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Review

Molecular imprinting: developments and applications in the analytical chemistry field

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Abstract

In analytical separation science, molecularly imprinted polymers have been applied in several analytical techniques, such as liquid chromatography, capillary electrochromatography and capillary electrophoresis, solid phase extraction, immunoassay, and as a selective sorbent in chemical sensors. A benefit of imprinted polymers is the possibility to prepare sorbents with selectivity pre-determined for a particular substance, or group of structural analogues. The application most close to a wider acceptance is probably that of solid phase extraction for clean-up of environmental and biological samples. The improved selectivity of imprinted polymers compared with conventional sorbents may lead to cleaner chromatographic traces in the subsequent analytical separation. Furthermore, the solid phase extraction application does not suffer from drawbacks generally associated with imprinted polymers in chromatography, such as peak broadening and tailing. Most liquid chromatographic studies have focused on using imprinted polymers as chiral stationary phases for enantiomer separations. Also, the use of imprinted polymers as selective sorbents in capillary electrochromatography has been presented. For this purpose, a protocol to prepare superporous, monolithic imprinted polymer-based capillary columns has been developed. Due to the high affinities and selectivities often achievable, imprinted polymers have been considered as alternative binding entities in biosensors and in immunoassay type protocols. Here, high stability, easy preparation and ability to be used for assay of both aqueous and organic solvent based samples are advantages of the polymers.

Keywords: Reviews; Molecular imprinting

Contents

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1. Introduction

The incessant need for new fast and efficient methods within the pharmaceutical and environmental sectors fuel research into better and more selective and sensitive analytical procedures. The increasing number of analytes requires fast method development and the increasing number of analyses requires fast methods amenable to automation. Trace analytical methods for complex matrices rely on efficient sample enrichment and selective assays. In the research into new analytical techniques molecularly imprinted polymers (MIPs) have gained interest as a novel type of sorbent with attractive properties.

Imprinting of molecules occurs by the polymerisation of functional and cross-linking monomers in the presence of a templating ligand (Fig. 1) [1–7]. The template-monomer system is chosen such that in solution the imprint molecule complexes one or several functional monomers, which then become spatially fixed in a solid polymer by the polymerisation reaction. The resultant imprints possess a steric (size and shape) and chemical (spatial arrangement of complementary functionality) memory for the template. Following removal of the imprint molecules these imprints enable the polymer selectively to rebind the imprint molecule from a mixture. Two principally different approaches to molecular imprinting may be distinguished. The non-covalent, or self-assembly, approach where complex formation is the result of non-covalent or metal ion coordination interactions. The covalent, or pre-organised, approach which employs reversible covalent bonds, usually involving a prior chemical synthesis step to link the monomers to the template. Whereas it is generally perceived that non-covalent imprinting is more flexible in the range of chemical functionalities which can be targeted and thus the range of templates that can be used, covalent imprinting yields better defined and more homogenous binding sites. The former is also much easier practically, since complex formation occur on mixing template and monomers in solution prior to polymerisation. Recent years have seen an increasing number of studies into the use of selective MIPs in the analysis of drugs and pollutants in biological and environmental samples (for reviews see [8–10]). Imprinted polymers have been used in several analytical techniques, including liquid chromatography, capillary electrophoresis and capillary electrochromatography, solid phase extraction, ligand binding assay, and sensor technology. Since thorough discussions on each type of application can be found in the many excellent reviews cited throughout the text, in-depth presenta-

