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Molecularly Imprinted Microgels as Enzyme Inhibitors

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Abstract: We demonstrate, on the example of trypsin, the use of water-soluble molecularly imprinted polymer microgels as specific enzyme inhibitors. Using a strong anchoring monomer, methacryloylaminobenzamidine, the growing polymer chains are confined to close proximity of the substrate recognition site of our model enzyme. The microgels bind selectively trypsin over other proteins of similar size and molecular weight, and show competitive inhibition of trypsin with an inhibition constant K_i of 79 nM, making them more potent inhibitors than the low molecular-weight competitive inhibitor benzamidine by almost 3 orders of magnitude. We believe that these tailor-made materials with biological activity have potential for future drug development that extends beyond enzyme inhibition.

Introduction

Molecularly imprinted polymers (MIPs) are synthetic materials that are able to specifically recognize and bind target molecules.[1−3] They are sometimes referred to as artificial antibodies[4] and are considered an alternative to antibodies because of their higher chemical and physical stability, easier availability, and lower cost. To date, most of the applications that have been proposed for MIPs are situated in the analytical chemistry and biochemistry fields. Chemical synthesis and catalysis are other application areas.[5,6] More recently, it has also been suggested that MIPs could be used for drug delivery.[7] However, there is no report on a MIP that exhibits a proper biological activity and that could thus be used as a drug itself.

Enzyme inhibition or activation is one of the most important principles for the control of metabolic reactions and thus for the regulation of biological processes. Therefore, efficient enzyme inhibitors are often potential drug candidates, and the discovery of new inhibitors is an important research area. Commonly, small organic molecules are used as inhibitors, which can be obtained in large scale through chemical synthesis. However, they often lack specificity, resulting in side effects. On the other hand, antibodies have been proposed as inhibitors because of their high specificity for the target antigen. Alas, they are more expensive and tend to be physically and chemically unstable. There have been attempts to replace antibodies with artificial receptors, such as aptamers or oligopeptides, obtained through selection from a large library. In that context, the question whether MIPs can be tailor-made that exhibit enzyme inhibition appears logical and of great interest. These MIPs may have some advantages over biomacromolecules; for example, when used in vivo, they will not be degraded by proteases.

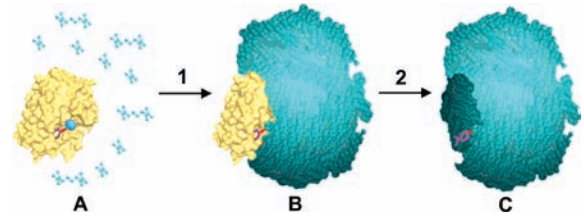

Figure 1. Schematic representation of the molecular imprinting of trypsin using a polymerizable inhibitor as an anchoring monomer. The enzyme is put into contact with the anchoring monomer and comonomers (A); polymerization is conducted; (1) a cross-linked polymer is molded around the substrate binding site (B); the enzyme is removed (2), revealing a specific recognition site with inhibitory properties (C).

In this paper, we propose, for the first time, the use of molecularly imprinted polymer microgels as enzyme inhibitors. We employ an original synthesis strategy where the polymerization is conducted in the proximity of the protein. This is possible by employing a strong anchoring point, in this case a low-molecular mass inhibitor, which is coupled to a polymerizable moiety and thus incorporated in the growing polymer chain (Figure 1). As a first model target, we have chosen the protease trypsin.

Results and Discussion

To be used as an enzyme inhibitor and potentially as a drug, a MIP should be water-compatible and be synthesized from biocompatible building blocks. There have been a number of reports on MIPs that can recognize proteins[8,9] as well as a few

