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Review

Molecularly imprinted polymers in pseudoimmunoassay

Richard J. Ansell*

School of Chemistry, University of Leeds, Leeds LS2 9JT, UK

Abstract

Immunoassays are a class of analytical techniques based on the selective affinity of a biological antibody for its antigen. Competitive binding assays, of which the radioimmunoassay (RIA) was the first example, are based on the competition between analyte and a labelled probe for a limited number of binding sites. Molecularly imprinted polymers (MIPs) have been shown to be suitable replacements for biological antibodies in such techniques. Molecularly imprinted sorbent assays (MIAs) similar to RIA have been developed for a range of analytes of clinical and environmental interest. Limits of detection and selectivities of such assays are often similar to those using biological antibodies. Some assays have been used for measurements directly in biological fluids. The field is reviewed and it is shown that some perceived disadvantages of MIPs do not hinder their application in competitive binding assays: many MIAs have been demonstrated in aqueous solvents, and it has been shown that the quantity of template required to prepare imprinted polymers can be drastically reduced, and that binding site heterogeneity is not a problem as long as the sites which bind the probe most strongly are selective. Finally, recent developments including assays in microtitre plates, the use of enzyme-labelled probes, flow-injection assays and a scintillation proximity MIA are discussed.

Keywords: Reviews; Molecularly imprinted polymers

Contents

* Tel.: +44-113-343-6415; fax: +44-113-343-6565.
E-mail address: r.ansell@chem.leeds.ac.uk (R.J. Ansell).

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1. Introduction

1.1. Biological immunoassays

Biological antibodies are exploited as reagents in many different analytical techniques: immunoassay is a class thereof [1–3]. The archetypal examples are the radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). Both rely on readily measurable labelled species (radiolabelled in the case of RIA, enzyme-labelled in the case of ELISA). ELISA is an example of a *non-competitive* assay (Fig. 1a): the sample analyte binds to a first, immobilised capture antibody, and simultaneously or in a subsequent step to another, second, antibody, carrying the enzyme label. The antibodies in this case should be present in excess such that the binding sites are not saturated. The bound labelled antibody can be physically separated from the unbound (by discarding the solution phase) and quantified by adding a substrate which in the presence of the enzyme produces a coloured product. The signal (colour) produced is then proportional to the analyte concentration originally present.

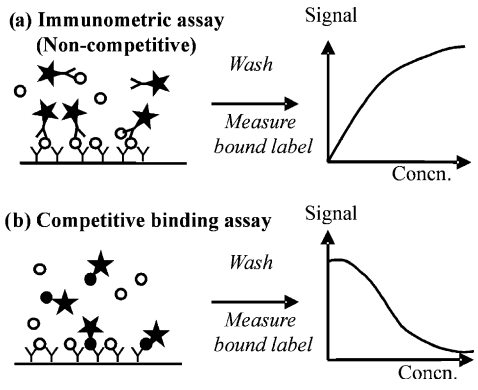

Fig. 1. Schematic representation of: (a) heterogenous non-competitive (e.g. ELISA) and (b) heterogenous competitive (e.g. RIA) immunoassays. The non-competitive format involves the formation of antibody–antigen–antibody 'sandwich'. Signal is proportional to the sites occupied by analyte. The competitive format involves the analyte competing with probe (here, labelled antigen) for the available sites. Signal is proportional to sites not occupied by analyte.

Because the capture antibody is immobilised to a solid support, ELISA is a heterogenous assay. Because the bound and unbound labelled antibody must be physically separated by washing it is also a *separation* assay. *Homogenous* and *non-separation* non-competitive binding assays are also possible, where all the reagents are in the solution phase and physical separation of bound and unbound second antibody is not needed. However, all non-competitive assays depend on an excess of antibody and produce a signal proportional to the analyte concentration.

RIA is an example of a *competitive* assay (Fig. 1b), the sample analyte and a probe, here a radiolabelled form of the analyte itself, compete for a limited number of antibody binding sites: in this case, there must be a deficit of antibody present. Again the antibody is bound to a surface so the assay is heterogeneous. The bound probe is separated from nonbound by washing: again, it is a separation assay. Finally, bound probe is quantified via radiometric counting. The bound activity is inversely related to the concentration of analyte present in the sample: the more unlabelled analyte present in the sample, the less sites are available for the labelled form. The first immunoassay using a labelled probe was a RIA developed to measure human growth hormone (HGH) by Catt et al. [4]. The concentration of HGH in unknown samples was determined by comparing the bound radioactivity with a standard curve determined for known concentrations, similar to that in Fig. 1b. Homogenous, non-separation forms of competitive binding assay are also possible, where the physical proximity of antibody and probe is sufficient to generate signal, such as the scintillation proximity assay. However, all competitive assays depend on a shortfall of antibody and produce a signal inversely related to the analyte concentration.

