Synthesis of an Enzyme-like Imprinted Polymer with the Substrate as the Template, and Its Catalytic Properties under Aqueous Conditions

Zhiyong Cheng, Liwei Zhang, and Yuanzong Li*[a]

Abstract: Transition state analogues (TSAs) have long been regarded as ideal templates for the preparation of catalytically active synthetic imprinted polymers. In the current work, however, a new type of molecularly imprinted polymer (MIP) was synthesized with the substrate (homovanillic acid, HVA) as the template and hemin introduced as the catalytic center, with the use of plural functional monomers to prepare the active sites. The MIP successfully mimicked natural peroxidase, suggesting that it may not be imperative to employ a TSA as the template when preparing enzyme-like imprinted polymers and that the imprinted polymer matrix provided an advantageous microenvironment around the catalytic center (hemin), essentially similar to that supplied by apo-proteins in natural enzymes. Significantly, by taking advantage of the special structure of hemin and multiple-site interactions provided by several functional monomers, the intrinsic difficulties for MIPs in recognizing template molecules in polar solutions were overcome. The newly developed polymer showed considerable recognizing ability toward HVA, catalytic activity, substrate specificity and also stability, which are the merits lacked by the natural peroxidase. Meanwhile, the ease of recovery and reuse the MIP implies the potential for industrial application.

Keywords: catalysts · enzyme models · hemin · homovanillic acid · peroxidase-like activity · substrate-imprinted polymer

Introduction

Over the past decades, many scientists have investigated the design and the construction of "macromolecular" synthetic receptors, in order to mimic the molecular recognition ability of biological molecules such as antibodies, enzymes, and receptors.[1,2] Among the techniques applied, molecular imprinting has come to be regarded as one of the most potentially promising and convenient methods by which to create a three-dimensional network with a "memorized cavity" specific to the shape and functional group positions of the template molecules.[3,4] However, intrinsic difficulties have made recognition of polar molecules in water a challenging subject, since, as is well known, polar functional groups are better hydrated and hydrogen-bonding and electrostatic/salt bridge interactions between polar functional groups are weakened by hydration when compared with the interactions in non-polar solvents. These disadvantages have greatly hindered the advancement of molecular imprinting technology in mimicking enzyme models where biocatalytic reactions proceed under aqueous conditions.

Metalloporphyrins and their analogues, known to recognize both hydrophilic and hydrophobic guests with significant selectivity and particular affinity in water,[5] may offer resolution of the above problems if used as co-monomers in the preparation of molecularly imprinted polymers (MIPs). These receptors have Lewis acidic sites (metal ions), electrostatic recognition site/salt bridge sites (-COO^- groups), and hydrophobic binding pockets (the porphyrin frameworks and hydrophobic groups such as alkyl chains and aryl groups).[5] Taking advantage of such unique structures, some groups have prepared optical sensor materials with metalloporphyrins or porphyrins.[6–9] Porphyrinosilica and metalloporphyrinosilica templates have also been obtained by sol–gel processing in which functionalized porphyrin and metalloporphyrin "building blocks" were assembled into a three-dimensional silicate network.[10–12] Nevertheless, no work on the employment of metalloporphyrins as co-monomers in the preparation of a catalytic MIP with typical enzyme-like characteristics—such as Michaelis–Menten kinetics, substrate specificity and saturation, and competitive inhibition—has yet been reported. In the current work, a new type of MIP with considerable peroxidase-like activity was prepared with hemin, a metalloporphyrin with two −C=C groups, as a co-monomer and homovanillic acid (HVA, **1**) as a specific template/substrate. Batch catalysis demon-

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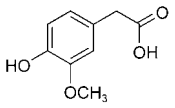


HVA (**1**)

strates that this type of MIP can efficiently catalyze the oxidation of HVA, and exhibits typical Michaelis–Menten kinetics and substrate specificity under mild aqueous conditions. In addition, the imprinted polymer host shows good stability and can be easily recovered and reused, indicating the potential for applications in industry.[13]

Results and Discussion

Hemin content in the MIPs: Since imprinted polymer recognition sites are heterogeneous in nature, not all the hemin sites are active, nor are they all accessible to solvent and substrate.[14,15] The concentration of hemin present in the MIP samples was defined as the difference between the initial total hemin involved in the preparation of the MIP and the hemin content in the eluents. It was found that the concentration of hemin was 38.2 $\mu mol\,g^{-1}$ of HVA-imprinted polymer and 39.5 $\mu mol\,g^{-1}$ of blank polymer. These data provide a theoretical maximum number of "active" sites in the assayed polymers (i.e., on the assumption that all available hemin units present in the polymer are active). Since hemin is the essential factor for the catalytic reaction, its content is used here as an index of "active" sites, and all further experiments are carried out on the basis of equal hemin content.