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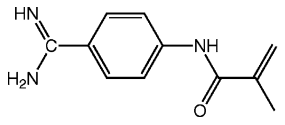
(1) Wulff, G.; Sarhan, A. *Angew. Chem., Int. Ed.* **1972**, *11*, 341.
(2) Arshady, R.; Mosbach, K. *Makromol. Chem.* **1981**, *182*, 687–692.
(3) Haupt, K. *Chem. Commun.* **2003**, 171–178.
(4) Vlatakis, G.; Andersson, L. I.; Müller, R.; Mosbach, K. *Nature* **1993**, *361*, 645–647.
(5) Hedin-Dahlstrom, J.; Rosengren-Holmberg, J. P.; Legrand, S.; Wikman, S.; Nicholls, I. A. *J. Org. Chem.* **2006**, *71*, 4845–4853.
(6) Liu, J. Q.; Wulff, G. *J. Am. Chem. Soc.* **2008**, *130*, 8044–8054.
(7) Hilt, J. Z.; Byrne, M. E. *Adv. Drug Delivery Rev.* **2004**, *56*, 1599–1620.
(8) Turner, N. W.; Jeans, C. W.; Brain, K. R.; Allender, C. J.; Hlady, V.; Britt, D. W. *Biotechnol. Prog.* **2006**, *22*, 1474–1489.
(9) Hansen, D. E. *Biomaterials* **2007**, *28*, 4178–4191.

papers on molecularly imprinted microgels (also occasionally referred to as nanogels), that is, highly solvated cross-linked polymer particles (the solvent is chosen such that the polymer chains formed never precipitate), with sizes well below 1 μm.[10,11] The latter format appears the most suitable for the preparation of MIP-based enzyme inhibitors if it can be adapted to aqueous solvents. Thus, we first had to find conditions for the synthesis of cross-linked polymer microgels in water. We have selected a number of water-soluble functional monomers and cross-linking monomers, and synthesized a range of polymers with all possible combinations (see Supporting Information, SI). In addition, we varied the total monomer concentration in the reaction mixture and the cross-linking degree of some of the polymers. The sizes of the particles were determined by dynamic light scattering. The smallest particles (mostly below 500 nm) were obtained with dihydroxyethylene bisacrylamide, but also ethylene bisacrylamide was a suitable cross-linker. Cross-linking of 60% or lower, and the lowest monomer concentration tested (0.5%), yielded the smallest particles.

Trypsin hydrolyses polypeptides at the carboxyl-side of a Lys or Arg residue. One of its best-known inhibitors is benzamidine. We have therefore used benzamidine as a ligand and strong anchoring point for MIP synthesis. It was rendered polymerizable by coupling 4-aminobenzamidine (AB) to methacrylic acid (see SI for synthesis details):



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The inhibition constant K_i of the resulting inhibitor monomer **1** was determined and compared to that of free 4-aminobenzamidine. Competitive inhibition with a K_i of 40.3 $\pm$ 8.9 μM was obtained for **1**, whereas 4-aminobenzamidine had a K_i of 18.4 $\pm$ 7.2 μM. Both values are in the same order of magnitude, and the affinity of trypsin for **1** is strong enough so that **1** can be used as an anchoring monomer in a stoichiometric ratio with trypsin during molecular imprinting. The concentration of **1** in the reaction mixture is about five times higher than its inhibition constant, so that a large percentage of **1** will be complexed during polymerization (about 65%, as calculated by a mass balance using the inhibition constant). This was a compromise, since preliminary experiments showed that when using a higher ratio of **1**/trypsin nonspecific binding increased and the molecular imprinting effect decreased. In fact, benzamidine-bearing monomers have been described earlier for the synthesis of polymers for affinity separation of trypsin.[12,13] Later, Vaidya et al. used a benzamidine monomer (acrylic acid coupled to AB) for the synthesis of a MIP for trypsin purification.[14] Their approach was different of ours, though; they used a large (127 times) excess of benzamidine monomer over trypsin, and synthesized bulk polymer monoliths with a lower degree of cross-linking that were then mechanically ground into particles with diameters between 250 and 500 μm. For our application, using such a large excess of inhibitor monomer **1** was not appropriate since that would result in excess inhibitor groups randomly distributed in the polymer (about 360 times more than with our MIP recipe as described above), thus yielding a multiligand affinity material where the imprinting effect would be masked by simple interactions of trypsin with these groups.