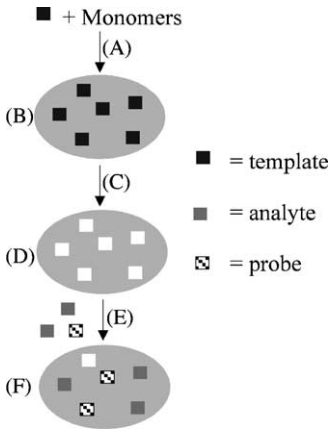

Fig. 2. Schematic of molecular imprint sorbent assay (MIA): (A) molecular imprinting process; (B) imprinted polymer containing trapped template-monomer complexes; (C) extraction of template; (D) isolated MIP; (E) analyte and probe are added to the MIP; (F) analyte and probe compete for the available binding sites. In the conventional radiolabel MIA, the analyte is identical to the template, and the probe is the radiolabelled form of the analyte.

1.2. The first MIA

The first molecularly imprinted sorbent assays (MIAs) were reported by Vlatakis et al. [5] (Fig. 2) and emulated the RIA design. Two assays were described, one for the sedative diazepam, and one for the bronchodilator theophylline, based on diazepam- and theophylline-imprinted molecularly imprinted polymer (MIPs), respectively. Unlike the biological RIA, sample plasma containing the drugs was not added directly to the MIP (/antibody), but rather the drug was first extracted from the plasma into organic solvent, this solvent was evaporated, the residue reconstituted in a different organic solvent, and this solution added together with radiolabelled ('hot') drug to the plasma. The extra solvent extraction and solvent exchange steps were necessary because the MIPs were expected to perform better in organic solvents than an aqueous matrix. 'Hot' and 'cold' drug competed for the available binding sites, and in this case, rather than directly measuring the bound radioactivity, the MIP was removed by centrifugation and the free radioactivity present in the supernatant was determined by liquid scintillation counting. Comparing the bound radioactivity (total added-free) for unknown samples with standards of known cold drug concentration enabled calculation of the concentrations of cold drug in the unknowns. The results were impressive: for theophylline, the cold drug was measured in blood in the range 14–224 μM, the assay results correlated excellently with a commercial enzyme-multiplied immunotest based on biological antibodies, and cross-reactivity of related molecules was similar to that of the biological antibodies.

Since this first work many more MIAs have been developed and the approach has been reviewed elsewhere [6–11].

1.3. Potential advantages of MIPs

The potential advantages of MIPs as replacements for biological antibodies in immunoassays include:

(i) It is difficult to produce and select antibodies for small molecules (they must be coupled to a carrier protein before inoculation). However, such compounds are best suited to molecular imprinting.
(ii) It is hard to raise natural antibodies against highly toxic compounds or immunosupressants. But there are no additional problems in making MIPs for such analytes [12].
(iii) In some cases, it may be desirable to perform binding assays in non-aqueous media. Protein antibodies function poorly in such conditions but MIPs often function better in organic solvents than in aqueous conditions.
(iv) MIPs are stable and rugged in comparison with biological antibodies.
(v) Production of MIPs does not require the sacrifice of animals.

The potential disadvantages include:

(vi) Reliable methods for imprinting large molecules are still not available: consequently for protein analytes, antibodies remain the receptor of choice.
(vii) Because the binding sites of MIPs (at least of conventional macroporous acrylate/vinyl based MIPs) tend to be within a macroporous structure they are expected to be inaccessible to large molecules. This might prohibit the use of an enzyme-labelled molecule, for instance, as a probe. However, recent work has shown that MIP formats where the binding sites are more readily accessible, can be used in assays with enzyme-labelled probes (Section 4.2) [13,14].
(viii) Similarly, MIPs are probably incompatible with non-competitive binding assays such as ELISA in which a antibody–analyte–antibody sandwich must be formed since such a sandwich with MIPs will be sterically very hindered. However, even with biological antibodies, methods like ELISA are limited to larger analytes such as proteins since the analyte must have two independent binding sites. Since MIPs are better suited to small molecules, the competitive format is likely to be preferred anyway.
(ix) A common misconception is that MIPs only work in organic solvents. Laborious extraction and reconstitution steps were required in Vlatakis et al.'s assay [5]. However, in more recent work, binding assays have been performed either directly after the first extraction step in the same solvent (which may actually be advantageous in that it serves to concentrate the analyte) [12], or directly in aqueous systems including diluted plasma [15] (Section 3.2).
(x) The inefficiency of molecular imprinting (i.e. the distribution of binding sites in a MIP, ranging from very strong and selective for the analyte to very weak and