Optimization of the reaction conditions: To optimize the conditions for the reaction, we investigated the effects of the tris-HCl buffer solution and the reactant concentrations on the reaction rate (v). Both the buffer solution pH and concentration were, in their manner, shown to play key roles in the HVA dimerization reaction (Figure 1). Significantly, the neutral shift of optimum pH value (pH 8.3) was obtained for the MIP-catalyzed HVA dimerization, where the optimum pH value was ca. 11.0 in the case of hemin as catalyst,[16] and pH 8.5 for natural horseradish peroxidase (HRP).[17–19] This implied that, through molecular imprinting, we had succeeded in mimicking the microenvironment around hemin, which is essential to the catalytic activity of natural HRP. The reaction rate initially increased with an increase in buffer concentration, and then decreased after passing through a maximum (15 $mmol\,L^{-1}$). A similar result was observed in the case of HVA concentration versus the reaction rate, presumably due to the concentration-induced decay effects. Hydrogen peroxide of extremely high concentration led to an obvious decrease in reaction rate and increasing noise. We attributed this to the fact that a large excess of H_2O_2 can destroy the planar rings of the porphyrins irreversibly.[20–22] Under the optimum experimental conditions ($7.35\times10^{-2}\,mol\,L^{-1}$ of H_2O_2, $3.66\times10^{-4}\,mol\,L^{-1}$ of HVA, and 0.13 $mg\,mL^{-1}$ of MIP), we recorded the excitation and emission spectra of the oxidation product (Figure 2), and the related dynamic curve was also measured and illustrated in Figure 3.

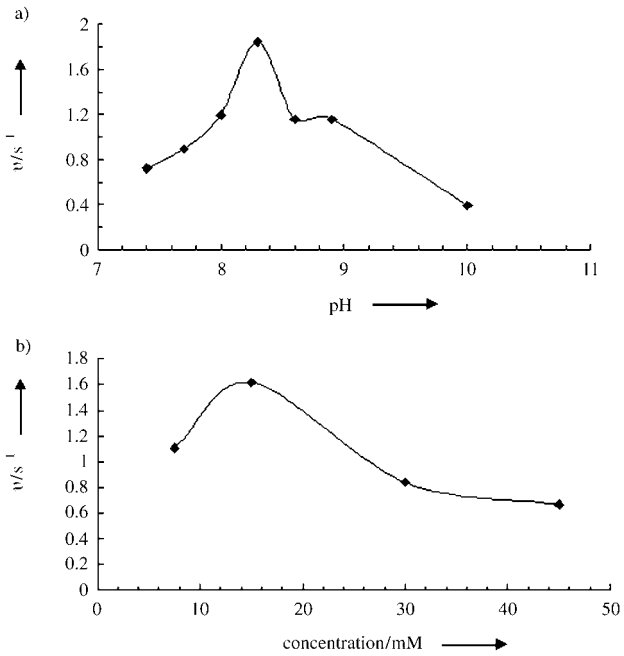


Figure 1. The effects of a) pH and b) concentration of Tris-HCl buffer solution on the catalytic activity of the HVA-imprinted polymer. Other conditions are as specified in the Experimental Section.

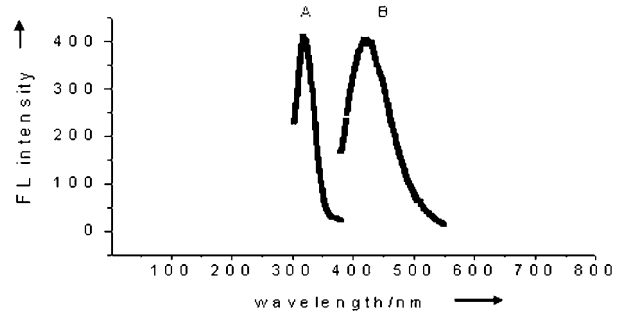


Figure 2. Excitation (A) and emission (B) spectra of the dimer product of catalysis by the peroxidase-like MIP.

