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REVIEW

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Molecularly imprinted polymers as antibody and receptor mimics for assays, sensors and drug discovery

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Abstract Biological receptors play an important role in affinity-based drug assays, biosensors, and at different stages during the modern drug discovery process. The molecular imprinting technology that has recently emerged has shown great potential for producing biomimetic receptors that challenge their natural counterparts. In this paper, we will overview recent progress in the use of molecularly imprinted polymers for drug assays, assembly of biomimetic sensors, and screening of combinatorial libraries. In addition, examples of using artificially-created binding sites to control synthetic reactions will be discussed. The "screening-of-building blocks" approach is expected to accelerate generation of valuable lead compounds, without the costly synthesis of large chemical libraries.

Keywords Molecularly imprinted polymer · Immunoassay · Biosensor · Library screening · Drug discovery

Molecular imprinting: a synthetic approach to biological-receptor mimics

The design and synthesis of biomimetic receptor systems capable of binding a target molecule with similar affinities and specificities to their natural counterparts has long been a goal of bioorganic chemistry. One technique that is being increasingly adopted for the generation of artificial macromolecular receptors is molecular imprinting. A target molecule, acting as a molecular template, is utilized to direct the assembly of specific binders (Fig. 1), usually followed by a polymerization step [1, 2, 3]. The term "molecular imprinting" is normally associated with the preparation of specific polymeric materials, although the general concept is similar to a large variety of strategies using target-directed synthesis. The simplicity in separating molecularly imprinted polymers (MIPs) from the soluble template makes this approach very attractive, because the resulting MIPs can be easily recovered and directly used as an artificial immobilized antibody, receptor or enzyme mimic for applications covering (bio)chemical analysis, separation and catalysis. In addition, MIPs have a higher chemical and physical stability compared to biomacromolecules. For example, the stability of theophylline-imprinted methacrylic acid–ethylene glycol dimethacrylate co-polymers has been investigated [4]. The polymers were shown to withstand 24 hours exposure to temperatures up to 150 °C without loss of affinity for the template. The polymers also exhibited remarkable resistance to extremes of pH, organic base, and to autoclave treatment.

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MIPs as antibody mimics in immunoassays

In a number of studies, MIPs have been shown to possess binding characteristics (in terms of affinity and specificity) similar to those of antibodies and biological receptors; the seminal paper in this application area being a report by Mosbach's group on the development of a MIP-based immunoassay against theophylline and diazepam [5]. In this and other examples, MIPs have been used as substitutes for antibodies in radioimmunoassays (RIA) for drugs, showing strong binding to the target analytes and cross-reactivity profiles similar to those of antibodies [5, 6, 7]. The dissociation constants that have been measured by some authors were found to be in the nanomolar to micromolar range [5, 6, 7]. This is in the same range as the average antibody, although antibodies exist that have an affinity for their antigen several orders of magnitude higher. Although originally restricted to use in organic solvents, Andersson and co-workers have shown that MIP-assays can also be performed directly with diluted blood plasma [8]. This mole-

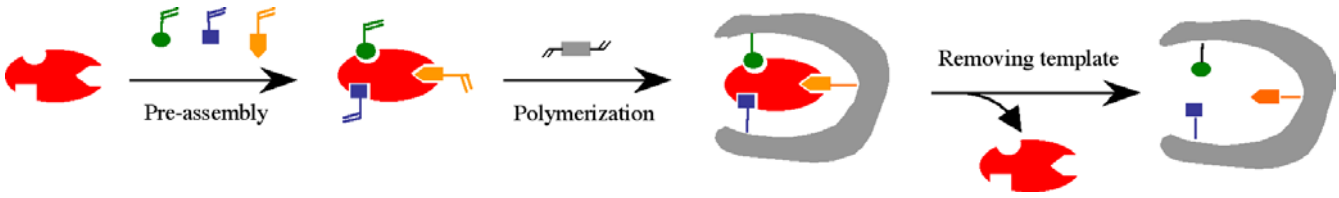


Fig. 1 Schematic representation of molecular imprinting principle. Pre-assembly of functional monomers around the template is driven by their molecular interactions. The subsequent co-polymerization with the cross-linker "freezes" binding groups within the imprinted "cavity". Removal of the template by solvent extraction or chemical cleavage affords a binding site complementary in size and in chemical functionality to the original template

