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Molecularly imprinted polymers in analytical chemistry

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1 Introduction

1.1 General principle of molecular imprinting

The design and synthesis of biomimetic receptor systems capable of binding a target molecule with similar affinity and specificity to antibodies has been a long-term goal in bioorganic chemistry. One technique that is being increasingly adopted for the generation of artificial macromolecular receptors is molecular imprinting of synthetic polymers. This is a process where functional and cross-linking monomers are copolymerized in the presence of a target analyte (the imprint molecule), which acts as a molecular template. The functional monomers initially form a complex with the imprint molecule, and following polymerization, their functional groups are held in position by the highly cross-linked polymeric structure. Subsequent removal of the imprint molecule reveals binding sites that are complementary in size and shape to the analyte. In that way, a molecular memory is introduced into the polymer, which is now capable of selectively rebinding the analyte (Fig. 1). The complex between monomers and imprint molecule can be formed *via* reversible covalent bonds or *via* non-covalent interactions such as hydrogen bonds, ionic bonds, hydrophobic interactions, van der Waals forces, *etc*. A combination of the two can also be used. In order to compare the covalent and non-covalent imprinting approaches, different aspects have to be taken into account. The non-covalent imprinting approach, which was pioneered by Mosbach and co-workers,[1] is more flexible concerning the choice of functional monomers, possible target molecules and the use of the imprinted materials. After polymerization, the imprinted molecule can be removed from the polymer by simple solvent extraction. However, the prepolymerization complex is an equilibrium system, the stability of which depends on the affinity constants between imprint molecule and functional monomers. This may yield a certain heterogeneity of the binding sites. For covalent imprinting, a polymerizable derivative of the imprint molecule has to be synthesized, and after synthesis of the polymer, the imprint molecule has to be removed by chemical cleavage. If upon use of the polymer the covalent bonds have to be reformed, the association kinetics may be low. On the other hand, owing to the greater stability of covalent bonds, covalent imprinting protocols should yield a more homogeneous population of binding sites. Moreover, the yield of binding sites relative to the amount of imprint molecule used (imprinting efficiency) should be higher than that with non-covalent protocols. This approach was developed primarily by Wulff and co-workers.[2] Protocols have also been suggested that combine the advantages of both covalent and non-covalent imprinting, that is, the target molecule is imprinted as a stable complex with the functional monomers formed *via* covalent interactions, whereas upon later use of the MIP, only non-covalent interactions come into play. As an example, Whitcombe and co-workers reported the imprinting of a tripeptide (Lys–Trp–Asp) using a sacrificial spacer (*o*-hydroxybenzamide) between the imprint molecule and monomers. In addition to these covalent bonds, non-covalent interactions have also been used. After polymerization, the covalent bonds between the imprint molecule and the monomers are hydrolyzed, leaving precisely positioned carboxy groups (Fig. 2). During rebinding the

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peptide interacts with the polymer only *via* non-covalent interactions.[3]

1.2 Target molecules

One of the many attractive features of the molecular imprinting technique is that it can be applied to a wide range of target molecules. The imprinting of small, organic molecules (*e.g.*, pharmaceuticals, pesticides, amino acids, peptides, nucleotide bases, steroids and sugars) is now well established and considered almost routine. Metal and other ions have also been used as templates to induce the specific arrangement of functional groups in the imprinting matrix.[4–8] Larger organic compounds (*e.g.*, peptides) can also be imprinted *via* similar approaches, whereas the imprinting of much larger structures is still a challenge. Specially adapted protocols have been proposed to create imprints of proteins in a thin layer of acrylic polymer on a silica surface,[9] of cells using a lithographic technique[10] and even of mineral crystals.[11] Fig. 3 shows an interesting approach to create imprints of proteins in a surface.[12] The protein of interest is first adsorbed on an atomically flat mica surface. It is then spin-coated with a disaccharide solution that upon drying forms a thin layer (1–5 nm) attached *via* multiple hydrogen bonds to the protein. This protective disaccharide shell is then covered with a fluoropolymer layer *via* glow-discharge plasma deposition, which covalently incorporates the sugar molecules. Finally, the polymer layer is attached to a glass substrate using an epoxy glue. After peeling off the mica, the protein is removed by treatment with aqueous NaOH–NaClO, leaving nanocavities as revealed by tapping-mode atomic force microscopy. The authors reported that the cavities were complementary in size and, it seems, to some extent also in functionality, to the template protein. For example, they could show that a surface imprinted with bovine serum albumin (BSA) preferentially adsorbed the template protein from a binary mixture with immunoglobulin G (IgG). Moreover, a ribonuclease A imprint preferentially adsorbed the template protein over lysozyme that is similar in size and isoelectric point, and *vice versa*. This protocol, although complex, might be more or less generally applicable to creating imprints of proteins in surfaces.

1.3 The imprinting matrix

1.3.1 Acrylic and vinyl polymers. At the present time, the majority of reports on molecularly imprinted polymers (MIPs) describe organic polymers synthesized by radical polymerization of functional and cross-linking monomers having vinyl or acrylic groups and using non-covalent interactions. This can be attributed to the fairly straightforward synthesis of these materials and to the vast choice of available monomers. These can be basic (*e.g.*, vinylpyridine) or acidic (*e.g.*, methacrylic acid), permanently charged (*e.g.*, *N*,*N*,*N*-trimethylaminoethyl methacrylate), hydrogen bonding (*e.g.*, acrylamide), hydrophobic (*e.g.*, styrene) and others. These fairly 'simple' monomers normally have association constants with the template that are too low to form a stable complex. Thus, they have to be used in excess to shift the equilibrium towards complex formation. Somewhat more sophisticated monomers are also starting to appear that form stable interactions with the template molecule or substructures thereof, and that can sometimes be used in a stoichiometric ratio.[13–17] A few examples of monomers recognizing amino and carboxy groups are depicted in Fig. 4. Another possibility for obtaining stronger interactions in the pre-polymerization complex, in particular in polar solvents such as water, is by using coordination bonds with metal chelate monomers.[9,18,19]

