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Molecularly Imprinted Polymers Having Amidine and Imidazole Functional Groups As an Enzyme-Mimetic Catalyst for Ester Hydrolysis†

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Abstract : A molecularly imprinted polymer (MIP) having both amidine and imidazole functional groups in the active site has been prepared using *p*-nitrophenyl phosphate as a transition state analogue (TSA). The imprinted polymer MIP with amidine and imidazole found to have the highest hydrolysis activity compared with other MIPs with either amidine or imidazole groups only. It is postulated a cooperative effect between amidine and imidazole in the hydrolysis of *p*-nitrophenyl methyl carbonate (NPMC) as a substrate when both groups were arranged in proximity by molecular imprinting. The rate enhancement of the hydrolysis by MIP was 60 folds over the uncatalyzed solution reaction and two folds compared with the control non-imprinted polymer CP1 having both functional groups. The enzyme-mimetic catalytic hydrolysis of *p*-nitrophenyl acetate by MIP was evaluated in buffer at pH 7.0 with K_m of 1.06 mM and k_{cat} of 0.137 h^{-1}.

Keywords : molecularly imprinted polymers, molecularly imprinting catalysts, enzyme-mimetic reaction, amidine functional, imidazole functional, transition state analogue imprinting.

Introduction

Molecular recognition by the use of molecularly imprinted polymers (MIPs) has drawn extensive attention.[1-5] Molecular architecture for specific binding sites capable of molecular recognition can be easily tailored in the network polymers through the molecular imprinting technique. Because of its molecular recognition capability, MIPs have found various applications, such as chromatographic separation, assays and biomimetic sensors.[6-8] In addition, similar to antibody catalysts,[9] which are raised against an analogue of the transition state of a catalytic reaction, enzymatic active sites can be designed in the MIP networks by employing the transition state analogue (TSA) as a template molecule.[1]

There have been some reports on MIPs as enzyme-mimetic catalysts.[10-13] Based on the theory of transition state stabilization for ester hydrolysis, phosphonic monoester as a TSA is generally used as a template molecule in molecular imprinting.[13] Since the shape of the catalytic cavity caused by TSA does not alone provide a strong catalytic effect, additional effective groups are needed. Very recently, the Wulff's group[14-18] utilized a functional monomer having an amidine moiety, *N,N'*-diethyl(4-vinylphenyl)amidine (DEVPA), to prepare an enzymatic MIP catalyst. The strong ionic interaction between amidine and phosphonic monoester enables DEVPA to form a stable complex with the TSA and additionally, the amidine group also plays an important role in a catalytic reaction. The first DEVPA-based catalytic imprinted polymers displayed some of important characteristics in the enzyme-mimetic reaction[16]: 100-fold rate enhancements of the ester hydrolysis, Michaelis-Menten kinetics, selectivity and TSA inhibition of activity.

In this study, as a part of our continuing study on TSA-induced MIPs for artificial enzymatic catalysis,[14, 17] both the imidazole and amidine functionalities were introduced to construct an active site for catalytic ester hydrolysis. The imprinted polymers, having both the amidine and imidazole as functional groups, were expected to have improved hydrolytic activity compared with other MIPs having the corresponding single functional group.

Experimental

Materials and Instruments. Two functional monomers: 4-vinyimidazole (VIm) was prepared from urocanic acid[19]

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and DEVPA was synthesized according to the known method.[18] *p*-Nitrophenyl phosphate (NPP) was prepared by acidification of disodium *p*-nitrophenyl phosphate and extraction with ether. Ethylene dimethacrylate (EDMA), hydroxylethyl methacrylate (HEMA), *N,N'*-azobisisobutyronitrile (AIBN), disodium *p*-nitrophenyl phosphate and 2-[4-(2-hydroxyethyl)-1-piperazino]ethanesulfonic acid (HEPES) were purchased from Aldrich Chemical Co. ^{1}H NMR spectra were taken on a Varian Cemini 2000 (200 MHz) spectrometer. UV-Vis absorbance measurement was performed on a Shimadzu UV-240 spectrophotometer.

