in gel I.¹¹ Both AFM and TEM images (Fig. 3) indicate that Hb molecules mainly locate at the crosslink sites of the nanofibers, which agrees with the rheological data. Little release of Hb from gel I into



Scheme 1 The preparation procedure of gels I, II and III.

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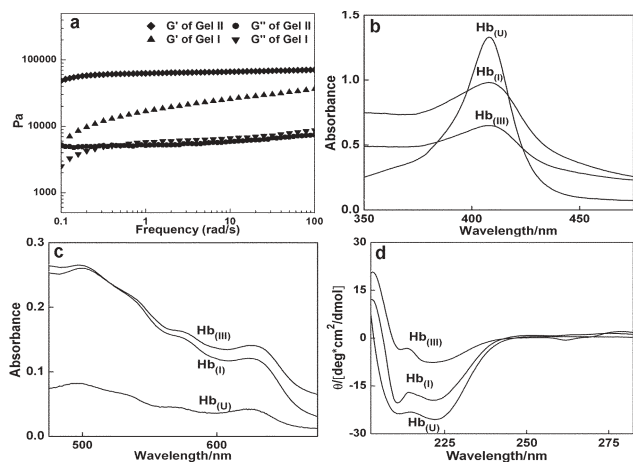


Fig. 2 (a) Frequency dependence of the dynamic storage modulus (G') and the loss modulus (G'') of hydrogel with and without Hb at a strain of 2%. (b) and (c) UV-Vis spectra of the $Hb_{(U)}$, $Hb_{(I)}$ and $Hb_{(III)}$ at various wavelengths. (d) CD spectra of $Hb_{(U)}$, $Hb_{(I)}$, and $Hb_{(III)}$.

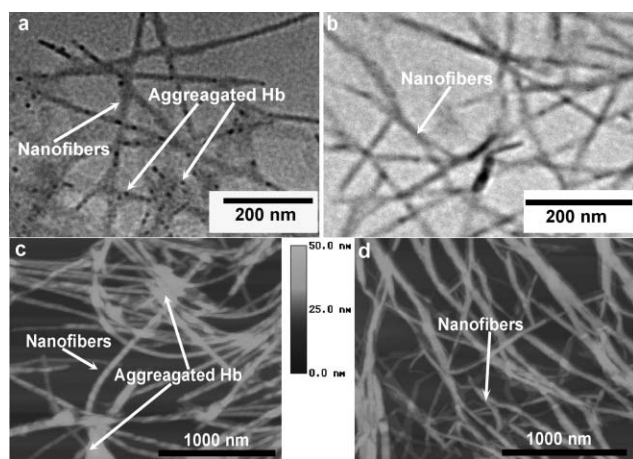


Fig. 3 TEM images of (a) gel I and (b) gel II. AFM images of (c) gel I and (d) gel II.

solvents¹¹ also confirms that the non-covalent interaction between the enzyme and the nanofibers is strong enough to ensure the immobilization.

We used UV-Vis and circular dichroism (CD) spectra to probe the state of Hb in different matrices. As shown in Fig. 2(b), the almost same absorption peaks at 408 nm of the unconfined Hb ($Hb_{(U)}$), gel I bound Hb ($Hb_{(I)}$), and gel III bound Hb ($Hb_{(III)}$) indicate the undisturbed spectral activity of the active heme group.¹² $Hb_{(U)}$, $Hb_{(I)}$ and $Hb_{(III)}$ all have a series of weak absorbances at about 500 and 630 nm (Fig. 2(c)), which indicate the same $HbFe^{III}$ form of the three Hbs. The CD spectra of the three Hbs (Fig. 2(d)) show the double absorbance at 210 and 222 nm, which are characteristic peaks of the α -helix. No change in the peaks of the CD spectra indicates little or no structural difference among $Hb_{(U)}$, $Hb_{(I)}$ and $Hb_{(III)}$. The employed spectroscopic methods have confirmed that the Hb remains at the same state as the unconfined form in water when it was immobilized by the hydrogels.