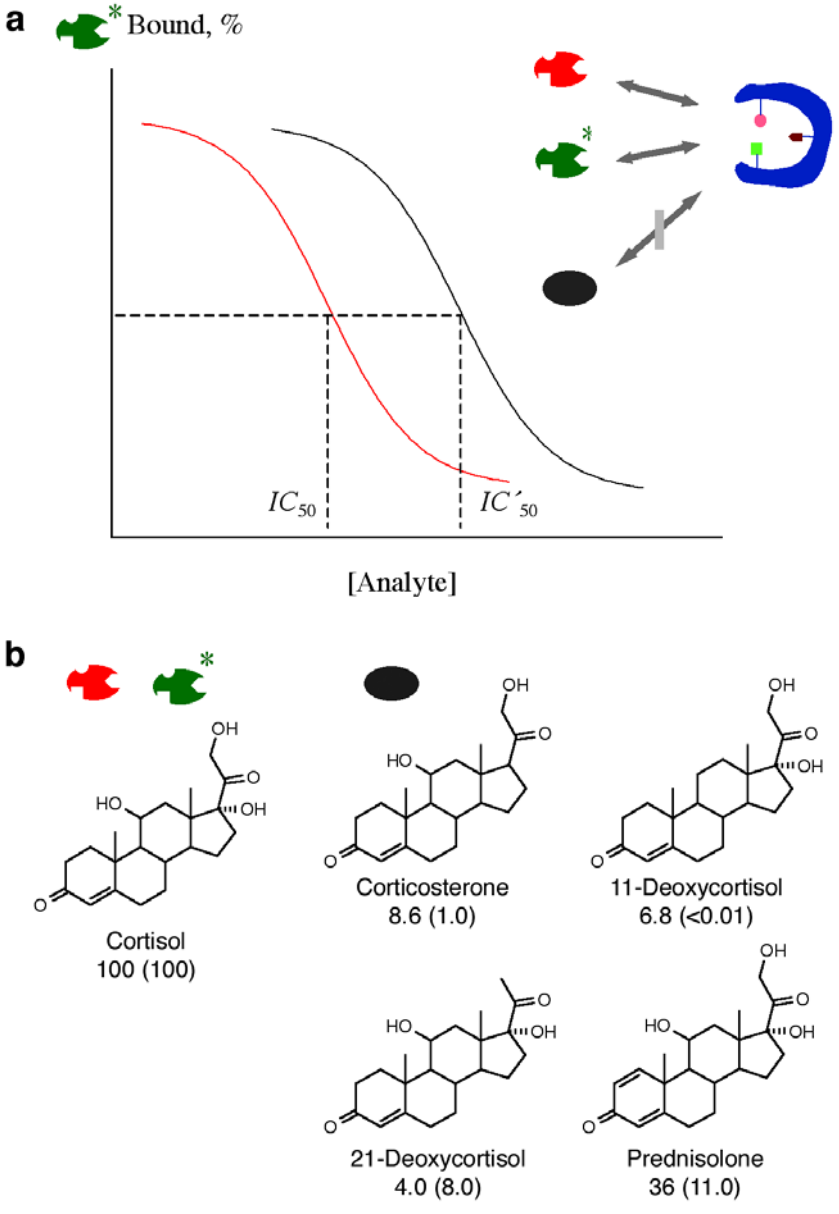


Fig. 2 **a** Principle of a MIP-based radioimmunoassay. The non-labeled template (in red) inhibits binding of the radioisotope-labeled template (in green, marked with *) to a limited number of sites on the MIP, whereas a compound with a slightly different structure (in black) has much less effect. **b** Binding selectivity of a MIP prepared against cortisol. The displacement curves obtained are utilized to calculate the MIP's cross-reactivity (in %) towards structurally related compounds. Data in parentheses: cross-reactivities (in %) obtained with an antibody-based radioimmunoassay for cortisol [7]

cularly imprinted sorbent assay format was later used to develop assay systems for other compounds as well, such as herbicides [9, 10]. Figure 2a schematically depicts the principle of a MIP-based RIA. In a competitive assay, the radioisotope-labeled target analyte is incubated with increasing amounts of non-labeled target (in green) to compete for binding to a limited amount of MIP. After the equilibrium is reached, the amount of label bound to the MIP, which is inversely related to the concentration of non-labeled analyte, is quantified by radioactivity measurements. A plot of bound label against the concentration of non-labeled analyte gives a typical sigmoidal calibration

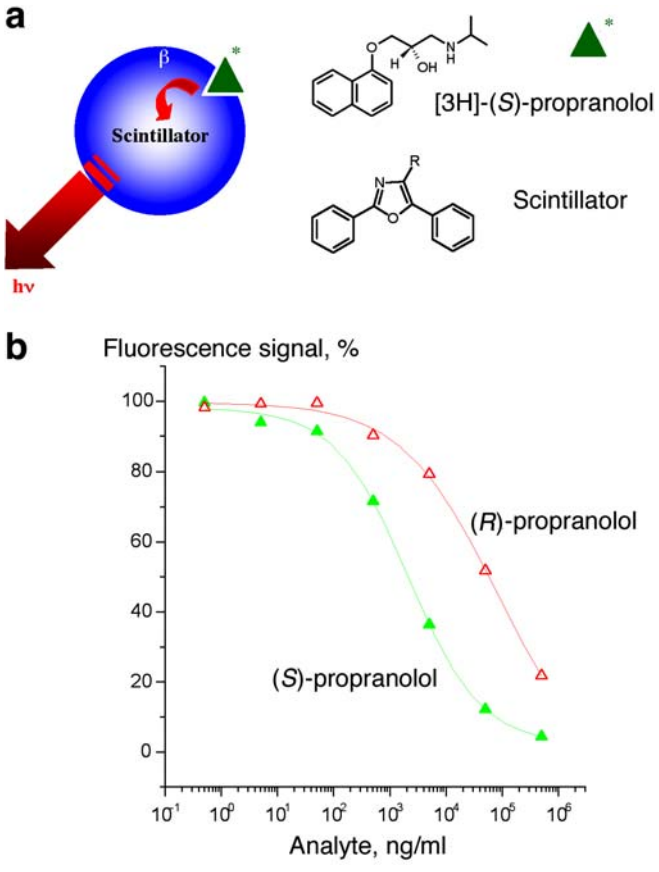


Fig. 3 MIP-based proximity scintillation assay. **a** The *S*-propranolol-imprinted microspheres contain a scintillation reporter located in proximity to the specific binding site. Binding of [^{3}H]*S*-propranolol makes the β-electron from the radioisotope decay stimulate the reporter to generate long wavelength fluorescence. **b** Calibration curve. In competitive mode, the non-labeled *S*-propranolol displaces the [^{3}H]*S*-propranolol, and so reduces the fluorescence signal. In an aqueous solvent, the MIP's cross-reactivity towards the other enantiomer, *R*-propranolol, is less than 2% (data from reference [12])

curve, which can be used to calculate the MIP's binding affinity and site population. The concentration of non-labeled analyte displacing 50% of the label is defined as the IC_{50} value. The same experiment can be repeated using other related drugs as the competing ligand, which gives displacement curves (and IC'_{50}) shifted to a higher concentration range. The MIP's cross-reactivity for the new ligand is defined as the percentage of IC_{50}/IC'_{50}. In Fig. 2b, we list the cross-reactivities of a cortisol-imprinted polymer towards certain related corticosteroids, in comparison with biological antibodies. The values obtained are essentially in the same order of magnitude, although the antibody is slightly more selective [7]. The dissociation constants (K_D) and the maximum binding capacities for cortisol (B_{max}) of the anti-cortisol MIP were determined using a two-site binding model. A high-affinity class of binding sites yielded 5.7×10^{-7} M and 0.21 µmol/g, and the low-affinity sites 1.6×10^{-3} M and 280 µmol/g, respectively.

