

# Combinatorial methods in molecular imprinting

Dolly Batra and Kenneth J Shea\*

Molecular imprinting is a general method for synthesizing robust, network polymers with highly specific binding sites for small molecules. Recently, combinatorial and computational approaches have been employed to select an optimal molecularly imprinted polymer (MIP) formulation for a targeted analyte. The use of MIPs in the combinatorial field, specifically their use for screening libraries of small molecules, has also been developed.

## Addresses

Department of Chemistry, University of California, Irvine, CA 92697, USA  
\*e-mail: kjshea@uci.edu

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## Abbreviations

<b>AMPSA</b>	2-acrylamido-2-methyl-1-propanesulfonic acid
<b>EGDMA</b>	ethylene glycol dimethacrylate
<b>FIA</b>	flow-injection analysis
<b>MAA</b>	methacrylic acid
<b>MIP</b>	molecularly imprinted polymer
<b>TFMAA</b>	trifluoromethacrylic acid

## Introduction

Molecular imprinting is a process by which polymeric materials are synthesized with highly specific binding sites for small molecules [1–3]. Molecularly imprinted polymers (MIPs) have been developed for a variety of applications including chromatography [4,5], enzymatic catalysis [6], solid-phase extraction [7,8], and sensor technology [4,9,10]. Intermolecular forces that develop during polymerization between the template molecule (T), functional monomer (M) and developing polymer matrix are responsible for creating a polymer microenvironment for the template or imprint molecule (Figure 1). The resulting polymer network contains synthetic receptors that are complementary in size, shape and functional group orientation to the template molecule. The polymers typically employed in imprinting are complex thermosets, an insoluble, highly crosslinked network polymer. Because both the morphology of the bulk polymer and the chemical microenvironment of the binding site are critical to the overall performance, the number of experimental variables that influence these factors are large. Imprinted polymers are,

therefore, ideal candidates for combinatorial synthesis and its screening technologies. Recently, combinatorial methods have been used to develop highly selective MIPs. Synthetic receptors produced by this technology, in turn, have been used in the screening of libraries of small molecules. This review covers both these emerging areas of MIP technology.

## Optimization of MIP formulations

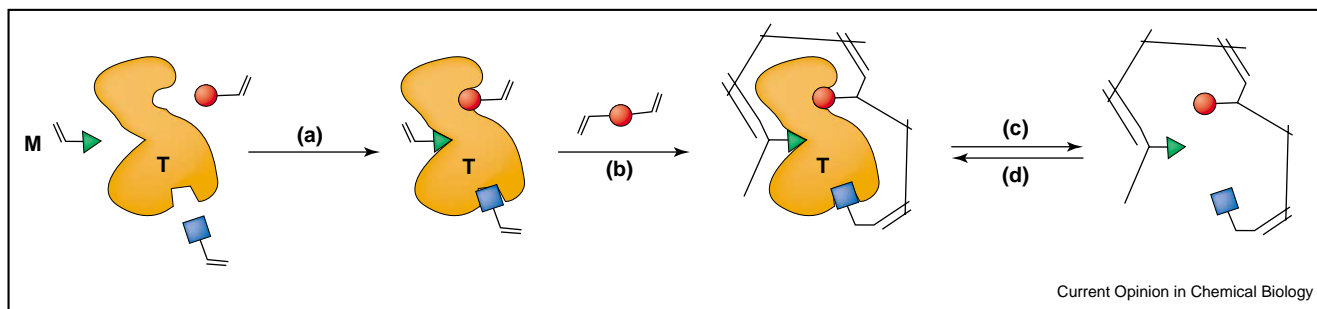
Many variables of the imprinting process influence the selectivity and capacity of a MIP. First, complementary interactions between the template and the functional and crosslinking monomers are necessary to create short-range molecular organization at the receptor site. These interactions include hydrogen bonding, electrostatic and/or van der Waals forces. Second, the stoichiometry and concentration of the template and monomers influences both polymer morphology and MIP selectivity. Third, the solvent used in the polymerization process, also known as the ‘porogen’, plays a dual role. In addition to mediating the interactions between the functional groups and the template molecule, the porogen determines the timing of the phase separation during polymerization [11] (Figure 1b), which is an important determinant of polymer morphology, porosity and ultimately accessibility of the binding site. Finally, the temperature of polymerization influences the timing of phase separation. Also, the temperature dependence of the equilibrium between the functional monomers and template (Figure 1a) affects MIP selectivity and capacity.

A typical imprinting protocol involves thermally or photochemically induced, free-radical polymerization of a concentrated solution of monomers to produce bulk, monolithic insoluble polymers that are crushed, ground and sieved to micron size for analysis. Selectivity and binding are evaluated in the chromatographic mode by using the MIP as stationary phase for HPLC columns, or by batch rebinding studies. These methods can be tedious and time-consuming, and obtaining an MIP with optimal binding properties can take several days to weeks, especially if the variation in the formulation is made by trial-and-error. As a result of the number of variables that effect MIP performance, there has been an overuse of certain ‘standard’ formulations. The process of determining an optimal MIP formulation, therefore, is an ideal candidate for a combinatorial approach for screening various formulations.

