Generation of New Enzyme Inhibitors Using Imprinted Binding Sites: The Anti-Idiotypic Approach, a Step toward the Next Generation of Molecular Imprinting

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Molecular imprinting can be characterized as a synthetic method toward the formation of a molecular host via template-guided synthesis in a self-assembly mode.[1] In this communication we describe the use of an already-made molecularly imprinted polymer (MIP) to generate bioactive compounds mimicking the original template, which may be, for example, an enzyme inhibitor or a receptor antagonist. This "double imprinting" is analogous to the formation of anti-idiotypic antibodies in the immune response system, where the combining site of the secondary antibody is an "internal image" of the original antigen.[2] We envision that our synthetic anti-idiotypic imprinting approach may be useful for finding new drug candidates, especially when the three-dimensional structure of a biological target, a prerequisite for the rational drug design, is unresolved.[3]

In this study we chose the medicinally interesting proteinase kallikrein[4] as a model system. Known inhibitors of tissue kallikrein have the common feature of a positively charged amino or guanidine group coupled to a hydrophobic moiety. It is believed that the guanidine and the hydrophobic moieties simulate the side chain functionalities of the peptide sequence Phe-Arg, which binds to the S_2-S_1 pocket of the enzyme's active site.[5] Inhibitor (**1**)[6] was chosen as the original template for the preparation of an imprinted polymer leading to specific binding "cavities" mimicking the enzyme's active site. These cavities were then used to direct the synthesis of the original template, as well as that of new inhibitors (Figure 1).

(2-Trifluoromethyl)acrylic acid (TFMA) and divinylbenzene (DVB) were used as the functional monomer and cross-linker, respectively, for the preparation of the MIP. TFMA was chosen because of its strong acid−base interaction with the amidine functional group of the template, while DVB is believed to form attractive $\pi-\pi$ interactions with the aromatic moieties of the template. To evaluate the imprinting effect, the imprinted and the control (nonimprinted) polymer were packed into HPLC columns, on which retentions of different test compounds structurally related to **1** were measured (Table 1). As reflected from the capacity factor (k'),[7] the MIP bound the template stronger than the control polymer. For the compounds lacking the phenylethylamine or the benzamidine moiety, specific binding by the MIP decreased. These results confirmed the essential contributions of the ionic and the $\pi-\pi$/hydrophobic interactions for the MIP to specifically bind to the analyte. In a batch-mode binding experiment, the template was incubated with various amounts of polymer, which resulted in the binding curves shown in Figure 2. It is seen that the MIP could bind much more of the template than the control polymer.