By incorporating an appropriate reporter element, MIPs can be designed to directly generate a specific physicochemical signal upon binding of an analyte [11, 12]. In Fig. 3, we depict the principle of using a "universal" scintillation reporter embedded in molecularly imprinted microspheres. The MIP containing the scintillation reporter is imprinted against a β-adrenergic antagonist, *S*-propranolol. When tritium-labeled *S*-propranolol binds to the MIP, its β-radiation triggers the nearby reporter to emit long wavelength fluorescence that can be directly quantified. When used in competitive-assay mode, the fluorescence signal decreases due to the non-labeled analyte competing for the limited number of binding sites. This MIP-based scintillation proximity assay (SPA) has the potential to provide a very high sample throughput, since it is a quasi-homogeneous assay that does not require washing steps to separate unbound radioligand from its bound fraction before quantification.

Imprinted polymer-based assays are conveniently performed using radiolabels, because the labeled analyte has the same structure as the original template. However, this involves the handling of radioactive materials and produces radioactive waste, which is sometimes undesirable. Interest is therefore increasing in the development of alternative assay formats based on other detection methods that could use, just like immunoassays, an enzyme reaction or fluorescence for detection. Several years ago, we proposed competitive immunoassays that use a fluorescent probe [13] or an electroactive probe [14] for detection. These assays were based on a polymer imprinted with the herbicide 2,4-D, and the probes had some structural similarity with it. It was shown that although binding of the probes to the polymer was only a few percent compared to the analyte, specificity and selectivity of the assay were on a par with a competitive radioligand binding assay using the same polymer and the radiolabeled analyte. The fluorescent assay could be performed in aqueous buffer as well as in organic solvents such as acetonitrile.

Others have proposed a quasi-homogeneous system where a fluorescent reporter group, which acts at the same time as the functional monomer, is incubated into the MIPs binding sites. A fluorescent functional monomer, *trans*-4-[*p*-(*N*,*N*-dimethylamino)styryl]-*N*-vinylbenzylpyridinium chloride, has been used together with another functional monomer to prepare a polymer imprinted with cyclic adenosine monophosphate [15]. Upon binding to the imprinted sites, the analyte interacts with the fluorescent groups, and their fluorescence is quenched, allowing the analyte to be quantified. Since the fluorophore acts at the same time as a functional monomer that recognizes the analyte, it has to be specifically designed for each analyte.

For us, a great challenge has always been to use enzyme labels. Although most common with immunoassays, enzymes seemed to be less practical in MIP assays for two reasons: first, they often only work in aqueous buffers, whereas the use of many imprinted polymers used to be restricted to organic solvents. Second, the rather hydrophobic nature and highly cross-linked structure of the polymer limits the access of the imprinted binding sites by the large protein molecules. However, during the last few years, MIPs that perform well in aqueous solvents have been de-

veloped [6, 8, 10, 16], and we have shown that the problem of binding site accessibility might be circumvented by using, instead of large porous MIP particles, imprinted microspheres that have binding sites at or close to their surface. We have developed ELISA-type assays where the analyte was labeled with the enzyme peroxidase. Therefore, colorimetry or chemiluminescence [17] could be used for detection. A colorimetric MIP-assay has also been reported by Piletsky and colleagues [18]. They have developed a method where the polymer is synthesized in situ in the wells of a polystyrene microtiter plate. Aminophenylboronic acid was polymerized in the presence of epinephrine (the target analyte) using oxidation of the monomer by ammonium persulfate. This process resulted in the grafting of a thin polymer layer onto the polystyrene surface. The polymer was then used in a competitive enzyme-linked assay with a conjugate of horseradish peroxidase and norepinephrine.

High-throughput assays

There is an ever-increasing demand for automated, high-throughput assaying and screening of natural products, as well as of biological and chemical combinatorial libraries. MIPs, owing to their specificity, ease of preparation, low price and high chemical and physical stability, could provide a useful complement or alternative to biological receptors for use as recognition elements in such assays. This is especially true in cases where a natural receptor does not exist or is difficult to obtain in large quantities. Our group has recently developed a high-performance MIP-based assay using a chemiluminescence-imaging format [17]. Microtiter plates (96 or 384 wells) were coated with MIP microspheres using polyvinyl alcohol as glue. The analyte is added together with a small amount of enzyme (tobacco peroxidase) labeled analyte and incubated until the equilibrium is reached. After washing, the amount of polymer-bound 2,4-D-peroxidase conjugate is quantified using luminol as the chemiluminescent substrate. Light emission is quantified with a CCD camera-based imaging system. This format allows for the simultaneous measurement of a large number of samples.