## Combinatorial imprinting

One of the first applications of a combinatorial method to optimize the performance of MIPs was reported by

Figure 1



Schematic showing the formation of a MIP. **(a)** Pre-polymerization complex formation between template (T) and functional monomers (M). **(b)** Co-polymerization with an excess of crosslinking monomer. **(c)** Extraction of template from imprinted binding site. **(d)** Rebinding of template molecule to imprinted binding sites.

Takeuchi and co-workers [12\*\*]. The authors utilized a semi-automated approach to prepare an array of MIPs by varying the proportions of functional monomers. Polymer synthesis and analysis employed programmable liquid-handling equipment to prepare small-scale MIPs (~30 mg) on the bottom surface of glass vials. The polymers were analyzed *in situ* by binding assays (Figure 2).

MIP libraries were synthesized for triazine herbicides, ametryn (**1**) and atrazine (**2**) (Figure 3). In each library, the ratio of two functional monomers, methacrylic acid (MAA) and trifluoromethacrylic acid (TFMAA) were varied to optimize performance. Ethylene glycol dimethacrylate (EGDMA) and 2,2'-azobis(isobutyronitrile) were used as the crosslinker and initiator, respectively. The 'combinatorial molecular imprinting robot' dispensed standard solutions of these reagents, pre-dissolved in the porogen, into 1.5 ml vials to make the 2D array of pre-polymerization mixtures.

The vials were purged with nitrogen and irradiated to initiate polymerization. Automated sampling was used to perform two screening steps on the resulting MIPs. The 'instant first screening' quantified the amount of free template desorbed from the MIPs after an acetonitrile incubation, enabling a rough estimation of the affinity of the template to the MIP. The second 'regular screening' step involved a more systematic batch rebinding study that took place *after* a thorough wash of the MIPs to extract the remaining template species. This screening step allowed the determination of the capacity of each MIP for the imprinted triazine as well as its selectivity for the imprinted molecule versus the non-templated triazine.

Despite the structural and functional similarities between ametryn and atrazine, the optimal imprinting formulations were quite different. Ametryn-imprinted polymers that were TFMAA-rich (selectivity factor = 2–2.5) showed

better selectivity for ametryn over atrazine than MAA-rich polymers (selectivity factor = 1–1.5). ('Selectivity', here, is defined as the capacity of template divided by the capacity of an analogous compound on the imprinted polymer.) On the other hand, atrazine-imprinted polymers that were MAA-rich (selectivity factor = 0.8–1.2) showed better selectivity for atrazine over ametryn than TFMAA-rich polymers (selectivity factor = 0.6–0.8). As the authors note, it is difficult to predict these competing interactions without experimentation, making this semi-automated approach ideal for assessing these factors.

Takeuchi *et al.* [13] later used this approach to develop an MIP catalyst for the decomposition of a soil and ground water pollutant atrazine (**2**) to atraton (**3**), a biologically inactive molecule (Figure 3b). Five libraries were constructed with varying concentrations of MAA and one of five functional monomers. MAA was expected to provide the high-fidelity binding sites [12\*\*], whereas the second monomer was expected to provide the catalytic activity.

Using the same automated screening protocols developed earlier [12\*\*], only the combined use of 2-sulfoethyl methacrylate (SEMA) with MAA provided MIPs with activity towards the decomposition of atrazine. The formulation of atrazine:MAA:SEMA in a mole ratio of 1:6:2 was found to be optimal. In addition, blank polymers, made with the same formulation but without the presence of the template, yielded less atraton, showing that the combination of functional monomers enhanced atrazine-decomposition activity. Unfortunately, the authors did not report the details of the catalytic efficiency of these polymers or the specific enhancement achieved by the imprinting process.

Although this combinatorial approach provided an improved method over the conventional trial-and-error approach in developing selective MIPs, the authors noted