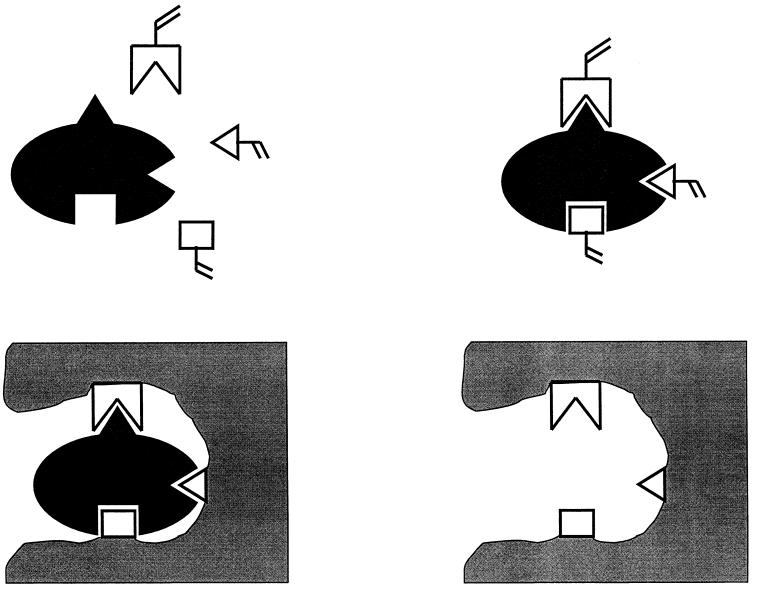

Fig. 1. Schematic depiction of the preparation of molecular imprints.

tions are beyond the scope of this review. Instead, the following discussion will concentrate on benefits and problems associated with the use of MIPs in analytical separations.

2. Applications in analytical techniques

2.1. Solid phase extraction

The application of molecular imprinting in the analytical separation field most close to practical realisation is probably that of solid phase extraction, SPE. Several groups have already applied MIP-based solid phase extraction to biological and environmental samples and this technique may well be accepted generally in the not-to-distant future. The technique has variously been referred to as MIP-SPE or MISPE and has been reviewed recently [10,11].

Following the first study on MIP-SPE employing a MIP selective for pentamidine in a model on-line sample enrichment of a spiked urine sample [12], a number of groups have presented SPE applications for various types of analytes (Table 1). The sample matrices have been various biological fluids in neat, diluted or protein-precipitated form [12–17], and organic solvent extracts of water samples [18,19], biological tissues and fluids [18,20–22], chewing gum [23], tobacco [24] and fermentation broth [25]. The experimental set-up has varied from different modes of on-line SPE [12,21,24,27], conventional SPE where the MIP is packed into columns or cartridges [13–17,20,23,26,28–30] to batch mode SPE where the MIP is incubated with the sample [22].

Benefits of the technique is the selectivity of the MIP can be pre-determined by the choice of template employed for its preparation, which combined with the high selectivity of the sorbent lead to efficient sample clean up. Chromatographic traces obtained with and without prior MIP treatment are shown in Fig. 2. Also, the ability to improve sensitivity by extracting larger sample volumes has been mentioned. This is particularly interesting for trace analysis of environmental samples. Being a novel technique most studies published until now have dealt with the preparation of the selective MIP, and optimisation of experimental conditions to obtain quantitative extraction of the sample and elution of the analyte into a small volume. The MIP often show strong affinity for the analyte and difficulties in effecting quantitative elution of the analyte have

Table 1
Summary of studies in which MIPs have been applied to solid phase extraction

Analyte	Sample	Refs.
Atrazine	Choroform extract of beef liver homogenate	[20]
Bentazone	Aqueous standard	[29]
Darifenacin	Plasma–acetonitrile (1:1, v/v)	[17]
Hydroxycoumarin	Urine	[15]
Indoleacetic acid	Chloroform standard	[30]
Monascus pigments	Ethyl acetate extract of fermentation broth	[25]
Nicotine	Ethyl acetate extract of chewing gum	[23]
Nicotine	Methanol extract of tobacco	[24]
Propranolol	Dog plasma, rat bile and human urine	[13,16]
Propranolol	Aqueous standards	[28]
Pentamidine	Diluted urine	[12]
Simazine	Aqueous standards	[26]
Sameridine	Heptane–ethanol (9:1, v/v) after extraction of human plasma	[22]
Tamoxifen	Human plasma and urine	[14]
Theophylline	Chloroform extract of human serum	[21]
Theophylline	Chloroform standards	[27]
Triazine herbicides	Organic solvent after C_{18}-extraction of tap water	[19]
Triazine herbicides	Acetonitrile after C_{18}-extraction of urine, apple extract and water containing humic acid	[18]

