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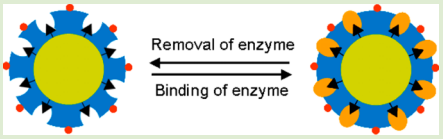
# Efficient Synthesis of Molecularly Imprinted Polymers with Enzyme Inhibition Potency by the Controlled Surface Imprinting Approach

Huiqi Zhang,* Jingshuai Jiang, Hongtao Zhang, Ying Zhang, and Pingchuan Sun

Key Laboratory of Functional Polymer Materials, Ministry of Education, College of Chemistry, Nankai University, Tianjin 300071, P. R. China.

Supporting Information

**ABSTRACT:** A facile, general, and highly efficient approach to prepare uniform core−shell molecularly imprinted polymer (MIP) particles with enzyme inhibition potency is described for the first time, which involves the combined use of molecular imprinting and controlled/"living" radical polymerization (CRP) techniques as well as surface-anchoring strategy. The thickness of the enzyme-imprinted surface layers of the core−shell MIP microspheres had a significant influence on their binding properties, and only those with their thickness comparable with the diameters of the targeted enzymes could afford enzyme-MIPs with optimal specific bindings. The as-prepared enzyme-MIPs were found to have homogeneous binding sites and high template binding capacities, affinity, and selectivity, and they proved to show much higher enzyme inhibition potency than the small inhibitor by 3 orders of magnitude (i.e., the enzyme inhibition constant of every binding site of the MIP microspheres was about one-thousandth of that of the small inhibitor), mainly due to the formation of strong long-range secondary interactions between enzymes and imprinted pockets. In addition, the general applicability of our strategy was confirmed.




Recent years have witnessed considerable interest in the design and synthesis of potent enzyme inhibitors due to their great potential as drugs. Some versatile techniques have been developed for this purpose, such as the well-known fragment-based strategy[1−3] and "two-prong" approach.[4,5] They can provide more potent enzyme inhibitors by introducing to relatively weak inhibitors certain functional groups capable of forming secondary interactions with the enzymes' binding pockets or with functional groups on the enzyme surfaces, respectively. The design of such "localized" secondary interactions, however, normally requires detailed knowledge of the binding pockets or surfaces of the enzymes and strict control over the structures of the designed inhibitors, which significantly limits their more general applications.

In nature, many biological processes are governed by protein−protein interactions that utilize a large interface area (approximately 600 Å$^2$) and multiple points of functionality to ensure tight binding.[6] Moreover, many potent enzyme inhibitors developed by nature (e.g., the soybean trypsin inhibitor) are themselves macromolecules, and they use long-range multiple interactions between their polypeptide chains and the targeted enzymes to achieve very high binding affinity.[7] It can be envisioned that the use of long-range secondary interactions in drug design should provide some distinct advantages over "localized" interactions such as the less requirement for the detailed knowledge of enzyme structures and greater flexibility in the synthesis and structural design of inhibitors. From this viewpoint, the molecular imprinting technique appears to be a good choice to address the above issue because it has proven highly versatile for the preparation of synthetic receptors (or namely molecularly imprinted polymers (MIPs)) with high and specific affinity toward biomacromolecules such as peptides and proteins,[8−23] mainly through the long-range collective interactions between such biological entities and the imprinted pockets.

Herein, we report a facile, general, and highly efficient approach to obtain MIPs with enzyme inhibition potency by the combined use of molecular imprinting and controlled/"living" radical polymerization (CRP) techniques as well as surface-anchoring strategy, which involves the first synthesis of uniform, highly cross-linked, surface-functionalized, and "living" polymer microspheres via our recently developed atom transfer radical precipitation polymerization (ATRPP) technique,[24−26] their surface modification to introduce strong enzyme-anchoring groups, the enzyme immobilization onto the polymer microspheres by their interaction with the anchoring groups, and the subsequent controlled surface molecular imprinting by surface-initiated atom transfer radical polymerization (SI-ATRP)[27,28] (Figure 1). The versatility of ATRPP for the one-pot synthesis of uniform "living" polymer microspheres with adjustable sizes and various surface functionalities,[24−26] the high efficiency of SI-ATRP for the controlled grafting of ultrathin polymer layers of a wide range of functional monomers,[27,28] and the good compatibility of ATRP with biomacromolecules[29,30] make this strategy highly applicable. Although a great number of MIPs have been developed up to now, those showing biological activity and enzyme inhibition potency are rare. It is only recently that Haupt and co-workers reported the synthesis of MIP microgels with enzyme inhibition