Figure 2

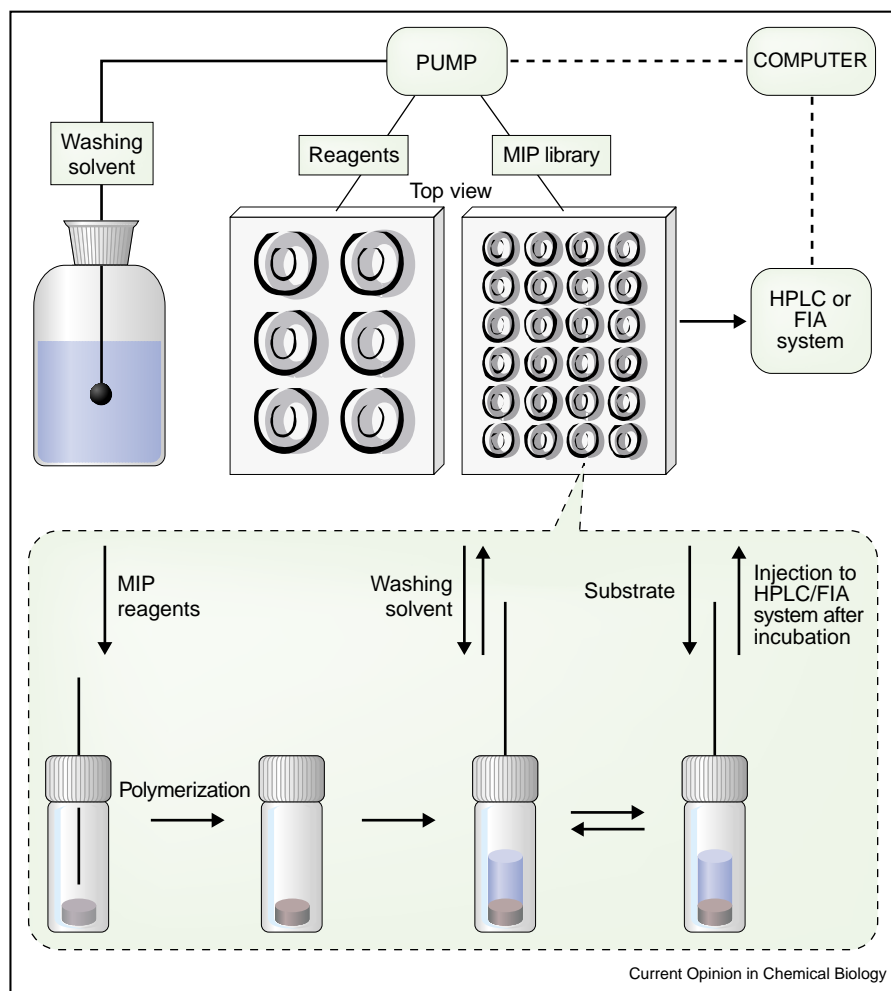


Illustration of an automated programmable liquid-handling system used to prepare and analyze small-scale MIPs. Adapted from reference [12\*\*].

that because of the slow HPLC or flow-injection analysis (FIA) for the binding studies, the process was still time-consuming and did not allow for the development of a high-throughput combinatorial library [12\*\*,13]. To combat this problem, Takeuchi *et al.* [14] attempted to develop a faster screening method that would allow evaluation of binding capacity by fluorescence measurements using a microplate reader. By using a fluorescent alkaloid, cinchonidine, as the template, the authors compared FIA rebinding results with the fluorescence of the MIPs after the template was rebound to the polymer. A correlation between the FIA measurements and fluorescence intensity was observed at cinchonidine levels of less than 0.02  $\mu\text{mol}$ . At high cinchonidine levels, the fluorescence became saturated. There are other limitations to this method, such as the requirement for a non-volatile porogen and the necessity for a fluorescent analyte. Also, the cinchonidine capacity was not compared with non-imprinted, blank polymers, and other analogues were not tested, so the