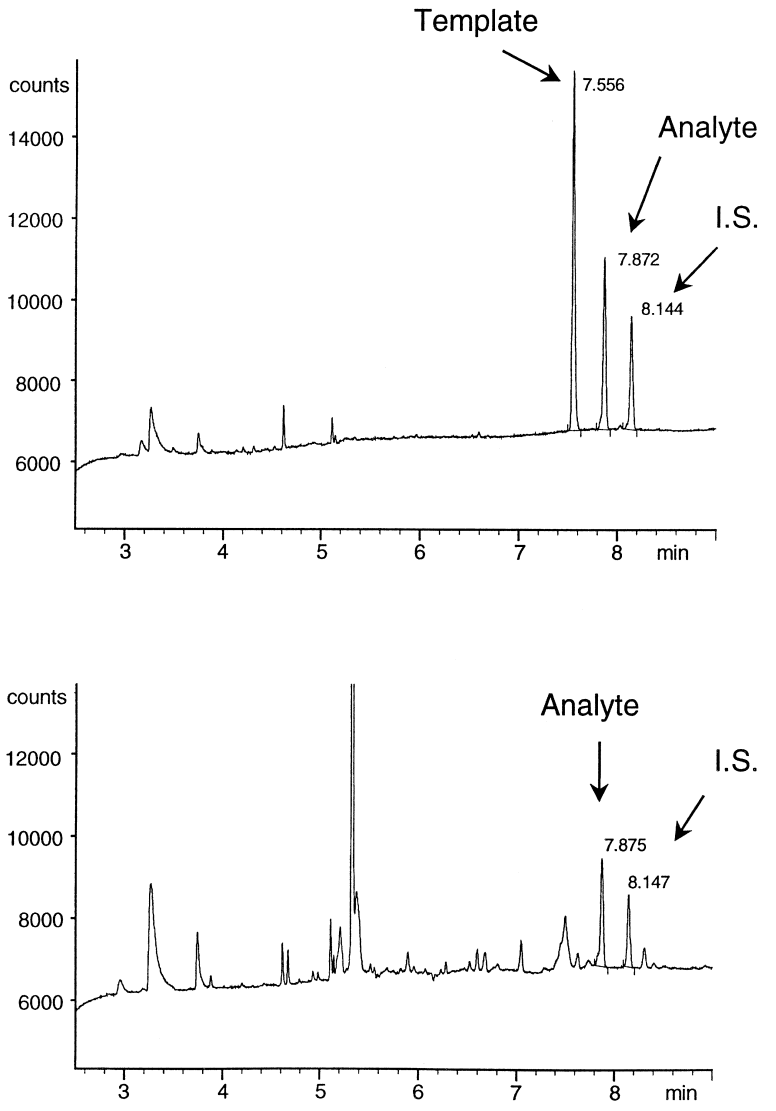

Fig. 2. Representative GC-traces of human plasma spiked with 66.8 nmol/l of sameridine and 50.2 nmol/l of internal standard. The sample enrichment method was either (top) liquid–liquid extraction followed by MIP-based solid-phase extraction or (bottom) liquid–liquid extraction only. Adapted and reprinted with permission from Ref. [22]. Copyright (1997) Vieweg Publishing.

been observed in a few cases [13,22,28]. While strategies to optimise conditions for selective binding using organic solvents are fairly well established the same is not yet true for aqueous samples, and especially not for biosamples. Due to the hydrophobic nature of the polymer, problems with non-specific adsorption are often encountered. This can be reduced by the use of small amounts of MIP and appropriate washing schemes prior to elution. Also, a switch-mode technique, involving lipophilic mode adsorption from an aqueous medium followed by washes with organic solvent to retain the analyte selectively through hydrogen bonding interactions, has been suggested [26]. In one instance, an evaluation of the analytical performance of a MIP-SPE based method was done and it was found to be

equivalent to that of the standard method based on the use of liquid–liquid extraction for sample cleanup [22].

