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Thermal responsive microgels as recyclable carriers to immobilize active proteins with enhanced nonaqueous biocatalytic performance†

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We describe the preparation of a thermoresponsive microgel, which can non-covalently immobilize active proteins with enhanced biocatalytic performance in organic solvents and easy reusability due to the porous microstructure and temperature responsive property.

The immobilization of enzymes aims at solving the problems of catalytic efficiency, stability and reusability of enzymes to some extent,[1] which is exceedingly important for the application of enzymes in various fields, including tissue engineering, fine chemical industry, bioremediation, and so on.[2] One important approach to improve the activity and stability of the immobilized enzymes is to explore suitable carrier materials with low diffusional limitation, large specific surface area, and high enzyme loading. The entrapped proteins can interact with solid supports by covalent coupling and non-covalent forces. The robust chemical crosslinking method includes glutaraldehyde molecules[3] and Lentikats® polymers,[4] which exhibit higher stability but heavy activity loss due to the change in enzyme structure. Recently, nano-structured materials[5] have been applied to mildly immobilize proteins by non-covalent forces. Hamachi *et al.* have reported that supramolecular hydrogels can preserve the active sites of enzymes and serve as a transportable scaffold for biological activity.[6] Therefore, non-covalent incorporation of enzymes into hydrogels is an alternative method.[7] Moreover, the enzymes immobilized within peptide-based supramolecular hydrogels can even exhibit apparent "superactivity" due to their hydrophilic nature in the network.[7a,8]

Relative to bulk hydrogels, microgels have several important advantages, namely, good control over the particle size, fast response to external stimuli and low diffusional limitation. In addition, due to the uniqueness of exhibiting stimulus-responsive behaviour to external stimuli like temperature, pH and ionic strength, the responsive microgels are particularly attractive carriers for encapsulating drugs and proteins for drug delivery and biosensor applications.[9] Therefore, the enzymes immobilized in responsive microgels incorporating these properties would exhibit particularly fascinating performance in biological catalysis.

Here, a typical thermoresponsive microgel was prepared by copolymerization of *N*-isopropylacrylamide (NIPAM) with acryloylated bovine serum albumin (BSA), non-covalently immobilized bioactive hemoglobin (Hb) or horseradish peroxidase (HRP) *via* a swelling effect at low temperature. This microgel serves as an efficient matrix for heterogeneous biocatalysis in various solvents. As a cyto-compatible component, BSA is the most ample protein in plasma and acts as a carrier for various compounds in the blood stream. As a thermo-sensitive component, poly(*N*-isopropylacrylamide) (PNIPAM) has been attracting increasing attention due to its uniqueness of undergoing a volume phase transition around 32 °C; moreover, this transition is perfectly reversible.[10] Therefore, the loading of Hb can be mildly realized by lowering the temperature to approx. 4 °C. After raising the temperature to room temperature (approx. 25 °C), the immobilized active proteins (Hb and HRP) within the microgel will not be expelled from the network due to the non-covalent interaction with the microgel. Sequentially, the enzymes entrapped in the microgel would be removed out of the network because of the weakening of hydrogen bonds by raising the temperature above the lower critical solution temperature (LCST). At last, the reproducible shrinking and re-swelling is beneficial to the recyclability of both enzymes and the matrix in biocatalysis.

Preparation of this microgel is very simple. Fig. 1 illustrates the detailed procedure to prepare the microgel *via* an inverse emulsion polymerization method.[11] The W/O inverse emulsion was created by dropwise addition of aqueous solution to the continuous oil phase and then sonication. Span80 (1.25%, w/w) dissolved in mineral oil constituted the continuous oil phase, while the aqueous phase was formed by acryloylated BSA and NIPAM. The emulsion was homogenized by sonication and then initiated by γ-ray irradiation for polymerization. Incorporation of enzymes into the microgel was carried out by alternating changes in the external temperature. A constant amount of microgel was mixed with a certain concentration of enzyme solution and stored at 4 °C for 24 h to achieve equilibration. Then, the whole solution containing the microgel was transferred to room temperature (approx. 25 °C). At last, the Hb containing microgel was separated from the solution *via* centrifugation. The amount of the

