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The role of molecular recognition in regulating the catalytic activity of peroxidase-like polymers imprinted by a reductant substrate

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Abstract

Due to the unique structural features of hemin as well as the controlled interactions between functional monomers and the substrate homovanillic acid, a new type of catalytically active polymers was allowed to be well designed and prepared by molecular imprinting with hemin introduced as the catalytic center. Subsequent to a previous work on mimicking peroxidase by molecular imprinting techniques, this paper is focused on the regulating role of molecular recognition in substrate-binding and enzyme-like activity of the imprinted polymers, and a mechanistic elucidation was made on the catalytic reactions. The results demonstrated that the catalytic activity of the molecular imprinted polymers (MIP) was largely determined by substrate-binding sites and the molecular recognition process. Hemin was proven to not only serve as the catalytic center but also play an essential role in molecular recognition; the multiple-site interactions between the plural co-monomers and the substrate may overcome the interference of water molecules during molecular recognition and confer on the MIP an ideal substrate specificity toward template and catalytic activity even under aqueous conditions.

Keywords: Molecularly imprinted polymers (MIP); Peroxidase-like activity; Molecular recognition; Multi-site interaction; Mechanistic elucidation

1. Introduction

The unique features of metalloporphyrins and their controlled interactions with the surrounding protein make metalloporphyrins and related aromatic macrocycles important prosthetic groups and coenzymes, working as redox and rearrangement reaction catalysts (cytochrome P-450, catalase and peroxidase), photoreaction centers (chlorophy ll) and oxygen carriers (hemoglobin), which has stimulated considerable interest in developing new catalysts and polymer receptors with metalloporphyrins introduced [1–7]. These receptors can recognize/bind both hydrophilic and hydrophobic guests with significant selectivity and particular affinity in water because porphyrins can provide a useful framework for artificial receptors and catalysts with several unique features [8,9]. Based on the strategy to recognize polar functional groups in water (e.g., preparation of a host with both hydrophobic binding pockets and polar recognition groups), the challenge in host–guest chemistry under aqueous conditions [10,11] is likely to be resolved. Meanwhile, more promising enzyme models may be developed to clarify the mechanisms of molecular recognition and catalysis where the catalytic reactions proceed under aqueous conditions. Our interest is thereby focused on such a topic, i.e., to develop new peroxidase models by taking advantage of the unique structural features of hemin as well as the controlled interactions between functional monomers and the substrate [9]. A peroxidase-like molecularly imprinted polymer (MIP) has been reported to efficiently catalyze the oxidation of homovanillic acid (HVA) with considerable substrate specificity in the presence of hydrogen peroxide [9]. In this paper, we tried to figure out the essential role of molecular recognition in regulating the enzyme-like activity of the imprinted polymer, and mechanistic elucidation was presented on the catalytic reactions with the new type of polymers imprinted by a potential substrate.

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2. Experimental

2.1. Chemicals

Acrylamide (ACM), 4-vinylpyridine (4-Vpy) and ethylene glycol dimethacrylate (EGDMA) were distilled immediately prior to use, Azo-bis-*iso*-butyronitrile (AIBN) was recrystallized from ethanol. Hemin was from Sigma Chemical Co. (St. Louis, MO, USA), and homovanillic acid (4-hydroxyl-3-methoxyphenylacetic acid, HVA) from Kanto Chemical Co., Inc. (Chuo-Ku, Tokyo). All solvents and other chemicals are of analytical grade.

2.2. Typical preparation of molecularly imprinted polymers [3–6,9,12,13]

To a solution of hemin (1 mmol) in chloroform/DMSO (1/4, v/v, 10 ml) was added HVA, ACM, 4-Vpy and EGDMA at various molar ratios as indicated in Table 1. The mixture was purged with nitrogen for 10 min, then connected to a vacuum line and evacuated at liquid nitrogen temperature. Afterwards, the polymerization reaction was triggered by AIBN (48 mg) at 60 °C for 24 h under vacuum. The polymers were ground and sieved to collect the portion of 32–50 μm, and extracted with methanol/acetic acid (7:1, v/v) [13], washed with methanol to remove the acid, dried under vacuum (60 °C) and used as catalysts in batch experiments.