The $HbFe^{III}$ form of the three Hbs allow them to serve as a substitute for peroxidase¹² for catalyzing oxidation in different

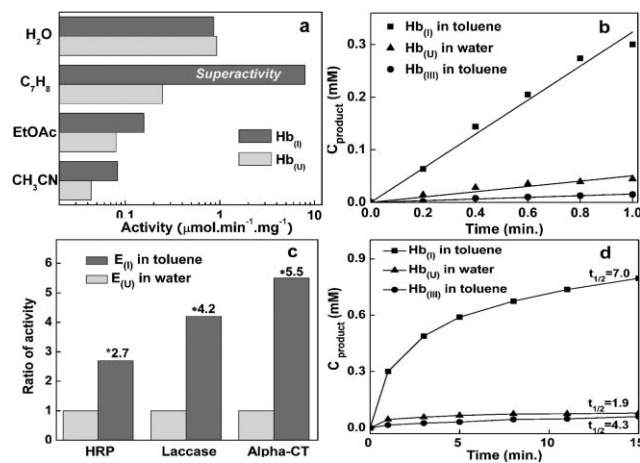


Fig. 4 (a) Comparison of activities of $Hb_{(I)}$ and $Hb_{(U)}$ in various media. (b) The reaction course in the first minute of pyrogallol (10 mM) and H_2O_2 (30 mM) catalyzed by various Hb (0.1 g L^{-1}) displays zero-order kinetics (all $r > 0.99$), and is therefore used to calculate the initial rate. (c) The ratios of activities of $E_{(I)}$ (I: immobilized by the molecular hydrogel) in toluene and $E_{(U)}$ (U: unconfined) in water.¹⁰ The observed activities of $E_{(U)}$ in water are indicated. (d) The extended 15 min reaction course of (b). All the concentrations were calibrated according to the molar extinction coefficient of the product in different solvents.¹¹

solvents at room temperature. Using the oxidation of pyrogallol by H_2O_2 as the model reaction, we obtained the activity of Hb by monitoring the concentration of purpurogallin.¹¹ The control, gel II, exhibits no activity.¹¹ As shown in Fig. 4(a), $Hb_{(I)}$ exhibits almost the same activities as $Hb_{(U)}$ in water, suggesting that the structures of $Hb_{(I)}$ and $Hb_{(U)}$ differ little, which agrees with the UV-Vis and CD analysis. $Hb_{(I)}$ exhibits higher activities than $Hb_{(U)}$ in the same organic media tested, confirming the protective effect of the aqueous microenvironment provided by gel I. The activities of both $Hb_{(I)}$ and $Hb_{(U)}$ increase with a decrease of the polarity of the organic solvent (from acetonitrile to toluene), which agrees with the established trend of the activity of an enzyme in an organic solvent.¹³ The Lineweaver–Burk plots constructed by the initial reaction rates at different pyrogallol concentrations are used to estimate their kinetic constant values. As shown in Table 1, the activity of $Hb_{(I)}$ in toluene ($7.98 \mu\text{mol min}^{-1} \text{mg}^{-1}$) is eight times that of $Hb_{(U)}$ in bulk water ($0.92 \mu\text{mol min}^{-1} \text{mg}^{-1}$). According to Fig. 4(b), the initial rate of $Hb_{(I)}$ at 10 mM pyrogallol concentration is much larger than that of $Hb_{(U)}$ and $Hb_{(III)}$. These results represent the first observation of the superactivity of an enzyme immobilized in a medium other than reverse micelles.⁸ Other enzymes immobilized in the molecular hydrogel also display superactivity in organic media (Fig. 4(c) and Table 1), indicating that the superactivity is generally conferred by the molecular hydrogel.

We suggest that it is likely that several factors contribute to the superactivity of $Hb_{(I)}$. (i) Hydrophilicity promotes the substrate (*i.e.*, pyrogallol) across the microinterface to enter the hydrogel, similar to the case of reversed micelles.^{7,8} (ii) Amphiphilic character¹⁴ and/or the molecular superstructure of the self-assembled nanofibers in gel I may assist the substrates to approach Hb and the products to leave Hb. This assumption agrees with the much lower activity of $Hb_{(III)}$ (*i.e.*, Hb immobilized by a randomly-crosslinked poly(acrylamide) hydrogel) than that of $Hb_{(I)}$. (iii) The