non-specific) has been perceived as a problem, but although it may be so for other applications such as chromatography, it is not at all for MIA: as long as the sites that bind the probe most strongly are selective (Section 3.3).

(xi) Another perceived weakness has been the quantity of template required to prepare a useful amount of MIP. However, dramatic advances have been achieved in reducing the quantity of template used [16,17] and furthermore MIPs unlike antibodies can be cleaned (even by autoclave) and reused.

Thus, biological antibodies and MIPs are in many ways complementary, biological antibodies having the upper hand for assays of large molecules such as proteins, but MIPs having several advantages for assays of small molecules.

2. Setting up a MIA

It is important to distinguish initially the *template*, which is used to produce the MIP, the *analyte*, and the *probe*. The probe competes with the analyte for binding to the MIP. The bound fraction of the probe can be quantified (Fig. 2). In this section, a typical procedure is outlined for establishing a conventional MIA, such as those listed in Table 1, where the analyte itself is used as template, and the probe is simply a radiolabelled form of the analyte.

2.1. Preparation of the MIP

The MIP can be produced in different formats, though most MIAs reported are still based on conventional acrylate/vinyl based particulate polymers. MIA development also requires a reference polymer, which may be a non-imprinted polymer (NIP) made similarly to the MIP but without template or a control polymer (CIP) imprinted with a very different template but otherwise similar to the MIP. It is important to wash the MIP thoroughly in order to remove as much of the template as possible before use.

2.2. Initial choice of solvent

If the polymer has been produced by non-covalent imprinting it is usual to begin by studying binding in an organic solvent, commonly the same as used for the polymer

Table 1
Reported MIAs employing radiolabelled probes

Template	Probe	Assay solvent	Competitors	Reference
Diazepam	^{3}H-Diazepam	MeCN/AcOH (99:1)	Diazepam, related diazepine derivatives	[5]
Theophylline	^{3}H-Theophylline	(a) MeCN/AcOH (99:1), (b) toluene/THF (9:1), (c) AcN, (d) toluene	Theophylline, theobromine, caffeine, related xanthines	[5,17,20–24]
Octyl-α-D-glucoside	^{14}C-Methyl-α-D-glucoside	MeCN/AcOH (199:1)	Methyl-α-D-glucoside, other sugar derivatives	[25]
Atrazine	^{14}C-Atrazine	(a) MeCN, (b) toluene, (c) phosphate pH 7/Tween 20 (9985:15)	Atrazine, related triazines	[26,27]
Morphine	^{3}H-Morphine	(a) Toluene, (b) citrate pH6/EtOH (9:1)	Morphine, related opiates	[16,28]
Leu-enkephalin anilide	^{3}H-Leu-enkephalin	(a) MeCN, (b) citrate pH 4.5/EtOH (9:1)	Leu-enkephalin, related peptides	[28]
S-Propranolol	^{3}H-*S*-Propranolol	(a) Toluene, (b) various aqueous buffers, (c) 60% plasma	*S*-Propranolol, *R*-propranolol, racemates of related drugs	[15,29–33]
Cortisol	^{3}H-Cortisol	THF	Cortisol, related steroids and sterols	[34]
Cortisone	^{3}H-Cortisone	THF	Cortisone, related steroids and sterols	[34]
Yohimbine	^{3}H-Yohimbine	(a) MeCN/AcOH (199:1), (b) phosphate pH5.0	Yohimbine, corynanthine	[35]
Cyclosporin A	^{3}H-Cyclosporin A	Diisopropyl ether	Cyclosporin A, metabolites, unrelated drugs and proteins	[12]
2,4-D	^{14}C-2,4-D	Phosphate pH7/Triton X-100 (999:1)	2,4-D, related acids and esters	[13,36]
17β-Estradiol	^{3}H-17β-Estradiol	MeCN	17β-Estradiol and related diols	[20,21,23]
Caffeine	^{14}C-Caffeine	(a) Heptane/THF (3:1), (b) MeCN	Caffeine, theophylline	[18,21]
NacGIIIB	^{14}C-NacGIIIB	H_2O? Unspecified	NacGIIIB, related peptides	[37]
17α-Ethynylestradiol	^{3}H-17α-Ethynylestradiol	Toluene	17α-Ethynylestradiol and related steroids	[38]
Bupivacaine	^{3}H-Bupivacaine	(a) Toluene/AcOH (995:5), (b) MeCN/H_2O (2:8), (c) MeCN/H_2O (9:1), (d) citrate buffer pH5/ethanol/Tween 20 (9495:500:5)	Bupivacaine, related local anaesthetics	[39]
4-Nitrophenol	^{14}C-4-Nitrophenol	MeCN	4-Nitrophenol	[40]