In order to obtain an optimized polymer for a given target analyte, combinatorial approaches to MIP synthesis have been used[20–22] where the ingredients of the imprinting recipe, in particular the kind and molar ratio of the functional monomers, are varied. This is ideally done using automated procedures.[20] As an example, an MIP selective for the triazine herbicide terbutylazine was optimized using a combinatorial approach where a number of different MIPs were synthesized on a small scale (*ca*. 55 mg).[21] The functional monomer was selected from a library composed of six different molecules (methacrylic acid, methylmethacrylate, hydroxyethyl methacrylate, trifluoromethylacrylic acid, 4-vinylpyridine and *N*-vinyl-α-pyrrolidone). An initial screening was performed for the type of functional monomer that retained the template most strongly. Among the six monomers tested, methyl methacrylate, 4-vinylpyridine and *N*-vinyl-α-pyrrolidone led to polymers from which the imprint molecule was rapidly and quantitatively extracted, whereas

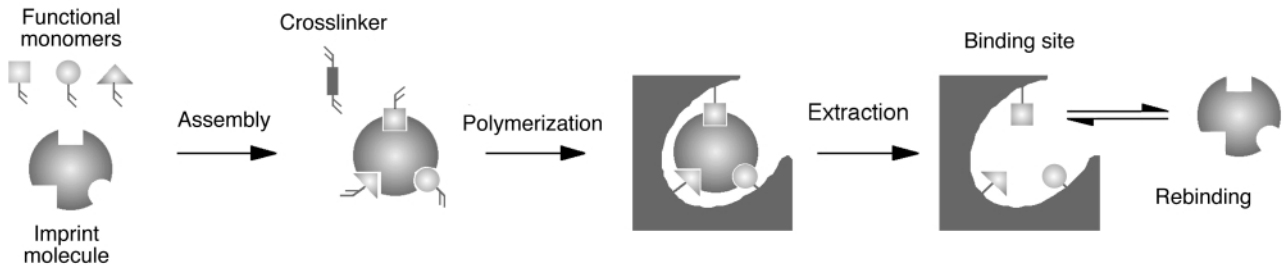

Fig. 1 Schematic representation of the molecular imprinting principle.

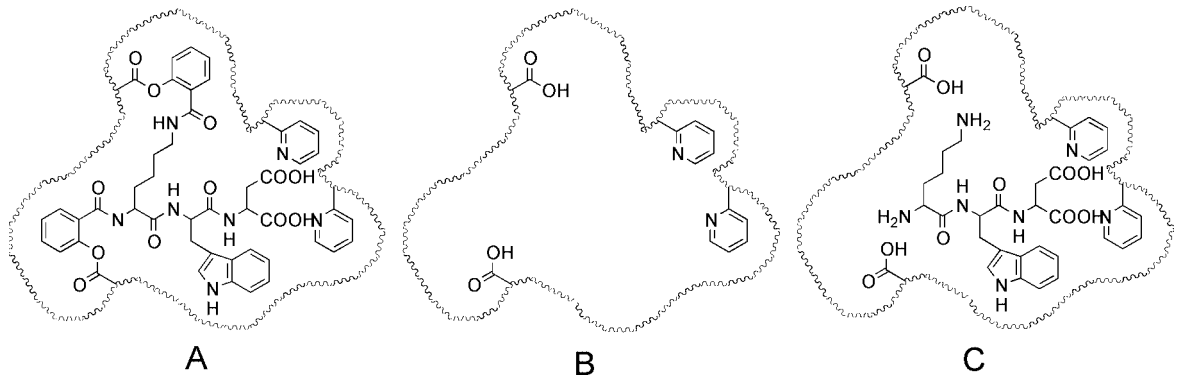

Fig. 2 Molecular imprinting of the tripeptide Lys–Trp–Asp using both covalent and non-covalent interactions. (a) Binding site with covalently bound imprint molecule; (b) binding site after chemical cleavage and extraction of the imprint molecule; (c) rebinding of the imprint molecule *via* only non-covalent interactions. Adapted with permission from ref. 3, Copyright 1999 Wiley-VCH.

methacrylic acid and trifluoromethylacrylic acid led to polymers that retained the template more strongly. Based on these two monomers, secondary screening for selectivity was performed. For that purpose, non-imprinted control polymers were also prepared and analyte binding to the MIPs and control polymers was evaluated in batch mode. The polymer showing the highest selectivity was found to be that based on methacrylic acid.

Others have attempted to select the best functional monomer(s) for a given template using a computational approach based on molecular modeling (Piletsky *et al*., communication at the *MIP 2000 Conference*, Cardiff, UK, July 2000). Although the results obtained are still preliminary and the method needs further improvement, it is nevertheless promising as it might allow considerable shortening of the development and optimization time for imprinted polymers.

1.3.2 Other organic polymers. In recent years, other polymers have started to appear that are either better suited for a specific application or easier to synthesize in the desired form. For example, polymers such as polyphenols,[23] poly(aminophenyl boronate),[24] poly(phenylenediamine),[25], poly(phenylenediamine-co-aniline),[26] polyurethanes,[27] overoxidized polypyrrole[28] and others have been used. Compared with polymers based on acrylic and vinyl monomers, the use of the above-mentioned polymers seems to be somewhat restricted owing to the limited choice of functional monomers.

1.3.3 Other imprinting matrices. Silica has been used as the imprinting matrix for the imprinting of inorganic ions[7] and organic molecules.[29–35] Thereby, either the bulk material can be imprinted by the sol–gel method, thus creating microporous materials with specifically arranged functional groups,[7,30,34,35] or an imprinted polysiloxane layer is deposited on the silica surface.[29,33,36-38] Another material that has been imprinted using the sol–gel technique is titanium oxide.[39–41]

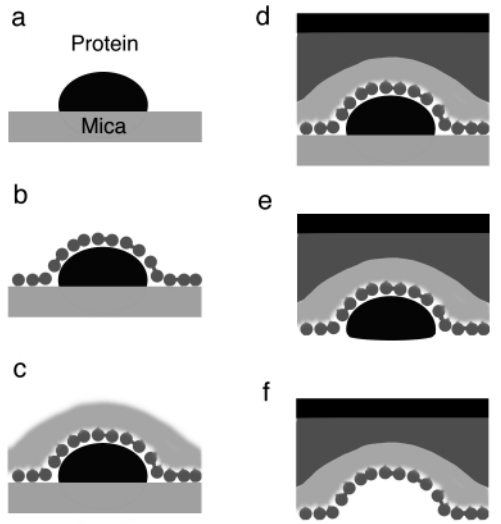

Fig. 3 Creation of a protein imprint: (a) the protein is adsorbed on mica, (b) coated with disaccharide, (c) a fluoropolymer layer is overlaid by plasma deposition (d), the polymer is glued to a glass substrate (e), the mica is peeled off and (f) the protein is removed leaving a binding site.[12]

