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Review

# Current status of molecularly imprinted polymers as alternatives to antibodies in sorbent assays

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**Abstract**

The literature on the use of molecularly imprinted polymers (MIPs) in antibody-like sorbent assay using radio, fluoro and enzyme-linked approaches is comprehensively reviewed, and their current status discussed. Although immunoassays are still commonly carried out using antibodies, recent developments have demonstrated that molecularly imprinted polymers can be viable alternatives. It is predicted that both traditional antibody-based and MIP sorbent assays will continue to develop in parallel, with each having superiority in certain areas.
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*Keywords:* MIP; Imprinted polymers; Immunoassay; Radioassay; Fluorescence; ELISA

## 1. Introduction

During the last decade, molecularly imprinted materials have been used in a range of techniques (liquid chromatography, solid phase extraction, capillary electrochromatography, biosensors, catalysts, bio imprinting, binding assays) and a number of approaches (covalent, non-covalent, sacrificial spacer, metal-coordination, stoichiometric non-covalent interactions) have been applied. Here we review the current state of the use of molecularly imprinted polymers (MIPs) as replacements for antibodies in sorbent assays. Immunoassays, which rely on specific interactions between antibodies and antigens, are in routine laboratory use for quantification of many analytes in biological fluids [1]. With the advent of production of monoclonal antibodies through hybridoma cell technology [2] and significant technical progress in detection systems, assays with high sensitivity and specificity have been developed [3]. However, use of animals in the production of antibodies is of concern in some areas so that development of alternative methods is preferable.

Antibody-based assays still present several fundamental limitations due to the nature of the recognition element involved. Antibodies are relatively chemically and physically unstable, which shortens their storage lifetime, limits their use to aqueous media, and makes them very sensitive to pH and temperature [4]. Furthermore, in the case of small molecules, the antigen itself is not immunogenic and addition of a carrier hapten moiety, such as bovine serum albumin (BSA), is needed to elicit an immune response [5]. It is not possible to produce immunogenic derivatives of all potential analytes. Depending on the choice of the hapten derivative, the immunisation procedure will lead to antibodies with different properties and affinities [6]. Although considerable efforts have been made to find ways of preparing antibodies with improved stability (antibody fragments, recombinant antibodies, etc.) [7,8], the current techniques may still not be time and cost effective.

MIPs result from the polymerisation of monomeric units in the presence of a template molecule (Fig. 1). Subsequent removal of the template results in cavities whose shape, size, functionality and spatial arrangement are complementary to the imprinted molecule [9–11]. These recognition sites with predetermined selectivity, enable MIPs to bind target molecules with similar affinities and specificities to their natural counterparts [12]. Furthermore, MIPs are stable and extremely robust. Their ability to recognise their template and related molecules can remain unaffected after treatment with strong base, strong acid, all common organic solvents and extremes of temperature [13,14]. This unique physical and chemical stability allows their use under con-

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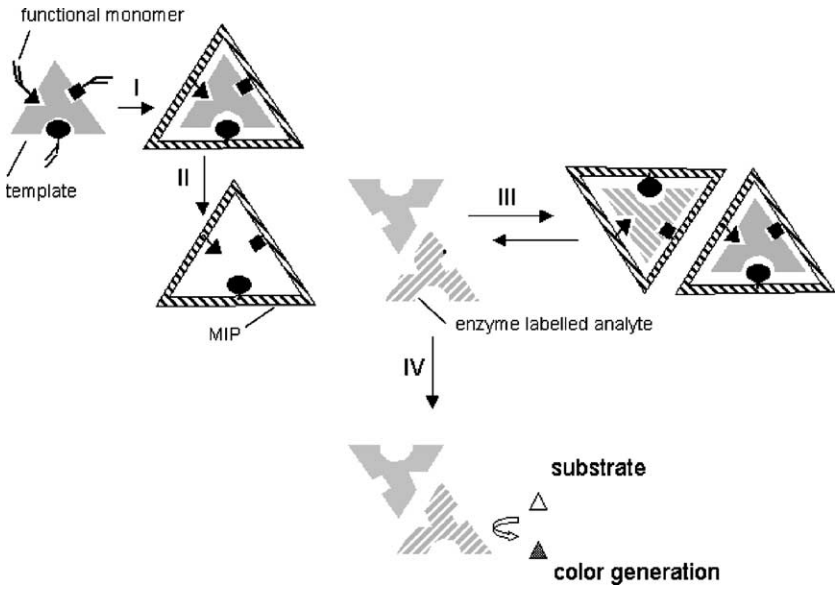