2.3. Catalytic properties of the MIPs and related polymers

The catalytic activity of MIPs was studied according to previous work [9,14–16]: the catalytic reaction was carried out in a suspended solution of the polymers and Tris–HCl buffer (15 mmol/l, pH 8.3) with stirring at 25 °C, with the final concentration of the polymer being 1.3×10^{-4} mg/ml, and the concentration of H_2O_2 being 7.35×10^{-2} M. The dynamic curve of the dimerization reaction of HVA catalyzed by the MIP was recorded by a LS-50B luminescence spectrometer (Perkin-Elmer, USA), with varying concentrations of HVA in the range of 2.4×10^{-4} to 3.2×10^{-2} M for MIPs and 4.8×10^{-3} to 1.2×10^{-1} M for blank polymer. The pseudo-first-order rate constants were obtained from linear plots of fluorescence intensity versus time. Triplicate runs showed a measurement error of less than 5%.

3. Results and discussion

3.1. The effect of cross-linker on the catalytic activities of the MIPs

The cross-linker, ethylene glycol dimethacrylate, demonstrated its important role in regulating the catalytic activity of the HVA-imprinted polymers. By comparing P_1, P_2 and P_3, one can see that K_m^{app} values decreased first with increasing content of cross-linker, whereas excessive EGDMA led to lower activity (Table 1). The reaction rate showed similar dependence on the cross-linker content in the MIPs. This can be explained by the fact that EGDMA plays an important role in maintaining the rigid framework that confers the favorite conformation essential to molecular recognition and catalytic activity. That is, the rigid structure is necessary for MIP to recognize and bind substrate, which contribute greatly to the enzyme-like activity. On the other hand, excessive EGDMA will reduce the flexibility of the backbone of the MIP leading to unfavorable mass transfer, and thus the decreased reaction rate.

3.2. The effect of 4-vinylpyridine on the catalytic activities of the MIPs

Proximal ligands are known to influence the activity of hemin-containing enzyme [17], and many studies have revealed that even free ligand contributed greatly to the catalytic activities [18–23]. In the present work, we employed 4-vinylpyridine (4-Vpy) as a co-monomer to prepare the MIPs so that dual functions were achieved, i.e., the recognition site and the axial ligand. Obviously, the presence of 4-Vpy greatly improved the recognition ability of MIP toward homovanillic acid as shown by the reduced K_m^{app} values (compare P_4 with P_2 and P_5). In addition, an increase in the 4-Vpy content parallels further decrease in the K_m^{app} value, presumably because the presence of 4-Vpy produced another interaction site for HVA to promote the molecular recognition to a greater extent [9] (see Fig. 1 for the proposed scheme). The more binding sites, the stronger the synergic interaction force, the more favorably the molecular recognition is

Table 1
Catalytic activity related parameters of the MIPs and related polymers in the oxidation of homovanillic acid by H_2O_2

Polymer	HVA:hemin:ACM:4-Vpy:EGDMA	K_m^{app} (M)	V_{max} (s^{-1})	κ_{cat} ($M^{-1}\,s^{-1}$)	ν_{obs} (s^{-1})[a]
P_1	1:1:2:2:10	$(4.63 \pm 0.22) \times 10^{-3}$	13.9 ± 0.5	$(2.80 \pm 0.06) \times 10^6$	1.81 ± 0.06
P_2	1:1:2:2:20	$(3.18 \pm 0.10) \times 10^{-3}$	31.9 ± 1.3	$(6.41 \pm 0.23) \times 10^6$	3.76 ± 0.08
P_3	1:1:2:2:40	$(4.07 \pm 0.19) \times 10^{-3}$	21.1 ± 0.6	$(4.24 \pm 0.18) \times 10^6$	2.36 ± 0.09
P_4	1:1:2:0:20	$(9.25 \pm 0.38) \times 10^{-3}$	8.15 ± 0.25	$(1.64 \pm 0.05) \times 10^6$	0.93 ± 0.04
P_5	1:1:2:4:20	$(2.32 \pm 0.08) \times 10^{-3}$	11.2 ± 0.4	$(2.26 \pm 0.04) \times 10^6$	1.39 ± 0.04
P_6	1:2:2:2:20	$(2.51 \pm 0.12) \times 10^{-3}$	44.6 ± 1.6	$(8.98 \pm 0.42) \times 10^6$	5.16 ± 0.24
P_7	1:0:2:2:20	–	–	–	–
P_8	0:1:2:2:20	$(9.89 \pm 0.36) \times 10^{-2}$	4.18 ± 0.12	$(8.42 \pm 0.32) \times 10^5$	0.51 ± 0.02

The concentration of H_2O_2 was fixed at a saturating level of 7.35×10^{-2} M. HVA, homovanillic acid; ACM, acrylamide; 4-Vpy, 4-vinylpyridine; EGDMA, ethylene glycol dimethacrylate. All the measurements were repeated three times to give the mean ± S.D. ($n = 4$).

