

ORGANIC MOLECULARLY IMPRINTED POLYMERS AS MIMICS OF HYDROLYTIC ENZYMES

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INTRODUCTION

Enzymes catalyze a wide variety of chemical and biochemical reactions with very high reaction rates and specificity under relatively mild conditions.¹ Thus, chemists have sought to create mimics that could match the catalytic properties of enzymes. Early approaches to enzyme mimics involved the design of macromolecular receptors such as cyclodextrins bearing appropriately placed functional groups that mimic the amino acid residues known to be involved in catalysis.² In a more recent approach, combinatorial chemistry was used to screen generated catalysts for enzyme-like activity. A third strategy was to generate a host that was capable of binding to a transition state analogue (TSA) of a reaction; upon removal of the template the host should behave as an artificial enzyme for the reaction chosen.³ This strategy has met considerable success with catalytic antibodies,⁴ and has inspired the use of molecularly imprinted polymers (MIPs) to achieve the same effect.⁵

The first examples of molecular imprinting by use of synthetic organic polymers were independently reported by Klotz and Wulff in 1972 and have since found applications in bio- and chemosensors, microreactors, solid-phase extractions, affinity chromatography, and catalysis.⁶ The principle underlying molecular imprinting is the assembly of a cross-linked polymer matrix around a template; when the template is removed, recognition sites are created which should be complementary to the template (Figure 1). Organic MIPs are characterized by the cross-linking monomers, which are either aliphatic or aromatic such as divinylbenzene (DVB).

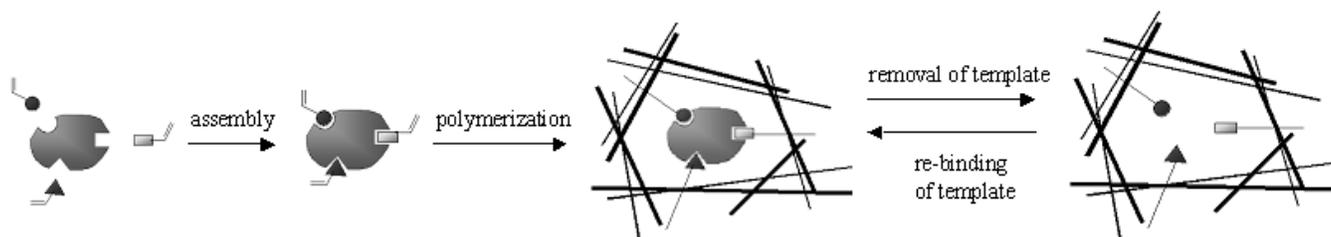


Figure 1. General scheme for MIP construction.

MIPs have been made to recognize small organic molecules, carbohydrates, peptides, proteins, nucleosides and even whole cells.⁸ The interaction between the polymer and template can be covalent or non-covalent, and the type of interaction influences the type of monomer used for polymerization. MIPs have received considerable attention because of their stability under extreme temperatures and pH where natural enzymes might normally degrade. Despite years of efforts to achieve such catalysis with MIPs, the results have thus far been modest.

Transition-State Theory

It was proposed by Pauling as early as 1946 and the concept was later expanded by Jencks that the basic principle behind enzyme catalysis is the molecular recognition and stabilization of the transition state of a reaction, relative to those of the ground state.⁷ In a simple enzyme-catalyzed reaction scheme (Figure 2), the reaction coordinate diagram shows that in order for the enzyme to catalyze the reaction, ΔG_{ETS} must be greater than ΔG_{ES} . MIPs must also be able to do the same to be efficient catalysts, and are thus made by use of a transition state analogue (TSA) as the template.