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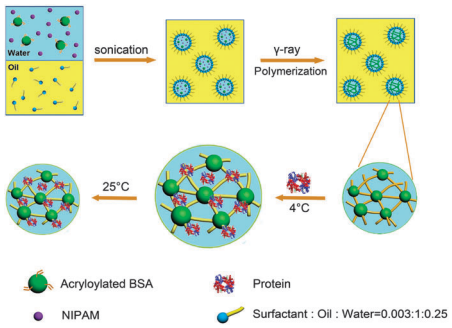

Fig. 1 The scheme for the preparation of microgels and their application as active protein carriers.

bound active protein was calculated by subtracting the residue from the total amount in solution analyzed by UV-Vis spectroscopy. The bound active protein has less than 5% loss in the 24 h incubation period.

We investigated the temperature-dependent sizes of the selected microgels using SEM and DLS measurements. Fig. 2a and b show the SEM morphology of the freeze-dried microgel particles, indicating preferably spherical shapes and monodispersity. Fig. 2c and d display the temperature-dependent variation in the average diameter of the particles from 10 °C to 55 °C. DLS measurements were executed to determine the hydrodynamic diameter (D_H) of the microgels and their size distribution at different temperatures varying from 4 °C to 55 °C. The measurements were executed after allowing the sample to equilibrate for 5 min at each temperature. The change in hydrodynamic diameter (D_H) as a function of temperature is displayed in Fig. 2c. It reveals that D_H of the microgel becomes smaller with the increase of temperature, illustrating that the microgel exhibits pronounced thermosensibility. The value of the swelling ratio (α) was found to be 53.0 by calculating the volume ratio before and after phase transition according to the literature.[12] The high value of α indicates the excellent swelling behavior of the microgel, which makes the microgel suitable to encapsulate the enzymes by lowering the temperature to approx. 4 °C.

The H_2O_2 oxidation of *o*-phenylenediamine (OPD) to phenazine was selected as a model reaction to explore the catalytic activity of

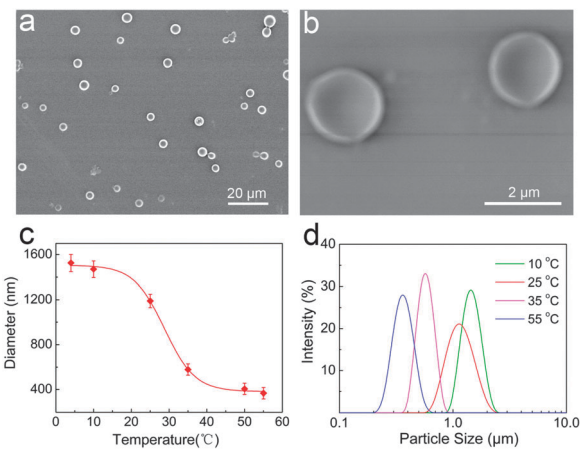

Fig. 2 SEM images of the microgels (a and b), hydrodynamic diameter of the microgels as a function of temperature (c), and particle size distribution of the microgels from 10 °C to 55 °C (d).

