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Enzyme Mimetics

A Supramolecular-Hydrogel-Encapsulated Hemin as an Artificial Enzyme to Mimic Peroxidase**

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A challenge in chemistry is an artificial enzyme[1] that mimics the functions of the natural system but is simpler than proteins. The intensive development of artificial enzymes that use a variety of matrices,[2] the rapid progress in supramolecular gels,[3] and the apparent "superactivity" exhibited by supramolecular hydrogel-immobilized enzymes,[4] prompted us to evaluate whether supramolecular hydrogels will improve the activity of artificial enzymes for catalyzing reactions in water or in organic media. To demonstrate the concept, we used hemin as the prosthetic group to mimic peroxidase, a ubiquitous enzyme that catalyzes the oxidation of a broad range of organic and inorganic substrates by hydrogen peroxide or organic peroxides. The structures of the active site as well as the reaction mechanism of peroxidases are well studied.[5] Much effort has been focused on incorporating metalloporphyrins[6] into protein-like scaffolds[7] in the quest towards peroxidase mimetics, which do not show satisfactory activity and selectivity mainly owing to the lack of the peptidic microenvironment that exists in the native peroxidase. The shape of the protein pocket and the amino acid residues or functional groups that surround the active site bring about the special inclusion behavior between the enzyme and the substrate. As a result, β-cyclodextrins (β-CDs), which are a frequently used model system, act as an excellent enzyme model owing to their appropriate size and their fairly rigid and hydrophobic cavities that provide favorable binding of the prosthetic group as well as substrates. Experimentally, β-CD-modified hemins have showed higher activity relative to free hemin,[8] suggesting that supramolecular hydrogels may act as an alternative matrix to encapsulate hemin for the mimetic of peroxidase.

Supramolecular hydrogels, formed by the self-assembly of nanofibers of amphiphilic oligopeptides[9,10] or small molecules,[11–13] have served as scaffolds for tissue engineering,[9] a medium for screening inhibitors of enzymes,[12,14] a matrix for biomineralization,[15] and as biomaterials for wound healing.[13] The application of supramolecular hydrogels as the skeletons of artificial enzymes has yet to be explored. Similar to peptide chains that form active sites in enzymes, the self-assembled nanofibers of amino acids in the supramolecular hydrogels could act as the matrices of artificial enzymes. Thus, the supramolecular-hydrogel systems serve two functions: 1) as the skeletons of the artificial enzyme to aid the function of the active site (e.g., hemin) and, 2) as the immobilization carriers to facilitate the recovery of the catalysts in practical applications.

Herein, we mixed hemin chloride (**3**) into the hydrogel formed by the self-assembly of two simple derivatives of amino acids (**1** and **2**). The activity of this new type of artificial enzyme is higher than the activity of free hemin, hemin in β-CD, or hemin in polymeric hydrogels. This artificial enzyme shows the highest activity in toluene for an oxidation reaction, reaching about 60% of the nascent activity of horseradish peroxidase (HRP). This result is particularly interesting because it implies that the control of the structure of hydrogelators could tailor the nanofibers as an adjustable microenvironment around active centers and thereby affect the performance of artificial enzymes. Moreover, the supramolecular hydrogel acts as an effective carrier to minimize the dimerization and oxidative degradation of free hemin in the peroxidization reaction. Overall, the supramolecular-hydrogel-based artificial enzyme offers a new opportunity to achieve catalysis with high operational stability and reusability, which ultimately would benefit industrial biotransformation.

Scheme 1 illustrates the simple procedure of using supramolecular hydrogels to encapsulate hemin. Equal molar equivalents of **1** and **2** and two equivalents of Na_2CO_3 in water formed a suspension, which turned into a clear solution at about 60 °C. Then, **3** was added and dissolved immediately in the solution. The subsequent cooling of the solution to room temperature afforded a supramolecular hydrogel containing hemin molecules (Gel II). Without the addition of **3**, the same procedure gave the control (Gel I). As a point of reference, we made the artificial peroxidases by using β-CD or a polyacrylamide hydrogel to encapsulate hemin according to the literatures.[16]

As shown in the TEM and AFM images in Figure 1, Gel I and Gel II have different morphologies. The networks of Gel I have 50–500-nm pores formed by the nanofibers (roughly 20 nm in diameter) of the self-assembled **1** and **2**. Besides the relatively large pores, the TEM image of the nanofibers in Gel II, however, shows two distinct regions: the dark part (fibers of approximately 20 nm in diameter) and the gray part (surface layer of approximately 6 nm thickness).