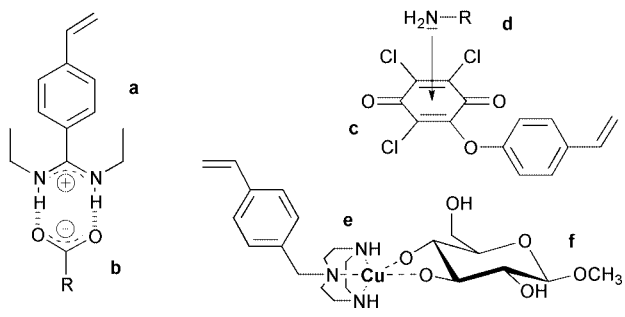

Fig. 4 (a) Amidine functional monomer binding to (b)a carboxy group;[15] (c) tetrachloroquinone monomer complexing (d) an amino group;[17] (e) metal chelate monomer coordinated by (f) a sugar diol.[130]

1.3.4 Physical forms of MIPs and new preparation methods. Traditionally, MIPs have been prepared as bulk polymer monoliths followed by mechanical grinding to obtain small micrometer-sized particles. Whereas the materials obtained through this somewhat inelegant method still seem to be useful for many applications, other applications require MIPs in defined physical forms for which specially adapted synthesis methods are needed. During the past few years, three aspects have mainly been addressed: the synthesis of small, spherical particles of below micrometer size, the synthesis of thin layers and the creation of surface imprints. MIP nanobeads can be synthesized by different methods such as precipitation polymerization and emulsion polymerization. Precipitation polymerization can be performed with similar prepolymerization mixtures as for bulk polymers, except that the relative amount of solvent present in the mixture is much higher. When polymerization progresses, imprinted nano- or microspheres precipitate instead of polymerizing together to form a polymer monolith. The method has the drawback that because of the dilution factor, larger amounts of imprint molecule are needed, although this may be compensated for by the typically higher yields. This method was successfully used by Ye *et al.* to prepare imprinted particles for binding assays,[42,43] and it has been shown that in some applications these particles performed better than particles obtained by grinding.[44] Whitcombe's group has followed a different approach based on emulsion polymerization, *i.e.*, small beads are created in an oil-in-water biphasic system stabilized by a surfactant. The particularity of their protocol is that the imprint molecule (here cholesterol) is part of the surfactant [pyridinium 12-(cholesteryloxycarbonyloxy)dodecane sulfate].[45] This results in all binding sites being situated at the particle surface [Fig. 5(A)], which was demonstrated by flocculation experiments using PEG–bis-cholesterol. Another protocol for the creation of surface binding sites has been reported recently by our group. The imprint molecule is immobilized on a solid support such as porous silica beads, prior to polymerization.[46] Following imprinting polymerization in the pores, the silica is removed by chemical dissolution, which leaves behind a porous polymeric structure. The binding sites are now all situated at the surface of the polymer and should be uniformely oriented [Fig. 5(B)].

Thin imprinted polymer films have been reported on several occasions and seem be useful or even necessary in many applications of MIPs. For example, they can be synthesized *in situ* at an electrode surface by electropolymerization,[23,25,26,28] or at a non-conducting surface by chemical grafting.[24,47] An elegant way would be to apply the soft lithography technique[48] to create thin MIP layers and surface patterns. There has been a first report on the use of this technique in combination with molecular imprinting, although no details were given concerning the binding performance of the obtained MIP microstructures.[49] It seems also that the current imprinting recipes are not always compatible with the poly(dimethylsiloxane) stamps used for soft lithography, hence more development efforts are needed.

2 Applications of imprinted polymers in analytical chemistry

2.1 Separation

2.1.1 Chromatography. The first application of MIPs was as stationary phases in affinity chromatography, in particular for

the enantioseparation of racemic mixtures of chiral compounds, and much of the early work on MIPs was devoted to this aspect. The imprinting process introduces enantioselectivity into polymers that are synthesized from (in most cases) non-chiral monomers. The particularity of MIPs compared with conventional chiral stationary phases is that they are tailor-made for a specific target molecule, hence their selectivity is predetermined. For example, if a polymer is imprinted with the L-enantiomer of an amino acid, an HPLC column packed with the MIP will retain the L-enantiomer more than the D-enantiomer and *vice versa*, whereas a column containing an identical but non-imprinted polymer will not be able to separate the enantiomers. Typical values for the enantioseparation factor α are between 1.5 and 5, although in some cases much higher values have been obtained. A very pronounced stereoselectivity has been observed with an MIP specific for the cinchona alkaloids cinchonidine and cinchonine, resulting in chromatographic α values of up to 31.[50] It is even possible to obtain chromatographic supports selective for compounds containing several chiral centers. For example, a polymer imprinted with the dipeptide Ac–L-Phe–L-Trp–OMe was able to recognize specifically the imprint isomer over the three other stereoisomers, which means that the LL form was more retained on an HPLC column packed with the MIP than the DD, DL and LD forms (separation factors: α = 17.8, 14.2 and 5.21, respectively).[51] If the molecule of interest contains more than two chiral centers, as is the case with carbohydrates, these properties of molecularly imprinted materials become even more relevant; in a study in which polymers were imprinted against a glucose derivative, very high selectivities between the various stereoisomers and anomers were recorded.[52]

The above figures are impressive and suggest that good enantioseparations should be achievable. Unfortunately, reality is often rather different, as the corresponding resolution factors and plate numbers are typically rather low (2000–5000 plates m^{-1}). This is due to often severe peak broadening and tailing, especially of the more retained enantiomer, which in turn can be attributed to a heterogeneous population of binding sites with respect to their affinities and accessibilities, as well as to a low functional capacity of the material.[53] One has to keep in mind that for each binding site to be created through imprinting, at least one template molecule has to be present in the polymerization mixture. In reality the 'imprinting efficiency', that is, the number of sites created divided by the number of template molecules, is much lower. Part of the template molecules can often not be extracted from the MIP after polymerization because they are deeply buried in the cross-linked matrix. Even if extraction is possible, part of the sites have such a low accessibility that they are useless in chromatographic applications. In order to obtain a mechanically stable material suitable for chromatography, a large percentage of the monomers has to be cross-linker (typically 80–90% for bifunctional cross-linkers), which limits the amount of functional monomers and thus template molecule that can be added. Moreover, if a non-covalent imprinting protocol is used, the functional monomer has to be in excess in order to shift the equilibrium towards complex formation. This inevitably results in a fraction of the monomers not being situated in a binding site but randomly distributed in the polymer, thus creating weak-affinity non-specific sites.