fabrication, to ensure the polymer swelling is the same as under the fabrication conditions. A modifier such as acetic acid may be added to reduce the strength of the interactions, and minimise non-specific binding.

If the assay is to be optimised for performance in an aqueous solution, it is usual to begin with a buffer such as phosphate pH 7. Since the MIP itself is usually relatively hydrophobic a small amount of a non-ionic surfactant such as Triton X-100 or a miscible organic solvent such as ethanol is often added to lessen the surface tension and minimise hydrophobic interactions, which may be less specific than hydrogen bonds or ion-pairs.

2.3. Optimising the binding of the probe

The amount of probe added to each assay should be the minimum such that it can be reliably quantified when the percentage bound ranges between 0 and 80%. Using more probe is wasteful and reduces sensitivity.

Competitive assays work best when in the absence of any analyte 30–80% of the probe binds to the antibody/MIP ($0.3 < B_0/T < 0.8$, where T is the total probe added, B is the bound probe and subscript '0' indicates no analyte is present). In MIA, as in biological immunoassays with polyclonal antibodies, a range of binding sites of differing strength and selectivity are present. It is desirable that the probe binds only to the most selective, which will generally be the strongest ones. Thus, the ideal conditions are as represented by the centre point in Fig. 3. Clearly, the amount

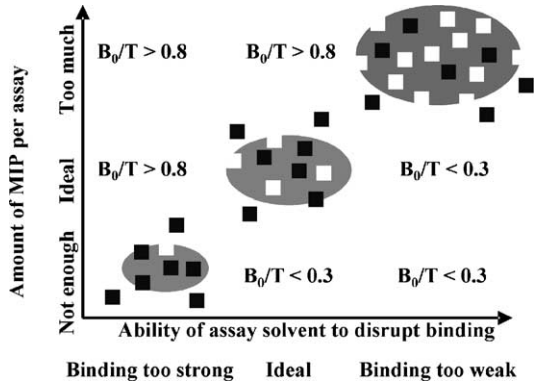

Fig. 3. Schematic of the process of optimizing the probe binding to MIP for a MIA. The figure represents a series of conditions investigated with in each case the same amount of total probe, the aim being to optimize the amount of polymer and assay solvent. MIP particles are represented by grey ovals. An increase in amount of MIP is represented as an increase in the size of MIP particle for simplicity. Along the diagonal, conditions are such that $B_0/T = 4/7$, which is in the range desired. At the lower left hand corner, too little MIP is used and although the solvent is such that binding interactions are strong and $B_0/T = 4/7$, some of the binding sites occupied by the probe are poorly imprinted, weak and non-selective. These conditions will give a MIA with poor selectivity and sensitivity. At the top right hand corner, too much MIP is used and although the solvent is such that binding interactions are weak and $B_0/T = 4/7$, these conditions would give a good MIA but are wasteful of MIP. The conditions in the center are ideal: $B_0/T = 4/7$ but the probe binds only to the best imprinted, strongest and most selective sites.

of MIP and the solvent must be optimised in tandem. (Having stated above that competitive immunoassays rely on a shortfall of binding sites, it may seem contradictory to suggest that an excess of MIP binding sites is required. The reason is that in the ideal situation as described by Fig. 3 there is a shortfall of *strong binding sites*. These are the only sites of interest in the MIA—if analyte or an interferent displace the probe from these sites, it will not bind to the weaker sites, but will be truly displaced. We can consider the weaker sites to be ignored.)