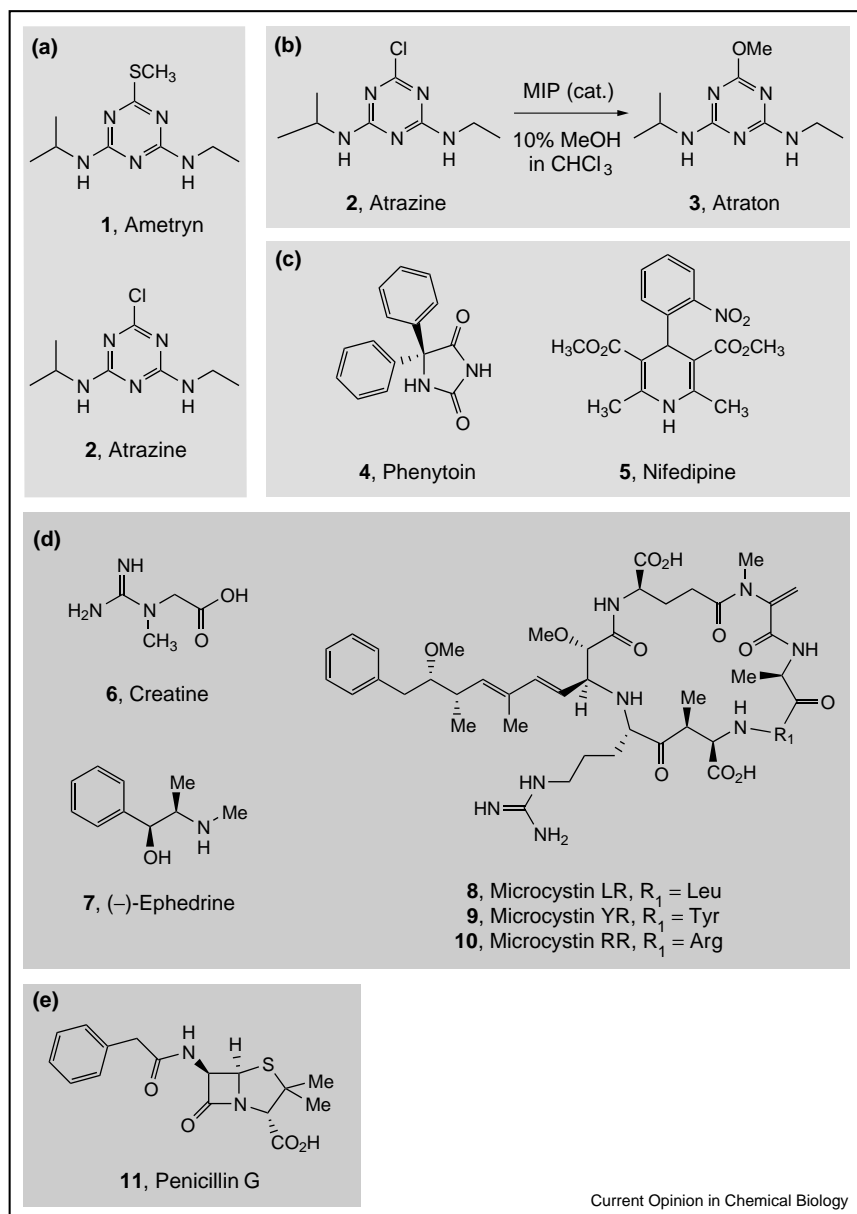
utility of this procedure was not rigorously demonstrated. The low correlation notwithstanding, the study shows the potential of this method for a high-throughput combinatorial library analysis.

### Parallel synthesis of MIPs

Independent of the Takeuchi work, Lanza and Sellergren [15\*\*] also developed a method for screening MIPs targeting a triazine herbicide, terbutylazine. Although not a strictly 'combinatorial' approach, the Sellergren method employed a semi-automated, scaled-down production of MIPs allowing *in situ* processing and evaluation of the synthesized materials to optimize the choice of functional monomer (Figure 4).

Six imprinted polymer formulations were prepared with different functional monomers. After photochemical irradiation of the vials, the resultant 'mini-MIPs' (~55 mg) were subjected to two screening steps, similar to the

Figure 3



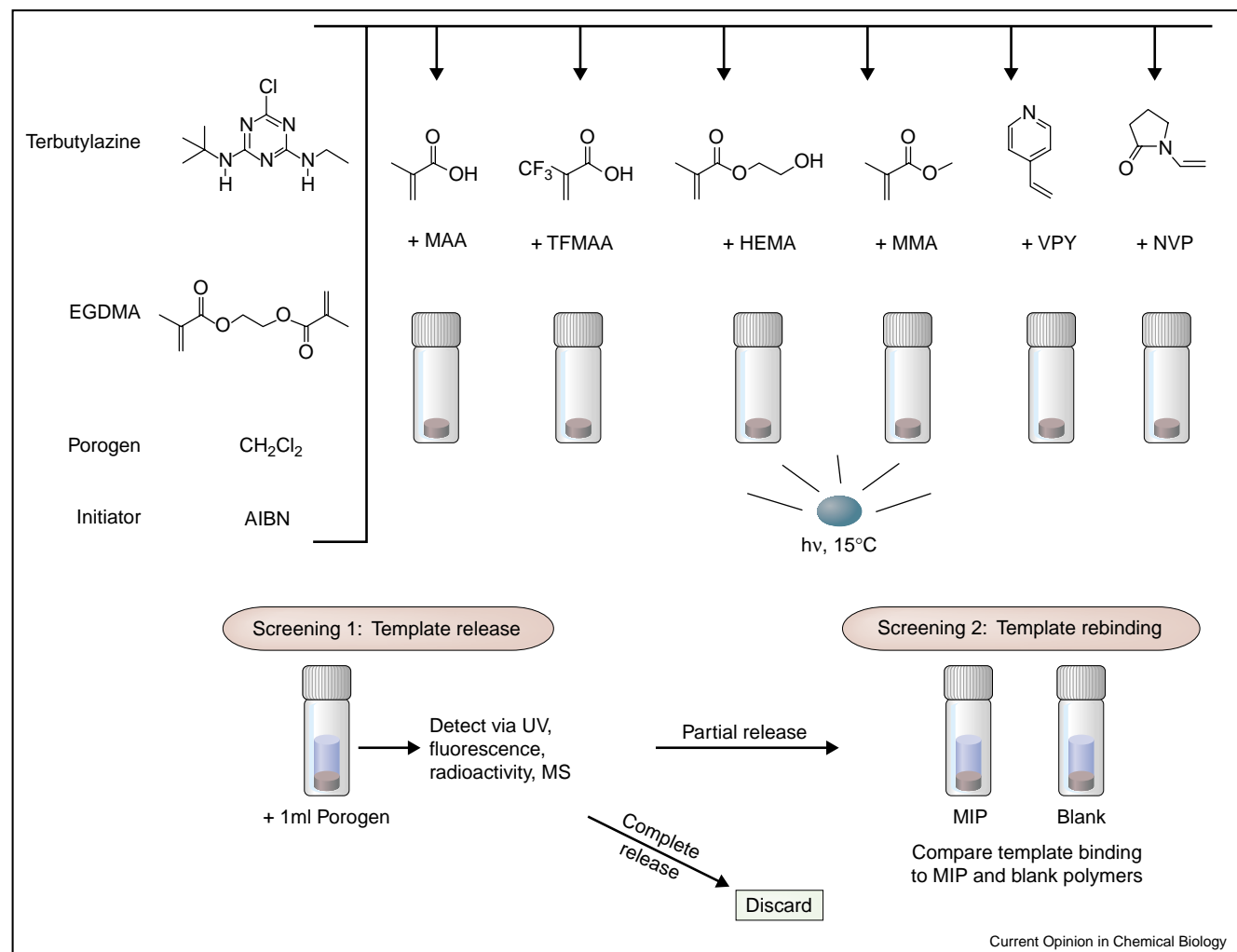
Structures of molecules discussed in the main text. **(a)** Triazine herbicides used for the synthesis of MIP libraries. **(b)** Decomposition of the pollutant atrazine to atraton, a biologically inactive molecule. **(c)** Template molecules phenytoin and nifedipine used by Lanza *et al.* [16]. **(d)** A computational approach was used to develop selective MIPs for the molecules shown. **(e)** Imprinted polymers containing recognition sites for targeted analyte molecules, such as penicillin G, may be used for rapid selection of ligands from a library of compounds.