Preparation of Imprinted Polymers. To a solution of NPP (110 mg, 0.5 mmol) in acetonitrile (3.0 mL) were added VIm (47 mg, 0.5 mmol) and DEVPA (101 mg, 0.5 mmol). The resulting salt in acetontrile was copolymerized with EDMA (1.98 g, 10 mmol) and HEMA (130 mg, 1.0 mmol) using AIBN (20 mg, 0.12 mmol, 1 mol%) at 60 °C for 24 h. The obtained polymer was crushed, ground and sieved to be 32-45 μm particles. The polymer particles were extracted continuously with acetonitrile, phosphate buffer and methanol until no color appeared in the extract. Then the polymer was dried at 50 °C under vacuum and kept in a vacuum desiccator. Control polymers, CP1, CP2 and CP3, containing the same ratio of the components except NPP, VIm or DEVPA, respectively, were prepared by the same method as described in Table I.

Evaluation of Hydrolysis. *p*-Nitrophenyl methyl carbonate (NPMC) or *p*-nitrophenyl acetate (NPA) was chosen as the substrate to evaluate the hydrolytic activity of the imprinted polymers in a 1 : 1 (v/v) solution of HEPES buffer (0.05 M) and MeCN at 25 °C. The polymer particles (20 mg) were mixed with 2.0 mL of buffer solution. The concentration of the substrate was 0.5 mM. After a definite time, the reaction mixture was filtered with a syringe filter and 1.0 mL of the solution was diluted with 2.0 mL of 0.1 M HEPES buffer (pH 8.0). The release of *p*-nitrophenol was monitored by UV absorbance at 400 nm, with the reference being the solution prepared with the same way in the absence of substrate. The concentration of the released *p*-nitrophenol was analyzed according to a standard curve. Twice runs showed a measurement error less than 5%. The initial rate of hydrolysis was determined by measuring the concentration of *p*-nitrophenol released at definite time. The pseudo-first order rate constants were obtained from the following equation:

$$\ln[A_{max}/(A_{max}-A_t)] = kt$$

where A_{max} is the absorbance of *p*-nitrophenol when all of the substrate has been hydrolyzed, A_t is the absorbance of *p*-nitrophenol at the definite time t, k is the pseudo-first-order rate constant.

Table I. Preparation of the Molecularly Imprinted Polymers in This Study*

Polymers	NPP	DEVPA	VIm
MIP	0.5 mmol	0.5 mmol	0.5 mmol
CP1	none	0.5 mmol	0.5 mmol
CP2	0.5 mmol	0.5 mmol	none
CP3	0.5 mmol	none	0.5 mmol

*All of the polymers were prepared by copolymerization of HEMA (1.0 mmol) and EDMA (10 mmol) with the above components using AIBN (20 mg) as an initiator in 3.0 mL of acetonitrile at 60 °C for 24 h.

Results and Discussion

VIm was generally used as a functional monomer for preparing MIPs to investigate catalytic hydrolysis activity, since the active site of the chymotrypsin esterase comprises a hydroxyl group from serine, a carboxyl group from aspartic acid and an imidazole group from histidine.[20] However, the catalytic enhancement of the synthetic MIP enzymes is not satisfactory. Importantly, G. Wulff *et al.* utilized DEVPA having amidine groups as a functional monomer for the preparation of catalytic MIP and achieved the highest enhancement factor in hydrolysis of ester so far.[15,16,18] Hereby, as depicted in Figure 1, for enhancing the catalytic activity through stronger interaction with the phosphate TSA as well as the ester substrate, the well-known imidazole functionality was introduced into the polymer networks in addition to the amidine functionality by employing NPP as a TSA template. It is postulated that the two acidic hydroxyl groups in NPP form a complex together with VIm and DEVPA. After copolymerization in the presence of large excess of a crosslinker, the crosslinked polymer underwent thorough extraction to remove the template molecules inside. Thus the active cavities complementary to the template were generated in MIP with both the amidine and imidazole groups in proximity.