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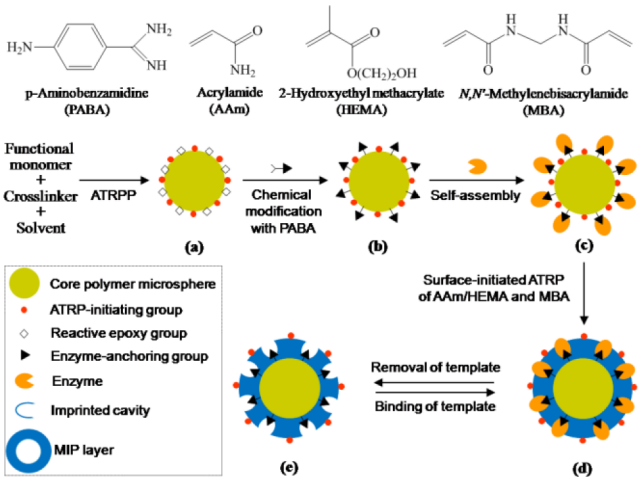


**Figure 1.** Schematic protocol for the synthesis of enzyme-imprinted core–shell polymer microspheres with high inhibition potency by the controlled surface molecular imprinting approach: "living" polymer microspheres with surface-bound epoxy groups (a), enzyme-anchoring groups (b), or enzymes (c) and the core–shell polymer microspheres with either immobilized enzymes (d) or enzyme-imprinted pockets (e) on the surface layers.

function by traditional precipitation polymerization using a strong anchoring monomer.[19] The described approach, however, suffers from time-consuming optimization of reaction conditions for achieving MIP microgels with suitable enzyme binding properties and particle sizes, relatively low binding capacities owing to the entrapment of enzymes inside the MIP particles, and the presence of randomly distributed enzyme-inhibiting groups on the MIP surfaces. In comparison, our strategy presented here can overcome the above-mentioned problems and allows the more efficient synthesis of uniform MIP particles with high enzyme inhibition potency.

The general principle of our strategy was first demonstrated by synthesizing hydrophilic MIP microspheres with trypsin inhibition potency. Trypsin is a well-characterized serine protease (molecular weight MW = 23 800), which is highly important to the human body, and its functional disorder can cause such diseases as diabetes.[31] Benzamidine and its derivatives are well-known trypsin inhibitors, whose inhibition effects on trypsin depend upon their structures.[32] Therefore, the benzamidine group was chosen here as the anchoring functionality for trypsin. Two hydrophilic functional monomers (FM) (i.e., acrylamide (AAm) and 2-hydroxyethyl methacrylate (HEMA)) and a hydrophilic cross-linker *N*,*N*′-methylenebisacrylamide (MBA) were utilized in the molecular imprinting because they can form hydrogen bonding with trypsin and induce collective interactions between enzyme and the formed binding pockets.

Uniform, highly cross-linked, and "living" polymer microspheres with surface-bound alkyl halide groups (ATRP-initiating groups) and epoxy groups were prepared by the one-pot ATRPP of glycidyl methacrylate and ethylene glycol dimethacrylate following our previous report.[25] *p*-Aminobenzamidine (PABA) was readily attached onto these polymer microspheres by the reaction of its amino group with the epoxy groups of the "living" polymer microspheres under mild conditions. The resulting "living" polymer microspheres with benzamidine groups on their surfaces were then allowed to bind trypsin in aqueous buffer media through the stoichiometric interaction. Finally, the obtained "living" polymer microspheres with surface-bound trypsin were used as the immobilized initiator for the SI-ATRP of MBA and AAm or HEMA for different times ($t$ = 3, 6, and 12 h), leading to a series of core–shell MIP microspheres (i.e., trypsin-MIP-FM-$t$ (FM = AAm and HEMA)) after the removal of trypsin by their being thoroughly washed with methanol and SDS/acetic acid (Figure 1). Control polymers trypsin-CP-FM-$t$ (FM = AAm or HEMA) were also prepared and purified following the same protocols but in the absence of trypsin.

A scanning electron microscopy (SEM) study revealed that the "living" polymer microspheres with surface-bound epoxy groups and those with benzamidine groups as well as trypsin-MIP-FM-$t$/trypsin-CP-FM-$t$ (FM = AAm and HEMA, $t$ = 3, 6, and 12 h) microspheres were all uniform microspheres with their number-average diameters ($D_n$) around 1 $\mu$m (Figure 2a–h and Figure S2 and Table S1, Supporting Information). In addition, the MIP/CP microspheres showed somewhat larger diameters than the core polymer microspheres (i.e., those with benzamidine groups on the surfaces) with their $D_n$ increasing with the polymerization time of SI-ATRP (Figure 2i). This, together with the presence of the characteristic peaks of the bonded AAm and HEMA in their FT-IR spectra (Figure S3, Supporting Information), clearly indicated the successful grafting of MIP/CP layers on the core polymer microspheres. Furthermore, both the dynamic laser scattering (DLS) and elemental analysis characterization also confirmed the successful preparation of core–shell MIPs/CPs (Tables S2 and S3, Supporting Information).

