An Esterolytic Imprinted Polymer Prepared via a Silica-Supported Transition State Analogue

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In this work we describe a new preparation method for an esterolytic imprinted polymer with catalytic sites on the surface. A template was prepared by immobilizing a transition state analogue (phosphoramidic acid derivative) of an esterolytic reaction within porous silica particles. Polymerization within the pores was carried out using 4-vinylimidazole as a functional monomer and divinylbenzene as a cross-linker. The polymer was released by dissolution of the silica support with hydrofluoric acid and catalytic properties were studied by incubation with three different 4-nitrophenylesters and spectrophotometric determination of the released 4-nitrophenol. For 4-nitrophenyl acetate an activity of 211 nmol min^{-1} mg^{-1} and a K_m value of 2.2 mmol L^{-1} was obtained.

Introduction

There is growing scientific activity and commercial interest in the development of robust enzymes for industrial applications.[1] However, the inherent limited lifetime of natural biocatalysts and their incompatibility with toxic and organic compounds has led to the search for novel biomimetic materials such as engineered polymers with enzyme-like properties.[2,3] Such biomimetic polymers display distinct advantages over their biological counterparts in terms of robustness, ease of application, and production costs. In particular, the technology of molecular imprinting has been intensively investigated in recent years for various applications in diverse areas of biotechnology.[4] The method of molecular imprinting entails the copolymerization of functional and cross-linking monomers in the presence of a target molecule.[4,5] After removal of target molecules, the remaining cavities of the molecularly imprinted polymer (MIP) can exhibit specificities and affinities comparable to those of antibodies.[6,7]

Imprinted polymeric materials have been successfully employed as stationary phases in chromatography[8,9] and solid-phase extraction,[10,11] as artificial receptors in immunoaffinity-type assays,[12] and in sensor applications.[13] The development of catalytically active MIPs has received an upsurge in interest recently and has been summarized in numerous reviews.[14−16] Although many of the MIPs described perform well in organic media,[17,18] an increasing number of examples show that MIPs display good[19] or sometimes enhanced recognition in aqueous media.[20]

In the present work we describe a novel way to obtain catalytically active MIPs. More specifically, the MIPs are produced in a similar way to that employed for catalytic antibodies, namely, by use of immobilized transition state analogues as templates.[21−23] Reactions for which catalytic antibodies have been produced in the past have also been demonstrated with MIPs, for example, ester hydrolysis, Aldol condensation, Kemp elimination, Diels−Alder cycloaddition, β-elimination, and redox reaction.[15,16,24−27] However, the reaction rates are still modest. The reason for this may include the

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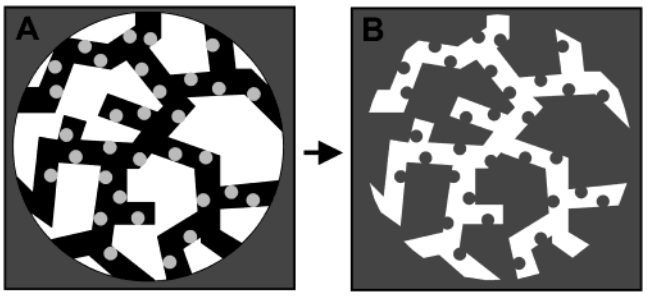

Figure 1. Scheme of molecular imprinting with immobilized transition state analogue (gray balls) within the pores of silica gel (A) and resulting polymer with catalytic pockets on the surface (B).

inflexibility of the highly cross-linked polymer or the slow rates of substrate binding and product release. Increasing the polymer flexibility can be achieved by decreasing the degree of cross-linkage, but at the expense of selectivity.[28] On the other hand, the latter aspect can be addressed by generating the catalytic sites near the polymer surface by using a recently developed approach, where the template is immobilized on a solid support during the imprinting step. The polymerization is carried out within the channels of porous silica gel with the covalently immobilized template on the surface (Figure 1). This approach has been shown to work with small organic molecules,[29] amino acids,[30] and short peptides.[31] One aspect of the present work was to extend this new methodology of immobilized templates to the synthesis of catalytic MIPs by immobilizing a TSA onto the surface of a porous silica support. Our aim was to produce novel catalytic MIPs with improved performance and with highly accessible binding sites that are compatible with aqueous solutions.