Takeuchi protocol (see above), to determine MIP capacity. To establish selectivity, the template capacity on the imprinted polymer was compared with that on similar non-imprinted, blank polymers, made in the absence of template.

The TFMAA-MIP showed the highest capacity for the template, whereas the MAA-MIP had the highest selectivity. The MAA-MIP was then scaled up to synthe-

size a bulk polymer that was ground, sieved and packed into an HPLC column, and the retention of various structurally related triazines measured (Table 1). Most triazines showed some retention, but terbutylazine and atrazine showed the highest binding and selectivity over the other triazines. In this case, the batch rebinding selectivity results using mini-MIPs correlated well with the chromatographic binding observed in the HPLC mode.

Figure 4



Schematic for the synthesis and analysis of 'mini-MIPs' through a semi-automated approach. Adapted from reference [15\*\*]. AIBN, 2,2'-Azobis(isobutyronitrile). HEMA, 2-hydroxyethyl methacrylate; NVP, *N*-vinylpyrrolidinone; VPY, 4-vinylpyridine.

In their search for optimal functional monomers for the templates phenytoin (4) and nifedipine (5) (Figure 3c), Lanza *et al.* [16] demonstrated a limitation for using this

technique. Their results showed that the formulation for an optimized small-scale MIP did not always perform well in large-scale HPLC studies. The authors

Table 1

## Capacity factors (K) for various triazines\*.

Triazine	Imprinted polymer $K_{MIP}$		Non-imprinted polymer $K_{blank}$		Separation factor $\alpha = K_{MIP}/K_{blank}$	
	10 nmol	100 nmol	10 nmol	100 nmol	10 nmol	100 nmol
Terbutylazine	>25	18	1.46	1.25	>17	14
Ametryn	9.7	6.7	2.46	2.25	3.9	3.0
Prometryn	9.9	6.3	2.47	2.25	4.0	2.8
Atrazine	22	10	1.23	1.08	18	9.3

\* Data obtained in the chromatographic mode using as stationary phase a normal scale version of an MIP imprinted with terbutylazine, and a blank polymer, prepared in the absence of template [15\*\*]. The columns were injected with two different loads of the triazine (10 or 100 nmol).

concluded that small-scale rebinding results obtained at equilibrium cannot always be used to predict selectivity of MIPs in the dynamic HPLC mode.

Despite these limitations, this semi-automated method has been successfully used to search for optimal functional monomers in developing MIPs for applications in solid-phase extraction [17,18], chromatography [19] and binding assays [20].

### Computational approach

A computational approach has been suggested as a method to search for optimal imprinting conditions. This approach has been used to develop selective MIPs for the molecules creatine (6) [21\*], ephedrine (7) [22], and microcystin-LR (8) [23\*\*] (Figure 3d). Molecular modeling software was used to design and screen a virtual library of functional monomers against the desired template, and a good correlation was found between the computational studies and the performance of the materials prepared in the laboratory.