The catalytic activity of the imprinted polymers was evaluated under different pH conditions. For investigating the effect of the imidazole functional group in proximity with amidine in the catalytic active sites, three different kinds of polymer, CP1, CP2 and CP3, were prepared with the same molar ratio of each component but without using NPP, VIm or DEVPA, respectively. As shown in Figure 2, CP3 with VIm as a functional monomer revealed higher activity than CP2 with DEVPA as a functional monomer. Thus imidazole groups are more active than amidine in ester hydrolysis by MIP. The initial rate of CP2 increased with pH increasing, while that of CP3 increased with increasing pH initially and reached highest at pH 7.0 and kept nearly constant at pH 8.0. This could be explained by the ionization of imidazole group at different pH conditions. An imidazole group (pK_a = 6.5 - 7.0) is known to undergo such equilibrium as follows[21]:

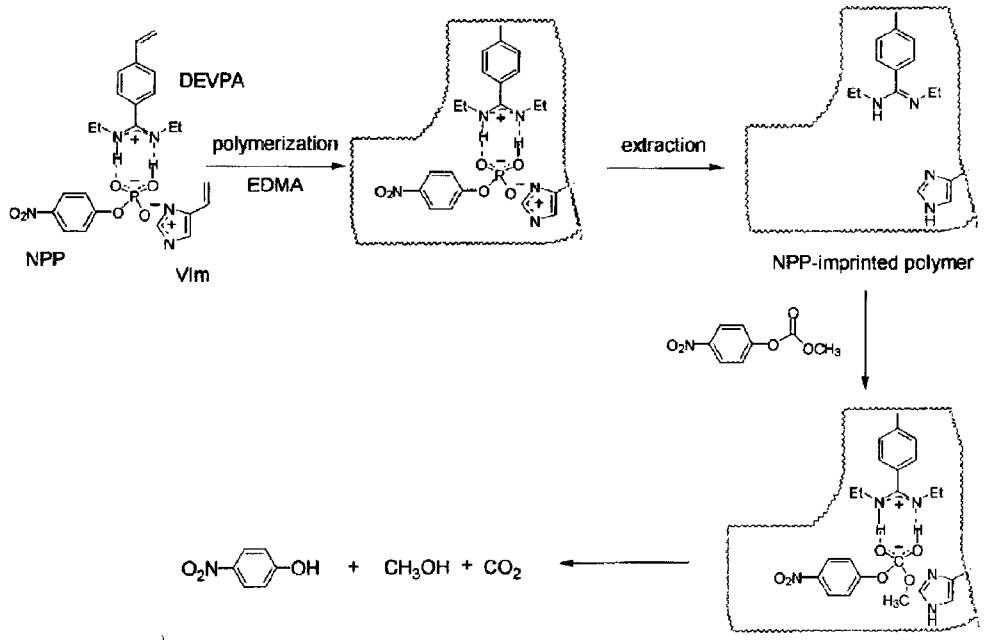


Figure 1. The schematic representation of preparation and catalytic hydrolysis by the NPP-imprinted polymer having both the amidine and imidazole functional groups.

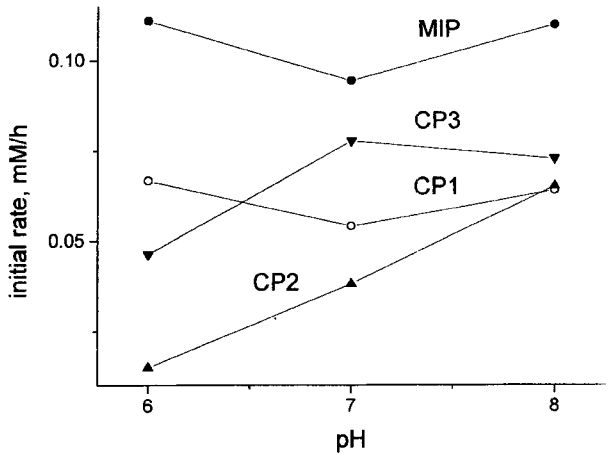
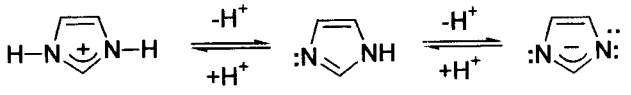