Another aspect in assay development is their possible use in automated systems for unattended monitoring. For such applications, flow systems are well-suited. Their combination with chemically and physically stable, regenerable MIP receptors seemed to us particularly promising. In a recent paper, we described the design of a flow-injection ELISA-type MIP assay [19] using the same polymer and

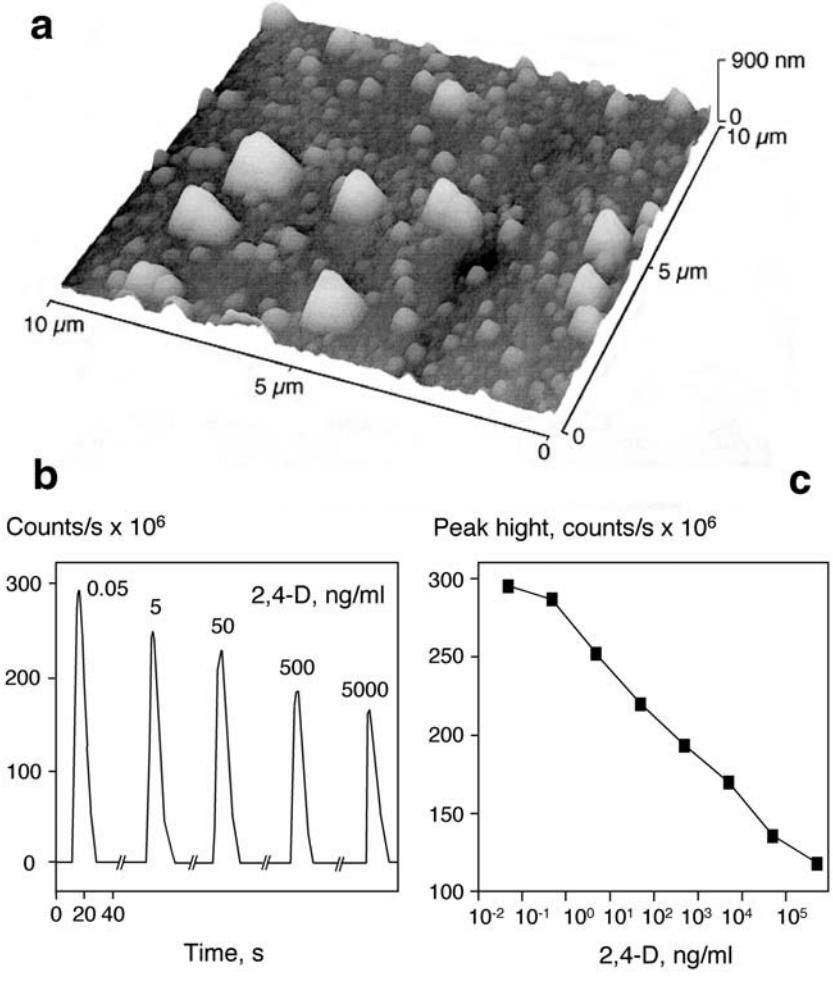


Fig. 4 MIP-based flow-injection capillary chemiluminescence ELISA. **a** Atomic force microscopy image of the polymer-coated inner capillary wall. **b** Typical readout of the PMT obtained from the competitive assay in FIA mode at different 2,4-D concentrations. **c** Calibration curve for 2,4-D (data from reference [19])

detection mode as described above for the imaging assay. A glass capillary was coated with the imprinted polymer and mounted in a flow system. A photomultiplier tube (PMT) was used for detection (Fig. 4). Calibration curves corresponding to analyte concentrations ranging from 0.5 ng/mL–50 µg/mL (2.25 nM–225 µM) were obtained, making the system one of the most sensitive MIP-based assays reported so far. A further increase in sensitivity by two orders of magnitude was obtained when detection was performed in discontinuous mode and the chemiluminescence light was conducted inside the photomultiplier tube by an optical fiber bundle, yielding a dynamic range of 5 pg/mL–100 ng/mL (22.5 pM–450 nM).

A different format for a flow-injection chemiluminescence assay using MIPs has been developed by Lin and Yamada [20]. A polymer selective for 1,10-phenanthroline was prepared based on a ternary metal complex of the analyte, 4-vinylpyridine–Cu(II)–1,10-phenanthroline, in combination with styrene and divinylbenzene, and packed into a glass tube. When the analyte was injected into a buffer stream containing H_2O_2, it was complexed by the pyridine–Cu(II) binding sites and encountered H_2O_2 molecules. The Cu(II)-1,10-phenanthroline complex was able to catalyze the decomposition of hydrogen peroxide, the analyte 1,10-phenanthroline acting at the same time as the chemiluminescent substrate. In fact, during the reaction, a superoxide radical ion is formed, which reacts with 1,10-phenanthroline and gives a chemiluminescent emission. The 1,10-phenanthroline is destroyed during the chemiluminescent reaction, liberating the binding site for another analyte molecule. Although technically elegant, this detection system unfortunately appears to be limited in terms of possible analyte molecules.

MIPs as recognition elements in sensors

In biosensors, a chemical or physical signal is generated upon the binding of the analyte to a biological recognition element like an antibody, a receptor or an enzyme. A transducer then translates this signal into a quantifiable output signal. The same general principle applies if a MIP is used as the recognition element instead of a biomolecule. Table 1 depicts the three different possibilities for transducing the binding event. In the simplest case, a change in one or more physicochemical parameters of the system upon analyte binding (such as mass accumulation) is used for detection. This principle is widely applicable and more or less independent of the nature of the analyte. In order to increase sensitivity and the signal-to-noise ratio, reporter groups may be incorporated into the polymer that generate or enhance the sensor response. If the analyte possesses a specific property (such as, fluorescence or electrochemical activity), this can also be used for detection.

Early attempts to utilize the recognition properties of MIPs for chemical sensing were, for example, ellipsometric measurements on thin Vitamin K_1-imprinted polymer layers [21], the measurement of changes in the electrical streaming potential over an HPLC column packed with a MIP [22], or permeability studies of imprinted polymer membranes [23]. Mosbach's group reported the first integrated sensor based on a MIP, a capacitance sensor consisting of a field-effect capacitor covered with a thin phenylalanine anilide-imprinted polymer membrane [24]. More recently, capacitive detection was employed by others in conjunction with imprinted electropolymerized polyphenol layers on gold electrodes [25].