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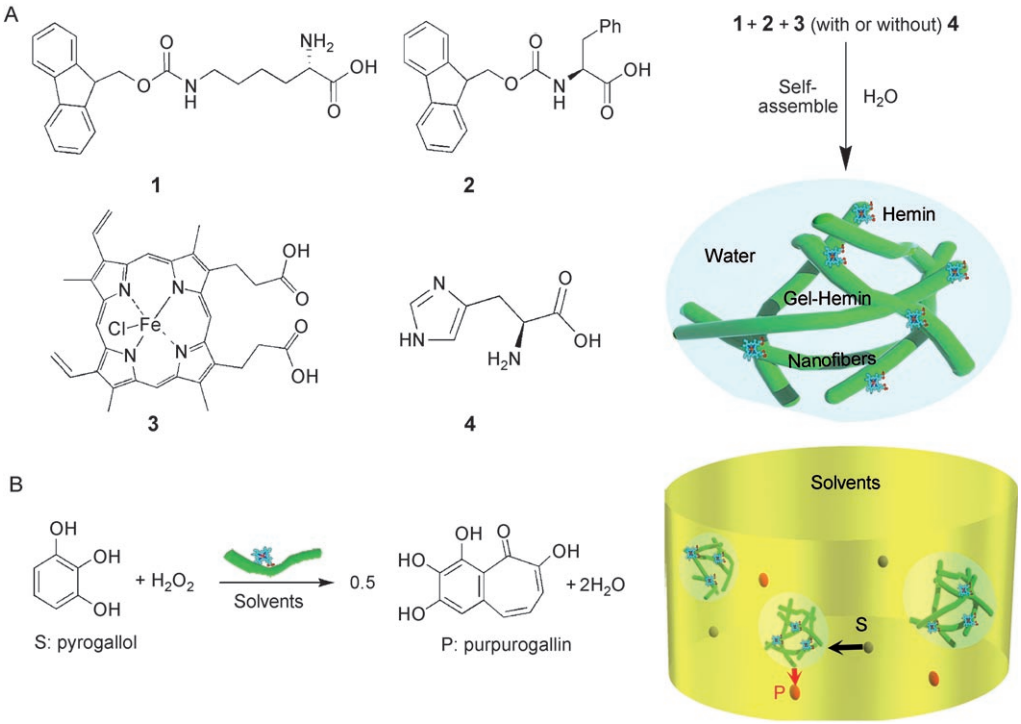

Scheme 1. A) Structures of the molecules and the procedure for making the supramolecular hydrogels containing hemin chloride. B) Illustration of the artificial enzyme-catalyzed peroxidation of pyrogallol to purpurogallin (S = substrate, P = product, and Solvent = aqueous buffer solution (0.01 M, pH 7.4, phosphate) or toluene).

The similar size of the dark part in Gel II compared with the diameter of the nanofiber in Gel I suggests that the dark part mainly consists of the nanofibers of **1** and **2**. The gray part likely consists of less-ordered aggregates of **1** and **2**. The AFM spectrum of Gel II also shows that a loose layer surrounds the dense nanofibers (Figure 1 C, the bright region is about 30 nm in height and agrees with the TEM result). The TEM image of Gel II clearly reveals that the dark nanofibers are surrounded by the gray part (Figure 1 D), which agrees with the AFM result. The energy-dispersive X-ray spectroscopic (EDX) analysis of different areas in the high-resolution (HR)TEM image of Gel II (areas E and F in Figure 1 D) indicates that the blank area (Figure 1 E) has no Fe signal, whereas the area around the nanofibers (Figure 1 F) has about 2.23 wt % Fe, confirming that the hemin molecules localize on the nanofibers. Moreover, the presence of hemin in the outer layer of the nanofibers would allow the substrate to approach hemin easily.