Experimental Section

Reagents and Equipment. Aminopropylsilica (APS), dimethylformamide (DMF, $H_2O \leq 0.01\%$), dichloromethane (DCM), hydrofluoric acid (HF), and α,α′-azoisobutyronitrile (AIBN) were purchased from Fluka and used as received. Succinic anhydride, *N*,*N*′-diisopropylcarbodiimide (DIC), propylamine, acetic acid anhydride, and methanol were purchased from Merck and used as received. Divinylbenzene (DVB) was purchased from Merck and purified over a basic aluminum oxide 90 column prior to use to remove polymerization inhibitors. 4-Nitrophenyl acetate, 4-nitrophenyl propionate, 4-nitrophenyl butyrate, methyl *N*-(4-nitrophenyl) carbamate, ethyl 4-nitrophenylcarbamate, 1-ethyl-3-(4-nitrophenyl) urea, and 1,1-diethyl-3- (4-nitrophenyl) urea were purchased from Sigma Chemical Co. and used as received. (4-Aminobenzyl)-phosphoramidic acid-4-nitrophenylester was synthesized according to Takaku et al. and Koizumi et al.[32,33] 4-Vinylimidazole (4-VI) was prepared according to Overberger and Vorchheimer.[34]

Procedures. *Silica Derivatization.* For the template-modified silica, 1 mmol of succinic anhydride was coupled to 1 g of aminopropylsilica (pores ~9 nm, particle size 35−70 μm, 0.9 mmol of NH_2-groups/g, pore volume 0.9 mL/g) in 10 mL of DMF. Onto this 0.1 mmol of template (4-aminobenzyl)-phosphoramidic acid-4-nitrophenylester) was immobilized using 0.3 mmol of DIC as a coupling agent. Finally, 1 mmol of propylamine was coupled to the silica to block the remaining carboxylic groups. Between all coupling steps the silica was washed with 100 mL of DMF, DCM, and methanol each and then dried at 60 °C. The control silica was made by coupling 1 mmol of acetic acid anhydride to 1 g of aminopropylsilica and processed as above.

Polymer Preparation. A pre-polymerization solution was prepared by dissolving 1 mmol of 4-VI and 0.3 mmol of AIBN in 14 mmol of DVB and 0.2 mL of methanol. This solution was purged with argon for 10 min and 0.4 mL was added to either 1 g of TSA-silica or blank-silica. By carefully mixing the pre-polymerization solution with the silica using a spatula, the pre-polymerization solution was completely absorbed by the silica. These monomer/silica mixtures were again purged with argon for 10 min and then kept at 4 °C for 19 h or were directly polymerized. Subsequent polymerization experiments took place at different temperatures (35, 45, 60, and 80 °C) for 72 h. For temperatures below 60 °C, the mixtures were treated afterward at 85 °C for 4 h for final curing of the polymer. The composite was then transferred to a plastic tube, suspended in 2 mL of acetone, and then cooled in an ice−water bath. To dissolve the silica, 4 mL of hydrofluoric acid (40% solution in water) were added carefully in 4−5 portions, while gently shaking the tube. The dissolution of the silica by HF was allowed to react overnight on a rocking table at ambient temperature. The obtained polymer particles were washed with 500 mL of water/ethanol (4:1, v/v) and 100 mL of ethanol, dried overnight at 50 °C, and stored at ambient temperature.

Determination of the Catalytic Activity. Activities were determined by adding appropriate amounts of a 2 mg mL^{-1} polymer suspension to the reaction buffer (20 mmol L^{-1} of phosphate buffer, pH 7.5 with 5% (v/v) methanol), and then starting the reaction with a substrate solution. Initial concentrations in the cuvette were 5% methanol and 5 mmol L^{-1} substrate.