During the last few years, mass-sensitive acoustic transducers, in particular the quartz crystal microbalance (QCM), have become very popular in combination with imprinted polymers. These sensors are based on the first group of transducers (Table 1). They consist of a thin quartz disk with electrode layers on both sides, which can be put into oscillation using the piezoelectric effect. A thin imprinted layer is deposited on one side of the disk. Analyte accumulation in the MIP results in a mass change, which in turn causes a decrease in oscillation frequency that can easily be quantified by frequency counting. Possible reasons for the success of this transducer type are its relatively low price, its robustness and its ease of use. In addition, it is relatively easy to interface the MIP with the sensor. A few years ago, we reported, in collaboration with Kutner's group in Warsaw, the first enantioselective MIP-based QCM sensor [26]. The sensor, coated with a poly(trimethylolpropane trimethacrylate-*co*-methacrylic acid) MIP imprinted with *S*-propranolol (a β-blocker), was able to discriminate between the *R* and *S*-enantiomers of the drug with a selectivity coefficient of α=5. Others have constructed an imprinted polymer-based sensor for glucose [27]. The polymer, poly(*o*-phenylene diamine), was electrosynthesized directly at the sensor surface in the presence of 20 mM glucose. In that way, a very thin (10 nm) polymer layer was obtained that could rebind glucose with certain selectivity over other compounds such as ascorbic acid, paracetamol, cysteine, and to some extent fructose.

Table 1 Different approaches to the transduction of the binding signal in MIP-sensors

	Signal is generated...		
	Directly through the binding event	By the analyte	By the polymer
What is measured	Change in general physiochemical properties of the system	Specific property of the analyte	Change in the signal emitted by reporter groups incorporated into the polymer
Examples	Mass change (QCM), capacitance change	Fluorescence, electrochemical activity, IR spectrum	Fluorescence, scintillation, spectral shift, proton release (pH)

Thin TiO_2 sol-gels have been used for imprinting of azobenzene carboxylic acid [28]. Nice work has recently been reported by Dickert's group [29]. They have produced imprints of whole yeast cells in polyurethane layers and in sol-gel layers at the surface of a QCM crystal using a stamping method. The sensor could be used to quantify yeast cells in suspension at concentrations between 1×10^4 and 1×10^9 cells/mL under flow conditions.

Other sensors belonging to the first group (Table 1) have been designed based on conductometric transducers [30, 31, 32]. Here, two electrodes are separated by an imprinted polymer membrane. Binding of the analyte to the polymer changes its conductivity, which is translated into an electrical signal. A sensing device for the herbicide atrazine, that is based on a freestanding atrazine-imprinted acrylic polymer membrane and conductometric measurements, has been constructed by Piletsky and coworkers [33]. According to the authors, the kind and molar ratio of crosslinking monomers used, and the relative amount of porogenic solvent in the imprinting mixture, were important factors not only for the flexibility and stability of the MIP membranes, but also because the conductometric response seemed to depend on the ability of the MIP to change its conformation upon analyte binding.

If the target analyte exhibits a special property such as fluorescence [34, 35] or electrochemical activity [36], this can be exploited for the design of MIP-based sensors (Table 1, second group). If the analyte lacks such property, a competitive or displacement sensor format may be used. In collaboration with Turner and coworkers, we have developed a voltametric sensor for the herbicide 2,4-D [14] where the electroactive compound 2,5-dihydroxyphenylacetic acid was used as a probe. MIP particles were coated as a thin layer onto a screen-printed carbon electrode. The electrode was then incubated with the sample to which the probe was added. In the presence of the analyte, some of the probe was displaced from the imprinted sites, whereas the remaining probe was directly quantified by differential pulse voltametric measurements. We believe that, because of the potential low production costs, the combination of screen-printed electrodes and MIPs is particularly well-suited for the design of disposable sensing elements.

An elegant way of designing the MIP/transducer couple is to have the signal generated by the polymer itself (Table 1, third group). This approach appears promising since it does not depend on a special property of the analyte. Moreover, it should facilitate the integration and production of the sensing device. One example for such a format is a polymer containing a fluorescent metalloporphyrin as the reporter group, which acts at the same time as one of the functional monomers [37]. Binding of the analyte 9-ethyladenine then results in quenching of the fluorescence of the polymer.

The signals generated by most transducer types are two-dimensional and provide only limited information about the composition of the sample. Although this is normally compensated by the high selectivity of MIPs, a different strategy is the use of transducer mechanisms that generate signals with higher inherent information content. One way to achieve this is to exploit the high molecular specificity of absorption spectra in the mid-infrared spectral region (3500–500 cm^{-1}). The combination of MIPs and FTIR spectrometry might allow analytical problems to be addressed where the selectivity of the MIP alone is not sufficient, for instance when samples with complex matrices are to be investigated, or when analytes that are structurally very similar are present in the sample. Together with Mizaikoff's group, we have combined imprinted polymers and infrared evanescent-wave spectroscopy in a chemical sensing device [38]. A polymer molecularly imprinted with 2,4-D was coated in the form of a thin film onto a ZnSe attenuated total reflection element, which was mounted in a flow cell. Accumulation of 2,4-D in the MIP layer could be followed on-line and in real time by FTIR spectrophotometric measurements. Analyte binding was concentration-dependent and could be quantified by integrating characteristic analyte bands.