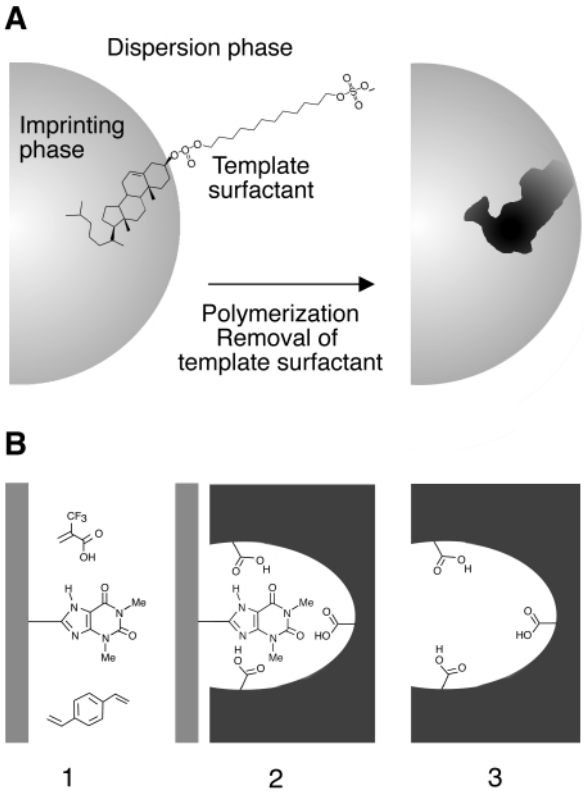

Fig. 5 (A) Synthesis of cholesterol-imprinted nanospheres by emulsion polymerization using a template surfactant.[45] (B) Molecular imprinting of theophylline immobilized on to a solid support: (1) immobilized template with monomers, (2) composite material after polymerization and (3) imprinted polymer after dissolution of the support.[46]

Numerous attempts have been made to improve the performance of MIPs and to avoid the problems mentioned above. The easiest way is what one could call the 'curative approach', that is, making the best out of the material available. This can be done by optimizing the separation protocol, including separation temperature, mobile phase, addition of competitors and the use of gradient elution protocols[54] to improve peak shapes. It has also been suggested to block the binding groups chemically in non-specific or low-quality sites[55] but the improvements in chromatographic behavior have been limited. More promising appear to be 'preventive' approaches in which efforts are made to synthesize better imprinted materials in the first place. During the last few years work has been focused mainly on two aspects: the synthesis of uniformly shaped and sized particles with narrow pore-size distribution and improved mass transfer, and the development of MIPs with better quality binding sites, ideally using stoichiometric template/functional monomer ratios. Uniformly sized spherical MIP particles for chromatography can be synthesized in a variety of ways, such as organic-in-water suspension polymerization, suspension polymerization using perfluorocarbon liquids as the dispersing phase,[56] and multi-step swelling procedures.[57] These materials should have a better chromatographic behavior than the commonly used ground bulk polymers. For example, is has been shown that a 25 cm $\times$ 4 mm id column filled with MIP beads prepared by suspension polymerization in perfluorocarbon could resolve 1 mg of Boc-DL-Phe at flow rates up to 5 mL min^{-1}, a result which is not easily obtained with a ground bulk polymer as column packing.[56]

2.1.2 Capillary electrochromatography. Capillary electrochromatography (CEC) might be one of the more promising chromatographic techniques to be used in combination with MIPs, in particular for chiral separations.[58,59] MIP-based CEC profits from the inherent separation power of this method; compared with MIP-based HPLC, appreciable resolution ($>100\,000$ plates m^{-1})[58] and separation factors can be achieved. In one study, enantioseparation of the β-blockers propranolol and metoprolol was achieved with MIP-CEC. The polymer was cast *in situ* in the capillary in the form of a macroporous monolith attached to the inner wall, and the capillary could be prepared and conditioned within a few hours.[60] The racemate of propranolol was resolved within only 120 s (Fig. 6), and when non-racemic samples were injected containing mainly the *R*-enantiomer, very small amounts (1%) of the *S*-enantiomer could be distinguished. Other possibilities

of using MIPs in combination with CEC or capillary electrophoresis is in the form of continuous polymer rods,[61] particles included in a gel matrix[62] and small particles suspended in the carrier electrolyte.[63]