For the estimation of K_m and V_{max}, different substrate concentrations (0.25−6 mmol L^{-1}) were added to the reaction buffer, resulting in a constant polymer concentration of 50 μg mL^{-1} and 5% methanol.

Substrate specificity was determined by adding different substrate solutions to reaction buffer. Resulting concentrations were 1 mmol L^{-1} substrate, 50 μg mL^{-1} polymer, and 5% methanol.

All kinetic measurements were monitored on a Shimadzu UV-1602 UV−visible spectrophotometer in stirred cuvettes by measuring the absorbance at 400 nm caused by the released nitrophenol in an initial time frame between 20 and 60 s of the reaction.

Results and Discussion

Preparation of TSA- and Blank-Silica. The transition state analogue (4-aminobenzyl)-phosphoramidic acid-4-nitrophenylester was immobilized to the carboxylic groups of succinic anhydride-modified porous aminopropyl silica gel using standard solid-phase peptide synthetic coupling methods (Figure 2).[35] As a control, acetic anhydride was immobilized onto aminopropyl silica instead of the TSA. Conversion of the amino groups was monitored with the Kaiser test.[36] Elemental

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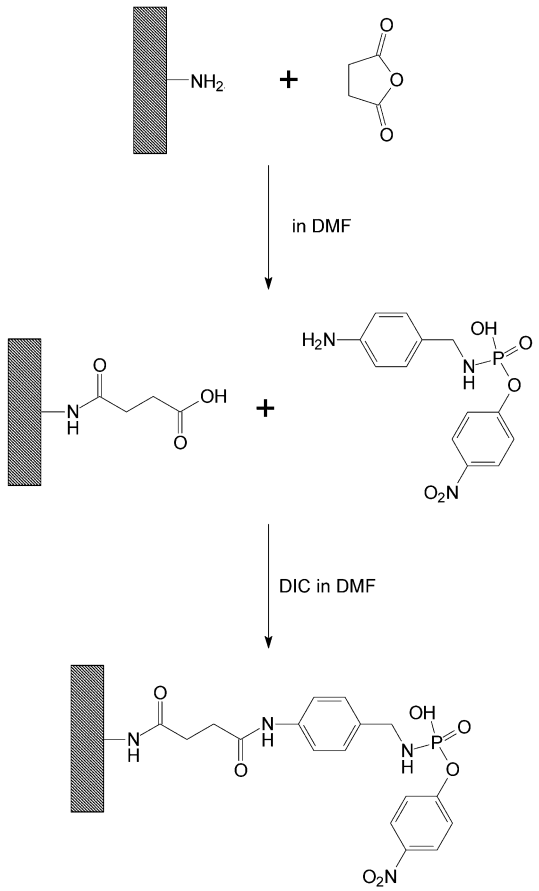

Figure 2. Schematic presentation of the immobilization of the transition state analogue (4-aminobenzyl)phosphoramidic acid-4-nitrophenylester to the silica support.

analysis showed modification of both TSA- and blank-silica. The immobilization yield for the transition analogue was about 69% (69 μmol g^{-1} silica), while about 96% of the amino groups of the control silica was modified with acetic acid. After surface modification, the pore volume was found to be approximately 0.4 mL/g silica.

Preparation of the MIP. For some time it has been known that imidazole can act efficiently as both a nucleophile and an electrophile and can form H-bonds to the oxygen of phosphoric ester groups.[37] For example, in 1957 Bruice and Schmir described imidazole catalysis of the hydrolysis of 4-nitrophenyl acetate.[38] The hydrolysis of 4-nitrophenyl esters was chosen as a simple model reaction because 4-nitrophenol is a good leaving group and the kinetics of the hydrolytic reaction can easily be followed by the increase in absorbance at 400 nm. Moreover, it has already been demonstrated that MIPs with esterase activity can be produced using templates of phosphoric acid derivatives as transition state analogues.[39,40]