Catalytic activities of the MIPs: On the basis of enzymological theory and methods, the hemin-containing MIPs were investigated for their catalytic activities with HVA and its analogues as substrates under the optimum conditions. A dynamic curve typical of a catalytic reaction was observed in the HVA/H_2O_2/MIP system (Figure 3), in the initial phase of which we obtained the characteristic parameters, such as Michalis–Menten constants (K_m), the maximum rate (V_{max}), and the transformation constant (K_{cat}) according to Lineweaver–Burk theory.[23,24] Since it was corrected that the concentration of hydrogen peroxide was saturated in the test system, the Michalis–Menten constants were expressed as K_m^{app}, and the detailed results are presented in Table 1.

According to the theory of enzymatic catalysis,[25] the Michaelis constant (K_m) represents the affinity of a given enzyme towards the substrate. The smaller the K_m, the stronger the affinity will be, and the "favorite" substrate of a given enzyme will show the lowest K_m value. The distinct

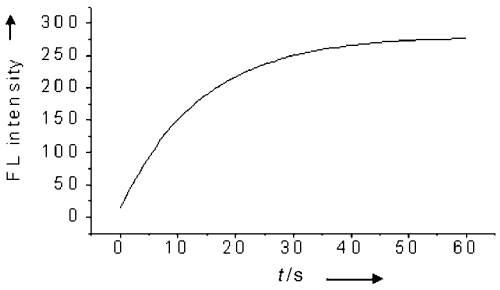


Figure 3. Typical dynamic profile of the dimerization reaction of homovanillic acid catalyzed by the peroxidase-like MIP.

same systems, *p*-HPA and *p*-HPPA had K_m^{app} values much higher than that of HVA. In contrast, the three reductant substrates showed similar affinities to N-MIP, with K_m^{app} values of 9.89×10^{-2}, 9.18×10^{-2}, and 9.42×10^{-2} M for HVA, *p*-HPA, and *p*-HPPA, respectively. At the same fixed concentration, HVA was oxidized most rapidly of the three compounds ($k_{cat} = 6.41\times10^{6}\,L\,mol^{-1}\,s^{-1}$). Interestingly, when $1.30\times10^{-4}\,mg\,L^{-1}$ of HVA-imprinted polymer (containing $4.97\times10^{-6}\,mol\,L^{-1}$ of hemin) was replaced by $4.97\times10^{-6}\,mol\,L^{-1}$ of free hemin as the catalyst, the relative reaction rates changed dramatically. The ratio of the reaction rates for HVA/*p*-HPA/*p*-HPPA was 7.7:1.4:1.0 when the HVA-imprinted polymer was employed as a catalyst, whereas the ratio with free hemin solution as a catalyst was 1.4:0.95:1.0. Conceivably, when prosthetic groups alone (metalloporphyrins such as hemin) served as catalysts, the multiple-site interactions provided by apo-proteins would be unavailable to the substrates. As a result, the essential mechanistic contributors—such as condensing effects deriving from the hydrophobic properties of the apo-proteins, the strain, orientation effects, and the substrate-specificity effects due to the multiple-site recognizing interactions, which are known to be in favor of the binding of substrates—would be absent from such a system,[27], and so these three substrates were not distinguished from each other in the rates of the oxidation reaction catalyzed by the free hemin solution. On the other hand, the HVA-imprinted polymer can provide multiple-site interactions and specific three-dimensional cavities for the recognition and binding of templates (also substrates in the case of enzyme-like MIPs, Scheme 1),[3,4,7] thus showing distinct catalytic activities in the oxidation reactions of HVA, *p*-HPA, and *p*-HPPA (Table 1).

Table 1. Catalytic activities of the HVA-imprinted polymers in the oxidation of the substrates.

Batch	Substrate	K_m^{app} [$mol\,L^{-1}$][a]	v_{max}^{app} [s^{-1}][a]	k_{cat} [$L\,mol^{-1}\,s^{-1}$][a]	v_{obs} [s^{-1}][b]	v_{obs} [s^{-1}][c]
1	HVA	3.18×10^{-3}	31.85	6.41×10^{6}	3.76	1.84
2	*p*-HPA	1.09×10^{-2}	9.69	1.95×10^{6}	0.68	1.27
3	*p*-HPPA	5.05×10^{-2}	5.37	1.08×10^{6}	0.48	1.33
4[d]	N-MIP	9.89×10^{-2}	4.18	8.42×10^{5}	0.51	–