Fig. 1. Schematic representation of MIP synthesis and a competitive enzyme-linked molecularly imprinted sorbent assays: (I) functional monomers and template interact via non-covalent interaction to form a complex, polymerisation is carried out after addition of the crosslinker and the initiator; (II) the template is removed leaving imprinted cavities in the polymer matrix; (III) after further processing the imprinted polymer can be used in an assay where the analyte (template) and an enzyme-labelled analyte compete for the available binding sites; (IV) upon separation of the free and bound fraction the enzyme activity is evaluated in the supernatant.

ditions where antibodies are unemployable (Table 1) and, as they are synthetic materials, they do not require the use of animals at any stage of the production, which make them more socially acceptable.

## 2. Radio-molecularly imprinted sorbent assays

In 1993, Mosbach's group reported the first molecularly imprinted sorbent assay (MIA) [12] in which polymers imprinted with either theophylline or diazepam replaced antibodies in competitive immunoassay-like experiments. Although the analyte needed to be extracted from human serum and transferred into a non-polar organic medium prior to analysis, these radioligand-binding assays were equivalent, in terms of cross-reactivity, to assays performed with monoclonal antibodies. Subsequent work using anti-morphine and anti-enkephalin polymers confirmed the potential of MIPs as antibody mimics in immunoassays [15] and, in the case of the morphine imprinted polymer, demonstrated that efficient binding could be achieved in an aqueous environment. Although affinity and selectivity were reduced compared with values obtained in organic solvents, they were still comparable with those obtained for other antibody systems (Table 2). These extremely encouraging results were followed by a plethora of reports of other MIP sorbent assays (Table 3). An (*S*)-propranolol MIP sorbent assay, demonstrated greater stereoselectivity than an antibody assay [16] (cross-reactivity towards (*R*)-propranolol 1% for MIP, compared with 5–7% for natural counterpart). Further to this

Table 1
Comparison between MIPs and antibodies

| MIPs | Antibodies |
|---|---|
| Can be used in both aqueous and organic media | Restricted to aqueous conditions |
| Synthetic: no use of animals | Biological production involving animals |
| High chemical, physical and thermal stability | Very fragile |
| Simple storage requirements | Need to be lyophilised and may denature upon long period storage |
| Preparation: fast | Preparation: time-consuming but monoclonal strategy allows large long-term production once antibody is optimised |
| Require a relatively large amount of template | Require a relatively small amount of antigen |
| Non-covalent approach: no need for derivatisation. Covalent approach: functional monomer–template complex needs to be synthesised | Necessary to derivatise small non-immunogenic molecules in order to produce immune response |
| Controllable batch to batch reproducibility | Polyclonal antibodies are specific to each animal. Monoclonal antibodies allow batch reproducibility |
| Reusable | Non-reusable |

Table 2
Cross-reactivity of opiates to the anti-morphine MIP and anti-morphine antibodies (adapted from reference [15])

| Ligand | Relative cross-reactivity (%) | | | | | |
|---|---|---|---|---|---|---|
| | MIP | | Antibody fragment | | | |
| | In buffer | In toluene | a | b | c | d |
| Morphine | 100 | 100 | 100 | 100 | 100 | 100 |
| Codeine | 25 | 4.7 | 18 | 104 | 36 | <0.1 |
| Hydromorphone | 15 | 6.0 | | 112 | 9.8 | |
| Normorphine | 9.9 | 8.3 | | | | |
| Heroin | 8.3 | 2.3 | | | | |
| Naloxone | 0.4 | <0.1 | <0.5 | 0.1 | 0.7 | <0.1 |
| Naltrexone | 0.3 | <0.1 | | ≪0.1 | 0.2 | ≪0.1 |
| Leu-enkephalin | ≪0.1 | | | | | |
| Met-enkephalin | ≪0.1 | | | | | ≪0.1 |