Figure 2 shows the UV/Vis spectra of hemin in a buffer solution and in the hydrogel. The free hemin chloride in pH 7.4 buffer solution displays a Soret peak at 385 nm along with a shoulder at 365 nm, indicating the presence of a mixture of the μ-oxo bihemin (the dimer of hemin) along with the monomeric hemin hydroxide (haematin).[17] Additionally, a low-intensity band around 610 nm also agrees with the Q band value of μ-oxo bihemin.[17,18] Therefore, the predominant structures of hemin in pH 7.4 phosphate buffer solution are the hemin dimers connected by μ-oxo bridges and a small amount of haematins. Gel II displays a broad Soret band at 400 nm with a shoulder at 365 nm and a weak band at 585 nm. The Soret bands of the hemin inside the hydrogel (at 400 nm, the bands are similar to hemin in aqueous micelle solutions and artificial proteins[19,20]) agree with the spectra of hemin chloride in dimethyl sulfoxide and methanol,[21] suggesting monomeric hemin chloride. As reported,[19,20] the weak absorbance band at 585 nm should be ascribed to charge-transfer transition. The weak shoulder at 365 nm in the spectra of Gel II indicates the presence of a small amount of monomeric haematin. Overall, the nanofibers in the supramolecular hydrogel effectively reduce the dimerization of hemin through supramolecular interactions to localize

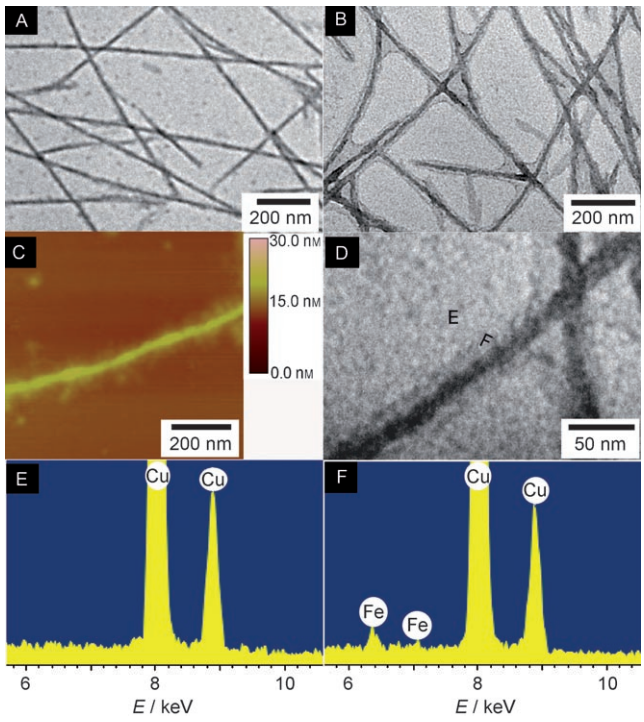

Figure 1. The TEM images of A) Gel I and B) Gel II. C) The AFM image of the nanofiber in Gel II. D) The high-resolution TEM image of Gel II. E) The EDX analysis of the selected areas (E and F) shown in (D).

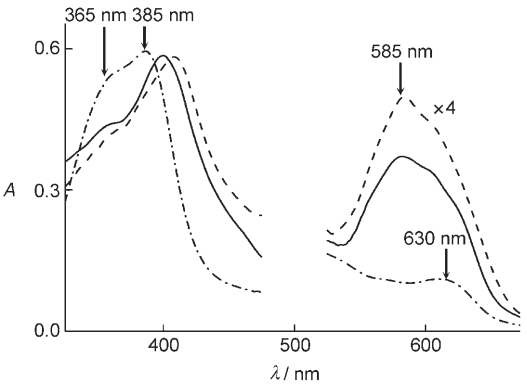

Figure 2. UV/Vis spectra of free hemin in pH 7.4 phosphate buffer solution (hemin$_{(Free)}$; –•–•), hemin in the hydrogel (hemin$_{(Phe)}$; ——), and hemin in the hydrogel with histidine (hemin$_{(Phe+His)}$; -----). The spectra at the weak absorption region (525–675 nm) are enlarged four times.

monomeric hemin chloride within/around the nanofibers. With the addition of histidine, the spectrum of Gel II shows a red shift in the Soret band from 400 nm to 406 nm, which is consistent with the hemin titrated by imidazole at 1:1 ratio in methanol,[20,21] indicating the formation of a hemin–histidine complex.