Screening of chemical libraries

As artificial receptors, MIPs have also been used to screen combinatorial chemical libraries, where compounds that are closely related to a known ligand could be easily identified by their relative binding strength to the imprinted polymers. Even though there have, until now, only been a few preliminary reports that demonstrated the feasibility of the approach [39, 40, 41, 42, 43], we believe that MIPs will find applications in drug screening and development, in particular for the initial screening of large libraries. In most of the examples, MIPs are used as stationary phases in an affinity chromatography mode. The relative retention times of the analytes reflect their molecular similarity with the template. Figure 5 shows the affinity profile obtained by screening a small steroid library using an estrogen receptor mimic prepared by molecular imprinting against the steroid hormone 17β-estradiol [42].

MIP-directed generation of bio-effective molecules

Using MIPs to control chemical reactions has been suggested by several authors. One aspect resides in the development of catalytic MIPs, where a strategy similar to the generation of catalytic antibodies has been followed. Typically, a transition state analogue (TSA) of a target reaction is used to prepare a MIP, which can now bind and stabilize the transition state and lower the activation energy barrier, resulting in an increased reaction rate [44, 45]. In related studies, MIPs were used to control the stereospecificity of particular reactions [46, 47], or as protecting reagents for selective modification of multi-functional compounds [48]. However, in all of these efforts, use of MIPs has been focused on single reactions or molecules, rather than to explore chemical spaces to look for new compounds.

Discovery of new bio-effective molecules is of paramount importance in modern drug development and bio-

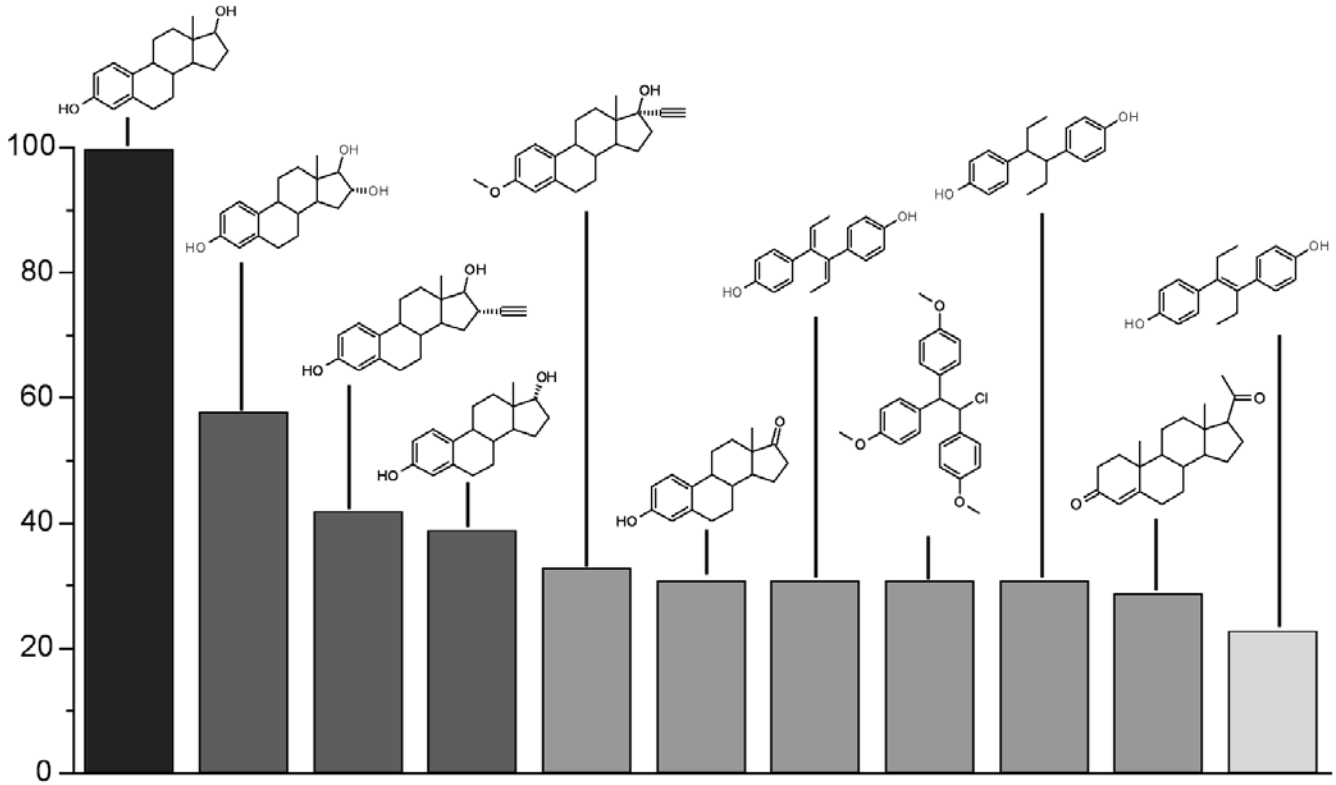


Fig. 5 Screening of a steroid library using an anti-17β-estradiol MIP as an artificial estrogen receptor. The MIP was used as an affinity stationary phase in chromatography mode. The graph represents the relative retention of the different analytes caused by their specific binding to the MIP. Non-specific binding was evaluated and corrected for by using a non-imprinted polymer as a control (data from reference [42])

technology. Different methodologies have been adopted to generate new enzyme inhibitors, receptor agonists/antagonists and DNA-binding molecules. In structure-based design, a thorough knowledge of the three-dimensional structure of a biological target is a prerequisite [49]. Although recent progresses in functional genomics and proteomics are expected to shorten the time required for target identification [50], characterization of the identified bio-target with x-ray crystallography or NMR spectroscopy is still a limiting factor. In addition, membrane-bound receptors are among the most difficult targets to be solved with the present techniques. Although combinatorial chemistry somehow reduces the stringent constraint encountered in rational design, and is expected to deliver hit molecules at a higher speed, the real output using the combinatorial approach alone is far from initial expectations. Because of the experimental difficulties in exploring an inclusive chemical space pertinent to a specific biological target, the current trend in searching for new drug candidates turns to the combination of structure-based design with combinatorial methodology. In this way, more focused chemical libraries are being investigated.