In one of the best examples of this approach, an imprinting formulation was designed for the aquatic cyanobacterial toxin, microcystin-LR (8) [23\*\*]. The Leapfrog algorithm was used to screen various monomers against the template for the optimal binding interactions, signified by certain 'binding scores'. The best monomers were then 'virtually annealed' to the template to optimize the arrangement of the functional monomers with energy minimization iterations.

Two imprinted polymers for microcystin-LR were then synthesized, one using a functional monomer with the best binding score, 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPSA) and the other using a functional monomer with one of the worst scores, MAA. MIP affinity for the template was performed using a competitive ELISA assay against microcystin-HRP analogues. It was found that the computationally designed MIP, prepared using AMPSA, had an affinity and sensitivity comparable with those of polyclonal antibodies (Table 2). Although the affinity was still lower than that of monoclonal antibodies, the synthetic MIPs showed superior chemical and thermal stabilities compared with those of biological antibodies. The MIP also performed better

**Table 2**

**Affinity and sensitivity range of MIPs and antibodies for microcystin-LR evaluated by a competitive assay [23\*\*].**

Receptor	$K_d$ (nM)	Sensitivity range ( $\mu\text{g l}^{-1}$ )
AMPSA MIP*	$0.3 \pm 0.08$	0.1–100
MAA MIP	$0.9 \pm 0.1$	0.8–100
Monoclonal antibody	$0.03 \pm 0.004$	0.025–5
Polyclonal antibody	$0.5 \pm 0.07$	0.05–10

\* Computationally designed.

**Table 3**

**Cross-reactivity of microcystin-LR imprinted polymers and antibodies [23\*\*].**

Receptor	MC-LR (%)	MC-RR (%)	MC-YR (%)	Nodularin (%)
AMPSA MIP*	100	$21 \pm 0.9$	$27 \pm 2$	$22 \pm 2$
MAA MIP	100	$19 \pm 0.8$	$30 \pm 3$	$36 \pm 0.5$
Monoclonal antibody	100	$106 \pm 0.3$	$44 \pm 2$	$18 \pm 0.8$
Polyclonal antibody	100	$92 \pm 2$	$142 \pm 0.8$	$73 \pm 1$

\* Computationally designed.

than the antibodies in selectivity studies for microcystin-LR compared with other toxin analogues (Table 3).

### The use of MIPs for screening 'combinatorial' libraries of compounds

In the above sections, the combinatorial approach was used for optimizing the performance of imprinted polymers. Imprinted polymers containing recognition sites for targeted analyte molecules may also be used as a crude preliminary screen and rapid selection of ligands from a library of compounds.

An example of the potential for this approach was recently demonstrated by Kempe and co-workers [24\*\*]. In this work, an MIP with specificity for penicillin G (11) was prepared using MAA and trimethylolpropane trimethacrylate as the functional monomer and crosslinker, respectively. The MIP formulation was selected by screening a library of large-scale MIPs made with various concentrations of different functional and crosslinking monomers. The optimal MIP had the best selectivity for penicillin G in a radioligand binding assay. This penicillin G imprinted polymer was then subjected to competition studies with several structural analogues of the imprinted antibiotic 11. The  $EC_{50}$  values of these antibiotics were determined by competing the non-labeled antibiotic with a fixed concentration of radioactively labeled penicillin G (Table 4).