Thus, for polymerization, 4-vinylimidazole was chosen as the functional monomer. Imidazole may mimic the function of His, which is a common catalytic component of the active site of numerous enzymes. It was anticipated that 4-VI would specifically interact with the phosphoramide group of the TSA and thereby lead to a conformation in the final polymer capable of exhibiting a catalytic effect. DVB and AIBN were used as a cross-linker and initiator, respectively. Methanol was added in a small quantity to solubilize the functional monomer and the initiator. DVB-based polymers are stable toward HF treatment[41] and have been shown to perform well with this cross-linking agent in similar catalytic MIPs.[42] The pre-polymerization mixture, being a solution of functional monomer, cross-linker, and initiator in methanol, was added to the silica gel in an amount sufficient only to fill out the pores of the gel (0.4 mL/g silica). After polymerization, during which the MIP solidifies inside the silica, the silica backbone was dissolved using aqueous HF. The residual polymer particles represent a negative image of the porous silica particles and also display an irregular shape with a size range of 35−70 μm (Figure 3). It can be seen from Figure 3 that there is no difference in shape or size of the modified silica compared to the silica/polymer composite and the final polymer. Similar imprinted materials where the final polymer reflects the negative image of the silica template have been described previously.[29−31] Agglutination was observed with the composite material but after HF treatment distinct particles were observed. The yield of the polymer particles obtained after dissolution and washing was very high (>95%). In contrast to standard MIPs,[4] these novel MIPs are obtained without any crushing, grinding, sieving, or sedimentation, and after washing, they can directly be used for catalytic studies in aqueous solutions.

All polymers were produced in triplicate to verify reproducibility of the polymer preparation. To improve the reproducibility of polymer synthesis, the pre-equilibration time of the pre-polymerization mixture with modified silica and the effect of the polymerization temperature were investigated. At temperatures of 45, 60, and 80 °C the polymerization of the basic monomer 4-vinylimidazole was unsuccessful in silica. Using lower polymerization temperatures improved the polymerization behavior, and accordingly the best results were obtained using 19 h of pre-equilibration at 4 °C, subsequent polymerization at 35 °C for 72 h, and final curing at 85 ° C for 4 h.

Catalytic Properties of MIP. To examine the catalytic behavior of the polymer, an activity profile by varying the pH of the buffer was performed. As can be seen from Figure 4, the catalytic activity rises with increasing pH and the most pronounced catalytic activity was found above pH 7.5. Phosphate buffer pH 7.5 containing 5% methanol was chosen for further studies. Methanol is important for wetting the highly hydrophobic MIP particles and facilitates the formation of a homogeneous suspension in the aqueous environment. Additionally, the solubility of the substrate is greatly enhanced in the presence of methanol.

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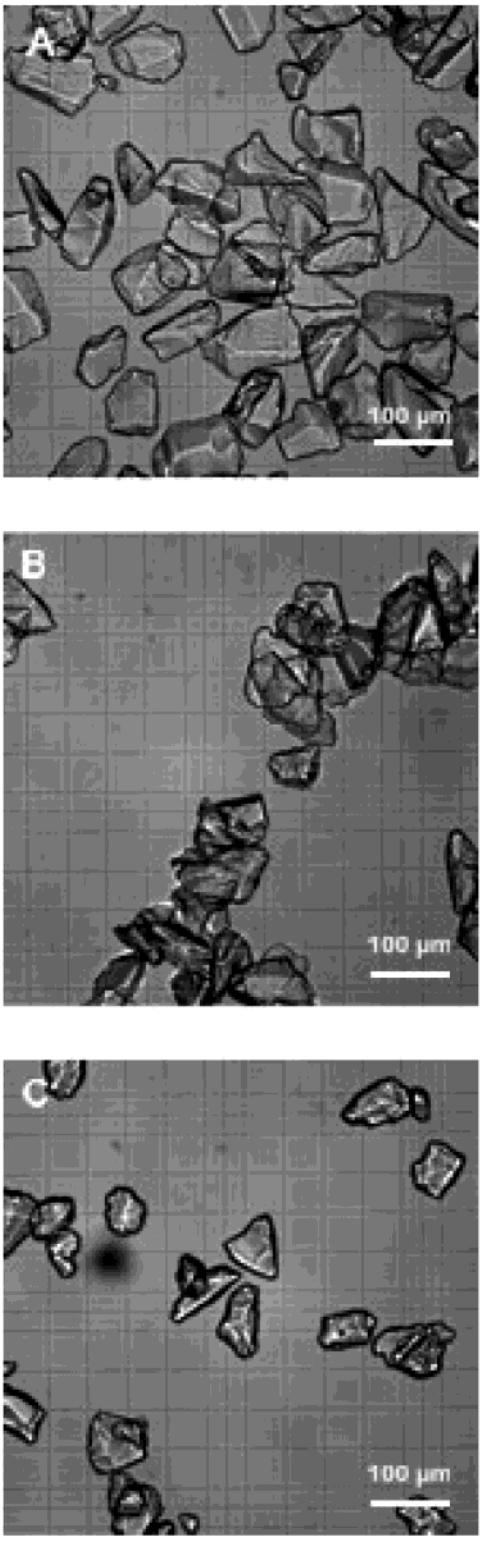