2.1.3 Solid phase extraction. The separation technique that has been most intensively studied in the past 3 years with respect to the possible use of imprinted materials is solid phase extraction (SPE).[64–70] The need for efficient methods for sample preconcentration and clean-up in medical, food and environmental analyses is constantly increasing. The advantages of SPE over liquid–liquid extraction (LLE) are that it is faster and more reproducible, cleaner extracts are obtained, emulsion formation is not an issue, solvent consumption is reduced and smaller sample sizes are required. Moreover, SPE can be easily incorporated into automated analytical procedures. In that context is not surprising that much of the current research in the molecular imprinting field is concentrated on SPE, as here the advantages of MIPs, especially their low price and their stability in different environments, come into play, whereas some of the limitations are less important than with other separation techniques. MIPs are not only more selective than common sample treatment methods using C_{18} or ion exchange materials, but are at the same time more stable than (also very selective) immunoextraction[71] matrices. Since MIPs are compatible with organic solvents, MIP-SPE can be applied directly after a solvent pre-extraction step. On the other hand, the low resolution factors are not an issue as SPE works in the adsorption–desorption mode. Thus, SPE seems to be one of the most promising application niches for MIPs today and at the same time the application that is closest to commercialization. This is also reflected in the comparatively large number of reports dealing with real samples. MIP-SPE has been used to extract the target analyte from blood plasma and serum,[68,72] urine,[73] bile,[68] liver extract,[65] chewing gum,[66] environmental water and sediment,[74] plant tissue,[70] *etc*. The quantification of the herbicide atrazine in beef liver is a good demonstrative example of the utility of imprinted polymers in SPE.[65] In a first step, atrazine was extracted from liver tissue with chloroform. The imprinted polymer was then used to clean up the chloroform extract and to concentrate the analyte further prior to quantification. In this specific example, the binding capacity of the polymer for atrazine in chloroform was found to be 19 μmol g^{-1}. The analyte was eluted from the polymer with a suitable solvent (acetonitrile containing 10% acetic acid) and quantified after drying and reconstitution in acetonitrile or buffer, by RP-HPLC or ELISA. On comparing the purified with the non-purified chloroform extracts in RP-HPLC, the SPE step with the imprinted polymer considerably improved the accuracy and precision of the HPLC method and lowered the detection limit from 20 to 5 ppb. This was achieved owing to the removal of interfering components in the sample, resulting in baseline resolution of the atrazine peak. Furthermore, the analyte recovery was increased from 60.9 to 88.7% (quantification by HPLC) and from 79.6 to 92.8% (quantification by ELISA).

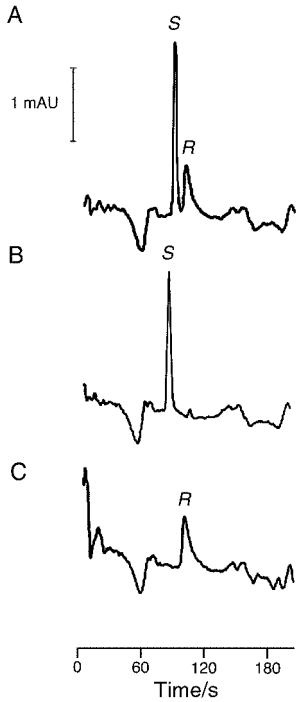

Fig. 6 Capillary electrochromatographic separation of (A) *rac*-propranolol, (B) (*S*)-propranolol and (C) (*R*)-propranolol on an (*R*)-propranolol-imprinted polymer. Adapted from Ref. 60 with permission.

When MIPs are to be used as SPE materials, one of their more troublesome features is template leakage. Generally, once an MIP has been synthesized, it is subjected to exhaustive solvent extraction to remove the template from the polymer matrix. The difficulty in extracting 100% of the template molecule from an imprinted polymer has long been recognized, although it was widely believed that the few per cent of template that did remain within the polymer was permanently entrapped. Recent work has clearly demonstrated that this is not necessarily the case. What can and does occur is slow leakage of a portion of the remaining template from the polymer matrix over a period of time, even after exhaustive extraction of the polymer beforehand. This can have serious implications when the polymer is to be used as an SPE sorbent in trace and ultra-trace analyses. A possible method of circumventing the bleeding problem entirely is to use a template analogue during the imprinting step rather than the template itself. The first demonstration of this approach was described by Andersson *et al.*[67] in a paper detailing the use of MIPs for the pre-concentration of the drug sameridine from human plasma, prior to its quantification *via* gas chromatography (GC). At the nanomolar concentration levels used in the study, leakage of template from the polymer matrix during sample handling was considerable and easily detectable *via* GC analysis, leading to large errors in the precision of the analytical measurement. As a remedy, a close structural analogue of sameridine [Fig. 7(A)] was used as the template molecule in the imprinting step, which yielded an imprinted polymer that still displayed a strong affinity for sameridine. Following SPE of sameridine from human plasma using this polymer, leakage of the analogue from the polymer matrix occurred, but sameridine and the analogue were readily resolved using GC. The analytical results obtained were similar to those obtained *via* a standard LLE method, with the added advantage that the sample injected in GC contained fewer matrix contaminants [Fig. 7(B)]. Similar approaches were later used by others.[75,76]

2.1.4 Other techniques. MIPs have also be used in other separation techniques, such as thin layer chromatography,[77–79] membrane-based separations[47,80-85] and adsorptive bubble flotation fractionation.[86]

2.2 Binding assays

The wide variety of techniques developed for the determination of analytes by immunoassay include various configurations of radioimmunoassays (RIA) and enzyme immunoassays (ELISA).[87–89] Since MIPs share with antibodies one of their most important features, the ability to bind a target molecule selectively, they could conceivably be employed in immunoassay-type binding assays in place of antibodies. This was first demonstrated by Mosbach's group, who developed MIP-based assays for the bronchodilator theophylline and the tranquilizer diazepam.[90] The format they used was analogous to the first solid-phase immunoassay, a competitive radioassay for human growth hormone.[91] The assay not only showed a very

good correlation with an antibody-based enzyme immunoassay currently used in analytical laboratories in hospitals but also, surprisingly, even yielded a cross-reactivity profile very similar to that of the natural monoclonal antibodies. From a selection of closely related substances, only 3-methylxanthine, which has one methyl group less than theophylline, was bound to the polymer to some extent, whereas caffeine, which has one additional methyl group, showed virtually no binding. The assay for the tranquilizer diazepam was also highly specific, with cross-reactivities comparable to those of antibodies. This molecularly imprinted sorbent assay (MIA) format has been adopted to develop assay systems for several other compounds such as drugs,[92,93] herbicides[94,95] and corticosteroids.[96] It has even been shown that MIP assays can be performed directly with diluted blood plasma.[97] A nice demonstration of the selectivity of MIPs was given with polymers imprinted with cortisol and corticosterone, which were very selective for their respective imprinting compounds and showed low binding of related steroids.[96] Table 1 shows the cross-reactivity patterns of these polymers and, for comparison, the cross-reactivities of mono- and polyclonal antibodies against the same target compounds. For example, the anti-cortisol polymer showed 36% cross-reactivity with the analogue prednisolone, which differs only in an additional double bond in the A-ring, and less than 1% cross-reactivity with cortisone, in which a hydroxy group in the original template is exchanged for a keto group. More recently, alternative assay formats that avoid the use of radiolabels have been reported. A competitive fluorescence immunoassay has been proposed that uses a fluorescent probe for detection.[98] The assay was specific for the herbicide 2,4–dichlorophenoxyacetic acid (2,4-D). The probe, a coumarin derivative, was unrelated to the analyte but had some structural similarity with it. The same principle could also be used with an electroactive probe.[99] Later, MIP-based ELISA-type assays were developed in which the analyte was labeled with an enzyme such as peroxidase. Thus, colorimetry[24,44] or chemiluminescence[44] could be used for detection. Since enzyme labels are rather bulky compared with a small analyte, special approaches have to be used to permit the analyte–enzyme conjugate to access the binding sites in the MIP. Piletsky *et al*.[24] developed a method in which the polymer is synthesized *in situ* in the wells of a microtiter plate. Aminophenylboronic acid was polymerized in the presence of epinephrine (the target analyte) using oxidation of the monomer by ammonium peroxodisulfate. This process resulted in the grafting of a thin polymer layer on the polystyrene surface. The polymer was then used in a competitive enzyme-linked assay with a conjugate of horseradish peroxidase and norepinephrine.