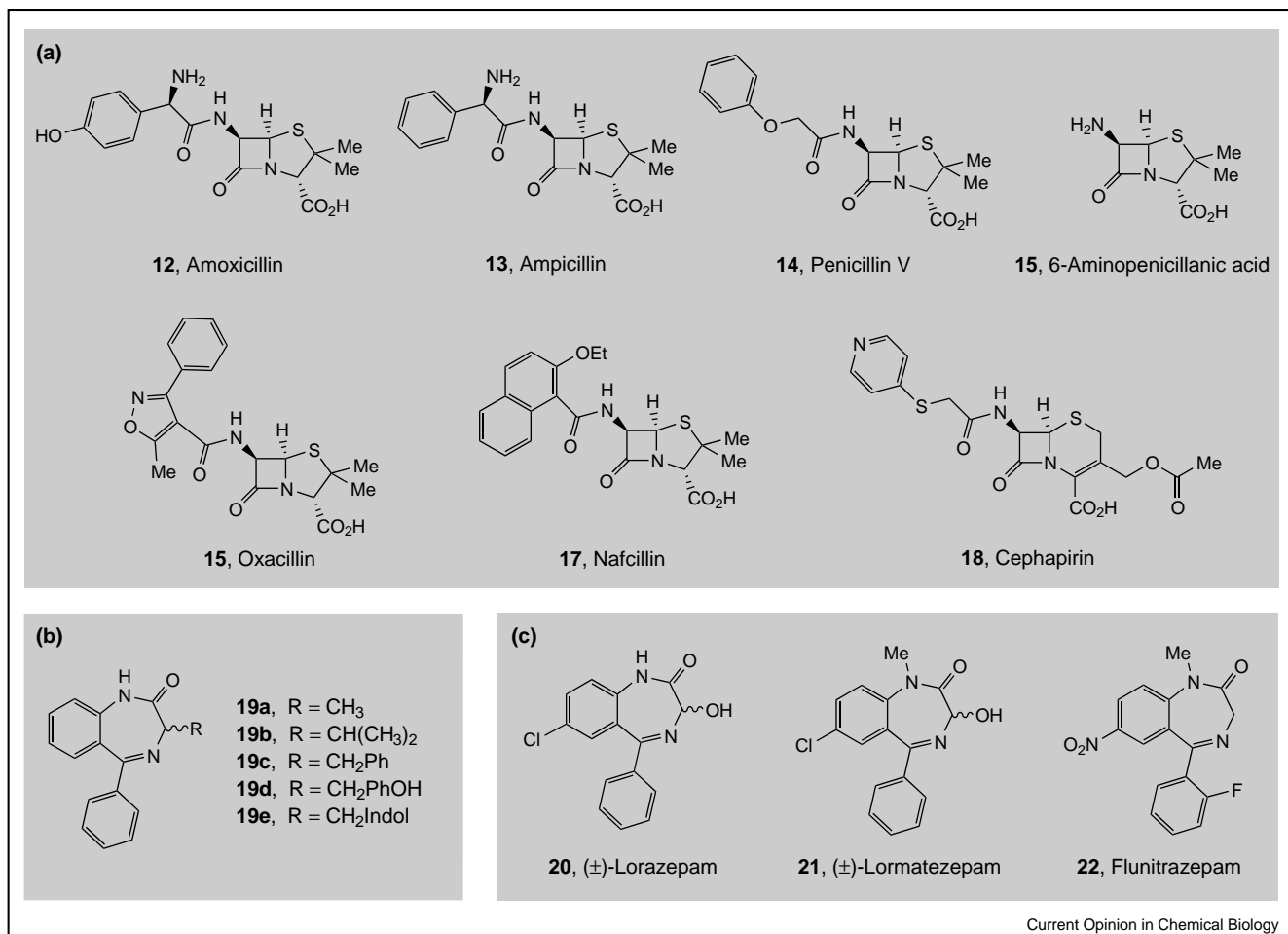
**Table 4**

**$EC_{50}$  and cross-reactivity of antibiotics competing with a penicillin G for an MIP imprinted for penicillin G\*.**

Antibiotic	$EC_{50}$ (nM)	Cross-reactivity (%)
Penicillin G (11)	60	100
Amoxicillin (12)	313	19
Ampicillin (13)	370	16
Penicillin V (14)	403	15
6-Aminopenicillanic acid (15)	1612	4
Oxacillin (16)	15 270	<1
Nafcillin (17)	196 300	<0.1
Cephapirin (18)	No competition	<0.01
Chloramphenicol	No competition	<0.01
Tetracycline	No competition	<0.01
Dapsone	No competition	<0.01
Erythromycin	No competition	<0.01

\* As reported by Kempe and co-workers [24\*\*].

Figure 5



Structures of molecules discussed in the main text. (a) Structural analogues of the imprinted antibiotic **11** (see Figure 3e). (b) Synthetic receptors for benzodiazepines. (c) Benzodiazepine derivatives.

The EC<sub>50</sub> of the competitor to that of penicillin G determined the cross-reactivity of the MIPs with the particular antibiotic.

Compounds with the highest cross-reactivity were structurally similar to penicillin G; for example, amoxicillin (**12**), ampicillin (**13**) and penicillin V (**14**), all derivatives of 6-aminopenicillanic acid (**15**) (Figure 5a). As the structural similarity decreased with antibiotics such as oxacillin (**16**), nafcillin (**17**) and cephapirin (**18**), the cross-reaction was worse (<1%). Antibiotics unrelated to penicillin such as chloramphenicol, tetracycline, dapsone and erythromycin showed no cross-reactivity (<0.01%).

These cross-reactivity studies showed the potential of this approach for using a MIP as a screen against a library of compounds structurally related to the template, to select molecules with the highest structural similarity. Another approach for this screening has been demonstrated in the

dynamic HPLC mode. These MIPs are ground, sieved and packed into an HPLC column and the 'library' of compounds is injected into the column to study structural similarity. This approach has also proved useful for creating crude mimics of biological receptors that are difficult to isolate or whose structure is not known.