Figure 3. Phase contrast microscopy of three different stages of polymer preparation: (A) modified silica, (B) silica/polymer composite, and (C) polymer.

A linear dependency of the hydrolysis rate on the amount of catalyst was observed (Figure 5).

The hydrolytic activity of the TSA-imprinted polymer was 3.5 times higher than that of the control polymer (Table 1). Compared to similar esterolytic MIPs, which were prepared with TSAs in solution, the relative catalytic activity of the MIPs to control polymer is as high or higher using the new approach. This improved

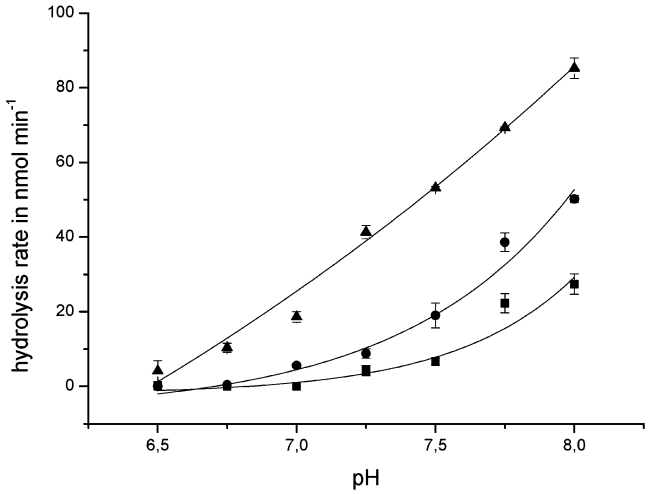

Figure 4. pH dependency of *p*-nitrophenylacetate hydrolysis. Final concentrations in the cuvette were 200 μg mL^{-1} polymer, 5% methanol, and 5 mmol L^{-1} substrate in 20 mmol L^{-1} phosphate buffer. Squares represent spontaneous hydrolysis, circles the control polymer, and triangles the imprinted polymer.