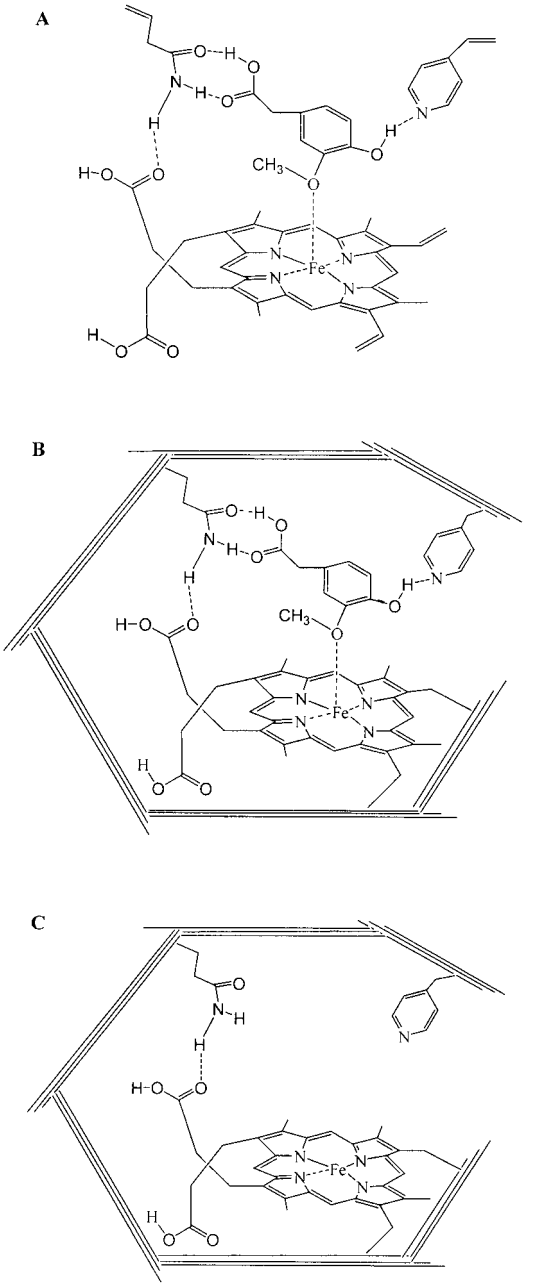
[a] The concentration of H_2O_2 was $7.35\times10^{-2}\,mol\,L^{-1}$, and the HVA-imprinted polymer at $1.30\times10^{-4}\,mg\,L^{-1}$ (hemin contained: $4.97\times10^{-6}\,mol\,L^{-1}$). [b] HVA, *p*-HPA, and *p*-HPPA were all fixed at $1.22\times10^{-4}\,mol\,L^{-1}$. [c] Experimental conditions are the same as in [b], except that the MIP was replaced by $4.97\times10^{-6}\,mol\,L^{-1}$ of free hemin. [d] HVA was used as the substrate.

increase both in recognition characteristics and in catalytic activity can be observed from Table 1, by comparing batch 1 and batch 4, in which the experiments were carried out in the presence of HVA-imprinted MIP and of blank polymer, respectively. The recognition ability of HVA-imprinted polymer towards its template (HVA) was about thirty times greater than that of blank polymer toward HVA. Significantly, the catalytic activity of the former polymer was nearly eight times greater than that of the latter, suggesting that the molecular imprinting process had greatly contributed to the high activity of the enzyme-like MIP,[15,26] presumably through a proximity, and orientation, and strain effects.[27] The lower selectivity for the alternative substrates, shown by comparison of batch 1 with batches 2 and 3, also lends support to the shape-recognition effects observed previously.[28]

Substrate-specificity of the enzyme-like MIP: It is known that natural peroxidases (e.g., HRP) show no substrate specificity toward hydrogen peroxide or toward reductant substrates,[29] and that they can catalyze the oxidation of many kinds of reductant compounds in the presence of H_2O_2. Such a feature has been shown to cause decreasing efficiency of enzymes, especially in the orientated synthetic reaction.[27] Notably, the peroxidase-like MIP developed in this work exhibited evident substrate specificity toward the template molecule (HVA), as shown by comparing the results of batch 1 in Table 1 with those of batch 4.