The amount of bound probe is usually determined by incubating a fixed amount of probe with varying quantities of polymer, separating the bound and non-bound fractions (for particulate MIPs, by centrifugation) and measuring the activity in a fixed volume of the solvent. Once the appropriate amount of MIP to give $0.3 < B_0/T < 0.8$ is known, the solvent can be adjusted to optimise the selectivity. It is desirable to maximise the difference in B_0/T between the MIP and CIP or NIP, binding to the latter being entirely non-specific. After optimising the solvent, it may be necessary again to vary the amount of polymer employed in each experiment until B_0/T (MIP) is in the desired range.

2.4. The competition assay with analyte and possible interferents

The competition assay is performed only with the MIP: the quantities of MIP and probe determined above are incubated with varying amounts of analyte or interferent (over as wide a range as possible, e.g. six decades of concentration). Controls are also set up, with probe only and with MIP and probe only. Ideally, a range of interferents should be examined, compounds structurally related to the analyte and also compounds which would be present as interferents in a real analytical application of the assay. However, it is often enough to demonstrate the potential of a MIA by considering only one or two close analogues, e.g. if the analyte is chiral, it may suffice to demonstrate the MIA distinguishes between the analyte and its enantiomer.

Samples and controls are incubated overnight, centrifuged and the supernatant measured for activity. For each concentration of analyte or interferent the ratio B/B_0 (probe bound in the presence of analyte or interferent/probe bound for control with no analyte or interferent) is calculated and plotted against concentration as shown in Fig. 4. The figure indicates that as the concentration of analyte, caffeine in this case, is increased, it competes more effectively with the probe and more is displaced from the MIP so B falls [18]. Furthermore, the interferent, theophylline in this case, also serves to compete with the probe and depress B, but only at higher concentrations than the analyte: theophylline is less effective at displacing the probe than is caffeine.

To obtain quantitative measures of the sensitivity and selectivity of the assay, the IC_{50} values (the concentrations at which $B/B_0 = 0.5$, i.e. 50% of the probe is displaced) may be calculated for the analyte and interferents. These values

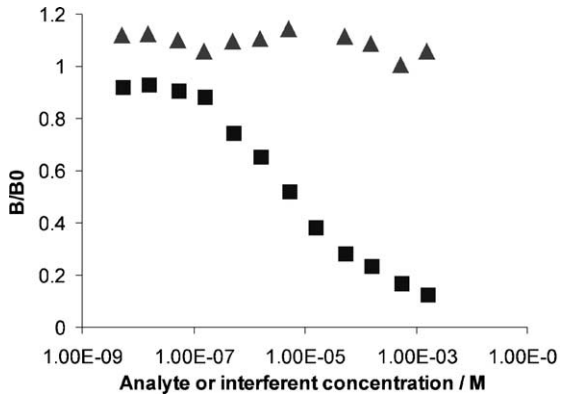

Fig. 4. Results of a MIA for caffeine performed in organic solvent (heptane/THF 3:1, v/v) [18]. Curves of B/B_0 vs. competitor concentration. Competitor: unlabelled caffeine (squares) or theophylline (triangles). Assays performed in 1 ml volume using 8 mg of anti-caffeine MIP. It can be observed that the IC_{50} value for caffeine is about 8 μM while theophylline even at 1 mM concentration does not displace 50% of the bound probe.

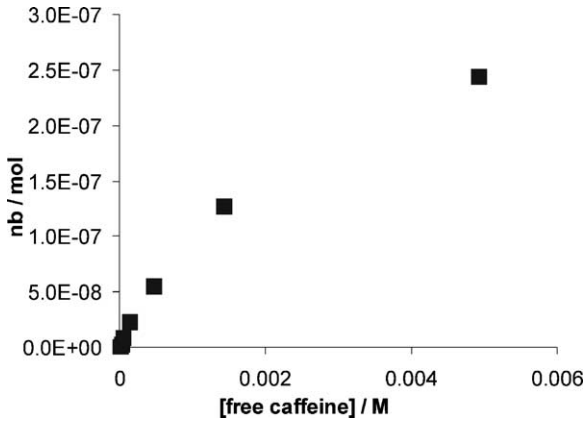

Fig. 5. Binding isotherm for caffeine binding to the MIP in the experiment shown in Fig. 4, derived from the B/T ratios and the added 'cold' caffeine concentration.

may be estimated from a graph such as Fig. 4 (for caffeine, the value is about 8.5 μM). For a greater degree of accuracy, the graph can be replotted in log/logit form ($\text{logit}(B/B_0) = \log((B/B_0)/(1 - B/B_0))$ versus log(concentration)). The cross-reactivity of interferents may be expressed as the ratio IC_{50} (analyte)/IC_{50} (interferent). In the MIA of Fig. 4, the IC_{50} value for theophylline cannot be reliably determined but certainly lies above 1 mM, so the cross-reactivity of theophylline is <0.8%.