There are some problems associated generally with the use of MIPs in analytical separations, such as peak broadening observed in liquid chromatography and leakage of imprint molecules. Since SPE employs an on–off type chromatography, which is less sensitive to poor chromatographic performance of the sorbent, MIP-SPE does not suffer to the same extent from the first of these drawbacks. Leakage of imprint molecules during elution has been evident on several occasions, which leads to an uncertainty in the concentration determination. Therefore, each method development must at one stage confirm the absence of interference from bleeding template species. One approach to circumvent this problem is the alternative imprint molecule approach [22]. A close structural analogue of the analyte(s) of interest is used as the imprint molecule. Then leaking template molecules present no problem as long as the subsequent analytical separation resolves the imprint species and the analyte(s). A second approach is heat-treatment of the polymer accompanied with excessive washes with strong eluents [23]. This treatment is claimed to greatly reduce or even eliminate bleeding of template from the MIP phase.

2.2. Liquid chromatography

The use of MIPs as stationary phases for LC is by far the most studied application of imprinted polymers and has been reviewed in some excellent recent reviews [31,32]. The objective of these activities is to profit on the easy access to stationary phases custom-made with selectivity for a predetermined ligand, or class of compounds. Especially, the separation of enantiomers has become a large subject area. As a result of the several types of chiral stationary phases (CSPs) presently available, the majority of enantiomer separation problems can ultimately be resolved. There are, however, still difficulties in the rapid identification of a suitable CSP and conditions for undocumented separations. By preparing a polymer imprinted with optically pure template, chiral discrimination can be introduced into a custom-made MIP designed for a specific separation. The predictable elution order of enantiomers is often emphasised since this is very rarely obtainable generally on conventional CSPs. In practice, however, most investigators have used LC as a convenient method for quantitative assessment of the quality of imprints produced by particular recipe or strategy. For this purpose, imprinting of enantiomers allows for an easy means of distinguishing between specific and non-specific binding. The fact that focus not has been primarily on the chromatographic and analytical performances of MIPs may in part explain the still poor improvement rate of these parameters. Another disadvantage relates to the requirement of a substantial amount of pure template, which itself may be expensive or difficult to obtain, prior to the polymerisation process. Whilst, for analytical separations this has to be taken under consideration, for preparative scale separations the cost of template may be sizeable.

A selection of studies in which MIPs have been applied to chromatographic separations of natural products, pharmaceuticals, and compounds of clinical and environmental interest is compiled in Table 2. Enantiomer separations accomplished include the resolution of several drug compounds [33–36], amino acid derivatives [31,37], and sugars [1], to mention just a few. The, often severe, peak broadening and tailing of peaks observed, however, presently hamper a wider use of imprinted phases. Factors claimed to contribute to peak broadening are mass transfer limitations, imprint heterogeneity with a distribution of sites from high to low affinity for the ligand, and variable association and dissociation kinetics [38,39]. Site heterogeneity leads to sample overload, which in turn leads to low capacity of the imprinted polymer. The best recognition sites become saturated and the separation predominantly occurs on poorer sites which subsequently results in decreased retention and unfavourable chromatographic performance. A second reason for peak broadening stems from the fact that MIPs are often prepared as bulk polymers, and then ground and sized into irregular particles of <25 μm before packing into LC-columns. A novel suspension polymerisation technique specifically developed for MIP preparation has been described [40,41]. The procedure involves polymerisation of small droplets of monomer solution in a liquid perfluorocarbon continuos phase, which do not interfere with the non-

Table 2
Examples of studies in which MIPs have been applied to chromatographic separations of natural products, pharmaceuticals, and compounds of clinical and environmental interest