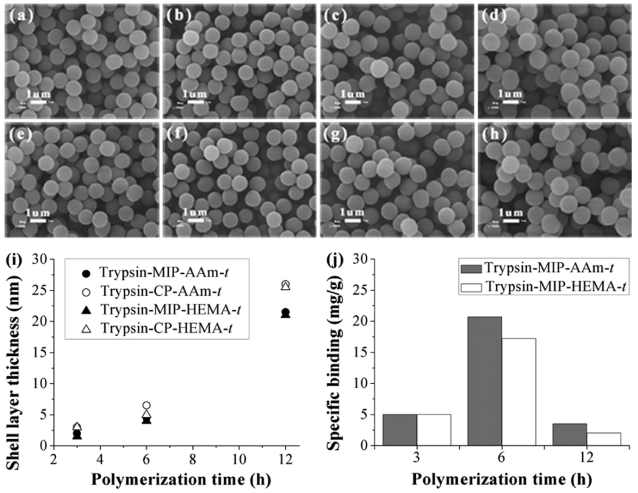


**Figure 2.** (a–h) SEM images of the "living" polymer microspheres with surface-bound epoxy groups (a) and those with benzamidine groups (e) as well as trypsin-MIP-AAm-$t$ (b,c,d)/trypsin-CP-AAm-$t$ (f,g,h) ($t$ = 3 h (b,f), 6 h (c,g), and 12 h (d,h)) microspheres. (i) Plot of the thickness of the surface-grafted polymer layers on the core–shell MIP/CP microspheres versus the polymerization times of SI-ATRP. (j) Dependence of the specific trypsin bindings of the MIP microspheres on the polymerization times of SI-ATRP (MIP/CP concentration, 5 mg/mL; trypsin concentration, 5 mg/mL).

With the trypsin-MIP-FM-$t$/trypsin-CP-FM-$t$ (FM = AAm and HEMA) in hand, we started to study the binding kinetics of the representative trypsin-MIP-FM-6h/trypsin-CP-FM-6h (FM = AAm and HEMA). The results showed that they could reach maximum binding capacities around 30 min (Figure S5, Supporting Information). Given that a rather high cross-linking


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degree (50%) was applied in our MIP/CP synthesis, the enzyme binding processes were very fast, which could be ascribed to their very thin surface-imprinted polymer layers.

The equilibrium binding properties of trypsin-MIP-FM-*t*/trypsin-CP-FM-*t* (FM = AAm and HEMA, *t* = 3, 6, and 12 h) were then studied. Both trypsin-MIP-AAm-6h and trypsin-MIP-HEMA-6h proved to bind more of the template than their corresponding CPs in the $CaCl_2$-containing Tris-HCl buffer (Figure S6, Supporting Information). This, together with their high selectivity toward trypsin in comparison with chymotrypsin, kallikrein, lysozyme, and bovine serum albumin (Figure S8, Table S5, Supporting Information), suggested the successful creation of specific trypsin binding sites in these MIPs. It is interesting to note that the specific trypsin binding (i.e., the binding difference between the MIP and its CP) of the studied MIPs first increased with increasing polymerization time of SI-ATRP from 3 to 6 h and then decreased with further increasing the polymerization time to 12 h (Figure 2j), demonstrating that the polymerization time (or the thickness of the MIP layers, Figure 2i) had a significant influence on the binding properties of the core−shell MIPs, and only those with appropriate MIP layer thickness (i.e., comparable with the molecular diameter of the globular trypsin (3.8 nm)[33]) could show best specific enzyme bindings. This result is easily understandable because only a suitable polymerization time can provide optimal enzyme binding pockets with strong collective interactions toward trypsin, while a too short polymerization time leads to too shallow imprinted pockets with weak collective interactions toward trypsin and a too long polymerization time results in the entrapment of enzymes inside the grafted shells and thus negligible enzyme binding pockets. In this context, it is worth mentioning that other polymerization conditions such as the monomer loadings in the surface imprinting systems also showed considerable influence on the MIP layer thickness of the resulting core−shell MIPs and thus their binding properties (Table S1, Figure S6c,d, Supporting Information).