By using the oxidation of pyrogallol as a model reaction, we obtained the catalytic rate of hemin in various environments. As shown in Figure 3, Gel I (i.e., the control) exhibits

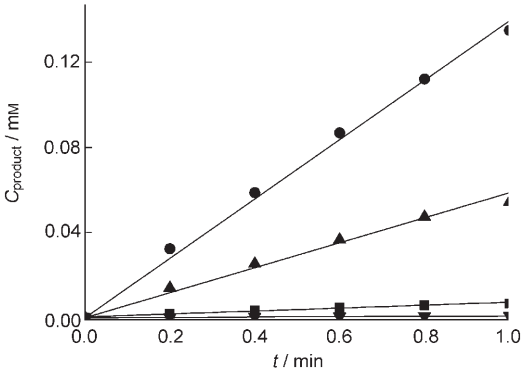

Figure 3. The initial reaction courses of pyrogallol (10.0 mM) and H_2O_2 (40.0 mM) in 0.01 M pH 7.4 phosphate buffer solution catalyzed by 5 μM hemin$_{(Phe+His)}$ (●), hemin$_{(Phe)}$ (▲), hemin$_{(Free)}$ (■), and Gel I control with 0.5 mL L^{-1} concentration (▼). The reactions in the first minute display zero-order kinetics, thus being defined as the initial activity.

no catalytic activity and Gel II (i.e., hemin$_{(Phe)}$) exhibits a higher activity than that of free hemin (i.e., hemin$_{(Free)}$) in the buffer solution (10 mM, pH 7.4, phosphate buffer solution), confirming the effectiveness of the artificial enzyme. The Lineweaver–Burk plots constructed by the initial rates of reactions give the activity of the artificial enzyme. As shown in Table 1, the artificial enzyme has a higher turnover number (k_{cat}) than that of free hemin (hemin$_{(Free)}$), β-CD-bound hemin (hemin$_{(β\text{-}CD)}$), or polyacrylamide-hydrogel-encapsulated hemin (hemin$_{(polymer)}$). Two factors likely contribute to the high activity of hemin$_{(Phe)}$ in pH 7.4 buffer solution: 1) localization of hemin on the nanofibers preserves the catalytic species, monomeric hemin, and 2) the nanoporous channels in the hydrogel facilitate the substrate across the hydrogel network to access the hemin in the hydrogel.

Table 1: Comparison of the activity of the artificial enzymes in water and toluene.[a]

	k_{cat} in water [min^{-1}]	k_{cat} in toluene [min^{-1}]
hemin$_{(Phe)}$	19.9	370.7
hemin$_{(Phe+His)}$	49.7	1045.3
hemin$_{(Free)}$	2.4	2.7
hemin$_{(Mix)}$	3.1	3.9
hemin$_{(β\text{-}CD)}$	4.8	6.1
hemin$_{(β\text{-}CD+His)}$	5.6	7.8
hemin$_{(polymer)}$	7.6	2.4
hemin$_{(polymer+His)}$	9.2	2.8
Met-Hb	37.4	1.0
HRP	1740.0	1.8

[a] The background of all carriers without hemin has been subtracted. Hb = hemoglobin.

A notable feature of the supramolecular-hydrogel-based artificial enzyme is its high activity in an organic solvent. The kinetic data in Table 1 indicate that the k_{cat} of hemin$_{(Phe)}$ in toluene (370.7 min^{-1}) is 136 times that of hemin$_{(Free)}$ in toluene (2.7 min^{-1}). Relative to hemin$_{(Phe)}$ in aqueous solution, the k_{cat} of hemin$_{(Phe)}$ in toluene increases about 17.6 times. The increase in the activity could be ascribed to the phase equilibrium of reactants and products between hydrophobic toluene solution and the aqueous environment of artificial enzymes and can specifically be attributed to two causes: 1) the enrichment of hydrophilic substrate (pyrogallol log$P$ 0.294) in the aqueous environment of the artificial enzyme directly enhances the activity, and 2) the easy diffusion of the hydrophobic product (purpurogallin log$P$ 2.416) to the similarly hydrophobic toluene (log$P$ 2.678) also promotes the catalytic conversion.

The supramolecular hydrogel greatly outperforms other hemin carriers in toluene (Table 1). Therefore, the unique molecular arrangement of the amino acids in the supramolecular hydrogels/nanofibers likely contributes to the high overall activity of the artificial enzyme in toluene. Specifically, the nanoporous channel and amphiphilic structure of the supramolecular hydrogel should assist the mass transfer between the hydrogel phase and the toluene solution. To further verify the effect of the supramolecular hydrogels, we tested the activity of hemin in the solution of N-acylated phenylalanine and lysine (see the Supporting Information). The k_{cat} values of this control (hemin$_{(Mix)}$, Table 1) are essentially the same as those of free hemin, indicating that the formation of the hydrogels is the major factor contributing to the activity of hemin in the artificial enzymes.