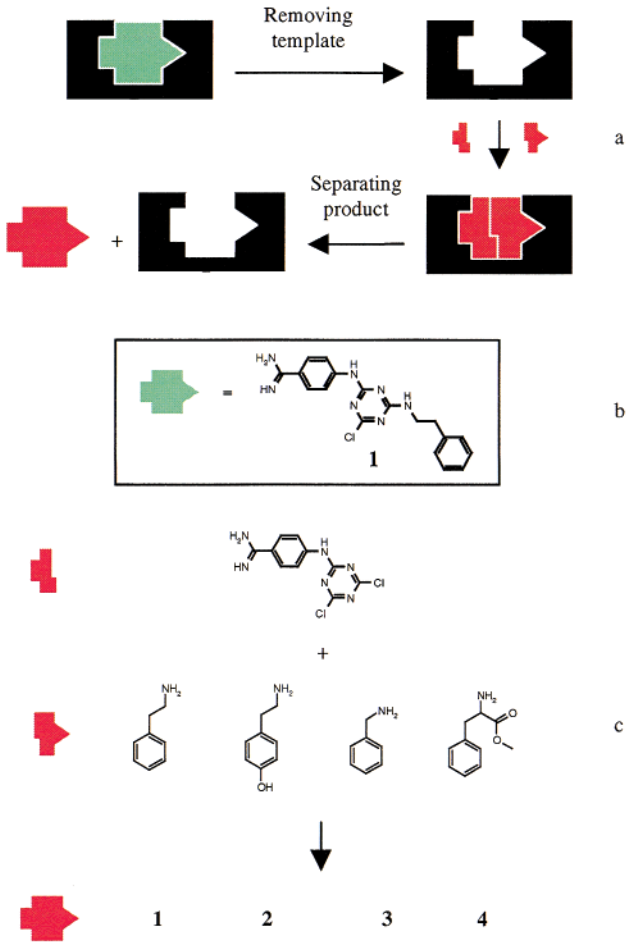

Figure 1. (a) Schematic representation of the anti-idiotypic imprinting approach. After removal of the template (in green) from the MIP (in black), the binding cavity was used to direct the assembly of the reactants (in red) to give products (in red) mimicking the original template (in green). (b) Chemical structure of the template, 2-(4-amidinophenylamino)-4-chloro-6-phenylethylamino-s-triazine (**1**). (c) Synthetic reactions investigated in the present study.

To test the feasibility of using imprinted sites to direct organic synthesis, we first incubated 2-(4-amidinophenylamino)-4,6-dichloro-s-triazine with the MIP and the control polymer, followed by addition of 10 equiv of phenylethylamine. After 8 h, all of the reactions reached maximum yields.[8] Acetic acid was added to release all the components from the polymer prior to HPLC analysis. The amount of **1** obtained with the MIP was 4 times higher than that with the control polymer.[9] It is noteworthy that the reaction carried out in free solution at the same reactant concentration did not give any product.

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(7) Capacity factor (k') = $(t - t_0)/t_0$, where t and t_0 are retention times of a test compound and of the void marker, acetone, respectively.

(8) This was investigated by stopping the reaction at different times by addition of acetic acid.

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Table 1. Chromatographic Evaluation of the Imprinting Effect

test compounds	capacitor factor (k') MIP[a]	capacitor factor (k') control	retention index (RI)[b]
1	12.5	9.0	100
2-(4-amidinophenylamino)-4,6-dichloro-s-triazine	7.0	6.2	81
4-aminobenzamidine dihydrochloride	2.6	3.6	52
cyanuric chloride	0	0	–

[a] For MIP preparation, **1** (free base, 0.3 mmol), (2-trifluoromethyl)-acrylic acid (2.4 mmol), divinylbenzene (12 mmol) and azobisisobutyronitrile (0.12 mmol) were dissolved in *N*,*N*-dimethylformamide (DMF, 2 mL). The solution was saturated with dry nitrogen, followed by polymerization at 60 °C for 16 h. The polymer monolith was ground and fractionated to give appropriately sized particles (10−25 μm). The template was removed by repetitive washing in methanol:acetic acid (90:10, v/v). The control polymer was prepared in the same way except for omission of the template. [b] Polymer particles were packed into HPLC columns (250 × 4.6 mm). Test compounds were applied (20 μL at 1.0 mg mL^{-1}) to both the MIP and the control columns, and eluted with a gradient of 1−10% acetic acid in acetonitrile (1 mL min^{-1}) within 30 min. Acetone was used as the void marker. The retention index was calculated as: $[k'(\text{MIP})/k'(\text{control})]/[k_1'(\text{MIP})/k_1'(\text{control})] \times 100$, where k'(MIP) and k_1'(control) were capacity factors of a test compound on the MIP column, and of the template on the control column, respectively.

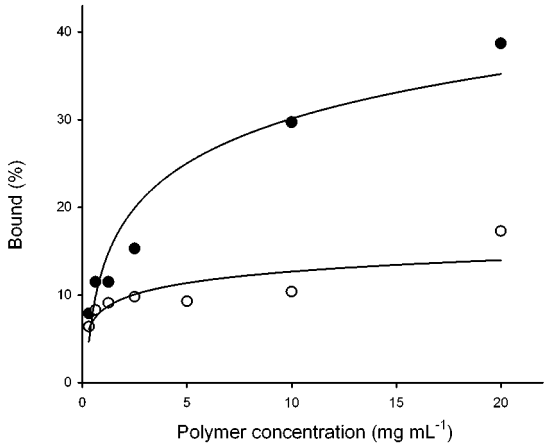

Figure 2. Titration of **1** with increasing amounts of the MIP (●) and the control polymer (○). The template (**1**, 0.25 μmol) was incubated with different amounts of polymer in DMF (1 mL) at 20 °C for 16 h. After centrifugation, the amount of template remaining in the supernatant was quantified by HPLC analysis. A Chromolith Performance column (RP-18e) from Merck (Darmstadt, Germany) was used, applying a gradient elution: 0−10 min, 20−50% acetonitrile in water, both containing 0.1% trifluoroacetic acid, at a flow rate of 1 mL min^{-1}.