We further performed the binding isotherm studies of trypsin-MIP-FM-6h/trypsin-CP-FM-6h (FM = AAm and HEMA). As expected, the MIPs showed higher template binding capacities than their corresponding CPs (Figure 3). The experimental data of both the MIPs and CPs proved to be fitted well with the single-site Langmuir isotherm model, suggesting the formation of homogeneous binding sites in the obtained MIPs. In addition, the binding parameters of the studied MIPs/CPs could be derived from the above fitting (Figure 3 and Table S4, Supporting Information). It can be seen that while the binding affinity of our trypsin-MIPs is several times higher than or comparable with that of the trypsin-MIPs developed by Haupt and co-workers the binding capacities of our MIPs are about 40 times higher than those of their MIPs.[19] These results clearly demonstrated the high efficiency of our strategy for the synthesis of trypsin-MIPs with largely improved binding properties.

Next, the trypsin inhibition by trypsin-MIP-FM-6h/trypsin-CP-FM-6h (FM = AAm and HEMA) was investigated by performing enzyme assays in the $CaCl_2$-containing Tris-HCl buffer with *N*-alfa-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (DL-BAPNA) as the substrate. The Lineweaver−Burk double reciprocal plots of the kinetic data revealed that the studied MIPs exhibited typical competitive inhibition behavior toward trypsin (Figure 4a and Figure S10b, Supporting Information),[34] indicating that the MIPs inhibited trypsin by binding to its active site. In contrast, noncompetitive

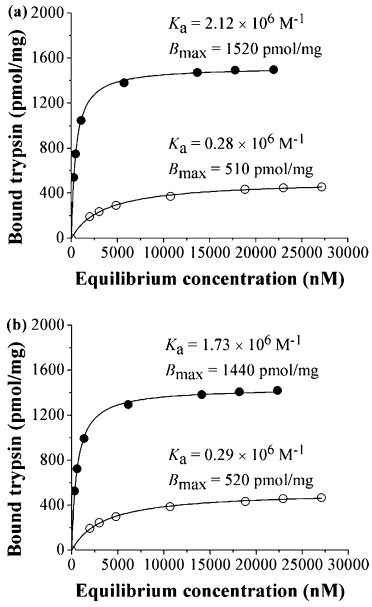


**Figure 3.** Binding isotherm plots of trypsin-MIP-FM-6h (filled symbol)/trypsin-CP-FM-6h (open symbol) (FM = AAm (a) and HEMA (b)) and their nonlinear regression with the single-site Langmuir isotherm model. Trypsin-MIP/CP microspheres (5 mg/mL) were incubated with a series of trypsin solutions with different concentrations (2940−29400 nM) in Tris-HCl buffer (50 mM, pH = 8.0) containing $CaCl_2$ (20 mM) at 25 °C for 6 h.

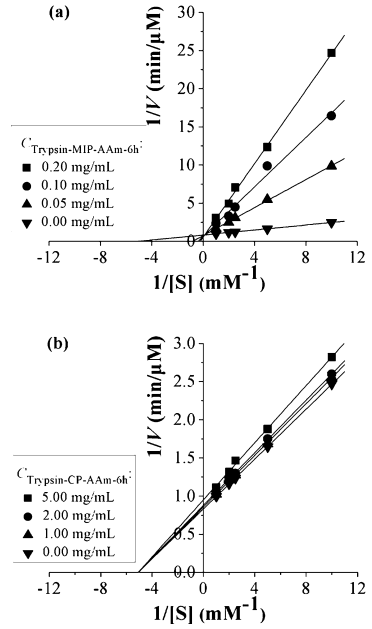


**Figure 4.** Lineweaver−Burk plots obtained for trypsin inhibition by trypsin-MIP-AAm-6h (a) and trypsin-CP-AAm-6h (b) at different concentrations of MIP/CP ($C_{Trypsin-MIP-AAm-6h}$ or $C_{Trypsin-CP-AAm-6h}$) and substrate DL-BAPNA ([S]). Enzyme assays were performed in Tris-HCl buffer (50 mM, pH = 8.0) containing $CaCl_2$ (20 mM) at 25 °C (trypsin concentration = 0.1 mg/mL).

type of trypsin inhibition was observed for the CPs (Figure 4b and Figure S10c, Supporting Information),[34,35] suggesting that CPs bound to a site on trypsin that is distinct from the substrate binding site (i.e., no benzamidine groups were present on the surfaces of our CP particles). The inhibition constants


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($K_i$) of the MIPs could thus be derived from the Michaelis–Menten equation developed for the competitive inhibition[19]

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

where $V$ is the initial velocity of the enzymatic reaction, $V_{max}$ the maximum velocity of the enzymatic reaction, $K_M$ the Michaelis constant, [I] the concentration of the inhibitor (i.e., the apparent maximum number of the binding sites on trypsin-MIP microspheres ($B_{max}$, Table S4, Supporting Information)), and [S] the concentration of the substrate.