study, the first assay of biological samples without prior clean up was carried out [17]. The assay was shown to be effective, reproducible and accurate with 60% plasma in the incubation mixture. However, although the analysis of the urine samples could be performed at pH 6 which was shown to be optimal for high specific binding [16], analysis of plasma samples under physiological condition (pH 7.4), resulted in a significant increase in non-specific binding and additional optimisation was needed. More recently, theophylline imprinted microspheres have been used to develop a competitive radioassay [18]. This approach resulted in higher binding site densities and more rapid kinetics.

Small molecules, such as atrazine, need to be linked to a protein in order to elicit an immune response [5] but such derivatisation is not needed when using a molecular imprinting technique. Anti-atrazine polymers have been synthesised [19] and, although the sensitivity of the MIP sorbent assay was less than a reference enzyme-linked immunosorbent assay (ELISA), it was able to differentiate the related herbicide propazine, whereas the ELISA showed a cross-reactivity of more than 200%. The solvent compatibility of molecularly imprinted assays is of particular significance. Whereas antibody-based systems are limited to aqueous environments, molecularly imprinted assays can be performed in apolar solvents which is particularly useful when the target analyte is hydrophobic. This advantage has been exploited in an assay for cyclosporin, which was developed in diisopropyl ether [20]. As cyclosporin is an immunosuppressive drug, it is difficult to raise antibodies against it. The analyte was recovered from haemolysed whole blood samples by liquid–liquid extraction. The concentration of cyclosporin was estimated using both MIA and