Figure 4 also shows the catalytic courses of the oxidation of pyrogallol catalyzed by hemin$_{(Phe+His)}$ in water and toluene, verifying that histidine significantly increases the performance of the artificial enzyme. As indicated by the kinetic parameters listed in Table 1, the addition of histidine dramatically enhances the activity of hemin in the artificial enzyme by about 2.5 times in water and 2.8 times in toluene,

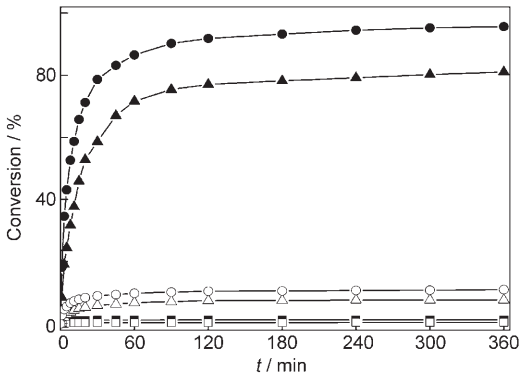

Figure 4. The conversion courses of pyrogallol (10.0 mM) and H_2O_2 (40.0 mM) catalyzed by $hemin_{(Phe+His)}$ in toluene (●), $hemin_{(Phe)}$ in toluene (▲), $hemin_{(Free)}$ in toluene (■), $hemin_{(Phe+His)}$ in water (○), $hemin_{(Phe)}$ in water (△), $hemin_{(Free)}$ in water (□).

indicating that the coordination of histidine to the Fe^{III} center in the hemin increases the activity in the artificial enzymes. As shown in Figure 3, the Soret band of $hemin_{(Phe+His)}$ is similar to that of methemoglobin (408 nm) and peroxidase (405 nm), both of which have a proximal histidine ligand coordinating the heme iron.[22] It is well known that the proximal histidine in preoxidase enhances the activity.[23] Therefore, the peroxidase-like hemin–histidine complex in the artificial enzymes should be the major cause of the high activity. This is evidenced in that the histidine-derived enhancements of activity are the same magnitude in different solvents and different carriers (Table 1).

The supramolecular-hydrogel-immobilized hemin also exhibits high stability and excellent reusability, which is particularly useful for industrial applications. Figure 4 shows the 360-min courses of the oxidation of pyrogallol catalyzed by various hemins in water and toluene. The hemins in the hydrogels all maintained high catalytic activity during the first 120-min course of the reaction in toluene or in water, whereas the free hemin lost most of its catalytic activity after about 5–10 min of reaction. The highest conversion of $hemin_{(Phe+His)}$ is about 96% after reaction for 360 min. One plausible explanation for the high stability of the artificial enzyme is that the hemin molecules are immobilized and isolated from each other within/around the nanofiber, and the hydrogels protect hemin from oxidative inactivation. To test its reusability, we compared the fresh and recovered artificial enzyme ($hemin_{(Phe)}$) during the 15-min peroxidization of pyrogallol in toluene. The result shows that the amount of product in the third run reaches 82% of that in the first run. Therefore, the artificial enzyme in toluene exhibits excellent reusability.

To verify the generality of this approach, we evaluated the activity of hemin in the hydrogels formed by other 9-fluorenylmethoxycarbonyl (Fmoc) amino acids. In either water or toluene, $hemin_{(Ala)}$, $hemin_{(Val)}$, and $hemin_{(Leu)}$ have activities similar to that of $hemin_{(Phe)}$. Various substrates also were employed to evaluate the catalytic ability of artificial enzyme in toluene. The result shows that the activity of $hemin_{(Phe+His)}$ in toluene can reach up to 20% and 4.2% of that of HRP in water with *o*-phenyldiamine and *o*-aminophenol as the substrate, respectively.

In summary, we have demonstrated that nanofibers in supramolecular hydrogels function as the skeleton of the artificial enzyme and serve as the immobilizing carrier to enhance the catalytic activity of hemin chloride for peroxidation in water or in organic media. The supramolecular hydrogels protect the hemin monomer by preventing dimmerization and degradation and facilitate the catalytic reaction by providing nanoporous diffusion channels, which possess unique flexibility to allow the transport of substrates. Although it is still difficult to obtain the complete molecular details in such artificial enzymes owing to current limitations on the characterization of supramolecular hydrogels, the noncovalent interactions among the hydrogelators and between the hydrogelators and water molecules would permit sufficient flexibility to assist the transport of the substrates and the products, which may be essential for this type of artificial enzymes.

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