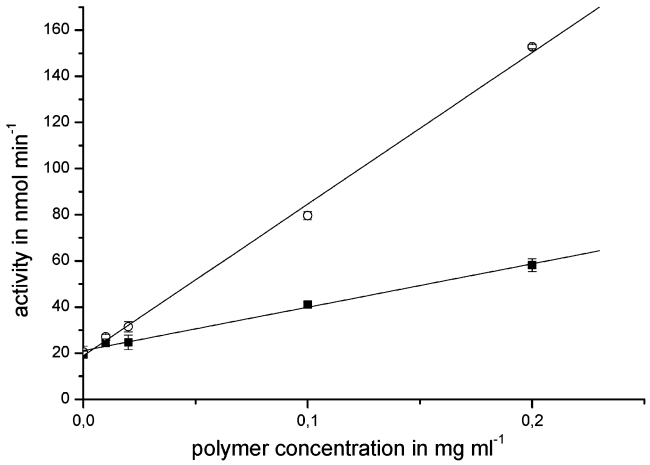

Figure 5. Rate of *p*-nitrophenylacetate hydrolysis dependent on the amount of polymer present in the sample. Final concentrations in the cuvette were 5% methanol and 5 mmol L^{-1} substrate in 20 mmol L^{-1} phosphate buffer pH 7.5. Squares represent control polymer and circles the imprinted polymer.

Table 1. Kinetic Parameters for *p*-Nitrophenyl Acetate Hydrolysis with Imprinted Polymers

	activity[a] (nmol min^{-1} mg^{-1})	K_m (mmol L^{-1})	V_{max} (nmol min^{-1})
control polymer	56.85 ± 1.86	4.13 ± 0.64	16.32 ± 1.33
imprinted polymer	211.15 ± 20.16	2.22 ± 0.28	29.22 ± 1.49

[a] Because of weak solubility, activities/amount polymer was not measured under substrate saturation, but at 5 mmol L^{-1}.

catalytic activity can be attributed to the higher concentration of functional groups on the surface of the MIP compared to that of the control polymer. In the latter, a higher portion of functional monomers is located randomly within the bulk of the polymer. As the present polymers are prepared with very low amounts of porogen, these nonspecifically positioned functional monomers are less accessible than in conventional control polymers prepared with higher contents of porogen.

For further characterization, the influence of substrate concentration on the reaction rate was studied. As shown in Figure 6, the MIPs follow Michaelis–

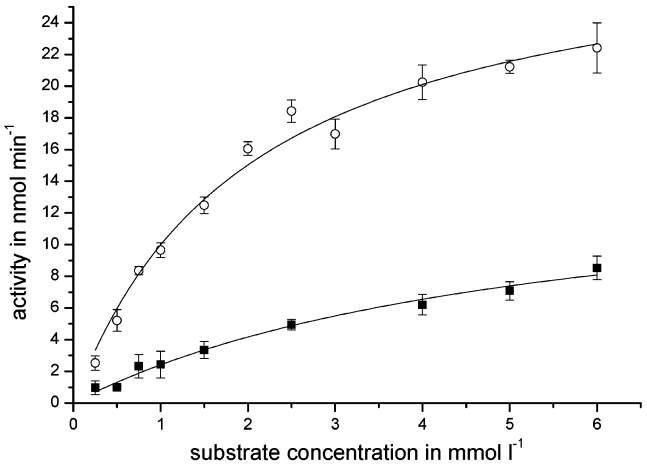

Figure 6. Concentration dependence of the rate of hydrolysis of *p*-nitrophenylacetate for the control polymer (squares) and the imprinted polymer (circles). Final concentrations in the cuvette were 50 μg mL^{-1} polymer and 5% methanol in 20 mmol L^{-1} phosphate buffer pH 7.5. Rates are corrected by spontaneous hydrolysis.

Table 2. Activities for Three Different Nitrophenylesters

	activity in nmol min^{-1}		
nitrophenyl−substrate	spontaneous hydrolysis	control polymer	imprinted polymer
acetate	3.1 ± 0.5	3.7 ± 0.9	7.0 ± 0.2
propionate	4.0 ± 0.8	4.4 ± 0.3	6.8 ± 0.8
butyrate	2.5 ± 0.1	3.5 ± 0.3	6.2 ± 0.5

Menten kinetics. It was not possible to apply concentrations higher than 6 mmol L^{-1} due to the weak solubility of the substrate, so V_{max} was calculated by nonlinear fitting. Nevertheless, it is shown that K_m values are about 2 times lower for the imprinted polymer than for the control polymer, and the calculated maximal activity V_{max} is 2 times higher. The higher affinity of the imprinted polymer, which is represented by the K_m value, is probably due to fact that the functional monomers used are in a much more specific arrangement and orientation to the template during the imprinting process.