Table 3
Examples of radio-molecularly imprinted sorbent assays

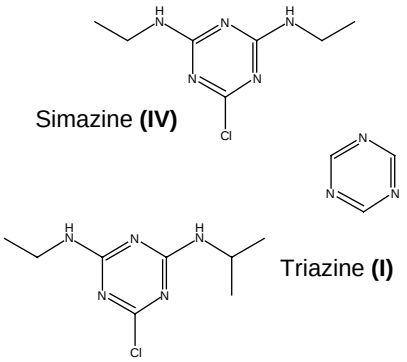
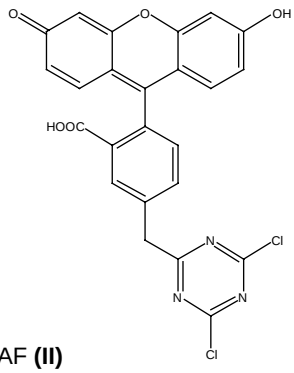
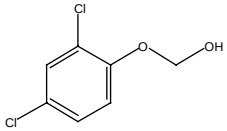
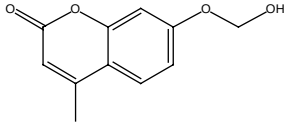
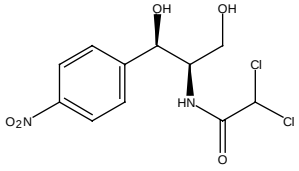
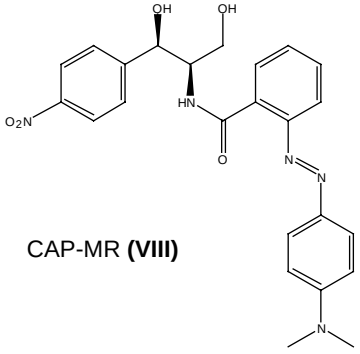
| Template | Polymer | Affinity, $K_D$ (μM) | Binding capacity (μmol/g) | Ref. |
|---|---|---|---|---|
| (*S*)-theophylline | MAA/EDMA/$CHCl_3$ | H: 0.35 ± 0.048, L: 65 ± 16 | H: 0.016, L: 1.3 | [9] |
| | TFMA/DVB | 0.01 | 0.023 | [18] |
| | MAA/TRIM/ACN | H: 0.32, L: 49.5 | H: 56.8 ± 11.8, L: 2120 | [23] |
| Diazepam | MAA/EDMA/$CHCl_3$ | H: 0.018, L: 60 ± 74 | H: 0.0062, L: 1.2 ± 0.93 | [9] |
| Morphine | MAA/EDMA/ACN | H: 0.092[a]/1.2[b], L: 8.9[a]/24[b] | H: 1.2[a]/0.78[b], L: 39[a]/6.9[b] | [15] |
| Leu–enkephaline | MAA/EDMA/ACN | H: 0.13[a]/0.1[b], L: 43[a]/440[b] | H: 0.017[a]/0.0038[b], L: 1.01[a]/36[b] | [15] |
| (*S*)-propranolol | MAA/EDMA/toluene | H: 0.04[a]/0.04[b], L: 23[a]/4.1[b] | H: 2.0[a]/0.63[b], L: 38[a]/28[b] | [16,17] |
| Proteins | Trehalose/$C_3F_6$ | nd | nd | [21,22] |
| Atrazine | MAA/EDMA/$CH_2Cl_2$ | 800[c] | 7.7[c] | [19,24] |
| 17-Oestradiol | MAA/TRIM/ACN | H: 2.1, L: 0.96 | H: 2.98 ± 0.75, L: 0.007 ± 0.76 | [18] |
| 2,4-D | 4-VP/EDMA/$CH_3OH/H_2O$ | nd | nd | [25] |
| Yohimbine | MAA/EDMA/$CHCl_3$/DMF | H: 0.06[a]/0.12[b], L: 4.8[a]/62[b] | H: 0.12[a]/0.06[b], L: 1.1[a]/11[b] | [26] |
| | MAA/EDMA/$CH_3OH$ | H: 47[a]/2.3[b], L: 5400[a]/150[b] | H: 0.96[a]/0.11[b], L: 59[a]/7.4[b] | [26] |
| Corynanthine | MAA/EDMA/$CHCl_3$/DMF | H: 37[a]/5.6[b], L: 1300[a]/170[b] | H: 70[a]/2.5[b], L: 840[a]/28[b] | [26] |
| | MAA/EDMA/$CH_3OH$ | H: 330[a]/25[b], L: 2500[a]/640[b] | H: 11[a]/3.9[b], L: 64[a]/57[b] | [26] |
| Corticosterone | MAA/EDMA/THF | H: 1.23 ± 0.43, L: 840 | H: 0.37 ± 0.12, L: 130 ± 60 | [27] |
| Cortisol | MAA/EDMA/THF | H: 0.57 ± 0.16, L: 1590 | H: 0.21 ± 0.05, L: 280 ± 120 | [27] |

nd: not determined; MAA: methacrylic acid; TFMA: trifluoromethacrylic acid; EDMA: ethylene glycol dimethacrylate; TRIM: trimethylolpropane trimethacrylate; 4-VP: 4-vinyl pyridine; DVB: divinylbenzene; ACN: acetonitrile; H: high affinity sites; L: low affinity sites.

[a] Aqueous environment.

[b] Organic solvent.

[c] Determined by frontal chromatography.

| Template | Fluorescent competitor | Ref. |
|---|---|---|
|  Simazine **(IV)** Triazine **(I)** Atrazine **(III)** |  DTAF **(II)** | [29] |
|  2,4-D **(V)** |  CMMC **(VI)** | [31], [32] |
|  CAP **(VII)** |  CAP-MR **(VIII)** | [34], [35] |

DTAF: 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein; 2,4-D: 2,4-dichlorophenoxyacetic acid; CMMC: 7-carboxymethoxy-4-methylcoumarin; CAP: chloramphenicol; CAP-MR: chloramphenicol methyl red.