Analyte	Polymer system[a] Monomer/Cross-linker/Polymer format	Refs.
Liquid Chromatography		
Amino acid derivatives	MAA/TRIM/Bulk	[37]
Amino acids	Cu(II)-N-(4-vinylbenzyl)iminodiacetic acid/EGDMA/ Polymer grafted on silica	[70]
Atrazine	MAA/EGDMA/Bulk	[71]
Cinchona alkaloids	MAA/EGDMA/Bulk	[35]
Ephedrine	MAA/TRIM/Bulk	[72]
Fructose, galactose	Vinylphenylboronic acid/EGDMA/Bulk	[73]
Nucleotide bases	MAA/EGDMA/Bulk	[74]
Naproxen	4VPy/EGDMA/Beads made by multi-step swelling and polymerisation, modified with hydrophilic external layer	[36]
Naproxen	4VPy/EGDMA/Bulk	[33]
Nicotine	TFMAA/EGDMA/Polymer rod in LC-column	[75]
Sialic acid	Vinylbenzeneboronic acid/EGDMA/Bulk	[76]
Steroids	MAA/EGDMA/Bulk	[77,78]
Synthetic estrogenics	Diethylaminoethyl methacrylate/TRIM/Bulk	[79]
Testosterone	MAA/EGDMA/Bulk	[80]
Timolol	Itaconic acid/EGDMA/Bulk	[34]
Thin-layer chromatography		
Ephedrine and analogues	MAA/EGDMA/Bulk	[81,82]
Capillary electrophoresis		
Propranolol	N-Acryloyl-alanine/EGDMA/Bulk	[56]
Capillary electrochromatography		
Amino acids	MAA/EGDMA/Bulk	[51]
Dansyl-phenylalanine	MAA+2VPy/EGDMA or TRIM/Thin film in capillary	[49]
Propranolol	MAA/TRIM/Superporous monolith in capillary	[53]
Local anaesthetics	MAA/TRIM/Superporous monolith in capillary	[54]
Pentamidine	MAA/EGDMA/Dispersion polymer made in situ	[52]

[a] Abbreviations used: MAA, methacrylic acid; TRIM, trimethylolpropane trimethacrylate; EGDMA, ethylene glycol dimethacrylate; 4VPy, 4-vinylpyridine; TFMAA, trifluoromethylacrylic acid; 2VPy, 2-vinylpyridine.

covalent monomer–template interactions employed. Uniformly shaped beads of defined size range can be made. A two-step swelling technique has been adapted for MIP synthesis and several stationary phases have been made using this technique [42,43]. Coating of MIP onto pre-made silica [44] and TRIM particles [45] have been presented. Also, several continuous rod type separation systems based on imprinted polymer inside LC stainless steel columns have been reported [46–48]. At best only partially improved chromatographic performance of imprinted columns has been achieved, and from a separation point of view these techniques still have to show any advantage over packed particle columns. However, a recent demonstration of the use of imprinted polymers in the open tubular liquid chromatography mode show promise of greatly improving column efficiency of MIP-based LC separations [49].

2.3. Capillary electrochromatography

Capillary electrochromatography (CEC) is a hybrid separation technique combining the stationary phase of liquid chromatography with the electro-osmotically driven mobile-phase transport of electrophoresis. Being an inherently more efficient chromatography technique, CEC-mode separations would yield improved performance of imprinted polymers compared with that achieved in LC. Another advantage over LC is the micro-column format leads to a minimal consumption of chemicals, including the imprint species. Hence, a future commercial scale production of MIP-based micro-columns for CEC may be more easily realised. Several studies into MIP-CEC have been published and the area has been reviewed recently [32,50]. The research has primarily focused on the adaptation of imprinted polymers to electrochromatography, including different means to enclose MIP stationary phases within fused silica capillaries. Conventional imprinted polymer particles have been immobilised inside capillaries using a polyacrylamide gel [51]. The imprinted polymer has been prepared in situ by a dispersion polymerisation procedure [52]. The, as yet, most successful approach has utilised capillary columns filled with a monolithic, superporous imprinted polymer obtained by an in situ photo-initiated polymerisation process [53,54]. Superporosity was introduced either by use of a suitable porogen or by careful timing of the polymerisation reaction. Prior to MIP synthesis the silica surface was derivatised with a methacrylate monomer–silane bifunctional reagent to permit covalent attachment of the polymer to the inner wall of the capillary. The same anchoring chemistry was used to prepare thin film coatings of MIP [55] and such capillaries could be used for enantiomer separations in the open-tubular CEC mode [49]. Also, MIP particles have been used as a mobile phase additive for enantiomer separations in the capillary electrophoresis mode [56].