In addition to 4-nitrophenyl acetate, the hydrolysis of 4-nitrophenyl propionate and 4-nitrophenyl butyrate was investigated (Table 2). Also shown is the spontaneous hydrolysis, which occurs in the same solution in the absence of any polymer. Catalytic activity toward the propionate ester of the imprinted polymer compared to that of the control polymer was 1.5 times higher and compared to spontaneous hydrolysis 1.7 times higher, while for the butyrate ester the ratios were 1.8 and 2.5, respectively. From this result we can conclude that the size of the acid component is not very crucial for recognition of the substrate and therefore for the turnover of the substrate. The result is consistent with the imprinting procedure where the template was immobilized in this part of the template molecule and only the phosphonate group with the nitrophenol moiety was exposed for imprinting (Figure 2). Moreover, it was interesting to see whether structurally similar but much more stable compounds can be hydrolyzed by the produced MIP. Therefore, hydrolysis of methyl-*N*-(4-nitrophenyl) carbamate, ethyl 4-nitrophenylcarbamate, 1-ethyl-3-(4-nitrophenyl) urea, and 1,1-diethyl-3-(4-nitrophenyl) urea were investigated (Figure 7). The

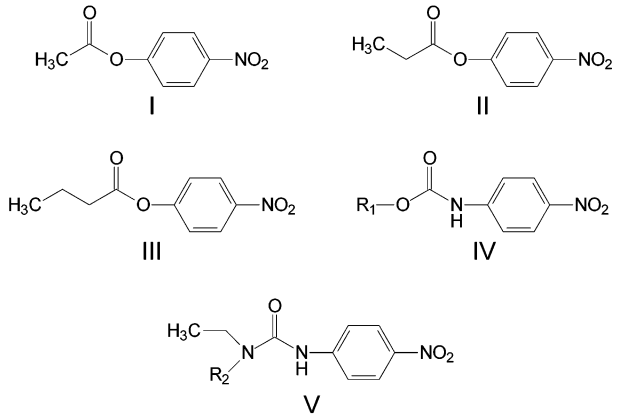

Figure 7. Different substrates used in this study. (I) 4-Nitrophenyl acetate, (II) 4-nitrophenyl propionate, (III) 4-nitrophenyl butyrate, (IVa) methyl-*N*-(4-nitrophenyl) carbamate (R_1: CH_3), (IVb) ethyl-4-nitrophenyl carbamate (R_1: C_2H_5), (Va) 1-ethyl-3-(4-nitrophenyl) urea (R_2: H), and (Vb) 1,1-diethyl-3-(4-nitrophenyl) urea (R_2: C_2H_5).

incubation time with polymers was extended to 24 h since after this period small amounts of *p*-nitrophenol could be detected due to spontaneous hydrolysis. However, no increase in hydrolysis was observed in the presence of polymers. The two carbamates and ureas were chosen because it was assumed that because of the bad leaving groups, the hydrolysis will also proceed via a tetrahedral intermediate ($B_{Ac}2$ mechanism). However, in contrast to the used TSA, which carries an aryl-O group, the tested carbamate and urea substrates carry an aryl-NH group. This could affect the proper orientation of the tetrahedral intermediate within the polymer and could be an explanation for the lack in activity. However, to understand the catalytic mechanism in more detail, further kinetic investigations are necessary.

Conclusions

In this study, we have shown that catalytic polymers can be produced by using an immobilized transition state analogue on the surface of porous silica gel as a template. The produced polymer carries easy accessibility catalytic sites on the surface of the polymer, it exhibits higher relative catalytic activities than conventional catalytic MIPs, and the reaction can be investigated in aqueous solutions. In future work, we would like to increase the catalytic strength by, for example, introducing metal ions and expand the investigations to the hydrolysis of organophosphate esters (pesticides and warfare agents), phenylcarbamates, and phenylureas and to integrate the MIPs into a sensor configuration.

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