As stable artificial receptors, MIPs prepared using known bioactive templates are expected to possess binding sites mimicking those of corresponding biological targets. We have demonstrated that these artificial sites can be used to control synthetic reactions, so that desired bioactive products can be enriched. This strategy is similar to the use of biological targets to generate small chemical ligands [51, 52, 53, 54], except that an artificial receptor (MIP) is used to direct the chemical reactions. Given their outstanding chemical stability, MIPs can be repeatedly used under various reaction conditions, including elevated temperature, low or high pH values, and in various organic solvents. Another advantage is that important orthogonal binding groups on the MIP can be designed so as to leave the reactive moieties in the building blocks that are used in coupling reactions unaffected. Upon completion, reaction products can be easily separated and characterized using standard analytical techniques. This will indicate the reaction routes that lead to the amplified hit compounds.

As a proof-of-principle, we have chosen a clinically interesting serine protease, kallikrein, as a model therapeutic target. This enzyme is known to be involved in a number of important biological processes: tissue kallikrein cleaves kininogens to release the vasoactive decapeptide, Lys-bradykinin, in several inflammatory processes including arthritis, asthma and rhinitis [55]. Specific kallikrein inhibitors present significant analgesic and anti-inflammatory activities [56]. In addition, a synthetic tissue kallikrein inhibitor has been found to suppress invasion of cancer cells [57].

It is known that inhibitors of tissue kallikrein have the common feature of a positively-charged amino or guanidino group connected to a hydrophobic moiety. The two

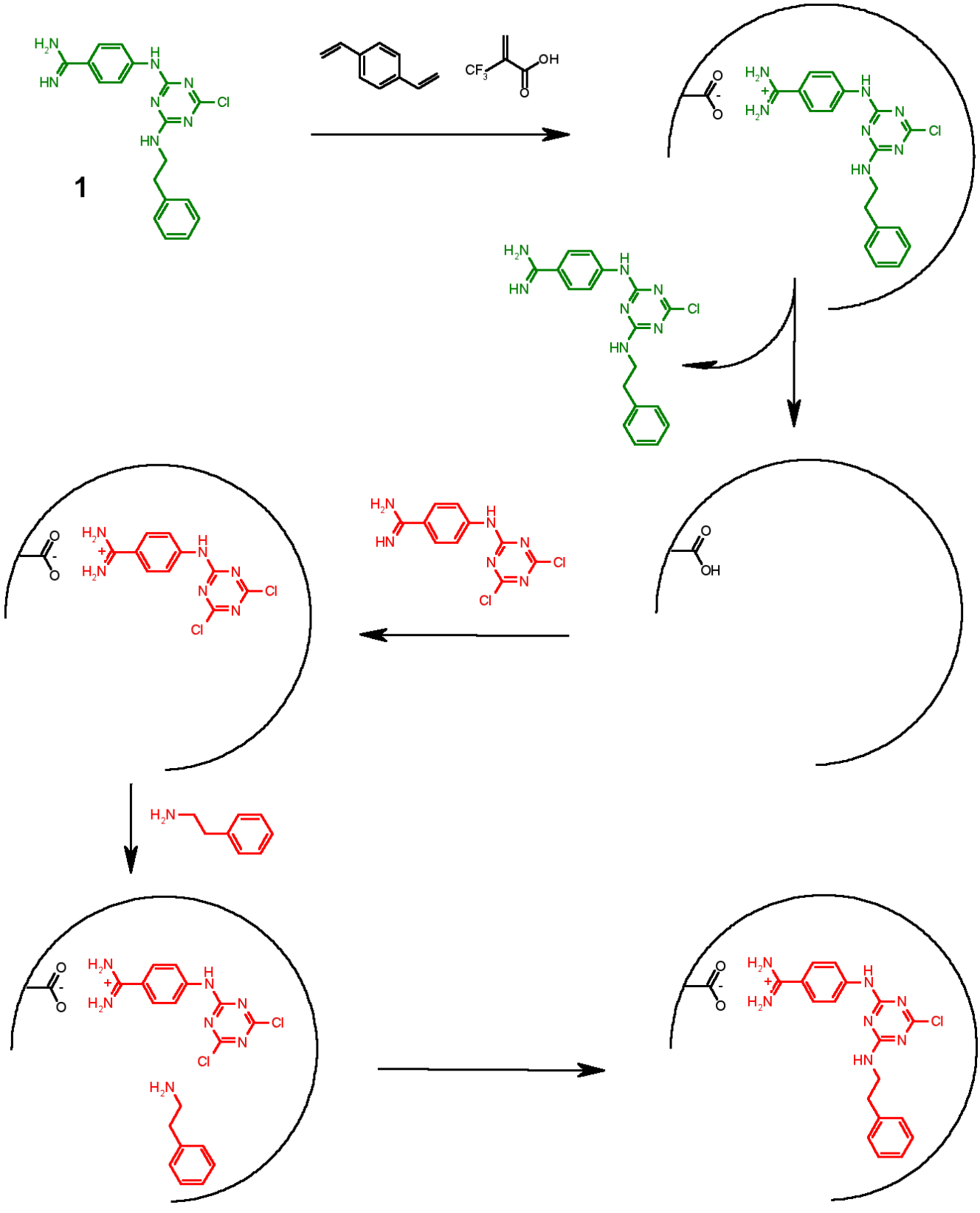


Fig. 6 Molecular imprinting against a kallikrein inhibitor (**1**), and the subsequent MIP-directed synthesis of inhibitor (**1**) [60]

residues resemble the side chains of the peptide sequence Phe-Arg, which bind to the S_2-S_1 pocket of the enzyme's active site [58]. A representative non-peptide inhibitor is 2-(4-amidinophenylamino)-4-chloro-6-phenylethylamino-*s*-triazine (**1**) [59], which we have chosen as a template for MIP preparation [60].