We then synthesized polymer particles imprinted with trypsin using anchoring monomer **1** and a range of water-soluble functional monomers together with ethylene bisacrylamide as the cross-linker. The imprinting factor (IF, trypsin bound to MIP/trypsin bound to nonimprinted control polymer) was determined by equilibrium binding experiments (see SI). We found that the best IF were obtained with the neutral hydroxyethyl methacrylate and methacrylamide monomers (IF = 2.8 and 2.5, respectively). Negatively charged monomers yielded high binding but no imprinting effect, while positively charged monomers yielded no binding, both due to the predominance of ionic attraction or repulsion. To facilitate the handling of the particles, for the following experiments, we chose a polymer recipe that yielded particles with diameters of around 1 μm as determined by dynamic light scattering (methacrylamide/ethylene bisacrylamide, 60% cross-linking, 5% total monomer concentration, see SI for dynamic light scattering data). An important issue was the complete removal of template enzyme from the polymers. Two methods were used to detect and quantify remaining trypsin in the MIP, SDS−polyacrylamide gel electrophoresis and determining the enzyme's activity in the MIP microgels through pH-stat measurements with *N*-*p*-tosyl-L-arginine methyl ester (TAME) as the substrate. pH-stat was used since the presence of micrometer-sized particles in the reaction mixture disturbed real-time activity measurements by spectrophotometry, due to light scattering. For the removal of trypsin from the polymers, different methods were tested (see SI for details): electromigration, 2 or 10 M urea, digestion by proteinase K, 5% SDS (w/w) in 10% acetic acid (v/v), autolysis at 40 °C. Only electromigration and proteinase K treatment resulted in the quantitative removal of trypsin from the MIP, whereas the other methods were less effective. We favored electromigration since it avoids both harsh conditions that might alter the polymer's conformation and thus the integrity of the binding sites, and the contact of the MIP with another protein (proteinase K) or with SDS that could give rise to artifacts.

The affinity of the polymers for trypsin and the selectivity were then determined through equilibrium binding assays. MIP microgels were incubated with trypsin. At equilibrium the particles were removed by centrifugation and the residual activity of trypsin in the supernatant was determined. This yields the amount of trypsin bound to the microgels. The results are shown in Figure 2. It can be clearly seen that molecular imprinting using the anchoring monomer **1** results in an increase in binding capacity when compared to the nonimprinted control polymer (NIP) (a). The imprinting factor is comparable to that reported by Vaidya et al.[14] The binding to the NIP may appear high, but that can be explained by the presence of benzamidine moieties that act as affinity ligands. Indeed, when the polymers were synthesized without the anchoring monomer, much less binding to the NIP and no imprinting effect were observed (b). When the anchoring monomer **1** was used but free AB was present as a competitor during imprinting, the binding of trypsin to both polymers was in the same range as that to the control polymer in experiment a, without imprinting effect, since the

(10) Wulff, G.; Chong, B.-O.; Kolb, U. *Angew. Chem., Int. Ed.* **2006**, *45*, 2955–2958.
(11) Carboni, D.; Flavin, K.; Servant, A.; Gouverneur, V.; Resmini, M. *Chem. Eur. J.* **2008**, *14*, 7059–7065.
(12) Male, K. B.; Luong, J. H. T.; Nguyen, A. L. *Enzyme Microbiol. Technol.* **1987**, *9*, 374–378.
(13) Vaidya, A. A.; Lele, B. S.; Kulkarni, R. A.; Mashelkar, R. A. *Biotechnol. Bioeng.* **1999**, *64*, 418–425.
(14) Vaidya, A. A.; Lele, B. S.; Kulkarni, M. G.; Mashelkar, R. A. *J. Appl. Polym. Sci.* **2001**, *81*, 1075–1083.