There is an ever-increasing demand for automated, high-throughput assaying and screening of natural products and 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 own group recently reported a high-performance MIP-based assay using a chemiluminescence imaging format.[44] Microtiter plates (96 or 384 wells) are coated with MIP microspheres using poly(vinyl alcohol) as a glue. The microspheres are obtained by precipitation polymerization and seem to have a higher number of accessible surface binding sites than a bulk polymer synthesized from the same monomers.[44] The analyte (in this example 2,4-D) is then added together with a small amount of enzyme (tobacco peroxidase)-labeled analyte and incubated until equilibrium is reached. After washing, the amount of polymer-bound 2,4-D–peroxidase conjugate is detected using luminol as the chemiluminescent substrate. Light

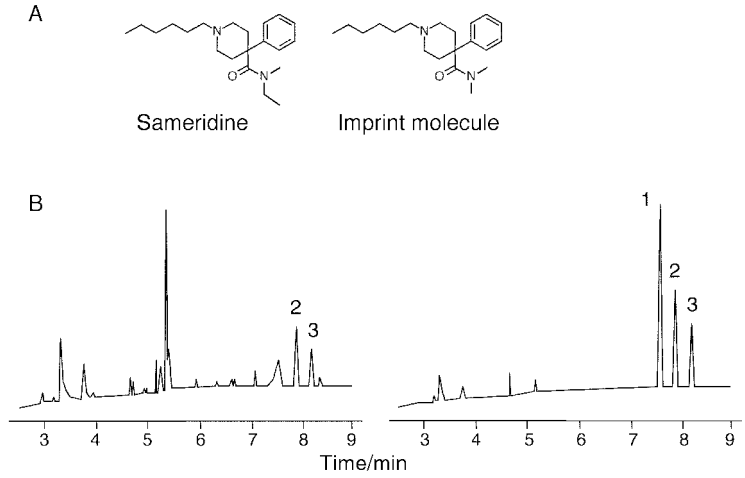

Fig.7 (A) Structures of the analyte sameridine and the imprint molecule. (B) GC trace of a solvent extract (left) and an MIP extract (right) of a plasma sample. Peaks: (1) imprint molecule, (2) sameridine and (3) internal standard. Adapted from Ref. 67 with permission.

Table 1 Cross-reactivities (%) for structurally related substances of imprinted polymers (MIP) and different monoclonal and polyclonal antibodies (ELISA, RIA) against cortisol and corticosterone[96]

	Cortisol assays[a]				Corticosterone assays[b]		
Ligand	MIP[c]	ELISA[d]	RIA[e]	RIA[f]	MIP[g]	RIA[h]	RIA[i]
Cortisol	100	100	100	100	10	2.7	0.03
Corticosterone	8.6	10	0.6	3.0	100	100	100
21-Deoxycortisol	4.0	< 0.1	0.3	—	0.54	—	—
11-Deoxycortisol	6.8	19	—	—	1.6	—	11
Prednisolone	36	13	46	—	5.7	—	—
Cortisone	0.89	—	—	16	0.38	—	< 0.01

[a] Cross-reactivities relative to binding of cortisol. [b] Cross-reactivities relative to binding of corticosterone. [c] MIP anti-cortisol. [d] Monoclonal mouse anti-cortisol antibodies.[131]. [e] Diagnostic Products (Los Angeles, CA, USA). [f] Polyclonal rabbit anti-cortisol serum.[132] [g] MIP anti-corticosterone. [h] Polyclonal rat anti-corticosterone serum.[133] [i] ICN Biomedicals (Costa Mesa, CA, USA).

emission is quantified with a CCD camera-based imaging system, yielding a calibration curve (Fig. 8). This allows for the simultaneous measurement of a large number of samples.

The competitive assays described above are heterogeneous formats, which means that the bound fraction of the labeled analyte has to be separated from the unbound fraction before quantification of either the bound or unbound fraction. Although a majority of the common immunoassays use this format, homogeneous assays are gaining interest, where the bound fraction of the labeled analyte can be quantified without separation from the free fraction. An approach has been proposed in which a fluorescent reporter group, which acts at the same time as a functional monomer, is incubated into the MIPs binding sites. Upon analyte binding, fluorescence is quenched and thus a calibration curve can be recorded (see under *Sensors* for details).[100] 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. An elegant way to introduce a universal reporter group into the polymer is by the use of proximity scintillation as the detection principle, a technique that has now been combined with MIPs.[101] A scintillation fluor is randomly covalently incorporated into a MIP by copolymerization. When the scintillation fluor is irradiated with β-rays, it emits fluorescent light which can be quantified with a photomultiplier tube (PMT). Small MIP microspheres were used that were stable in suspension for the time required for the measurement. Since the scintillation fluors are located in close proximity to the imprinted sites, binding of a radiolabeled analyte results in excitation of the fluor and emission of fluorescent light. In the presence of unlabeled analyte, some of the radiolabel is displaced from the MIP, resulting in reduced fluorescence, as the distance is now to long to excite the fluors (Fig. 9). This competitive homogeneous assay, although it requires the use of radiolabels, has some considerable advantages. Since the scintillation fluor does not need to interact with the template, the same fluor can be used for many different analytes. The assay is easier to automate as it does not require a separation step, and if a PMT array or a CCD camera is used, high throughput can be obtained. Another advantage is that binding can be followed in real time and binding kinetics can be investigated. A possible drawback, however, is that the requirements in terms of selectivity of the polymer are higher than with heterogeneous assays owing to the lack of a washing step.