[a] Results were obtained with 1.22×10^{-4} M HVA.

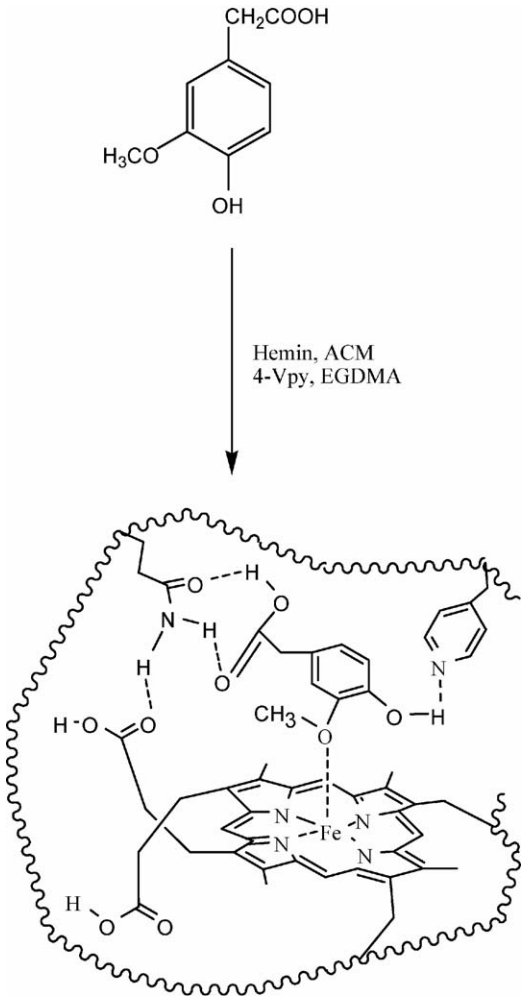
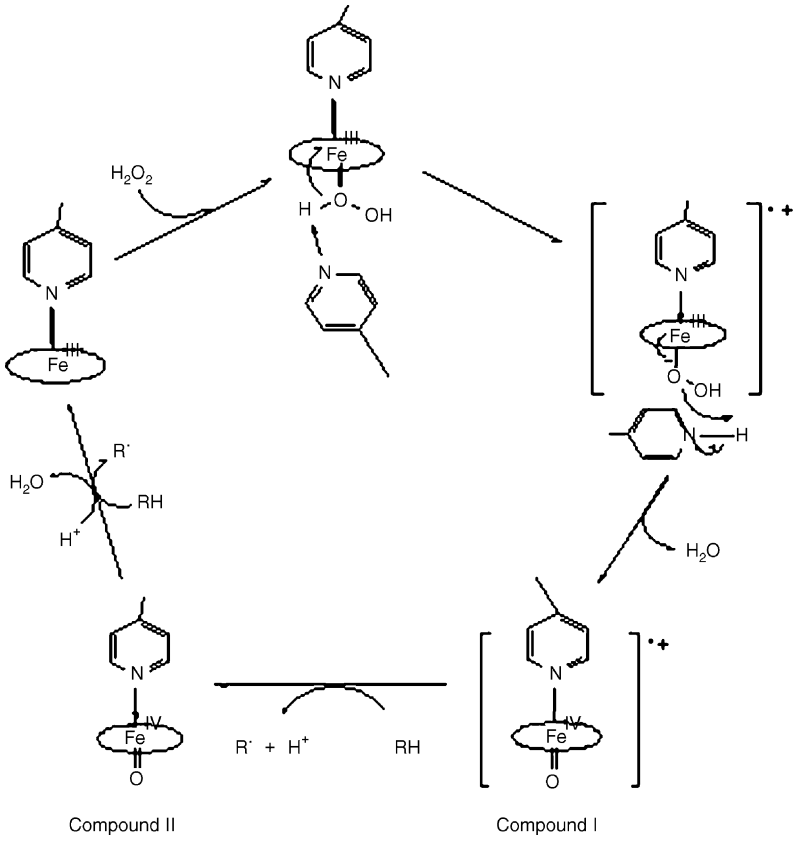