Fig. 2. Structures of some templates and their fluorescent counterparts used in competitive fluorescence molecularly imprinted sorbent assays.

an enzyme-multiplied immunoassay (EMIT). Optimisation of the MI assay showed that unknown matrix component effected its sensitivity and it was not possible to determined concentration $<100$ ng/ml with accuracy. However, unlike the EMIT, the MIA was able to measure the concentration of cyclosporin and its first generation metabolites (AM1, AM9 and AM4N) with approximately equal response. It was observed that this might have clinical value.

It is often considered that molecular imprinting is more suitable for use with small stable molecules rather than with more fragile and complex macromolecules. However, surface imprinting of proteins, such as albumin, lactalbumin, immunoglobulin, lysozyme and ribonuclease, has been achieved using carbohydrates and a plasma deposition technique [21]. Proteins were adsorbed onto a mica surface and coated with the disaccharide trehalose. A film of hexafluoropolymer created by plasma deposition was further attached to a solid support and, after removal of the mica and extraction of the template, an imprinted surface complementary to the protein was obtained. Competition experiments using $^{125}$I radiolabelled proteins demonstrated that these surfaces had a higher affinity for their template than other related molecules [22]. They also confirmed that specific recognition relied on a dynamic adsorption-exchange process, which, for larger less rigid molecules, could be an issue.

## 3. Fluoro-molecularly imprinted sorbent assays

Radiolabelled compounds are structurally virtually identical to their unlabelled counterparts, are often commercially available, and can give rise to sensitive assays. However, there are health and safety implications associated with the use of radioactive materials and attention has been directed to detection systems involving less hazardous materials. Fluorescence detection is a possible alternative [28] and pseudo-fluoroimmunoassays based on molecular imprinting have been developed.

### 3.1. Competitive and displacement assays

Piletsky et al. was the first to describe a competitive molecularly imprinted assay using a fluorescent reporter [29]. Triazine (**I**) and 5-[(4,6-dichlorotriazin-2-yl)amino]-fluorescein [DTAF (**II**)] (Fig. 2), a fluorescent triazine derivative, competed for the available binding sites, fluorescence of the supernatant was proportional to the triazine concentration, and the polymer was selective for triazine over atrazine (**III**) and simazine (**IV**). This group also developed a competitive assay using DTAF based on an atrazine imprinted membrane [30]. Following work using radiolabelling [25], Haupt et al. developed a competitive fluoroassay for 2,4-dichlorophenoxyacetic acid (2,4-D) (**V**) in both organic and aqueous solvents [31,32]. The detection limit was similar to that of a radioassay (0.1 μM) and the equilibration time was half that used for the triazines. Furthermore, as found with the MIP radioassay, cross-reactivity of the polymer towards 2,4-dichlorophenoxyacetic acid methyl ester (2,4-D-OMe) was lower than that reported for immunoassays.

As a comparator to the flow through format used in some immunoassays [33], displacement of a fluorescent probe from an MIP-HPLC stationary phase was successfully used to determine chloramphenicol [34,35] and L-phenylalaninamide [36] in biological samples, although no competition could be achieved between oestradiol and different fluorescent derivatives [37].

Chow et al. adopted an approach in which they derivatised DL-homocysteine with a pyrenyl moiety and imprinted this entire fluorescent molecule rather than the analyte [38]. Then, using the catalytic property of the imprinted polymer [39], they detected DL-homocysteine by in situ fluorescent derivatisation. The response of the polymer was linear in the concentration range found in plasma and the specificity of the detection was enhanced by the fact that the imprinted polymer acted as a "footprint catalyst" for the derivatisation reaction [39].