Further evidence for substrate specificity can be obtained if we compare the catalytic activity of the peroxidase-like MIP with that of blank polymer (N-MIP) in the presence of (*p*-hydroxyphenyl)acetic acid (*p*-HPA) and (*p*-hydroxyphenyl)propionic acid (*p*-HPPA) as substrates, because the oxidation products of these two compounds in an HRP/H_2O_2 oxidant system also show fluorescent characteristics similarly to HVA.[19] As expected, the HVA-imprinted MIP can catalyze the oxidation of *p*-HPA and *p*-HPPA and produce a fluorescence signal, but its behavior differed from that seen in the case of HVA oxidation (see Table 1). The Michaelis–Menten constants suggested that HVA was the favorite substrate and that, although easily oxidized in the

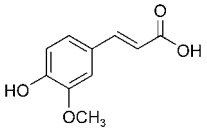
Overall, the HVA-imprinted polymer exhibited promising peroxidase-like activity with considerable substrate specificity. By comparing the K_m^{app} values of the four batches in Table 1—*p*-HPPA ($5.05\times10^{-2}\,mol\,L^{-1}$) < *p*-HPA ($1.09\times10^{-2}\,mol\,L^{-1}$) < HVA ($3.18\times10^{-3}\,mol\,L^{-1}$)—one can observe distinct recognizing effects among the three substrates. This suggested that the longer side chain of *p*-HPPA weakened the recognizing forces,[30] whereas the absence of -OCH_3 also reduced the affinity of MIP toward *p*-HPA. Notably, HVA shows affinity toward the MIP 30 times greater than it does toward non-imprinted polymer, indicating that the essential recognizing sites and cavity shapes are formed during the preparation of HVA-imprinted MIP. The recognizing ef-



Scheme 1. Schematic representation of self assembly (**A**), polymerization (**B**), and the highly specific recognition cavity generated after removal of the template (**C**) in the preparation of HVA-imprinted polymers.

fects, in turn, made positive contributions in the catalytic reactions, yielding higher reaction rates.

Inhibiting effects of ferulic acid on the peroxidase-like activity: To confirm that the imprint recognition site had indeed been formed, ferulic acid (FA, an analogue of homovanillic acid; **2**) was tested for its ability to inhibit the polymer-catalyzed reaction.



FA (**2**)

As can be seen from Figure 4a, the competitive inhibition of the catalytic reaction by FA showed obvious concentration dependence with the following properties: 1) an increase in the inhibitor concentration parallels an increase in the Michaelis constant K_m^{app}, which indicated a decrease in the affinity of MIP towards the template molecules, and 2) V_{max}, which ranges from 10.6 though 10.9, shows no significant variation with increasing inhibitor concentration from 10 to 30 mM. However, it should be noted that when the FA concentration exceeded 60 mM and was increased continuously, K_m^{app} reached a plateau paralleling an increase in V_{max}, presumably because excess FA occupied the sixth axial coordination site and led to the so-called non-competitive inhibition of the catalytic activity of MIP.[31] According to enzyme inhibition theory,[32] an increase in the concentration of substrate should recover the catalytic activity and attenuate or even eliminate the competitive inhibition. Our results in this current work demonstrated that HVA did reduce the inhibition caused by FA ($K_i^{app} = 3.47 \pm 0.18$ mM), and that increasing concentration of HVA invited an obvious weakening of affinity of MIP toward inhibitor. That is, the inhibition constant decreases with increasing HVA concentration (varying from 3.5 to 18.9 mM when HVA concentration was increased from 2.0 to 8.0 mM; see Figure 4b). Interestingly, when we investigated further by increasing the HVA concentration to 16 and 32 mM, the inhibition constant was maintained around 18.9 mM, with the reaction rate increased considerably. We ascribe this to the fact that FA may partially occupy the sixth axial sites of hemin. With an increase in the HVA concentration to 64 mM, reduction in catalytic activity was again observed, which confirmed the hypothesis that both excessive substrate and specific inhibitors will compete with hydrogen peroxide for the sixth proximal ligand-binding site through coordination interaction, thus hindering the formation of compounds I and II, which are essential for hemin-catalyzed reactions.[33,34] As a result, inhibition of catalytic activity occurred. In contrast, when we performed the inhibiting experiments with FA for N-MIP, no obvious decrease was observed either in the reaction rate or in the fluorescent product formation.

Notably, the peroxidase-like MIP did not exhibit absolute substrate specificity. For example, although the substrate analogue caused an obvious inhibiting effect on the catalytic activity of MIP, the characteristics of the inhibition are not completely consistent with those of competitive inhibition according to enzyme inhibition theory.[32] That is to say, nonspecific binding of substrate and its analogues is also in existence. This kind of binding may produce some degree of change in the micro-circumstances of the imprinted cavity shape and the recognition ability of MIP, thus influencing the properties associated with both catalytic activity and enzyme inhibition by FA.