Fig. 1. The schematic representation of molecular imprinting and recognition for the HVA-imprinted polymers.

realized [24]. A similar fashion was observed for the effect of acrylamide (ACM) on the affinity of MIP toward HVA; i.e., when the content of ACM changed from 0 to 2 mmol in the polymerization reaction with other component content kept constant, the MIPs showed an increasing ability to bind HVA, indicating that plural functional monomers promised an ideal affinity to template for MIPs. Considering that the preferred ratio of templates to monomers is 1:4 or 1:5 [25–31], we did not further investigate the effect of ACM on MIP's activity, and fixed its content at 2 mmol in 10 ml of porogen. On the other hand, the 4-Vpy content in the MIPs affected their catalytic activity in a different manner; catalytic activities increased first and then decreased with increasing 4-Vpy content. It is known that the central metal ion in metalloporphyrin can produce coordination sites accessible to base ligands, and the axial base ligand always enhances the catalytic activity of metalloporphyrins or metalloporphyrin-containing enzyme mimics if one axial site is available to hydrogen peroxide [18,19,21–23]. Hemin-containing mimic enzymes, for example, have been reported to show strong axial-ligand dependence on His [32], imidazole and other nitrogen-containing molecule [19]. It is conceivable that the pyridinyl residues of 4-Vpy would provide the base necessary for generation of coordination site of the Fe^{2+}-porphyrin. The attribution of the 4-Vpy content to the catalytic activities of HVA-imprinted polymers can be explained by the ligand effects at the axial position as established previously, while the negative contribution of 4-Vpy to catalytic activity as shown in Table 1 may arise from the occupation of the sixth axial position of hemin by excessive 4-Vpy. The sixth axial binding site is known to be reserved for hydrogen peroxide in the catalysis, where the compounds I and II are generated (see Scheme 1). In turn, the compounds I and II react with HVA to produce phenoxyl radicals that further form strong-fluorescent dimer products via coupling reactions [21,22,33]. Evidently, excessive 4-Vpy in the pre-mixture of the polymerization reaction can occupy the sixth axial site and prevent the essential interaction between H_2O_2 and hemin. As a result, the level of compounds I and II generation will be lowered, which inhibit the catalytic reaction to some extent.

3.3. The effect of hemin on the catalytic activities of the MIPs

Although considerable catalytic activity was obtained with the HVA-imprinted polymers, not all of them possessed such property. Polymer 7, for example, showed no peroxidase-like activity without introducing hemin into the polymer, demonstrating the key role of hemin in the activity of the enzyme-like MIP. As discussed above, hemin nucleus can form the so-called compounds I and II with hydrogen peroxide which are the primary steps for further catalytic reactions. Without hemin present in the polymer, compound I could not be formed; thus the catalysis was blocked. It is noteworthy that free hemin and the polymers prepared without HVA involved (control polymer) also exhibited catalytic activity in the HVA oxidation, but their catalytic properties were quite different from HVA-imprinted polymers in both catalytic constants and specificity [9]. Interestingly, an increase in the hemin content also caused a decrease in K_m^{app} values (compare P_7 with P_6 and P_2), implying that hemin is also essential to the molecular recognition in addition to being the catalytic center. It is known [3–8] that metalloporphyrins or their homologues are the ideal candidates as monomers for preparation of versatile hosts because of the special structural features as follows: (1) an approximately planar structure owing to the π-electron conjugation, which gives a facile design of receptor having a geometrically well-defined binding pocket consisting of a porphyrin framework and recognition groups; (2) the central metals incorporated with varying recognitions and catalytic activities; (3) several distinct functionalization sites. The presence of the meso- and β-positions, central metal and inner nitrogen atoms for functionalization sites made it likely that the monomers interact with HVA through π–π stacking, Van der Waals and coordination interactions, and aid in defining the recognition site topography of the imprinted polymers. In their work, Matsui et al. found a three-dimensional cavity constructed on a porphyrin plane in cross-linked polymers to which a ligand was specifically bound through multiple-point interaction, where a guest molecule was bound via coordination by a



Scheme 1. The catalytic mechanism of hemin-containing enzymes and their mimics with proximal ligand effects involved, where RH denotes reductant substrate such as HVA; R• denotes the resultant free radical from the substrate.

porphyrin metal center or hydrogen bonding and electrostatic interaction as well [3]. The coordination interaction or static salt bridge could be generated between the template and hemin. Those interactions, along with the other forces as aforementioned, would contribute greatly to the molecular recognition and substrate binding so that the catalytic reactions were accelerated via condensing and distorting/orientating effects [34]. That is why metalloporphyrins have shown promising applicable prospective in mimicking enzymes with varying recognition and catalytic activities, characteristic redox chemistry of both metal and ligand and their important photochemical behavior as an electron donors or acceptors [8].