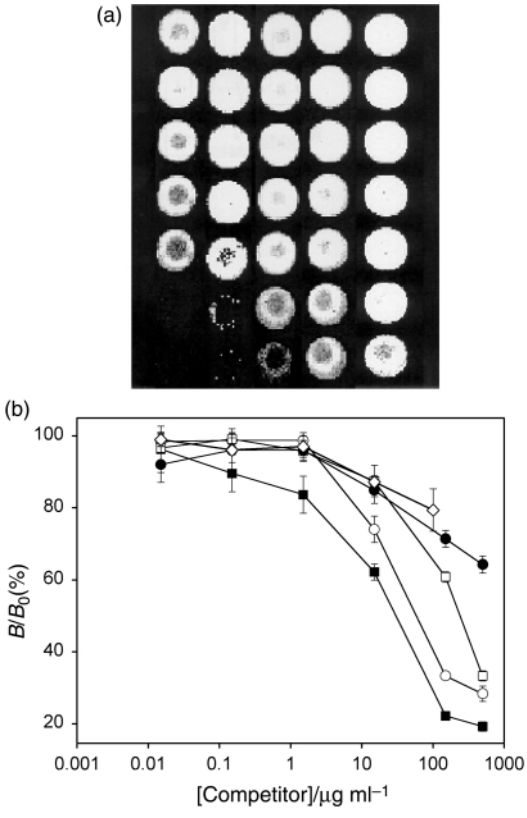

Fig. 8 CCD camera image of a chemiluminescence assay for 2,4-D in a microtiter plate (top). The analyte 2,4-D (left row) and different structurally related compounds were used as competitors in the assay. Calibration curves obtained by plotting the chemiluminescence intensities of the wells *versus* analyte concentration (bottom). 2,4-D (the imprint molecule) is the strongest competitor (full squares). Reproduced from Ref. 44 with permission.

2.3 Sensors

In chemical sensors and biosensors, a chemical or physical signal is generated upon the binding of the analyte to the recognition element, A transducer then translates this signal into a quantifiable output signal. The same general principle applies if an MIP is used as the recognition element instead of a biomolecule. Certain general properties of the analyte (such as its IR spectrum) or changes in one or more physico-chemical parameters of the system (such as mass accumulation or adsorption heat) upon analyte binding are used for detection. This principle is widely applicable and more or less independent of the nature of the analyte. Alternatively, reporter groups may be incorporated into the polymer to generate or enhance the sensor response. In other cases, the analyte may possess a specific property (such as fluorescence or electrochemical activity) that can 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,[102] the measurement of changes in the electrical streaming potential over an HPLC column packed with a MIP[103] or permeability studies of imprinted polymer membranes.[104] The first reported integrated sensor based on an MIP was a capacitance sensor. The device consisted of a field-effect capacitor containing a thin phenylalanine anilide-imprinted polymer membrane. Binding of this model analyte resulted in a change in capacitance of the device, thus allowing for the detection of the analyte in a qualitative manner.[105] More recently, capacitive detection was employed in conjunction with imprinted electropolymerized polyphenol layers on gold electrodes.[23] In another report, thin films of TiO_2 were imprinted with chloroaromatic acids such as 2,4-D and used as recognition layers in sensors based on ion-sensitive field-effect transistors.[39] Selective detection of the sodium salts of the imprint molecules was possible with

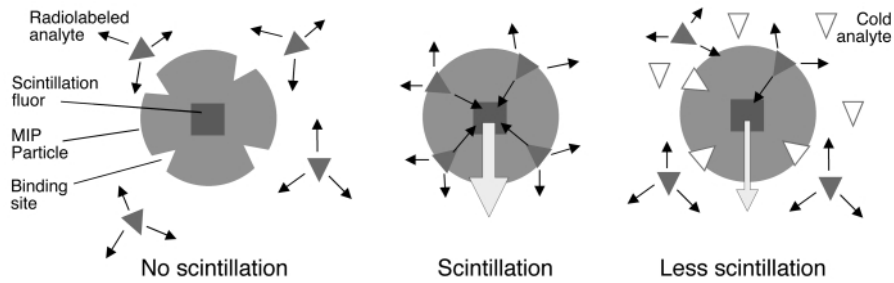

Fig. 9 Schematic representation of an competitive binding assay format based on proximity scintillation.[101]

detection limits in the micromolar range and an equilibration time of about 5 min.

During the last few years there has been a big boost in the use of mass-sensitive acoustic transducers such as the surface-acoustic wave (SAW) oscillator,[27,106] the Love-wave oscillator[107] and the quartz crystal microbalance (QCM)[25,27,106,108-112] for the design of MIP-based sensors. The QCM (Fig. 10) has been particularly popular probably because of its comparatively low price, robustness and ease of use. In one application, polymers of the polyurethane type were-synthesized at the surface of SAW and QCM oscillators in the presence of a certain organic solvent.[106] The polymer films subsequently showed a preferential uptake of the imprinting solvent over other solvents. This uptake could be quantified by piezoelectric microgravimetry, that is, *via* the change in oscillation frequency resulting from the mass change at the oscillator surface. A QCM has also been used by another group to construct an imprinted polymer-based sensor for glucose.[25] 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. However, only millimolar concentrations of the analyte could be measured. Others have relied on common acrylic polymers for the design of MIP-based QCM sensors.[109,110,112,113] With such polymers, it has been demonstrated that the sensor selectivities are similar to those obtained in other applications of acrylic MIPs. For example, a QCM sensor coated with an (*S*)-propranolol-imprinted polymer was able to discriminate between the *R*- and *S*-enantiomers of the drug with a selectivity coefficient $\alpha = 5$.[109]

Other sensors have been designed based on conductometric transducers.[114–116] 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 which is based on a freestanding atrazine-imprinted acrylic polymer membrane and conductometric measurements has recently been constructed.[117] The authors reported that the kind and molar ratio of cross-linking 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. Therefore, long and flexible cross-linkers (triethylene glycol dimethacrylate and oligourethane acrylate) had to be used. Attractive features of this sensor were the comparatively short time required for one measurement (6–10 min), its fairly low detection limit of 5 nM and its high selectivity for atrazine over structurally related triazine herbicides.