According to Wulff,[3] an imprinted polymer should show competitive inhibition by the template analogue, and this is a good indication of a molecular imprinting effect; competitive inhibition should occur with an imprinted catalyst, whereas it should either not occur or occur less strongly with a non-imprinted control polymer. The concentration-dependent inhibition of product formation by FA in this work implies the presence of specific active sites in the polymer matrix, including binding sites and reaction centers.

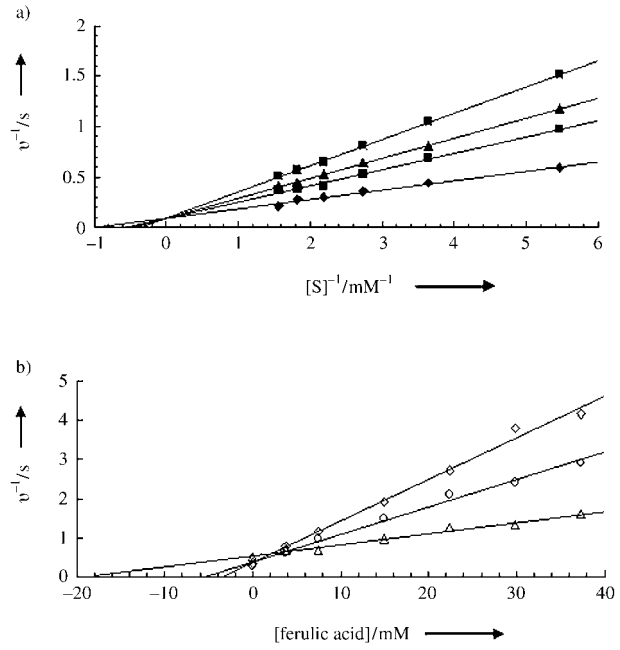


Figure 4. Plots for the production of fluorescent dimer product over a rang of ferulic acid concentrations. a) Lineweaver–Burk plot for the production of dimer over a range of inhibitor concentrations [V_{max} = (10.57 ± 0.21) s^{-1}, Michaelis constant K_m^{app} = (0.98 ± 0.03) mM.] ◆ 0; ■ 10 mM; ▲ 20 mM; ● 30 mM; b) Dixon plot over a range of HVA concentrations [K_i^{app} = (3.47 ± 0.18) mM]. ◇ 2 mM, ○ 4 mM, △ 8 mM. Other conditions are as specified in the Experimental Section.

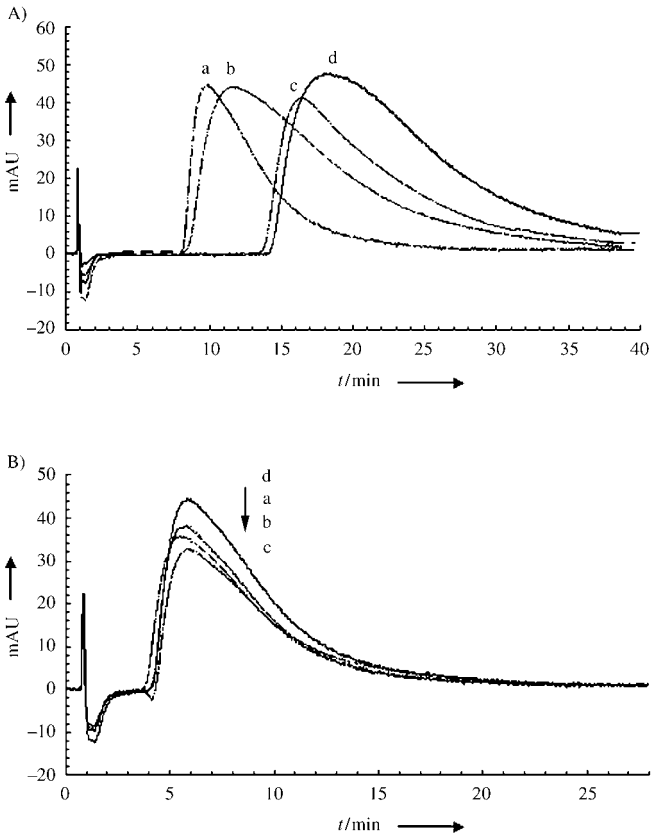


Figure 5. Chromatographic study on the affinity of HVA-imprinted MIP (A) and N-MIP (B) toward different substrates. The MIPs (32–50 μm) were packed into stainless columns (50 mm × 4.6 mm i.d.). The affinity of MIP toward substrate was analyzed in terms of their retention times by HPLC assay, and methanol was used as mobile phase, at a flow rate of 0.8 mL min^{-1}, the injection volume was 20 μL (1.5 mg mL^{-1}), and the detection was carried out at 280 nm. a) *p*-HPPA, b) *p*-HPA, c) FA, d) HVA.