Enzymes are efficient catalysts also because they exhibit rapid catalytic turnovers. That is, enzymes typically have lower affinity for product than substrate, and many undergo conformational changes that favor release of product. If the enzyme exhibited high affinity for product, then product inhibition would likely occur. In fact, product inhibition has proven to be a serious problem for many MIP catalysts due to their rigidity, especially when the substrate, intermediate, and product are all structurally similar.⁸

Hydrolysis Reactions

The hydrolysis of esters and amides is arguably one of the most common reactions found in nature and vital to the degradation of many biochemical substances. Many enzymes such as serine proteases, lipases, cholesterol esterases, and other hydrolytic enzymes share the same catalytic machinery and mechanism. Some of the earliest and most extensive efforts toward MIP catalysts have used the ‘catalytic triad’ motif of serine, histidine, and aspartic acid found in the family of serine proteases to serve as a model. Chymotrypsin, an enzyme with a well documented catalytic mechanism,^{1,8} has long been a model of choice for MIP catalysts. Rate enhancements by MIPs however, have yet to reach the catalytic rate of this enzyme, which enhances the rate of the hydrolysis of peptide bonds by a factor of $\sim 10^{10}$. This seminar will present recent efforts to synthesize and use organic MIP’s as mimics of hydrolytic enzymes, and the rate enhancements achieved by this approach.

ESTER HYDROLYSIS

Leonhardt and Mosbach were the first to synthesize an MIP chymotrypsin mimic.¹⁰ Imidazole residues were employed to hydrolyze amino acid nitrophenyl esters (Figure 3). The imidazole groups were coordinated by Co^{2+} ions and a picolinyl-*N*-Boc protected amino acid was used as the template. Copolymerization of divinylbenzene (DVB) and the imidazole monomers followed by removal of the

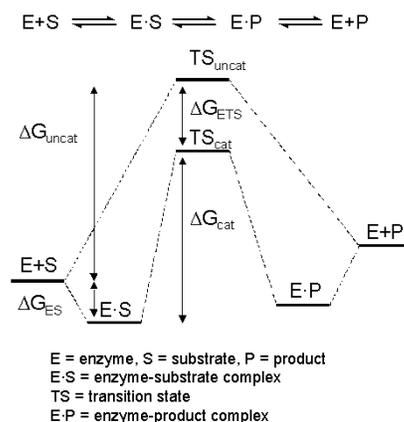


Figure 2. Reaction coordinate diagram for an enzyme-catalyzed reaction.

template resulted in an imprinted polymer network with catalytic activity. They found a 5 to 7-fold rate enhancement of the hydrolysis of Boc-met(or leu)-*p*-nitrophenylester by the imprinted polymer over the control with statistically distributed imidazole groups. Strictly speaking, however, the template molecule was not a TSA but an analogue of the substrate, which was likely the cause of the polymers' rather low rate enhancement.

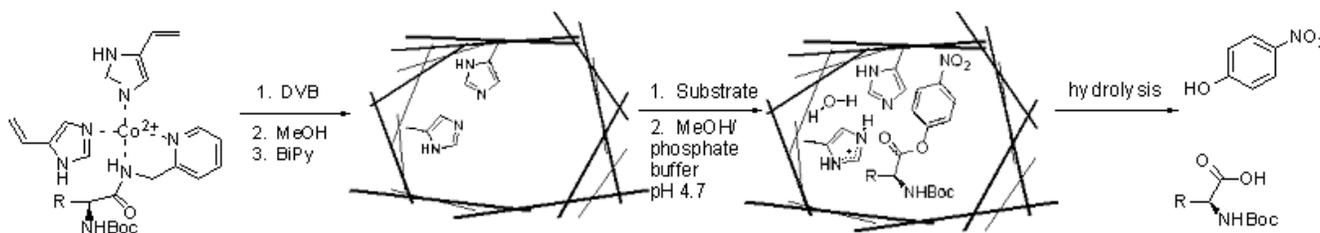


Figure 3. MIP chymotrypsin mimic developed by Leonhardt and Mosbach.¹⁰

Mosbach addressed this problem by using a phosphonate as a TSA for 4-nitrophenyl acetate, and was also attempted by Ohkubo et al.¹¹ The polymer catalysts developed by Mosbach and Ohkubo however, showed a lower rate of enhancement (6.7-fold for Ohkubo's catalyst) compared to the uncatalyzed hydrolysis in solution. The low enhancements were attributed to their inability to orient the imidazole groups selectively. No control polymers were tested and therefore it could not be determined if the imprinting effect was responsible for the catalysis.