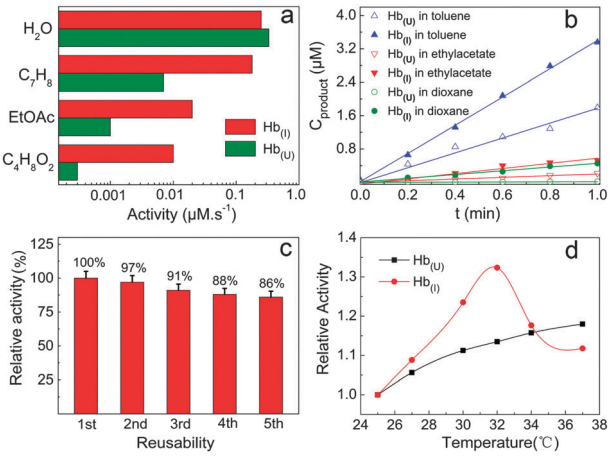

Fig. 3 (a) Comparison of activities of $Hb_{(U)}$ and $Hb_{(I)}$ in various media. (b) The initial reaction course of *o*-phenylenediamine (OPD) (10.0 mM) and H_2O_2 (40.0 mM) in four kinds of organic solvents catalyzed by various Hbs (0.1 g L^{-1}). (c) The reusability of $Hb_{(I)}$ in toluene. (d) The relative catalytic activity of $Hb_{(U)}$ and $Hb_{(I)}$ at temperatures from 25 °C to 37 °C in water.

unconfined Hb ($Hb_{(U)}$) and immobilized Hb ($Hb_{(I)}$). As shown in Fig. 3a and b, the OPD oxidation reaction was demonstrated in different solvents, *e.g.*, including toluene, ethylacetate, and dioxane, respectively. And the kinetic constants of $Hb_{(U)}$ and $Hb_{(I)}$ in various environments are listed in Table 1. From Fig. 3a, it can be observed that the activity of $Hb_{(I)}$ is almost the same as that of $Hb_{(U)}$ in water. The microgel without Hb has no activity under the same conditions. $Hb_{(I)}$ exhibits higher activity than $Hb_{(U)}$ in toluene, ethylacetate, and dioxane. The results indicate that the microgel can provide the aqueous microenvironment within organic solvents. The activities of both $Hb_{(U)}$ and $Hb_{(I)}$ increase with the decrease of the polarity of the organic solvent. As shown in Fig. 3b, $Hb_{(I)}$ exhibits the maximum initial rate in hydrophobic toluene. Relative to $Hb_{(U)}$ in other organic solvents, $Hb_{(I)}$ also shows observable enhancement in the initial reaction rate. Even in the most hydrophilic dioxane, $Hb_{(I)}$ still has a certain activity, while $Hb_{(U)}$ powder shows almost no catalytic ability.

Various kinetic constants of various Hbs were employed to demonstrate the effect of the carrier and the solvent. The kinetic constant values of Hb were calculated from the Lineweaver–Burk plots established by the initial reaction rates at different *o*-phenylenediamine (OPD) concentrations (Fig. S1, ESI†). As shown in Table 1, the activity of $Hb_{(I)}$ (V_{max} = 0.25 μM s^{-1}) is 0.76 times that of $Hb_{(U)}$ (0.33 μM s^{-1}) in bulk water, which does not display the positive effect of immobilization. However, in the organic solvents, $Hb_{(I)}$ exhibits enhanced activity relative to $Hb_{(U)}$. The K_{cat} values of $Hb_{(I)}$ in toluene

Table 1 Reaction kinetic constants of various Hbs in organic solvents and water

	Solvent	V_{max} (μM s^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})
$Hb_{(I)}$	Toluene	0.18	16.17	0.12	0.007
	Ethylacetate	0.02	2.85	0.014	0.005
	Dioxane	0.01	1.73	0.007	0.004
	Water	0.25	0.78	0.17	0.22
$Hb_{(U)}$	Toluene	0.007	3.57	0.005	0.002
	Ethylacetate	0.001	—	0.0007	—
	Dioxane	0.0003	—	0.0002	—
	Water	0.33	1.05	0.23	0.22