Analogous to the situation in LC, separation efficiencies characteristic of CEC are still to be achieved. Further improvements in peak shape are required before MIP-based stationary phases will become a true complement to the selectors presently employed in CEC. Thus far, the focus has, rightly, been on methodology for MIP-capillary preparation and investigations into optimisation of separation parameters have been addressed less. The chemical resistance of the polymer, however, permits use of organic solvents, of strong acidic and basic buffers, and of high voltages, which may lead to short separation times (Fig. 3).

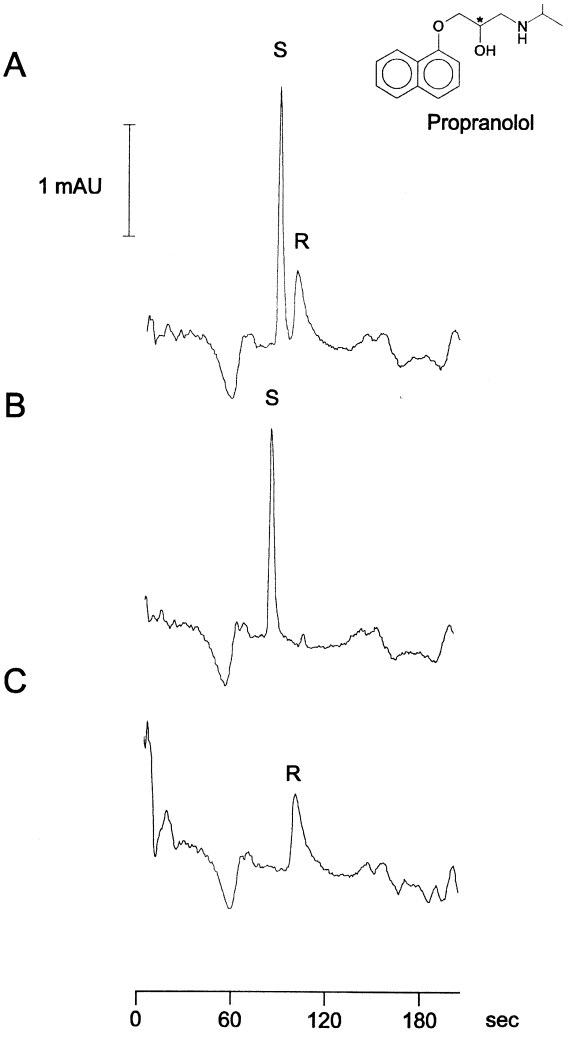

Fig. 3. Electrochromatogramme showing resolution of the enantiomers of propranolol on a capillary column containing a MIP imprinted against the *R*-enantiomer of propranolol. Samples, 100 μM *rac*-propranolol (A), 50 μM (*S*)-propranolol (B), and 50 μM (*R*)-propranolol (C), were injected electrokinetically and separated at a constant voltage of 30 kV at 60°C using an electrolyte composed of acetonitrile–4 M acetate pH 3.0 (4:1, v/v). Reprinted with permission from Ref. [53]. Copyright (1997) American Chemical Society.