Figure 2. The pH effect on the hydrolysis rate of NPMC in 1 : 1 MeCN/0.05 M HEPES buffer solution at 25 °C : polymer, 20 mg in 2.0 mL of buffer solution; substrate, 0.5 mM; initial rate obtained after 1 h.



Therefore, at low pH, the positively ionized form of imidazole has no nucleophilic activity. The neutral form and negatively ionized form has almost the same nucleophilic activity. Similarly, since amidine has pK_a of 11.6 in solution,[14] the fraction of the neutral form of amidine increased with increasing pH and consequently the hydrolytic activity of amidine is increased.

MIP having both amidine and imidazole as functional groups had highest hydrolytic activity compared with CP2 and CP3, which have only amidine or imidazole functional groups, respectively. However, while the tendency of the pH effect of MIP was the same as that of CP1, it was different from that of CP2 and CP3. At pH 6.0, a significant enhancement for the hydrolysis by MIP was observed in comparison with the initial hydrolysis rate of CP2 and CP3. Therefore, the hydrolytic activity of MIP with both amidine and imidazole could not be considered as simple addition of the activities induced by each functional group. Since the amidine and imidazole groups were brought into proximity in the polymer network through molecular imprinting with PNP as a template, as depicted in Figure 1, it is considered that the significant enhancement of the hydrolytic activity of MIP at low pH (pH = 6.0) is due to the result of the cooperative effect induced by the amidine and imidazole groups. Thus the imidazole group is suggested to serve for carbonyl attack, while amidine for stabilization of the transition state of hydrolysis reaction. In a neutral condition or basic condition, no cooperative effect was observed.

For evaluation of catalytic rate enhancement by molecular imprinting, the pseudo first order rate constants of hydrolytic reactions catalyzed by MIP and CP1 were determined and compared with the hydrolysis in background solution containing the same amount of amidine and imidazole. As shown in Figure 3, MIP achieved 60 times of rate enhancement for the ester hydrolysis compared with that of background solution and 2 times of enhancement compared with that of

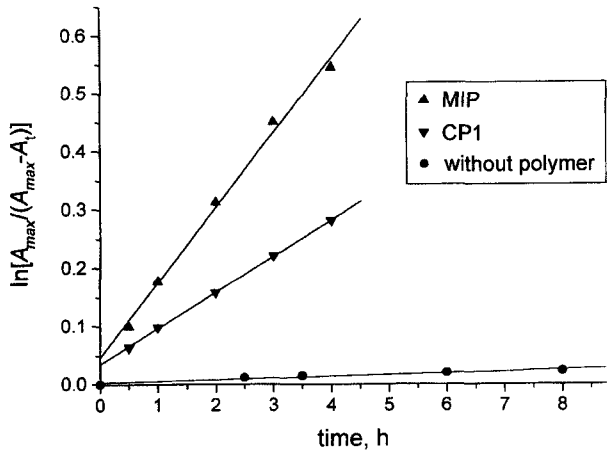


Figure 3. Catalytic hydrolysis of NPMC by MIP at pH 7.0: 20 mg in 2.0 mL of buffer solution containing 4.43 μmol of functional groups; substrate, 0.5 mM. k_{MIP} = 0.128 h^{-1}, k_{CP1} = 0.0618 h^{-1}, $k_{solution}$ = 0.00211 h^{-1}.