(log P = 2.5), ethylacetate (log P = 0.73), and dioxane (log P = −1.1) were 25.7, 20, and 33 times higher than those of $Hb_{(U)}$, respectively. This trend indicates that the activity of $Hb_{(I)}$ appears to be affiliated with the solvent polarity. The enhancement of the solvent hydrophobicity (log P) prompts the higher catalytic activity. Based on the V_{max} values presented in Table 1, $Hb_{(U)}$ follows evidently the same tendency as $Hb_{(I)}$ in biological catalytic activity. This correlation is consistent with the theoretical explanation, which illustrates the enzyme catalytic ability in organic solvents.[13] The low intrinsic activity of $Hb_{(U)}$ initiated by the polar solvent may be due to their high hydrophilicity to remove the essential water layer around the Hb surface. Thus, the enhanced activity of the $Hb_{(I)}$ in the microgel could be attributed to the aqueous environment which prevents the water extraction from the Hb surface by organic solvents. Another active protein, HRP, displays much higher catalytic ability and similar trends in organic solvents relative to Hb for the same biocatalytic reaction (Fig. S2 and Table S1, ESI†). The V_{max} of immobilized HRP can reach about 74% of that in water. In toluene, the immobilized HRP can achieve 10-fold activity compared to the unconfined one.

In the three selected organic solvents, the K_m (Michaelis constant) value for the encapsulated enzyme was found to increase with the enhancement of hydrophobicity of the solvent (from dioxane to toluene). Because of the low catalytic activity, the kinetic parameter K_m for $Hb_{(U)}$ can only be measured in toluene, among the three kinds of organic solvents used for investigation. It is apparent from Table 1 that $Hb_{(I)}$ has the minimum value for K_m in water solution, which suggests the increasing affinity of the enzyme toward substrate binding. Moreover, the catalytic specificity constant (K_{cat}/K_m) of the entrapped enzyme is almost the same as that of the free enzyme in water, also indicating the high binding capacity of the substrate to enzyme. These approximative kinetic constants confirm that the immobilization of the microgel evidently reduces the diffusional limitation of reactants and products within the microgel. This is significantly different from the enzyme encapsulated in other bulk carriers, which are usually subject to remarkable mass transfer limitations in an aqueous medium.[14]

The microgel can impressively improve the recyclability of $Hb_{(i)}$ in toluene. To test this activity, we compared the fresh and recovered bound Hb during the 15 min oxidation of 3 mM OPD in toluene at 25 °C, respectively. As shown in Fig. 3c, the amount of product in the fifth run reaches 86.0% of that in the first run in toluene. Moreover, the reaction product can be collected and purified easily by decantation to remove the microgel. Therefore, our microgel exhibits good reusability in toluene without denaturation. After raising the temperature to 55 °C, the Hb in the microgel would be removed out of the network because of the weakening of hydrogen bonds. The amount of Hb can only be less than 10% after this expulsion, which proves our hypothesis. At last, the incubation in Hb solution at 4 °C can reload fresh Hb into the microgel matrix. The activity of the reloaded Hb in the microgel can reach about 95% of that of the fresh $Hb_{(I)}$.

The thermal responsive property also affects the biocatalytic performance of the $Hb_{(I)}$ in our microgels. At first, the increasing slope of activity for $Hb_{(I)}$ is much higher than that for $Hb_{(U)}$ (Fig. 3d). The increased hydrophobicity of the polymer network at rising temperature leads to the amplification of hydrophobic interactions between the microgels and the substrates, which could be the reason for the excellent activity of $Hb_{(I)}$. This result is also supported by the investigation of the immobilization of enzymes, which differ in their sensitivity to hydrophobic environments.[15] The $Hb_{(I)}$ in the microgel shows a slowing down of the catalytic rate at temperatures above 32 °C. The suitable operative temperature should be from 25 to 32 °C. In contrast, $Hb_{(U)}$ always shows a slow increase of the catalytic rate along with the increasing temperature. The shrinkage of the microgel network above the transition temperature can lead to the inconvenient diffusion of the product and slow activity.

In summary, we demonstrate a simple and useful approach to immobilize enzymes in the thermo-sensitive microgel by changing the temperature from 4 °C to 25 °C. The desirable characteristics of microgel-immobilized Hb with enhanced catalytic performance have also been shown in aqueous and organic solvents. The catalytic activity of the enzymes can also be modulated by temperature over a certain range. Our microgel is emerging to be a recyclable enzyme-immobilizing carrier for a large variety of applications.

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