### 3.2. Homogeneous assays

An advantage of fluorescence detection is the possibility of developing homogeneous assays where it is not necessary to separate the bound and unbound analyte fractions [28]. A range of fluorescent functional monomers were incorporated and rebinding of the template was shown to modify the fluorescence of the matrix (Table 4). Where no significant fluorescence intensity change was observed [40,41], the alternative solution of using a quencher as a competitive agent was applied [41]. Piletsky et al. [42] and Wang et al. [43] adopted a more successful covalent approach in which the fluorescence was modified upon rebinding of the template. However, the sialic acid imprinted polymers showed a high cross-reactivity towards galactose [42]. Turkewitsch

Table 4
Examples of homogeneous pseudo-fluoroimmunoassays

| Template | Polymer | Fluorescent chromophore | Fluorescence | Ref. |
|---|---|---|---|---|
| Cholesterol | EDMA[a] | $M_1$ | 0[b] | [40] |
| (L)-Tryptophan | EDMA[c] | ((2-*N*-dansyl)ethyl) 3,3-dimethylacrylate | −[d], +++[d,e] | [41] |
| Sialic acid | $M_2$/aA/EDMA/DMF[a] | Primary amine/OPA | +++[d] | [42] |
| (D)-Fructose | HEMA/EDMA[a] | Anthracene/boronic acid conjugate | +++[d] | [43] |
| cAMP | HEMA/TRIM/$CH_3OH$[c] | vb-DMSAP | −−−[d,f] | [44,47,48] |
| Carboxamidrazone derivative | EDMA/$CHCl_3$[c] | 3-Acrylamido-5-(2-methoxy-1-naphthylidene)-rhodamine | −−−[b] | [45] |
| Carboxamidrazone derivative | TRIM/toluene[c] | | −−−[b] | [46] |
| 9-Ethyladenine | MAA/EDMA/$CHCl_3$[c] | Zn-porphyrin | −−−[b] | [49] |
| (−)-Cinchonidine | MAA/EDMA/$CHCl_3$[c] | Zn-porphyrin | −−−[b] | [50] |
| Histamine | MAA/EDMA/$CHCl_3$[c] | Zn-porphyrin | −−−[b] | [51] |
| Toluic acid | In solution (preliminary study) | 9-(Guanidinomethyl)-10-vinylanthracene | +++[b,g] | [52] |

+: increase in the fluorescence; −: decrease in the fluorescence; 0: no change in the fluorescence; aA: allylamine; $M_1$: acrylic acid 4-(2-{4-[2-(4-hydroxy-3,5-dimethoxy-phenyl)-vinyl]-phenyl}-vinyl)-2,6-dimethoxy-phenyl ester; $M_2$: sialic acid-*o*((4-vinylphenyl)boronate); OPA: *o*-phthalaldehyde; vb-DMSAP: trans-4-[*p*-(*N*,*N*-dimethylamino)styryl]-*N*-vinylbenzylpyridinium; HEMA: 2-hydroxyethyl methacrylate.

[a] Covalent approach.
[b] Organic solvent.
[c] Non-covalent approach.
[d] Aqueous environment.
[e] *p*-Nitrobenzaldehyde used as quencher.
[f] cAMP $K = 2.9\,\mu M$.
[g] Toluic acid $K = 8.3\,\mu M$.

et al. [44] designed and synthesised a fluorescent monomer able to interact with purine nucleotides (cAMP and cGMP) via electrostatic interactions. In an aqueous environment the anti-cAMP polymer was able to rebind the template selectively with a single affinity constant ($K_D = 2.9\,\mu M$), which was uncommonly high for non-covalently imprinted polymers. Rathbone et al. [45] synthesised imprinted polymers incorporating a fluorescent monomer able to interact with the template via hydrogen bonds and used these in high-throughput screening of a library of potentially antitubercular drugs [46]. Matsui et al. developed a homogeneous system based on cooperative interactions between methacrylic acid and porphyrin-based functional monomers [47] where binding of the template (9-ethyladenine or (−)-cinchonidine) altered the fluorescence of the porphyrin ring [48,49].