If the target analyte exhibits a special property such as fluorescence[27,118,119] or electrochemical activity,[120] this can be exploited for the design of MIP-based sensors. For example, a chromatographic flow system with fluorescence detection was used to construct a sensing device for the fluorescent analyte flavonol. The analyte was enriched in a flavonol-imprinted polymer contained in an optical detection cell, allowing for its detection at nanomolar concentrations.[119] If the analyte lacks such a specific property, a competitive or displacement sensor format may be used. A labeled analyte derivative or an unrelated probe is allowed to compete with the analyte for the binding sites in the MIP.[98,121–123] In one application, a voltammetric sensor for the herbicide 2,4-D was reported[99] in which the electroactive compound 2,5-dihydroxyphenylacetic acid was used as a probe instead of the labeled analyte. MIP particles were coated as a thin layer on a disposable 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 competed out of the imprinted sites, and the remaining probe was directly quantified by differential pulse voltammetric measurements.

An attractive design of the recognition element–transducer couple is to have the signal generated by the polymer itself, similarly to the above-mentioned homogeneous binding assays. This approach appears promising since it does not depend on a special property of the analyte and, moreover, should facilitate the integration and production of the sensing device. One example of such a format is an optical sensing system in which fluorescent reporter groups are incorporated into the MIP, the properties of which are altered upon analyte binding.[100,124] For example, a fluorescent functional monomer, *trans*-4-[*p*-(*N*,*N*-dimethylamino)styryl]-*N*-vinylbenzylpyridinium chloride, has been used together with a conventional functional monomer to prepare a polymer imprinted with cyclic adenosine monophosphate.[100] Upon binding to the imprinted sites, the analyte interacts with the fluorescent groups, and their fluorescence is quenched, thus allowing the analyte to be quantified. Others have used a similar system with a fluorescent metalloporphyrin as the reporter group, a polymerizable derivative of which was used as one of the functional monomers (Fig. 11).[125] Binding of the analyte 9-ethyladenine then resulted in quenching of the fluorescence of the polymer.

A very sensitive sensor for a hydrolysis product of the chemical warfare agent Soman has been described based on a polymer-coated fiber optic probe and a luminescent europium complex for detection.[126] The complex of europium ligated by divinylmethyl benzoate (ligating monomer) and by the analyte pinacoyl methylphosphonate was copolymerized with styrene, whereafter the analyte molecule was removed by washing. Rebinding of the analyte was quantified from laser-excited luminescence spectra. Although it is not clear whether imprinting has contributed to the selectivity of the sensor, this detection principle appears attractive as very low detection limits that can be obtained (7 ppt in this particular case).

The signals generated by most of the above-mentioned transducer types are two-dimensional and provide only limited

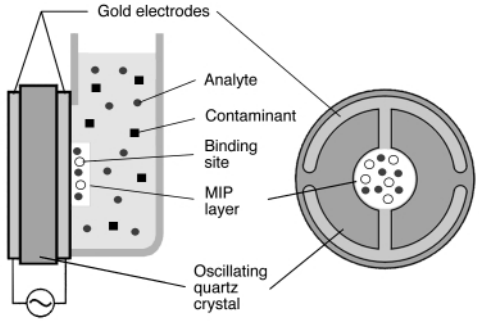

Fig. 10 Schematic representation of a MIP-coated quartz crystal microbalance sensor.

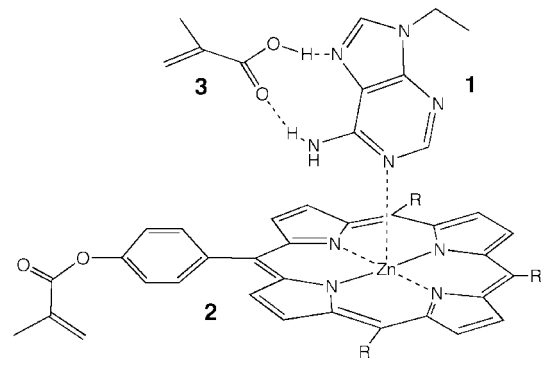

Fig. 11 Pre-polymerization complex of (1) 9-ethyladenine (imprint molecule), (2) a Zn-porphyrine signaling monomer and (3) methacrylic acid as a co-monomer.[125]

information about the composition of the sample. Although this is normally compensated for by the high selectivity of MIPs, a different strategy could conceivably be the use of 'intelligent' transducer mechanisms, which generate signals with a higher inherent information content. One way to achieve that 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, *e.g.*, when samples with complex matrices are to be investigated, or when structurally very similar analytes are present in the sample. A recent report described an approach towards a chemical sensor based on an imprinted polymer and infrared evanescent-wave spectroscopy.[127] A polymer molecularly imprinted with 2,4-D was coated in the form of a thin film on 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.

3 Outlook

As outlined above, a possible use of MIPs is as artificial receptors for the screening of combinatorial libraries. Even though so far there have been only a few preliminary reports that demonstrated the feasibility of the approach,[128,129] it is believed that MIPs might find applications in drug screening and development, in particular for the initial screening of large libraries. MIPs can be synthesized for molecules for which biological receptors cannot easily be obtained, and they seem to be perfectly adapted to automated high-throughput screening methods.

Even though MIPs have already found some niche applications that are close to commercialization, such as SPE, more work needs to be done to make them a real alternative or complement to biomolecules. In particular, what one hopes to achieve is the development of MIPs that contain a more homogeneous binding site population, have a higher affinity for the target analyte and can be routinely used in aqueous solvents. A considerable part of the current research efforts on MIPs already deals with these problems. On the other hand, the outstanding stability of MIPs and their low price are among the properties that make them especially suitable for applications in analytical chemistry. It appears that the development of imprinted polymer-based analytical methods is just about to leave the proof-of-principle stage, and researchers are starting to address specific analytical problems and to measure real samples. Fortunately, national and international funding agencies such as the European Commission have recognized the potential of MIPs for analytical chemistry, and several large research projects aimed at demonstrating the validity and practical usefulness of MIP-based analytical methods and devices are currently under way.

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