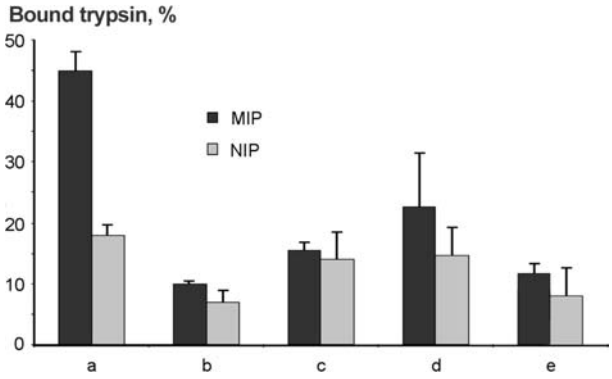

Figure 2. Binding of trypsin to trypsin−MIP and NIP microgels in 5 mM TRIS·HCl buffer pH 8.0, 10 mM $CaCl_2$. The MIP was a methacrylamide/ethylene bisacrylamide copolymer with 60% cross-linking. Trypsin concentration 600 nM, polymer concentration 10 mg/mL in all assays. Free trypsin in the supernatants was quantified by spectrophotometric measurements using TAME as the substrate: (a) anchoring monomer **1** was used; (b) anchoring monomer was not used; (c) anchoring monomer **1** was used plus 450 μM of free AB; (d) anchoring monomer **1** was used and trypsin was inactivated with PMSF; (e) anchoring monomer was not used but free AB (450 μM) was present during polymer synthesis.

anchoring monomer could not bind to trypsin during imprinting (c). When, however, the anchoring monomer was used but trypsin was irreversibly inhibited by the group-specific agent phenylmethylsulfonyl-fluoride (PMSF), a slight imprinting effect was obtained (d). This can be explained by the fact that PMSF reacts with the serine residue (Ser 195) in the active site to inhibit trypsin,[15] whereas the competitive inhibitor benzamidine binds to Asp 189 in the substrate recognition site[16] that is localized at a certain distance to the active site. Thus, blocking the active site serine with PMSF does not necessarily prevent the anchoring monomer **1** from binding to trypsin. Finally, when the polymer was synthesized without the anchoring monomer **1**, but nonpolymerizable AB was present during imprinting, binding was again low to both MIP and NIP, without imprinting effect (e). These experiments clearly show that molecular imprinting of polymer microgels with trypsin is possible when a strong anchoring monomer is used; moreover, comparison with the NIPs and the different other controls also confirm that the binding of trypsin by the MIP is not simply due to the presence of benzamidine as an affinity ligand.

Binding isotherms were recorded for the MIP and NIP microgels in a concentration range between 30 nM and 6 μM of trypsin. Nonlinear curve fitting of a single-site Langmuir-type binding isotherm yielded a good fit for the MIP in that concentration range (see SI), allowing to determine the binding parameters. A dissociation constant K_D of 1.5 ± 0.2 μM and a maximum binding capacity of 40.7 ± 2.1 pmol/mg of polymer were obtained. While the affinity of our MIP is lower, the binding capacity is comparable to that of the MIP reported by Vaidya.[14] The NIP microgel did not yield a typical single or double site Langmuir isotherm, and binding parameters could therefore not be determined with precision.

To study the selectivity of the MIP microgel for trypsin, we performed equilibrium binding experiments with a number of other proteins, namely, cytochrome c, RNase A, lysozyme, myoglobin, chymotrypsin, and bovine serum albumin (Figure 3). We observed specific binding to the trypsin−MIP only with trypsin. Other proteins of similar or smaller size and similar