High fluorescent backgrounds may impair the detection limit and sensitivity of fluoroassays and Wandelt et al. [50] adopted a different approach to circumvent this problem. They used the fluorescent functional monomer of Turkewitsch et al. [44] coupled with time-resolved fluorescence spectroscopy to study binding of cAMP [51] and found that the fluorescent lifetime decreased with increasing template concentration, resulting from the formation of tightly bound stable complexes within the cavities.

## 4. Enzyme-linked molecularly imprinted sorbent assays

Few enzyme-linked molecularly imprinted sorbent assays have been reported to date (Table 5). Surugiu et al. [53] first demonstrated that it was possible to use an enzyme-labelled conjugate as a competitive probe. Tobacco peroxidase (TOP) was used as the label and an assay developed for 2,4-dichlorophenoxyacetic acid (2,4-D) based on either colourimetric or chemiluminescent detection. By measuring the activity of the enzyme in the supernatant it was shown that 2,4-D was able to compete with 2,4-D-TOP for the available binding sites. Quantification of the analyte was possible at concentrations ranging from 40 to 60 μg/ml and 1 to 200 μg/ml using either colourimetric or chemiluminescence detection, respectively. This assay was later adapted to a high-throughput imaging format with a charge-coupled device (CCD) camera by coating microtitre plates with imprinted microspheres [54]. The concentration range of the calibration curve was lowered down to 0.01–100 μg/ml and the detection limit to 34 nM. Cross-reactivities were comparable with those obtained in a previous radioassay [25] and when compared to monoclonal antibodies the polymer demonstrated a lower cross-reactivity for methyl ester 2,4-D but higher for 4-chlorophenoxyacetic acid. Adapting this system to a flow injection ELISA-type chemiluminescent MIP assay [55], the same group further improved the detection limit of 2,4-D allowing the detection of picomole of analyte with cross-reactivities as previously reported [53,54]. Piletsky et al. [56,57] developed a technique for coating microtitre plates with a thin MIP film and performed competitive assays using epinephrine and atrazine labelled with horseradish peroxidase (HRP). In order to minimise the non-specific binding of the enzyme conjugate the assay was performed at pH 6–8. Although, in the case of atrazine, problems were encountered, related to ionic repulsion, promising results for the epinephrine assay were obtained as the polymer was highly specific and the affinity ($K_D = 9.2 \pm 2\,\mu M$) was similar to natural receptors ($K_D = 4.6 \pm 0.2\,\mu M$).

Table 5
Examples of enzyme-linked molecularly imprinted sorbent assays

| Template | Enzyme | Affinity, $K_D$ (μM) | | Ref. |
|---|---|---|---|---|
| | | MIP | NIP | |
| 2,4-D | TOP | nd | nd | [53–55] |
| Epinephrine | HRP | 9.2 ± 2 | 150 ± 15 | [56,57] |
| Atrazine | HRP | nd | nd | [56,57] |
| Phenylephedrine | HRP | 25 ± 2 | 100 ± 5 | [56] |

2,4-D: 2,4-dichlorophenoxyacetic acid; nd: not determined; TOP: tobacco peroxidase; HRP: horseradish peroxidase.

## 5. Conclusion

Although immunoassays are still commonly carried out using antibodies recent developments have demonstrated that molecularly imprinted polymers can be viable alternatives.

A number of significant issues and opportunities remain. In general, binding affinities of MIPs remain lower than antibodies, although the differential is progressively diminishing. Implementation of the polymer washing procedures, though reducing template leaching, should allow the use of analyte at lower concentration and give access to high affinity binding sites. Recognition by MIPs in aqueous media needs to be improved, but their excellent ability to perform in non-aqueous conditions and to recognise non-immunogenic analytes significantly extends the range of conditions under which analytes can be determined. Other issue concerns the use of MIA to analyse real biological samples. Although few assays analysing samples from serum were reported, to date only one was performed without prior sample clean up. This highlight the problems and interferences that can arise from the component of the biological matrix compare to the conditions used for fundamental studies.

It is probable that both traditional antibody-based and MIP sorbent assays will continue to develop in parallel, with each having superiority in certain areas.

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