Table 1 The catalytic data of the hydrogel-immobilized enzymes in toluene and the unconfined enzymes in water

Enzyme	Substrate	Product	Activity (half-life/min) of E _(U) in H ₂ O	Activity (half-life/min) of E _(I) in toluene	Ratio of activity ^a
Hb	Pyrogallol	Purpurgallin	0.92 ^b (1.9)	7.98 ^b (7.0)	8.7
HRP	Pyrogallol	Purpurgallin	802 ^c (2.8)	2165 ^c (5.3)	2.7 ^b
Laccase	<i>o</i> -Phenyldiamine	Phenazine-2,3-diamine	54.6 ^c (4.2)	229.3 ^c (13.6)	4.2 ^b
Alpha-CT	<i>N</i> -Benzoyl-L-tyrosine- <i>p</i> -nitroanilide	<i>p</i> -Nitroanilide	1.06 ^c (5.9)	5.83 ^c (28.4)	5.5 ^b

^a Ratio of activity: activity of E_(I) in toluene/activity of E_(U) in water. ^b μmol min⁻¹ mg⁻¹. ^c nmol min⁻¹ U⁻¹.

large pore sizes of the nanofibrous networks in gel I (TEM and AFM confirm 0.2–2 μm and 5–6 nm pores in gel I and III,¹¹ respectively) facilitate the mass transport in gel I.

We also found that molecular hydrogels significantly improve the stability of the enzymes. As shown in Fig. 4(d), Hb_(I) has improved stability in toluene compared with that of Hb_(U) in water. The quantitative analysis of their reaction course shows the highest stability of Hb_(I) in toluene, as indicated by their half lives (*t*_{1/2}) of Hb. To evaluate the potential industrial application of the system, we chose 2-aminophenol (**3**) as another substrate for Hb_(I) catalyzed oxidation in toluene because the oxidative product of **3** is 2-amino-3*H*-phenoxazin-3-one (**4**, a useful antibiotic, questiomycin A). The initial rate of Hb_(I) in toluene is slightly lower than that of Hb_(U) in water, indicating that the superactivity is also substrate dependent, a characteristic feature of enzymes. The molecular hydrogel significantly improves the stability of Hb_(I) in toluene (*t*_{1/2} = 27.8 min) and leads to the additional production of **4** in an hour (Fig. S6(a)¹¹). We also employed this reaction to test its stability as a recovered catalyst (Fig. S6(b)¹¹). The first run achieved 98% conversion of **3**, and the second and third runs of reused Hb_(I) obtained 97.0 and 95.0%, respectively. Almost the same conversion of the first and third runs indicates that the Hb_(I) in toluene can be reused without losing activity. Two plausible reasons can explain the observed high stabilities: first, the molecular hydrogel provides an aqueous microenvironment that protects the enzyme from deactivation by the organic solvent. Second, the relatively large pore size and amphiphilic nature of the molecular hydrogel facilitate the transport of the product back to the organic phase, thus reducing inhibition of the catalyst. The second reason also explains the short *t*_{1/2} of Hb_(III) in toluene (*t*_{1/2} = 14.1 min)¹¹ on account of the trapping of the product in the hydrogel due to small pore sizes.

As summarized in Table 1, for different enzymes and substrates, the immobilization of the enzymes in the molecular hydrogels all achieve superactivity in toluene relative to unconfined enzymes in water. The observation of overall (or apparent) superactivity⁸ indicates that molecular hydrogels provide a unique aqueous microenvironment in which to carry out enzymatic reactions in an organic solvent. Our observation also suggests that molecular hydrogels may lead to a general strategy, which combines the reusability of polymer hydrogels and the high activity of reversed micelles, to perform enzyme catalyzed biotransformations in organic media. The self-assembled nanofibers in molecular hydrogels also offer a new opportunity to engineer the immobilization medium in organic solvents for superactivity, high operational stability, and reusability of enzymes, which ultimately

will benefit industrial biotransformations. Moreover, the principle illustrated in this work may allow the immobilization of catalysts in organogels¹⁵ to carry out reactions in water.¹⁶ Our future work will expand this general strategy to a variety of catalysts and gels.

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