3.4. The effect of molecular imprinting on the catalytic activities of the MIPs

In our previous study, the substantial contribution of molecular imprints to catalysis was addressed for the HVA-imprinted polymers with a fixed composition [9]. In the present study, the polymers were prepared with varying compositions in the polymerization reaction. The study of these polymers in catalysis shows that the performance of the MIPs in both molecular recognition and catalysis are highly dependent on their compositions. As depicted in Table 1, a distinct improvement both in recognition characteristics and in catalytic activity were obtained with imprinting effects introduced (comparing P_2 with P_8). The exclusion of HVA from polymerizing reaction mixture made the recognition ability of P_8 reduce abruptly (nearly one thirtieths of that of P_2 in terms of K_m^{app}) with a concomitant decrease in the catalytic activity (one-eighth decay), suggesting that the imprint shape of a cavity attributed significantly to the strong catalytic activity of MIP. Meanwhile, steric property as well as multiple-point interaction for molecular recognition may account for the high reactivity of the MIP in catalytic reactions as discussed above and in previous studies [35–37]. In the case of P_4, P_8 and P_7 that are deficient in 4-Vpy, hemin and HVA in the polymerization reaction, respectively, only very weak recognizing ability and relatively low catalytic activity were observed for these polymers. These drastic changes in recognizing ability and catalytic activity must arise from the loss/attenuation of the optimized multi-point interactions between the host (MIP) and the gust (HVA) provided by plural monomers and imprint effects. By looking into P_1 through P_8, one can easily specify the essential factors for developing ideal peroxide-like MIPs with hemin and other functional monomers. Since the substrate-binding site and catalytic center are the prerequisites for an MIP of high substrate specificity and catalytic activity, hemin was introduced to ensure the formation of compounds I and II for the further catalytic reaction. In addition, hemin could contribute to the substrate-binding ability of MIP under aqueous conditions via coordination interaction and hydrophobic driving force. Optimal recognition of substrate was dependent upon both the presence of the coordinating metal and the complementary imprint site interactions, and distinctly high activity can be obtained only when

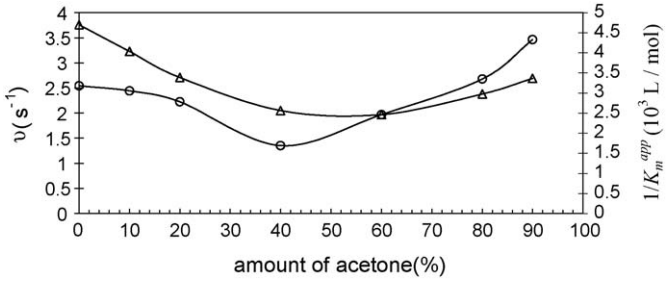


Fig. 2. The effect of acetone amount in reaction media on the catalytic (Δ: ν in s^{-1}) and recognizing (◯: $1/K_m^{app}$ in 10^3 L/mol) ability of HVA-imprinted polymer. Other reaction conditions are the same as specified in Section 2.

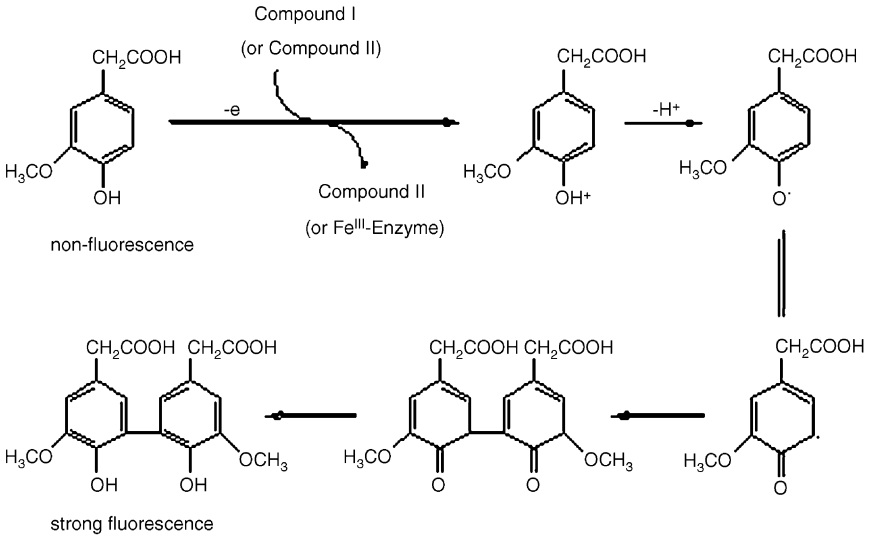
multiple-point interactions exist between the MIP and substrate by introducing hemin and 4-Vpy as co-monomers. Furthermore, the correctly positioned functional groups also play an important role in the catalytic mechanism [38,39].