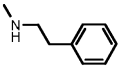
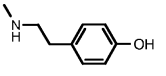
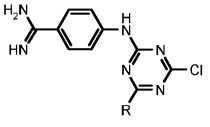
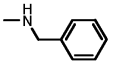
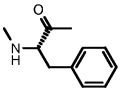
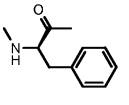
To obtain a MIP that presents specific binding to **1**, we have used (2-trifluoromethyl)acrylic acid (TFMAA) and divinylbenzene (DVB) as the functional monomer and cross-linker. The rationale for this was that TFMAA forms strong ionic interaction with the amidine group, while DVB provides the possibility of π-π stacking with the aromatic moieties in the template (Fig. 6). As verified by chromatographic analysis, the obtained MIP contained specific binding sites for **1**. These sites are thought to resemble the active center of tissue kallikrein, where the carboxyl group of the MIP should represent the negatively-charged Asp189 of the enzyme.

When the two building blocks, 2-(4-amidinophenylamino)-4,6-dichloro-*s*-triazine and phenylethylamine, were allowed to react in the presence of the MIP, the amount of **1** obtained with the MIP was four times higher than that obtained in the presence of a non-imprinted control polymer. Under the same condition, no product was obtained in free solution when no polymer was present. The fact that some product was also obtained with the control polymer is most likely due to the randomly distributed carboxyl groups and hydrophobic patches on the polymer itself. Based on these results, it seems reasonable to conclude that the reaction between the two building blocks mainly took place in the specific binding sites of the MIP (Fig. 6). This made it possible to utilize these specific sites to screen different building blocks for their suitability to furnish new inhibitors – without the time-consuming synthesis of a product library.

The MIP imprinted against **1** was then challenged with other building blocks, where the choice of the amine reactants was based on their similar molecular size and nucleophilicity to react with 2-(4-amidinophenylamino)-4,6-dichloro-*s*-triazine. Table 2 lists the different inhibitors obtained in the presence of the MIP, together with their inhibition constants for tissue kallikrein. While the additional inhibitors **2** and **3** were successfully synthesized with the MIP, neither of them were obtained with the control polymer. The low yield of **2** can be attributed to the extra 4-hydroxyl group in the amine, which introduces steric hindrance and local polarity of the building block. For the same reason, the bulky methyl ester group prevented L-phenylalanine methyl ester from entering the MIP's binding sites; therefore, no inhibitor **4** was obtained.

It appears reasonable to anticipate that the choice of the primary template in this approach is not limited to exactly the same bioactive molecule. For example, one may wish to create a MIP with a more spacious binding site, and a somewhat more flexible polymer backbone, to increase the number of hits. This can probably be achieved by using a chemically modified ligand as the primary template, and by decreasing the cross-linking level. A certain degree of "induced fit" for binding the building blocks would be expected.

Table 2 Target-directed synthesis of kallikrein inhibitors using molecularly imprinted polymers (anti-1 MIP and anti-4 MIP), and the target enzyme as the template. The relative yield (%) was calculated in comparison with 1 (data from reference [61])

Kallikrein inhibitor	R	Relative yield (%)			K_i (10^{-6} M)
		Anti-1 MIP	Anti-4 MIP	Kallikrein	
1		100	100	100	4.5
2		21	n.d.	2	40
 3		34	118	86	5.2
4		0	74	26	33
5		n.d.	59	14	47

In a more recent study, we have used the chiral and bulkier template **4** to explore the same building blocks, except for the addition of another chiral amine, D-phenylalanine methyl ester, a building block leading to **5**. As expected, the MIP prepared against **4** displayed chiral selectivity; that is, the synthetic yield of **4** was increased by 60% compared to **5** [61]. When this MIP was challenged with different building blocks, more inhibitors could be identified (Table 2). To further verify the MIP-directed synthesis, we also carried out a target-directed synthesis of inhibitors using kallikrein itself to direct the reactions. Porcine pancreatic kallikrein was first titrated with 2-(4-amidinophenylamino)-4,6-dichloro-*s*-triazine at a neutral pH. Under these conditions, no reaction between lysine residues on the enzyme and the building block could take place. After removing excess reagent, the enzyme complex was transferred into an amine solution at pH 10. This allowed the amine to be coupled to the 2-(4-amidinophenylamino)-4,6-dichloro-*s*-triazine already bound in the enzyme's active site. In addition to the hits of **1** and **2**, kallikrein facilitated formation of **4** to a larger extent than that of **5**, with a chiral selectivity similar to that obtained with the MIP (Table 2).

Conclusions

Molecular imprinting has been used to generate numerous artificial receptors that have demonstrated selective binding of their target molecule. Successful demonstrations of the use of MIPs for assays, sensors, and very recently for drug development, revealed the great potential of the technology. However, fundamental research needs to be intensified to develop MIPs with much-improved functions. The future of MIPs as biomimetic receptor for bioanalytical applications is promising, both for their potential in fundamental research and for the many potential industrial applications. For drug development, the "screening-of-building blocks" approach only requires a few known bioactive molecules to generate MIPs mimicking unresolved biological targets. The artificial target can be used to screen building blocks in order to find hit reactions and products, without involving costly synthesis of chemical libraries. Given the possibility of fine-tuning the binding strength and specificity of molecularly imprinted polymers, these artificial targets may result in faster and more cost-effective identification of new bioactive molecules.

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