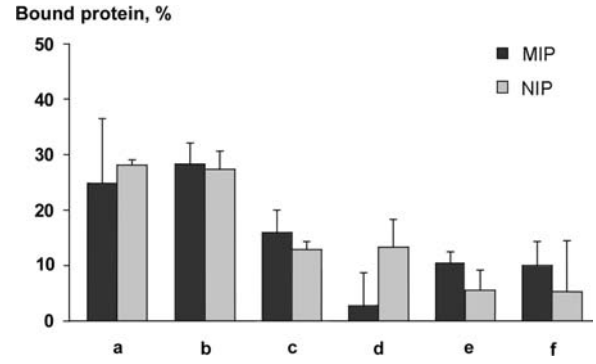

Figure 3. Binding of different proteins to trypsin (MW = 23800, pI = 10.5) MIP and the corresponding NIP microgels. Experiments were done in 5 mM Tris·HCl buffer pH 8.0. Protein concentration was 600 nM. Polymer concentration was 10 mg/mL in all assays: (a) cytochrome c (MW = 12400, pI = 10.2); (b) RNase A (MW = 13700, pI = 9.6); (c) lysozyme (MW = 14300, pI = 11.3); (d) myoglobin (MW = 17600, pI = 7.1); (e) chymotrypsin (MW = 25000, pI = 9.1); (f) bovine serum albumin (MW = 66000, pI = 4.8). All proteins were quantified using the BCA protein assay.

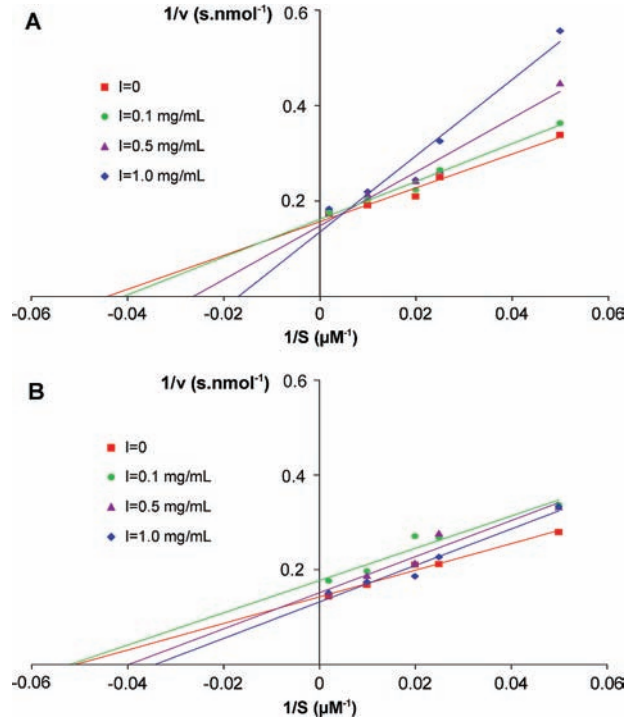

Figure 4. Trypsin inhibition by trypsin−MIP (top) and NIP (bottom) microgels (Lineweaver−Burk plot). Activities were determined by pH-stat measurements using TAME as the substrate, in a solution of 200 mM KCl and 10 mM $CaCl_2$. The polymer concentrations were 0.1, 0.5, and 1.0 mg/mL. Trypsin concentration = 30 nM.

isoelectric point (cytochrome c, RNase A, lysozyme, chymotrypsin) bind equally to MIP and NIP, whereas larger proteins like BSA and myoglobin bind to a lesser extent, and again no imprinting effect is observed.

The trypsin−MIP was now tested as an inhibitor for trypsin. Classical enzyme inhibition studies were performed where the Michaelis constant K_m and the maximum activity V_{max} were determined in solution in the absence and in the presence of different concentrations of MIP. Enzymatic activities were determined using pH-stat measurements. Figure 4 shows the results obtained as Lineweaver−Burk graphs. For the MIP, a typical competitive inhibition behavior was obtained. For the

(15) Gold, A. M. *Biochemistry* **1965**, *4*, 897–901.
(16) Mares-Guia, M.; Shaw, E. *J. Biol. Chem.* **1965**, *240*, 1579–1585.