2.5. Assessing the heterogeneity of binding sites

Non-covalent molecular imprinting inherently yields a distribution of binding sites of differing affinities and selectivities. In the case when the probe is the radiolabelled form of the analyte, further information about the heterogeneity of the MIP binding sites can be obtained from the results of the assays in part 2. This is based on the assumption that

$$\frac{\text{bound probe}}{\text{total probe}} = \frac{B}{T} = \frac{\text{bound analyte}}{\text{total analyte}}$$

This is not valid if the probe is in any way chemically different from the analyte. The plot of amount of bound analyte versus concentration of free analyte is the binding isotherm (Fig. 5) and can be fitted to a number of models including the Langmuir, bis-Langmuir, Freundlich and Langmuir–Freundlich isotherms—a full discussion of binding site heterogeneity and isotherm analysis appears elsewhere in this issue [19]. Such an analysis is not essential to the development of a MIA, but is often performed in order to gain an estimate of binding site numbers and affinities. Fitting of the data for the caffeine MIP to a bis-Langmuir model suggests a population of weak binding sites ($d_1 = 48.8 \pm 0.4\ \mu\text{mol g}^{-1}$ and $K_{a1} = 311 \pm 8\ \text{M}^{-1}$) and a smaller population of strong binding sites ($d_2 = 1.09 \pm 0.15\ \mu\text{mol g}^{-1}$ and $K_{a2} = 3.3 \pm 1.2 \times 10^5\ \text{M}^{-1}$). In reality, there is more probably a distribution of binding sites of continuously varying K_a values.

2.6. Validating the method with real samples

In practice this step has often been omitted where proof-of-principle of a MIA for a new analyte, or of a new MIA format, has been the main goal. Only in a few cases has a full assay procedure for real samples been developed, all involving drug measurements in serum, plasma or urine and most the work of Andersson and co-workers [5,12,15].

The first full procedures relied on extraction of the drug from plasma into an organic solvent. This solvent was either evaporated so the extracted residue could be redissolved in the assay solvent [5] or was chosen to be the assay solvent itself [12]. In both cases, the assay solvent and MIP quantity per assay could be optimised separately, as in Section 2.3, then the extraction step optimised. In the full MIA, drug-free plasma spiked with different known quantities of drug are used as standards, and plasma samples with unknown drug concentrations compared. For the standards, a calibration curve is drawn in the form of B/B_0 against drug concentration in the plasma, or, better, $\text{logit}(B/B_0)$ against log(drug concentration) which can be fitted to a straight line. The unknowns are determined from the calibration graph and the values obtained compared against those obtained via another method.

Only in one case [15], has the full MIA procedure been developed for measurement directly in plasma/urine (another *S*-propranolol assay, Section 3.2). The plasma/urine was diluted by the addition of MIP suspension and probe, so the amount of MIP and composition of the plasma/diluent mixture were first optimised as in Section 2.3.

3. Conventional radioligand MIAs

Since the initial work by Vlatakis et al. [5], great advances have been made in applying MIA to diverse analytes

Table 2
Cross-reactivities observed in organic and aqueous MIAs for *S*-propranolol [29]

Assay and competitor (*S*-propranolol MIA)	Cross-reactivity (organic MIA) (toluene/AcOH (199:1, v/v))	Cross-reactivity (aqueous MIA) (25 mM sodium citrate pH 6/EtOH (49:1, v/v))
S-Propranolol	100	100
R-Propranolol	1.5	17.3
R,S-Atenolol	18.1	<0.1
R,S-Metoprolol	6.3	0.7
R,S-Timolol	0.8	0.3

(Table 2) [12,13,15–18,20–40] and in improving the basic design, to enable assays in aqueous solvents and directly in real samples, to reduce the quantity of MIP required, and improve the selectivity and sensitivity.