2.4. Binding assays

MIPs have been employed as non-biological alternatives to antibodies in a competitive radiolabelled molecularly imprinted sorbent assay, MIA, (Table 3). The assay is analogous to that of a competitive immunoassay, or limited reagent assay (Fig. 4). Sample, containing analyte, and a fixed concentration of marker, a labelled derivative of the analyte, are incubated with a limited number of antibody binding sites or imprints. Analyte and marker compete for binding to the same sites and, hence, the amount of labelled marker bound to the antibodies or imprints is quantitatively related to the amount of analyte added to the incubation mixture. Interest in this technique is due to MIPs combine highly selective molecular recognition, comparable to biological systems, with typical properties of polymers such as high thermal, chemical and stress tolerance, and extremely long shelf-life without any need for special storage conditions. In MIA the most commonly used label is a radioactive isotope [57], but also detection systems based on fluorescence have been suggested [58]. For MIA to become accepted generally one critical point is the introduction of efficient and easy-to-use non-radioactive techniques. An innovative technical improvement is the use of magnetic MIP beads to facilitate separation of free and bound radiolabelled marker [59]. Also, sub-micron beads, more resistant to precipitation and aggregation and, hence, requiring less agitation during incubation, may simplify the assay procedure [60].

Some studies into the use of MIA for analysis of drug compounds in blood derived biofluids have already been presented [57,61]. Following liquid–liquid extraction of the biological sample the actual assay was performed using organic solvents as the incubation medium. Investigations into extending the scope of MIPs to aqueous conditions generally have led to some analyte-MIP systems can be employed equally well for organic solvent or aqueous buffer based assays [62,63]. These studies led subsequently to the demonstration of a MIA method by which plasma samples could be assayed directly [64]. From

Table 3
Studies in which MIPs have been applied to binding assay either for bioanalysis or fundamental studies on molecular recognition or optimisation of polymer preparation

Analyte	Sample	Refs.
Radioimmunoassay		
Atrazine	Acetonitrile standards	[83]
Atrazine	Standards in toluene–acetonitrile (19:1, v/v)	[84]
Corticosterone	Tetrahydrofuran standards	[85]
Cortisol	Tetrahydrofuran standards	[85]
Cyclosporin	Diisopropyl extract of human whole blood	[61]
Diazepam	Toluene–heptane (3:1, v/v) after extraction of human serum	[57]
2,4-Dichlorophenoxyacetic acid	Aqueous standards	[86]
17β-Estradiol	Standards in acetonitrile	[60]
Leu-enkephalin	Aqueous standards	[62]
Morphine	Aqueous standards	[62]
S-Propranolol	Human plasma and urine	[64]
S-Propranolol	Aqueous standards	[59,63]
Theophylline	Acetonitrile–acetic acid (99:1, v/v) after extraction of human serum	[57]
Theophylline	Standards in toluene–tetrahydrofuran (9:1, v/v)	[87]
Theophylline	Standards in acetonitrile	[60]
Fluoroimmunoassay		
2,4-Dichlorophenoxyacetic acid	Aqueous standards	[58]

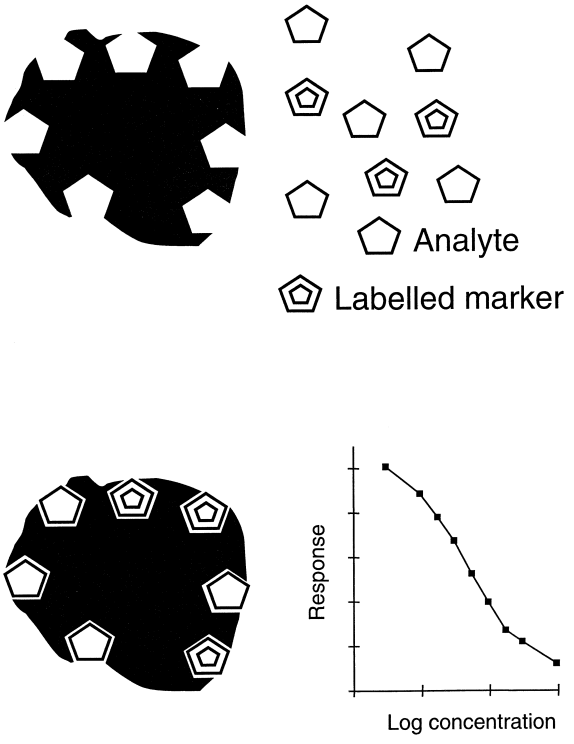

Fig. 4. General principle of a competitive radiolabelled binding assay. Analyte and labelled marker compete for binding to a limited number of binding sites, resulting in a sigmoidal dose-response curve where the signal is inversely related to the concentration of analyte in the sample. The response is the amount of labelled marker bound to the binding sites.