Stereoselective Hydrolysis

Ohkubo and co-workers investigated the rate enhancement of the hydrolysis of *Z*-*L*-Leu-4-nitrophenyl ester on a polymer imprinted with a racemic TSA using *L*-histidine as the functional monomer¹² (Figure 4). MIPs with cross-linkers of different lengths and hydrophobic styrene comonomer were synthesized to introduce better cooperativity with the hydrophobic substrate. They were shape- and stereo-selective polymers with hydrolytic activity; their most

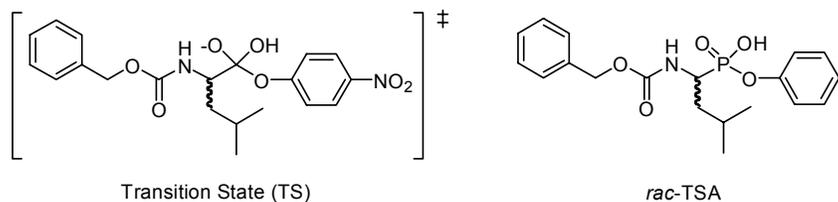


Figure 4. TS and *rac*-TSA used by Ohkubo and coworkers.¹² successful polymers showed a faster hydrolysis for the *L*-isomer over the *D*-isomer by factors of 1.15 and 2.54, and showed catalytic rate enhancements of 28.8 and 3.41, respectively.

Sellergren et al.^{13,14} studied the hydrolysis of *D*- and *L*-(*N*-*t*-butoxycarbonyl)phenylalanine nitrophenyl esters (BocPheONP). The MIP catalysts were constructed by copolymerization of methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), and the template monomer. The cross-linked polymer had appropriately placed hydroxyl, imidazole, and carboxyl functional groups to mimic the amino acid residues of chymotrypsin (Figure 5). One catalyst was constructed in which a

chiral phosphonate analogue of D-phenylalanine was used as the template. The two other catalysts were control polymers, one in which the carboxylic acid groups were randomized using an achiral template and without the

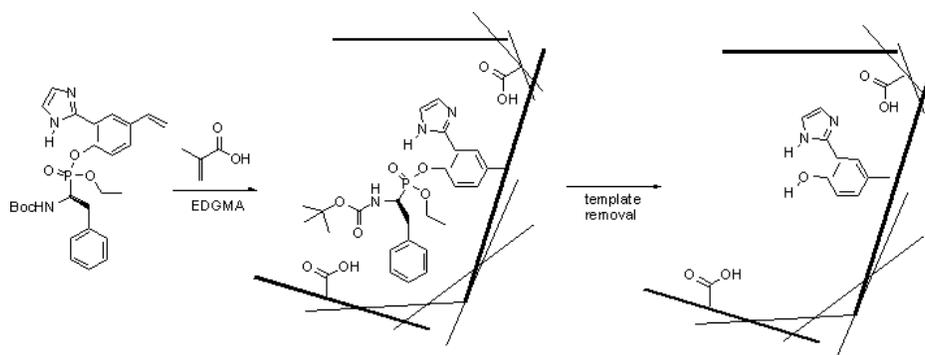


Figure 5. MIP chymotrypsin mimic prepared by Sellaergren et al.^{13,14}

tetrahedral phosphonate, and the other in which the phenylimidazole functionality was removed. The maximum rate enhancement for the hydrolysis of Boc-D-PheONP that was observed with the polymers was 10-fold over reaction in solution. As expected, the control polymers showed less activity, approximately 5.7-fold or less over the reaction in solution and complete loss of enantioselectivity. The polymer catalyst showed a 1.85-fold rate enhancement of the D-isomer over the L-isomer; the control polymers showed no preference of one isomer over the other.