This is in good agreement with the chromatographic data, which demonstrated a higher affinity of the MIP toward HVA than toward FA, *p*-HPA, and *p*-HPPA (see Figure 5).

Retaining of the catalytic activity of MIPs: One strategy in developing catalytic systems capable of mimicking catalytic oxidation has been the use of metalloporphyrins, which are analogues of monoxygenase: iron protoporphyrins IX.[16,35,36] However, the cost of these catalysts is such that methods to endure maximum product output per gram of catalyst have to be developed. If these metalloporphyrin-catalyzed oxidations are to be applicable to synthetic procedures for laboratory-, medium-, and large-scale reactions, two important difficulties must be overcome: catalyst recovery and reuse.[37] Iron porphyrins are known to react with hydrogen peroxide, displaying both catalase- and peroxidase-like activities, and have the advantage of forming intermediate species similar to those formed during enzymatic processes.[38,39] However, most of them tend to dimerize[40] and to suffer oxidative degradation by the reactive intermediates formed during the catalytic cycle.[20–22] Further evidence that hydrogen peroxide can cause the polymerization and degradation of metalloporphyrins and natural hemin enzymes in an irreversible way concomitant with H_2O_2 decomposition and loss of enzymatic activity has also been reported.[33,41,42]

In this work, we investigated the recovery of the catalytic activity of HVA-imprinted MIP over several reaction runs, and found that a number of polymer samples could be recovered from reaction mixtures, exhaustively re-extracted, and reused for similar reactions. Notably, under the reaction conditions and over the time course employed in these studies, negligible breakdown of MIP materials was observed in the first several runs when sufficient reductant substrate was present in the reaction system. Almost 95% of the previous activity could be retained by addition of HVA immediately after the completion of the reaction; the slight loss in activity may arise from the gradual decomposition of the polymeric matrix under such employed reaction conditions. If the MIP was incubated with excessive H_2O_2 in the absence of substrate for more than 15 min, or HVA was added 15 min after the completion of the reaction, no obvious fluorescence signal was observed upon addition of HVA to the mixture, whilst HVA would recover enzymatic activity by about 45% if added 2 min after the completion of the reaction. These observations suggest that the presence of reductant substrate in the reaction mixture plays an important role in retaining the enzyme-like activity of MIP. An earlier study indicated that ascorbic acid can inhibit the inactivation of hemin enzyme by H_2O_2.[43] Furthermore, microperoxidase degradation was almost completely prevented when the ascorbate concentration was greater than that of H_2O_2.[33] We ascribe this to a scheme in which a putative "compound I" of the hemin with H_2O_2 is reduced by reductant substrate

such as HVA and Vc into "compound II" and then back to their original redox state (ferric) in two one-electron steps; in the absence of reductants, the "compound I" reacts further with the hydrogen peroxide causing hemin degradation and evolution of O_2.[33,34]

Conclusion

Transition state analogues (TSAs) have long been regarded as ideal templates with which to prepare catalytically active synthetic imprinted polymers or catalytic antibodies,[44,45] according to the fact that transition state stabilization is the mechanism driving catalytic reaction forward.[3,46] In this work, we have used the substrate (HVA) as a template to prepare molecularly imprinted polymers and have successfully mimicked the natural peroxidase, suggesting that it may not be imperative to employ a TSA as the template when preparing enzyme-like imprinted polymers. Moreover, the imprinted polymer matrix provided an advantageous microenvironment around the catalytic center (hemin), as is done by apo-proteins in natural enzymes. As is known, when the substrate is efficiently bound into a binding pocket containing a metalloporphyrin, the enzymatic reactions selectively depend on the primary and higher-order structures of the surrounding environment, such as the protein or—in our studies—the polymeric matrix.[8] Therefore, the enzyme models derived here with the metalloporphyrins fixed in the imprinted polymer matrix should mimic the in vivo behavior of the metalloporphyrin residues more closely.

Notably, by taking advantage of the particular structures of hemin and the multiple-site interactions provided by plural co-monomers, the intrinsic difficulties inherent for MIPs in recognizing template molecules in polar solutions were overcome and a new type of polymer with considerable recognizing ability and catalytic activity was prepared by molecular imprinting. In addition, the MIP can efficiently catalyze oxidation of the template in a similar fashion to peroxidase under mild aqueous conditions, and showed considerable substrate specificity as well as stability, the merit that natural peroxidase lacks. Meanwhile, the ease of recovery and reuse of the MIP implies it has potential for industrial application.