a practical standpoint, and for a general acceptance of the technique, the ability of MIA to assay biosamples without any need for transfer of the analyte into an organic solvent is essential. In some circumstances, however, the ability to be used in both organic solvents and aqueous buffers adds flexibility to assay development, for instance in environmental analysis where poorly water-soluble analytes can be assayed. The relative lack of sensitivity of MIA methods needs to be improved. Leakage of remaining imprint molecules seems to be one factor which limits sensitivity, and the use of appropriate washing protocols in the MIP preparation is imperative.

As is obvious from Table 3 the MIA approach has been of greater significance in fundamental research into imprint-ligand binding reactions. The technique is more powerful than LC, which is the routine in most studies, in gaining detailed information about the molecular recognition system in a short period of time.

3. Preparation of imprints

A discussion on imprint preparation is beyond the scope of this overview, instead the reader is referred to the many excellent reviews published in recent years [1–7]. Some issues of particular interest for MIP applications in the analytical chemistry field have been discussed in a recent review [10]. The future is bound to see more of specialised development strategies for the optimisation of MIPs for each particular application area described above. Present issues that need to be focused upon include stabilisation of monomer–template complexes in the pre-polymerisation mixture, complete removal of template molecules, and appropriate design of polymer format for intended use. Better strategies for optimisation of methods of MIP preparation, which is done using organic solvents, for use under aqueous conditions is required. Furthermore, true for the whole molecular imprinting area, a better knowledge about experimental conditions for efficient use of MIPs in context with biofluids has to be gained.

Depending on the experimental design during polymerisation and subsequent rebinding, the monomers and polymer, respectively, interact with the template molecules by either non-covalent interactions [2–5,7] or reversible covalent interactions [1,2] (Fig. 1). While the non-covalent strategy is the one being most widely employed, both approaches have their strengths and weaknesses. A hybrid technique, which combines imprinting of well-characterised monomer–template assemblies with non-covalent rebinding conditions, may prove valuable. An ingenious approach is the introduction of a sacrificial spacer between monomer and template, the cleavage of which would leave monomer residue functionality in precise spatial arrangement to interact non-covalently with the template [65–67]. The expected advantages are a more homogenous population of imprints and less non-specific interactions between ligand and polymer matrix. However, the obvious drawback is the more demanding chemistry involved

with respect to design and synthesis of template–monomer complexes. A strategy, introduced recently, to optimise binding for non-covalent imprinting systems, is the synthesis and screening of libraries of MIPs to find the recipe of monomers and cross-linker giving the strongest binding [68,69].

4. Conclusions and future outlook

The, at present, almost exponential growth in literature published each year is an indicator of the growing interest in molecular imprinting technology. In addition, molecular imprinting is now maturing from a phenomenon of interest to academics to a technique of potential practical interest to the analytical chemist. As an example, among the first to study the solid phase extraction application were several research groups within the pharmaceutical industry. This process will continue, however, to involve fundamental research and some areas with high priority may be listed: to obtain a better knowledge base for optimisation both of MIP synthesis and use, mechanistic studies of imprint formation and imprint recognition will remain an important area. Extension of the types of chemistry available, especially for non-covalent imprinting, must be addressed. It is necessary to establish strategies for selection of the best recipe of monomers, cross-linkers and polymerisation conditions for a particular combination of analyte and application. This includes consideration which is the best format of the MIP, may it be as a thin film, monodisperse beads or macroporous monolith, with the optimal pore morphology. The end user of MIPs is interested in factors such as the elimination of template leakage and batch-to-batch reproducibility. Finally, development of MIP systems useful for aqueous samples, such as serum, plasma and ground water, continue to be a key focus.

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