NIP, some inhibition was also observed though to a much lesser extent, and it was not possible to clearly determine the type of inhibition. This is probably due to a much greater inhomogeneity, compared to the MIP, of the nonimprinted polymer concerning possible interaction sites with trypsin. The curves obtained for the MIP allowed the determination of the real and the apparent (in the presence of a given concentration of inhibitor) Michaelis constants and the maximum activity. The inhibition constant K_i of the MIP could thus be calculated from the Michaelis−Menten equation developed for competitive inhibition:

$$v = \frac{V_{max}[S]}{K_M(1 + I/K_i) + [S]}$$

For the calculation of the inhibition constant K_i, the concentration of the inhibitor (I) needs to be known. In our case this is the maximum binding capacity, that is, the concentration of accessible binding sites in the MIP (40.7 pmol trypsin/mg of polymer as determined through nonlinear curve fitting or 40.7 nM if 10 mg of MIP are used in 10 mL solution in an inhibition experiment, see SI). The calculated inhibition constant K_i is 79 nM, which demonstrates that the MIP is a much more potent inhibitor than free AB (K_i = 18.9 μM) or the anchoring monomer **1** free in solution (K_i = 40.3 μM) by almost 3 orders of magnitude. Even with the theoretical concentration of binding sites of 4.2 μM (the amount of anchoring monomer **1** used during imprinting, and assuming an imprinting efficiency of 100%), the calculated K_i of 4.8 ± 0.3 μM is still lower than that obtained with the anchoring monomer or with AB. This cannot simply be explained by the "multivalency" of the inhibitory polymer (several benzamidine moieties are present on one molecule), since the NIP, which is identical to the MIP in terms of chemical composition (monomers, cross-linker, initiator), does not yield the same effect. On the contrary, we believe that this strong increase in inhibitory potency can be attributed to the creation of specific binding sites by molecular imprinting, providing additional interactions and shape-specificity for the binding of trypsin. However, this is only possible thanks to the use of a strong anchoring monomer during imprinting, the polymerizable benzamidine, which seems to confine the formation of the MIP to the proximity of the substrate binding site of trypsin. Similar inhibition results were obtained with MIP microgels of below μm size. A copolymer of hydroxyethyl methacrylate and ethylene bisacrylamide, with 60% cross-linking and a monomer concentration of 2% (650 nm particle size) yielded competitive inhibition with an inhibition constant K_i = 44 nM (see SI for Langmuir isotherm to determine the binding capacity). No inhibition was obtained with the NIP microgel.

To verify the selectivity of the inhibition by the trypsin−MIP, we performed inhibition experiments with two other, closely related enzymes, namely chymotrypsin and kallikrein. Chymotrypsin is a serine protease of similar molecular weight and isoelectric point, but with a different substrate specificity compared to trypsin. It hydrolyzes polypeptide chains after aromatic residues and is not inhibited by benzamidine. Kallikrein is a serine protease of a molecular weight slightly higher than that of trypsin and with an acidic isoelectric point (38000 Da and 4.3, respectively). It has a similar substrate specificity as trypsin and is, like trypsin, inhibited by benzamidine derivatives.[17] Although some inhibition of kallikrein by the trypsin−MIP was observed, the effect was nearly negligible compared to the inhibition of trypsin, and no particular type of inhibition could be distinguished. We ascribe this to the presence of some randomly placed benzamidine groups at the particle surface. Chymotrypsin was not inhibited by the trypsin−MIP. These results demonstrate again the specificity of the trypsin−MIP acquired through the molecular imprinting process.

Conclusion

We have demonstrated for the first time that water-compatible molecularly imprinted polymer microgels can be used as specific enzyme inhibitors. Using a MIP synthesis method that relies on a strong anchoring monomer, the growing polymer chains are confined to close proximity of the substrate recognition site of our model enzyme trypsin. The MIPs showed a competitive inhibition behavior with an inhibitor potency almost 3 orders of magnitude stronger than the well-known low molecular-weight competitive inhibitor benzamidine. We believe that the potential of these materials is much larger than just enzyme inhibition, since the principle of the anchoring monomer is not limited to enzyme inhibitors, but other strong interaction points will work as well. Thus, these synthetic polymeric materials with biological activity have a potential in future drug development.

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Supporting Information Available: Details about experimental procedures, the synthesis of **1**, optimization of microgel synthesis, and equilibrium binding data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) Stürzebecher, J.; Prasa, D.; Wikström, P.; Vieweg, H. *Braz. J. Med. Biol. Res.* **1994**, *27*, 1929–1934.