In one example, synthetic receptors for benzodiazepines **19a–e** were prepared [25\*\*]. These template molecules affect sensory perceptions and are known to modulate the GABA<sub>A</sub> receptor. The structures of most benzodiazepine receptors are unknown ([25\*\*] and references therein). Several MIPs were synthesized for enantiomerically pure *S*-benzodiazepines **19a–e**, using EGDMA and MAA as the crosslinker and functional monomer, respectively. After extracting the template, each MIP was crushed, ground and sieved and the 25–38 μm particles were packed into HPLC columns. A 'library' of enantiomerically pure (*R*-) and (*S*-)benzodiazepines **19a–e** were then

Table 5

Capacity factors on an *S*-c (benzyl) benzodiazepine MIP column\*

Molecule	Capacity factor	Molecule	Capacity factor
<b>S-19c</b> benzyl	1.15	<b>R-19d</b> hydroxy benzyl	0.44
<b>S-19d</b> hydroxy benzyl	0.99	<b>R-19a</b> methyl	0.44
<b>S-19e</b> indolyl	0.82	<b>R-19c</b> benzyl	0.38
<b>S-19a</b> methyl	0.75	<b>R-19b</b> isopropyl	0.32
<b>S-19b</b> isopropyl	0.52	(±)-Lorazepam	0.29
(±)-Lorazepam	0.51	Benzylamine	0.27
<b>R-19e</b> indolyl	0.47	Flunitrazepam	0.19

\* As reported by Shea and co-workers [25\*\*].

injected individually into the columns and their retentions compared.

It was found that the amide nitrogen and the stereochemistry of the template played a major role for specific recognition at the synthetic binding site. For example, on the (*S*)-**19c** MIP column, the (*S*)-enantiomers of *all* benzodiazepines were retained longer than the (*R*)-enantiomers, showing that the template stereocenter seemed to be a more important factor in determining binding site recognition than the structure of the different side chains (Table 5). Also, benzodiazepine derivatives with a methylated amide group, lorazepam (**21**) and flunitrazepam (**22**), were not significantly retained on the MIP column, whereas lorazepam (**20**) and other benzodiazepine derivatives with a free amide group ((*R,S*)-**19a–e**) were retained longer on the column, showing the significance of the free amide group in determining selective recognition.

As the authors note, lorazepam is classified as an anxiolytic drug whereas lorazepam and flunitrazepam are classified as hypnotic drugs. It is suspected that these drugs bind preferentially to different benzodiazepine subtype receptors. Because an MIP analogue of one of these receptors was able to sort molecules on the basis of their pharmacological properties, this screening method could be used as a new technique for developing new drugs for a specific biological receptor.

In recent years, this approach has also been used to create synthetic receptors for screening libraries of steroids [26,27] and tricyclic antidepressants [28,29].

### Conclusions and future directions

Combinatorial methods have been successfully applied in the imprinting field to search for optimal MIP formulations. Although a semi-automated approach to synthesize and screen libraries of MIP formulations has been used mainly to find optimal functional monomers for various molecules [12\*\*,13,15\*\*,16–20], the technique can also be used to screen for other MIP variables such as the cross-linking monomer, porogen or initiator. The development of a faster screening method than the current HPLC or

FIA method could make this approach even more useful in high-throughput combinatorial applications [14]. Optimal formulations have also been discovered using molecular modeling software to ‘rationally design’ crosslinking and functional monomers for a particular template [21\*,22,23\*\*].

MIP synthetic receptors have found use in the combinatorial field for screening libraries of structurally related molecules. Using this method, MIPs mimicking biological receptors have been used to identify key interactions that are involved in selective molecular recognition [25\*\*,28,29]. Even though the basis of the affinity for a biological receptor and its MIP mimic may be completely different, this methodology shows potential as a preliminary screen for libraries of compounds to identify high-affinity ligands. The real value of this technique may be the outliers (i.e. molecules that do not have obvious structural similarity to the native ligand but bind well to a MIP receptor). These can give rise to new structural motifs as leads for drug candidates. It should be noted that this has *not* yet been demonstrated in the laboratory. However, the viability and success of this application for MIPs would be of special utility when the biological receptor is in limited supply or not isolable.

### Acknowledgements

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Using a computational approach, a highly selective MIP was 'rationally designed' for the cyanobacterial toxin microcystin-LR. A virtual library of functional and crosslinking monomers was designed and screened against the template using molecular modeling software. The optimal MIP formulation, synthesized in the laboratory, had an affinity and sensitivity comparable with those of polyclonal antibodies and superior chemical and thermal stabilities compared with those of biological antibodies.
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A highly selective MIP for penicillin G was synthesized using a MIP formulation that was discovered by evaluating a library of *large-scale* MIPs. The optimized penicillin G-imprinted polymer was found to have some cross-reactivity with structurally similar antibiotics in radioligand binding assays. This work demonstrated the potential of MIPs as a crude preliminary screen for the rapid selection of ligands from a library of structurally related compounds.
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