Taken together, the composition of HVA-imprinted polymers plays an important role in both the recognition ability and their peroxidase-like activity. Removal of hemin from the polymers (P_2, P_6, P_7 and P_8) will completely quench their catalytic activity due to elimination of the free radical-generating reagent Compounds I and II, as well as the reduced strength of multi-point interactions for host (MIP)-guest (HVA) recognition, which was also observed in the absence of 4-Vpy or ACM. Similarly, removal of the template molecule (HVA) from the polymerization reaction only led to poorly performed polymers. This difference is conceivably ascribed to the loss of multi-point interactions and the well-tailored cavity to recognize/bind HVA for an accelerated reaction. An optimized composition for the peroxidase-like polymers, by taking into consideration the host–guest interactions and mass transfer (the size of sieve, determined by the content of crosslinker EGDMA), was achieved by a ratio of HVA:hemin:ACM:4-Vpy:EGDMA (1:2:2:2:20) in the polymerization reaction. This optimized ratio, in essence, illustrated the key role of recognizing/binding of the guest/substrate (HVA) by the MIP host in the subsequent oxidation/dimerization of HVA via a free radical reaction mechanism. This host–guest interaction, notwithstanding, can be regulated by the reaction medium, especially the swelling effect induced by solvents with differential polarities (refer to the following section for further discussion).

3.5. The solvent effect on recognizing ability and catalytic activity of MIP

Solvent has been proven to influence not only the polymer morphology but also the strength of electrostatic interactions. In recent publications, Yu et al. observed that for MIPs prepared in acetonitrile, the recognition varied substantially with the mobile phase changed from acetonitrile-based to other organic solvent-based or to water/acetonitrile-based solvent systems [40–42]. However, in the present work, we found that organic solvent (acetone) did not always contribute positively to catalytic activity or recognition ability of HVA-imprinted polymers (Fig. 2). When acetone increased gradually in the reaction media, the recognition ability decreased considerably at the beginning and reduced to its minimum with 40% acetone present in the media. This implied that, although solvents of higher polarity (water) decrease somewhat the strength of electrostatic interactions and hydrogen binding interactions, an increase in solvent polarity does not always result in a decrease in recognizing ability of MIPs, which seemed to be contrary to the principle that the ideal rebinding condition for a given template should include porogen [43]. The discrepancy could be explained by the fact that the polymer swelling might change the three-dimensional configuration of functional groups participating in the recognition process [44]. Imprinted polymers are known to exhibit different swelling properties in solvents of different solvating properties [45], and the swelling effects may change considerably the morphology of the polymer network, the size, shape and relative positions of the essential functional groups of the recognition sites. The swelling effects in the buffered system of various polarities can conceivably re-arrange the recognition sites of the polymer and thus resume its affinity toward the template via different prime interactions. Under aqueous conditions, water molecules compete with the template molecule for the binding of the functional groups at recognition sites and weaken the specific polar interactions on one hand [42,46]; on the other hand, hydrophobic interactions have been proven to play a crucial role in the binding of many ligands to their receptors, and hydrophobic interaction could be optimized even at the expense of possible hydrogen bonds [47,48]. Direct evidence indicated that hydrophobic interactions appeared to be the dominant driving force, and tight binding of ligands such as drugs to receptors is achieved through the optimization of specific hydrophobic interactions [47]. Reasonably, the considerable binding and catalytic activity of the MIP in highly aqueous solution as illustrated in Fig. 2 can be explained at least in part by the attribution of optimized hydrophobic interaction. The recognition ability was recovered and increased with the further increase in the acetone content in the reaction media (ranging 40–90%), indicating the prime role switched from hydrophobic interactions to coordination and salt bridge in the molecular recognition. Notably, the catalytic activity of MIP showed a concomitant acetone-dependent recovery although it was not recovered as much as the recognition ability (Fig. 2). As such, two important implications could be obtained: first, recognition sites and the molecular recognition process are essential to the peroxidase-like activity of HVA-imprinted polymers; secondly, acetone makes significantly positive contribution to the recognition ability of MIP toward HVA at high concentration, but it attributes less to the catalytic activity. In dry acetone the MIP exhibited no catalytic activity presumably because aqueous and alkaline conditions favored the oxidation of homovanillic acid into its dimer products by facilitating the electron transfer and proton transfer during the free radical reaction [14,15,49,50].