Since the imprinted sites were able to direct resynthesis of the template, we wanted to know whether these sites could also be used to assist the synthesis of new compounds that have the same benzamidine moiety, but with slight differences at the hydrophobic end. Thus, we attempted to couple several different aromatic amines to 2-(4-amidinophenylamino)-4,6-dichloro-s-triazine. The choice of amine reactants was based on their molecular size and nucleophilicity, for example they should be able to fit into the active center of the MIP that had been preloaded with 2-(4-amidinophenylamino)-4,6-dichloro-s-triazine, and the reactivity of the amine with the cyanuric chloride should be similar. In Table 2 we list the different kallikrein inhibitors synthesized using the MIP's cavity as the reaction mold. While inhibitors **2** and **3** were successfully synthesized with the MIP (of which **3** represents a new inhibitor not previously reported), none of these were obtained with the control polymer. The low yield of **2**, relative to **1** and **3**, can be attributed to steric hindrance. Similarly, product **4** was not observed because the bulky methyl ester group could not be accommodated within the MIP cavity.

(9) We should point out that more than 90% of the isolated **1** was obtained by the MIP-assisted synthesis. The amount of template leakage was quantified by incubating the MIP with none or only one of the reactants followed by the same acid treatment.

Table 2. MIP-Assisted Synthesis of Kallikrein Inhibitors[a]

product	retention time (min)[b]	prod. concentrated (μM)	relative yield (%)[c]	K_i (μM)[d]
1	8.4	1.51	100	4.5
2	6.5	0.31	21	40
3	6.6	0.52	34	5.2
4	8.5	0	0	33

[a] 2-(4-Amidinophenylamino)-4,6-dichloro-s-triazine (31.3 nmol) was incubated with the MIP (10 mg) in DMF (600 μL) at 20 °C for 2 h. Different amine reactants (10 equiv) in 100 μL of DMF were then added, and the reactions continued for 8 h. After the reaction, acetic acid (100 μL) was added, and the mixture was incubated for another hour. Polymer particles were removed by centrifugal filtration. Product content in the filtrate was quantified by HPLC analysis. [b] A Chromolith Performance column (RP-18e) from Merck (Darmstadt, Germany) was used with a gradient elution: 0−10 min, 20−50% acetonitrile in water, both containing 0.1% trifluoroacetic acid at a flow rate of 1 mL min^{-1}. [c] Relative yield was normalized to that of **1**. [d] Inhibition constants of **1** and **2** for tissue kallikrein were adapted from ref 6, and those of **3** and **4** were determined following the same procedure as described in ref 6.

To further demonstrate that the coupling reactions took place in the specific binding cavities of the MIP, we carried out MIP-assisted synthesis of **2** and **3** in the presence of a competing amine, phenylethylamine. In the presence of the competing reaction that gave the original template, the relative yield of **2** was decreased to 16%, whereas that of **3** was affected much less (31%).

The test compounds were also subjected to enzyme inhibition tests. Compared with the K_i value for 2-(4-amidinophenylamino)-4,6-dichloro-s-triazine (>100 μM), all the compounds showed improved activity, among which **3** gave the strongest inhibition except for **1**, which was the original template used for preparing the MIP.

In this study we have demonstrated our "anti-idiotypic" approach of using imprinted polymer for the template-guided synthesis of bioactive molecules. As an example we have prepared an imprinted polymer specific for a known enzyme inhibitor and used the MIP to facilitate the synthesis of new inhibitors (mimics). In addition to using MIPs as artificial receptors for screening synthetic compounds,[10] here we used a MIP to find out useful reactants (or reaction conditions) leading to active products.[11] Since no prior product library is needed, our approach has the advantage of saving considerable synthetic effort, that is, only the hit reactions need to be scaled up for further investigation.

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Supporting Information Available: Scale-up synthesis of compounds **1**−**4**, MIP-assisted synthesis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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