Charged Hydrogen-Bonding and Ester Hydrolysis

The binding of a substrate to an enzyme is influenced by a variety of intermolecular forces, namely electrostatic forces, hydrogen bonding, and cumulative hydrophobic and van der Waals effects. Electrostatic forces and hydrogen bonding are slightly moderated however due to the fact that enzymes operate in water.³ The energy gained from a single neutral-neutral hydrogen bond in water is on the order of 1.5 kcal mol⁻¹. The charged hydrogen bond, however, contributes up to 4.7 kcal mol⁻¹.¹⁵ Model receptor **1** for glutaric acid (Figure 6) with neutral-neutral hydrogen bonding showed strong binding in chloroform ($K_{\text{assoc}} = 60,000 \text{ M}^{-1}$) but in DMSO binding is not observed.¹⁶ Receptor **2** with charged hydrogen bonding, however, showed binding nearly as strong with glutaric acid in DMSO and 5% THF as **1** did in chloroform ($K_{\text{assoc}} = 50\,000 \text{ M}^{-1}$).¹⁷

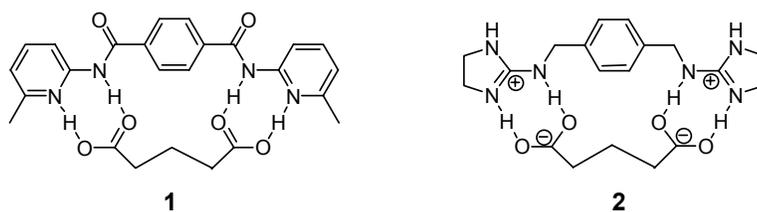


Figure 6. Model receptors with glutaric acid.

Benkovic et al. established that the positively charged guanidine group of arginine formed a similar charged hydrogen bonded complex with phosphonic acid monoesters, playing an essential role in the positioning of the substrate during the formation of an antibody and in catalysis.²⁰ In light of these findings, Wulff and coworkers decided to use *N,N'*-diethyl(4-vinylphenyl)amidine (DEVPA) as a functional monomer to bind to phosphonate TSA-imprint molecules (Figure 7).²¹ Wulff reasoned that a complementary shape to the transition state analogue itself may not be sufficient for catalysis, and that

an appropriately positioned amidine functionality similar to that of arginine had to be introduced for efficient catalysis to occur. Furthermore, the authors reasoned that the efficiency could be improved if the noncovalent bonds had sufficiently high association constants to give 1:1 complexes of template and binding site, an interaction termed the "stoichiometric noncovalent interaction."

The strong, double salt bridge of the DEVPA-phosphonate TSA complex provided an 'oxyanionic hole' for TS stabilization similar to that found in serine proteases. Basic hydrolysis of an aryl ester **3** (Figure 7) was shown to be accelerated >100-fold in the presence of the MIP catalyst. The catalyst exhibited Michaelis-Menton kinetics ($K_m = 0.60$ mM; $k_{cat} = 0.8 \times 10^{-4}$ min⁻¹). The low k_{cat} value was an indication of poor turnover; in fact, in addition to the template, the product was also found to be a competitive inhibitor. Product inhibition was attributed to the carboxylate group that also was capable of binding to the amidine group of DEVPA.

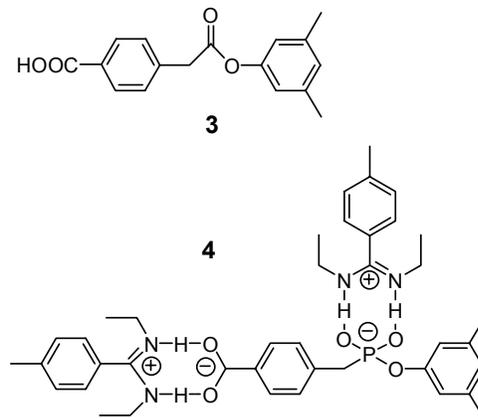


Figure 7. Ester substrate **3** and DEVPA hydrogen-bonded to TSA **4**.

CARBONATE AND CARBAMATE HYDROLYSIS

Wulff et al. investigated the possibility of using DEVPA to catalyze the hydrolysis of carbonates and carbamates (Figure 8).²² The hydrolysis of carbonates and carbamates does not yield carboxylic acids as esters do, but instead liberates CO₂ which does not bind strongly to the catalytic amidine site. Under pseudo-first order conditions, the hydrolysis of diphenyl carbonate and diphenyl carbamate showed rate enhancements of 588 and 1435, respectively, compared to the rates of the uncatalyzed reactions. Relative to that of the control polymer, the MIP catalyst showed rate enhancements of 10 and 24, respectively. These are the largest rate accelerations achieved by MIP catalysts, approaching that of catalytic antibodies.