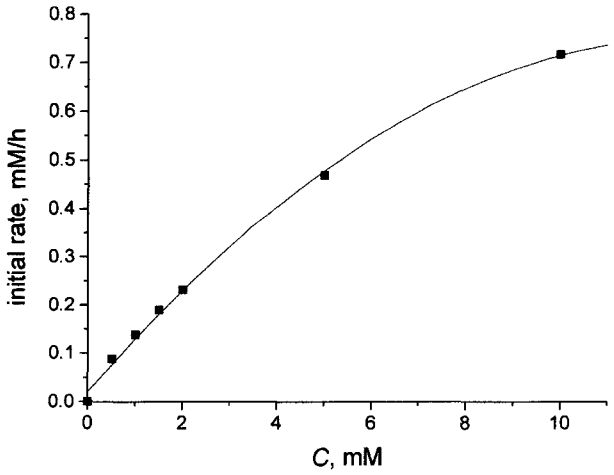


Figure 4. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl acetate at pH 7.0 by MIP: 20 mg MIP in 2.0 mL of buffer solution; initial rate obtained from the release of *p*-nitrophenol in 2 h; concentration *C* of *p*-nitrophenyl acetate.

control polymer CP1 prepared without using the template. This result suggests that the catalytic site containing both the amidine and imidazole functional groups and complementary to the transition state of ester hydrolysis, as depicted in Figure 1, should be formed by molecular imprinting of NPP as a template. Since only small fractions of such catalytic sites are exposed to the surface of the MIP particles for the catalytic reaction with substrates in the polymer network, the enhancement factor was limited.

The ability of the synthetic MIP to imitate enzymes has been evaluated by measuring the Michaelis-Menten kinetics of the hydrolysis with MIP. The reaction was performed with increasing concentration of *p*-nitrophenyl acetate as a

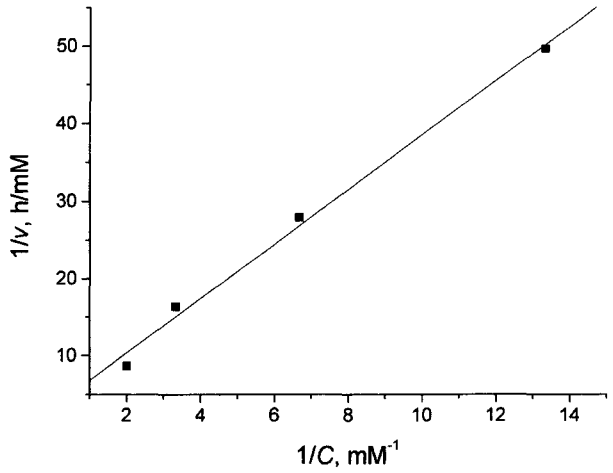


Figure 5. Lineweaver-Burk plot for hydrolysis of *p*-nitrophenyl acetate by MIP at pH 7.0 (*v* as the initial rate for 2 h, *C* as the concentration of *p*-nitrophenyl acetate): 20 mg of MIP containing 4.43 μmol of functional groups in 2.0 mL buffer solution. K_m = 1.06 mM, k_{cat} = 0.137 h^{-1}, k_{cat}/K_m = 0.129 h^{-1} mM^{-1}.

substrate. As shown in Figure 4, the initial rate was increased at first with increasing substrate concentration, but then leveled off at higher substrate concentration, and it remained constant when all active sites were occupied. The imprinted polymer exhibited Michaelis-Menten kinetics for hydrolysis of *p*-nitrophenyl acetate with K_m = 1.06 mM and k_{cat} = 0.302 h^{-1} as calculated in Figure 5.

Conclusions

The molecularly imprinted polymer having both the amidine and imidazole groups in specific microcavities designed for ester hydrolysis displayed enhanced activities with cooperative effect induced by the amidine and imidazole functionalities. The catalytic effect of the imprinted polymer was verified by its Michaelis-Menten kinetics in hydrolysis of the substrate. Thus the above results using the unique MIP system should be useful for the development of synthetic enzymes. The cooperative effect between amidine and imidazole in the synthetic active sites was reported for the first time and is valuable to study further. We shall continuously investigate the nature of the cooperative effect based on the amidine and imidazole functions for the enzyme-mimetic hydrolysis to achieve higher catalytic activity by molecular imprinting.

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