The $K_i$ values of the binding sites on trypsin-MIP-AAm-6h and trypsin-MIP-HEMA-6h were evaluated to be 18.6 and 30.2 nM, respectively, indicating that our MIPs had higher trypsin inhibition potency than the free PABA ($K_i$ = 25.4 $\mu$M) by about 3 orders of magnitude (Table S6, Supporting Information). It is worth mentioning here that these $K_i$ values are somewhat less than those reported by Haupt and co-workers (a $K_i$ value of 79 and 44 nM was obtained for their trypsin-MIP microgels prepared with methacrylamide and HEMA as the functional monomers, respectively[19]). In particular, the presence of benzamidine groups only inside the binding pockets of our trypsin-MIPs should lead to the accurate evaluation of their $K_i$ values. On the basis of these results and the observation of only noncompetitive trypsin inhibition by trypsin-CP-FM-6h (FM = AAm and HEMA), we can conclude that the significant increase in the trypsin inhibition potency of the MIPs should be attributed to their existence of specific trypsin binding sites, which provided strong collective interactions between trypsin surfaces and the binding pockets of the MIPs in addition to the interactions between the active sites of trypsin and the benzamidine groups inside the MIP binding sites. Moreover, trypsin-MIP-FM-6h (FM = AAm and HEMA) microspheres were found to show noncompetitive inhibition behavior toward two other closely related enzymes, namely, chymotrypsin (a serine protease of similar molecular weight and isoelectric point but with a different substrate specificity compared to trypsin) (Figure S12a,c, Supporting Information) and kallikrein (a serine protease with a molecular weight of 38 000 and also inhibited by benzamidine derivatives[36]) (Figure S11b,d, Supporting Information). These results revealed the highly selective and potent inhibition of trypsin by the obtained trypsin-MIPs. In addition, they also demonstrated that although trypsin-MIPs bound chymotrypsin and kallikrein to some extent (Figure S8, Supporting Information) these bindings were mainly relatively weak nonspecific ones, whereas the high inhibition activity of trypsin-MIPs was attributed to the strong specific bindings.

Finally, kallikrein-imprinted polymer/CP (i.e., kallikrein-MIP-AAm-6h/kallikrein-CP-AAm-6h) microspheres were also prepared following a similar protocol as trypsin-MIP-AAm-6h/trypsin-CP-AAm-6h (Figures S2 and S3, Table S1, Supporting Information). Kallikrein-MIP-AAm-6h microspheres proved to have homogeneous binding sites of high kallikrein affinity and selectivity (Figures S7 and S9, Tables S4 and S5, Supporting Information). In addition, their kallikrein inhibition potency increased 1000 times ($K_i$ = 261.8 nM for the binding sites on kallikrein-MIP microspheres) compared with that of the free PABA ($K_i$ = 295.8 $\mu$M) (Figure S11a,f, Table S6, Supporting Information). Furthermore, only noncompetitive inhibition behavior was observed for both kallikrein-MIP-AAm-6h toward trypsin (Figure S10d, Supporting Information) and kallikrein-CP-AAm-6h toward kallikrein (Figure S11g, Supporting Information). These results clearly verified the general applicability and high versatility of our strategy in preparing MIPs with high specific enzyme inhibition potency.

In conclusion, we have demonstrated for the first time a facile, general, and highly efficient approach to prepare MIPs with high enzyme inhibition potency by the controlled surface molecular imprinting approach. The thickness of the enzyme-imprinted layers on the core–shell MIP microspheres had a significant influence on their binding properties, and only those with their thickness comparable with the diameters of the targeted enzymes could afford enzyme-MIPs with optimal specific bindings. The as-prepared enzyme-MIPs proved to show higher enzyme inhibition potency than the free PABA by 3 orders of magnitude. In addition, the general applicability of our strategy was confirmed. In view of the versatility of controlled/"living" radical precipitation polymerization in the synthesis of uniform "living" core polymer beads with adjustable sizes and various functional groups, the easy attachment of enzyme-anchoring (or protein-interacting) groups onto such polymer particles, and the robust nature of surface-initiated CRPs for the controlled protein imprinting, we believe that the present strategy represents a promising way to develop advanced enzyme or other protein-imprinted polymer particles of uniform and desired sizes with great potential not only in drug development but also in many other areas.

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION


### Corresponding Author

*E-mail: zhanghuiqi@nankai.edu.cn.


### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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