Peroxidase catalyzes double-substrate reactions. The oxidation of HVA catalyzed by peroxidase is known to proceed via a free radical mechanism [14,51–53], and the resultant HVA free radicals generally evolve non-enzymatically to fluorescent dimers via rapid coupling reaction [34,54,55]. That is, to achieve



Scheme 2. The oxidation reaction of HVA catalyzed by horseradish peroxidase (HRP) or MIPs in the presence of H_2O_2.

HVA dimerization, the catalytic reaction must undergo the formation of HVA phenoxyl radicals and subsequent coupling reaction of the free radicals. Oxidation of hemin by H_2O_2 is the first step of the reaction and produces the active form of the mimetic enzyme "compound I", which in turn reacts with the second substrate HVA to generate HVA phenoxyl radicals (Scheme 2). The molecular imprinting method we used may locally direct the second substrate (HVA) in a preferred orientation proximal to the functional groups of hemin, and thus formed complex resembles the transition state of the reaction between HVA and "compound I". Meanwhile, any factors favoring the formation of HVA free radicals would be advantageous to the dimerizing reaction, which was well demonstrated by the MIP-constructed microenvironment around hemin in driving forward and accelerating the reactions. First, the proximity and orientation effects operated when the MIPs recognized HVA via multi-point interactions. Upon binding HVA specifically, the MIPs brought reducing substrates close to the oxo-ferryl intermediates and led to an increased rate of HVA oxidation because chemical reactions will be greatly accelerated when the reacting groups are combined within a single molecule [56,57]. The reacting groups, approaching in a preferred orientation as the MIP hosts bound guest molecules (HVA), would also contribute positively to HVA oxidation [57–59]. Secondly, the strain effect will grow when HVA is bound to MIPs to form "enzyme–substrate-like" complexes [26,28,31], where the electron-donating groups at the ortho-position and proximal base contribute to the decrease in HVA O–H bond dissociation energy (BDE), thus facilitating the formation of phenoxyl radicals [60–67]. Upon the specific binding of HVA to the MIP host, therefore, the phenolic O–H bond of HVA was more readily cleaved to generate HVA radicals after being stretched and weakened by strain/distortion, thus driving the reaction forward. Finally, the formation of oxo-ferryl intermediates (compounds I and II), which was essential to the transformation of HVA to phenoxyl radicals via acid-base catalysis [34], could be enhanced by the proximal pyridine residue of 4-Vpy. The generated HVA phenoxyl radicals, in turn, undergo rapid electron rearrangement to produce carbon-centered free radicals (Scheme 2). These free radicals approaching close to each other within the MIP host will readily in situ form the final dimer product that is structurally different from the imprinting template/substrate and thus excluded from the active center of the mimetic enzyme. This is very similar to the release of the reaction product from the microenvironment of the enzyme, and pushes the oxidation of HVA toward the dimer product. Once HVA radicals evolved to its dimer, the HVA-imprinted cavity shape would exclude the dimer thus promoting and recycling the reaction.

4. Conclusions

The results of this work demonstrated that the functions exerted by hemin and other co-monomers can somewhat mimic the controlled interactions between metalloporphyrin and the surrounding protein in natural peroxidase, thus leading to considerable catalytic activity along with ideal substrate specificity. The substrate-binding sites and molecular recognition process determined largely the catalytic activity of this new type of MIPs. Hemin was proven to not only serve as the catalytic center but also play an essential role in molecular recognition because of its unique and potentially useful framework for the artificial receptor. The multi-site interactions produced by plural monomers hemin, 4-Vpy and ACM, which could compensate for the negative contribution of water molecules to molecular recognition, promise the MIPs for ideal substrate specificity toward template as well as catalytic activity under aqueous conditions.

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