Experimental Section

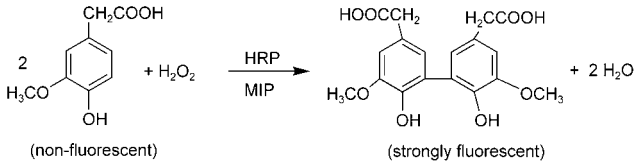
Chemicals: Ethylene glycol dimethacrylate (EGDMA) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Toshima, Kita-ku, Tokyo). Acrylamide (ACM) was from Sangon Co., Ltd., and 4-vinylpyridine (4-Vpy) from E. Merck (Darmstadt, Germany). Azo-bisisobutyronitrile (AIBN) was obtained from Nankai Chemical Plant (Tianjing, China). Hemin, (*p*-hydroxyphenyl)acetic acid, and (*p*-hydroxyphenyl)propionic acid were from Sigma Chemical Co. (St. Louis, MO, USA), and homovanillic acid ((4-hydroxy-3-methoxyphenyl)acetic acid) from Kanto Chemical Co., Inc. (Chuo-Ku, Tokyo). Ferulic acid (4-hydroxy-3-methoxycinnamic acid) was from Shanghai Chemical Plant (Shanghai, China). Monomers and cross-linker were distilled immediately prior to use, and initiator AIBN was recrystallized from ethanol. All solvents and other chemicals were of analytical grade.

Preparation of molecularly imprinted polymers: The polymer preparation was carried out as reported by Matsui et al.,[6,8,9] with a slight modification. HVA (1 mmol), ACM (2 mmol), 4-Vpy (2 mmol), EGDMA (20 mmol), and AIBN (48 mg) were added to a solution of hemin (1 mmol) in chloroform/DMSO (1:4, 10 mL). The mixture was purged with nitrogen in a glass tube for 10 min. This was then connected to a vacuum line, and the tube was sealed at liquid nitrogen temperature. The reaction was carried out in a 60 °C water bath over 24 h. The non-imprinted control polymer was generated in the same way, without the HVA template. Polymers were ground in a ball mill (Retsch, type S 100), and sieved to collect the 32–50 μm portions. The collected particles were extracted with methanol/acetic acid (7:1),[47] washed with methanol to remove the acid, dried under vacuum (60 °C), and used as catalysts in the batch experiments.

MIP Assay

Determination of the hemin content present in the polymers: The hemin present in the polymers was measured by quantitative spectrophotometric (398 nm) analysis based on subtraction of the extractable hemin from the hemin added for the reaction.

Catalytic reaction with MIP as catalyst: HVA can be oxidized to a dimer by hydrogen peroxide with HRP as catalyst, and this dimer shows strong fluorescent emission under alkaline conditions (Ex/Em = 315/425 nm) (Scheme 2).[17,18,48] In this work, the catalytic reactions were carried out in



Scheme 2. Dimerization reaction of HVA catalyzed by HRP or homovanillic acid imprinted polymer (MIP) in the presence of H_2O_2.

Tris-HCl buffer (15 mmol L^{-1}, pH 8.3), stirred with the suspended solution of the polymers at 25 °C. The final concentrations were fixed at 1.30×10^{-4} mg L^{-1} (i.e., hemin concentration is 4.97×10^{-6} mol L^{-1}) for the polymer and 7.35×10^{-2} mol L^{-1} for H_2O_2, with varying HVA concentrations. The dynamic curve of the dimerization reaction of HVA catalyzed by the MIP was recorded with a luminescence spectrometer (LS-50B, Perkin Elmer, USA) and the pseudo-first-order rate constants were obtained from linear plotting of fluorescence intensity vs. time. Triplicate runs showed a measurement error of less than 5%. Meanwhile, another two substrates—(*p*-hydroxyphenyl)acetic acid (*p*-HPA) and (*p*-hydroxyphenyl)propionic acid (*p*-HPPA)—were employed to study the substrate selectivity because they exhibit reactivity similar to HVA oxidation by H_2O_2 in the presence of peroxidase and yield fluorescent products.[19] To examine the potential inhibiting effects of HVA analogues on the enzyme-like MIP, the similar kinetic studies were carried out in the presence of an appropriate aliquot of ferulic acid solution.

Acknowledgment

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