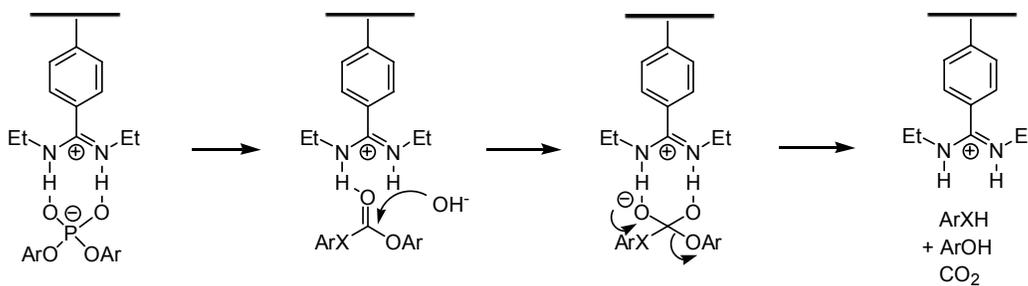


Figure 8. Proposed mechanism of the hydrolysis of carbonates and carbamates (X = O, NH).

MIP with Cholesterol Esterase Activity

The rate enhancements achieved in ester and carbonate hydrolysis by DEVPA inspired Wulff and co-workers to create an MIP catalyst for the hydrolysis of cholesterol 4-nitrophenyl carbonate, a reaction where catalytic antibodies have previously failed.²³ A phosphonate diester was used as the TSA (Figure 9). The resulting complex was copolymerized with ethylene glycol dimethacrylate (EGDMA) as cross-linker. Upon removal of the template with a 1:1 mixture of 0.1 N NaOH:MeCN, the polymer was tested for its catalytic ability. Under pseudo-first order conditions, the hydrolysis of the substrate by the MIP catalyst indicated a 27-fold rate enhancement over the uncatalyzed reaction, and a 2.4-fold rate enhancement over that of the control polymer containing statistically distributed amidine functionalities.

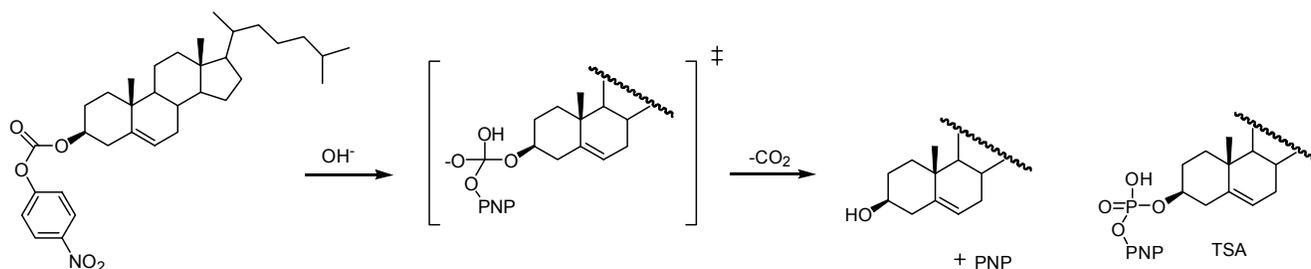


Figure 9. Hydrolysis of cholesterol 4-nitrophenyl carbonate, and TSA.

The MIP catalyst exhibited Michaelis-Menton kinetics ($K_m = 3.7 \text{ mM}$, $k_{cat} = 2.22 \times 10^{-4} \text{ min}^{-1}$), which was an indication of high substrate binding (K_m) and relatively good turnover (k_{cat}). The MIP catalyst was also competitively inhibited in the presence of the TSA ($K_i = 0.9 \text{ mM}$), which was a strong indication that catalysis occurred inside the imprinted cavities. Although this polymer did not show as high a catalytic efficiency as the hydrolysis of diphenyl carbonates or carbamates, it demonstrated the increasing versatility of MIP technology for use as catalysts.

ADVANCES IN POLYMER TECHNIQUES

MIP Catalysts in Bead Form

MIPs are generally prepared via bulk polymerization, a method that is not ideal considering that the irregular particle sizes give rise to reproducibility and scale-up problems. To circumvent these problems, Wulff and co-workers have investigated an aqueous suspension polymerization technique as a method of creating MIPs in bead form.²⁷ The hydrolysis of carbonates and carbamates was studied using DEVPA as the functional monomer and diphenyl phosphate as the template, similar to the hydrolysis studied by the MIPs produced in bulk. The pseudo-first order rate constants showed enhancements by factors of 293 for carbonate and 160 for carbamate compared to the uncatalyzed reactions. These results were somewhat poorer than those given by the MIPs produced in bulk. The rate enhancements with respect to the control polymer were 24-fold for carbonate and 11-fold for the carbamate; the rate

enhancements from the MIPs produced in bulk were only 10-fold in the case of carbonate and 5.8-fold for carbamate. The MIPs in bead form therefore showed a much higher imprinting selectivity than those produced in bulk.

Another study with suspension beads showed that the control polymers containing statistically distributed amidines also exhibited catalytic activity.²¹ Thus, nonspecific catalysis at the surface of the polymers must also play a role in the rate enhancements. The rate of diphenyl carbonate hydrolysis in DEVPA-HEPES buffer solution containing the same amount of amidine groups in the MIP catalyst was not at all higher than the rate constant of hydrolysis in buffer solution ($k_{\text{ami}}/k_{\text{soln}} = 0.93$); thus, the amidine functionality alone could not be responsible for catalysis.

Surface Molecular Imprinting Technique

The inapplicability of water-soluble substrates and slow diffusion of imprint molecules toward the catalytic sites with current MIPs prompted Goto et al. to investigate the "surface molecular imprinting technique."²⁸ Briefly, the MIP was prepared by polymerizing water-in-oil (W/O) emulsions containing the functional host molecule (oleyl imidazole), the template (N α -*t*-boc-L-histidine), and the cross-linking monomer (DVB). Co²⁺ ions were used to coordinate the imidazole residues of the host molecule. The host-guest complex was formed at the interior surface of the water droplets, and the surrounding organic layer was polymerized. Goto et al. investigated the hydrolysis of N-*t*-boc-L-alanine *p*-nitrophenyl ester (Figure 10). Curiously, they used a substrate analogue and not a TSA for the template. Nevertheless, a 1.8-fold increase in rate enhancement was found for the imprinted polymer over the control. Considering that the hydrolysis of the substrate did not occur in solution under the experimental

conditions, the results of the MIP catalyst showed a modest gain.

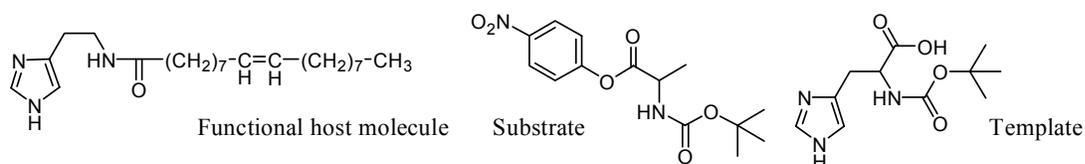


Figure 10. Functional host, substrate and template (substrate analogue).

CONCLUSION

Efforts toward enzyme-mimics of hydrolytic enzymes using molecularly imprinted polymers have been described. The current results still leave much to be desired in terms of practicality, but recent results in carbonate and carbamate hydrolysis indicate progress. MIP catalysts for other organic reactions including elimination reactions, aldol condensations, Diels-Alder reactions,^{5,8} and more recently, oxidative cleavages²⁸ have been reported. The development of molecularly imprinted polymers

for use as catalysts is still relatively new compared to that of catalytic antibodies and faces significant challenges before they find widespread use.

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