3.1. In organic solvents

'Traditional' non-covalent MIPs employ functional monomers such as methacrylic acid (MAA) and acrylate or vinyl cross-linkers. Molecular recognition relies on a combination of weak non-covalent interactions to create the binding sites during MIP fabrication and subsequently to rebind the template. Thus, most early MIAs were performed in organic solvents in which such interactions are expected to be strongest. Where MIAs were applied to 'real' samples such as plasma, these were first extracted into an organic solvent [5].

The use of an organic solvent as the assay medium should not automatically be considered a disadvantage. Many medical and environmental analytes may be present at such low concentrations that a pre-concentration and clean-up step is required for any assay including biological immunoassay. For nonpolar molecules liquid–liquid extraction may be the most convenient method.

Since the initial demonstration of the principle using theophylline and diazepam, organic-phase MIAs based on 'traditional' MIPs have been developed for a number of other drugs and medical targets including morphine [16,28], Leu-enkephalin [28], cortisol and cortisone [34], yohimbine [35], caffeine [18,21] and bupivacaine [39]. In each case, selectivity for the target has been demonstrated, and limits of detection are typically in the 100 nM to 1 μM range. Most of these studies were not optimised, but a thorough study was made of a MIA for *S*-propranolol and the detection limit lowered to 5.5 nM [29].

If a liquid–liquid extraction clean-up step is employed, it would clearly be most advantageous to be able to perform the MIA directly in the solvent into which the drug has been extracted. This was demonstrated in a MIA for cyclosporin where plasma was extracted into diisopropylether, then MIP and radiolabelled cyclosporin were added directly [12]. This MIA was also noteworthy because cyclosporin is an immunosuppressant drug, so that preparation of biological antibodies is not trivial. The assay had an impressive limit of detection of 4 nM, but although non-related structures exhibited minimal cross-reactivity, four first generation metabolites of the parent drug cross-reacted to 100%. This reflects the complexity of the imprint species, which is a cyclic peptide of molecular mass 1101. However, it was pointed out that a total measurement of the metabolites itself provided clinically useful information.

Organic phase MIA has also been applied to the herbicide atrazine, the groups of Muldoon and Stanker [26] and Mosbach and co-workers [27] publishing similar assays simultaneously. In both cases selectivity was demonstrated over related substituted triazines. The former assay performed in acetonitrile had a detection limit of 4.6 μM, the latter performed in toluene 250 nM. Other analytes of environmental interest for which conventional radiolabel MIAs have been developed include 2,4-dichlorophenoxyacetic acid [13,36] and 4-nitrophenol [40] although in the latter case interferents were not studied.

The stability of MIPs, in comparison to biological antibodies, was demonstrated in a recent study on a theophylline MIP, of identical composition to that used by Vlatakis et al. in the original MIA [24]. The affinity of ^{3}H-theophylline for the MIP in acetonitrile/acetic acid (99:1, v/v) was shown to be essentially unaffected by exposure of the MIP to temperatures up to 150 °C, to 5 M HCl or 15% ammonia solution: however, the selectivity of the MIP after treatment was not assessed.

3.2. In aqueous solvents

For many applications an extraction step into organic solvent is unwarranted and assays would be simpler, and more widely accepted, if they could be performed direct in the sample matrix, which whether for medical or environmental applications is usually aqueous. In applications such as chromatography and sensors traditional MIPs work poorly in aqueous conditions: this is in part because specific (polar) interactions between good imprinted sites and analyte are weakened, and in part because non-specific (hydrophobic) interactions between other small molecules and the polymer matrix are strengthened. However, for MIA non-specific interactions at weak binding sites are relatively unimportant provided the strongest recognition sites retain their selectivity (Section 2.3). Thus, in most cases, MIAs have been adapted to aqueous conditions successfully.

Early demonstrations of aqueous MIAs included those for morphine, Leu-enkephalin, atrazine and *S*-propranolol [27–29] (Table 1). In each case, the MIA was first developed in organic solvent, then investigations were conducted to find aqueous pH and additive conditions such that specific binding was maximised. This was generally found to require a pH where the analyte and polymer carried complementary charges (often pH 5–6 for MAA based polymers and basic analytes) and the addition of ethanol as cosolvent (1–10%) or a surfactant (0.1–1%) to wet the polymer surface. Sensitivities and selectivities comparable to the organic

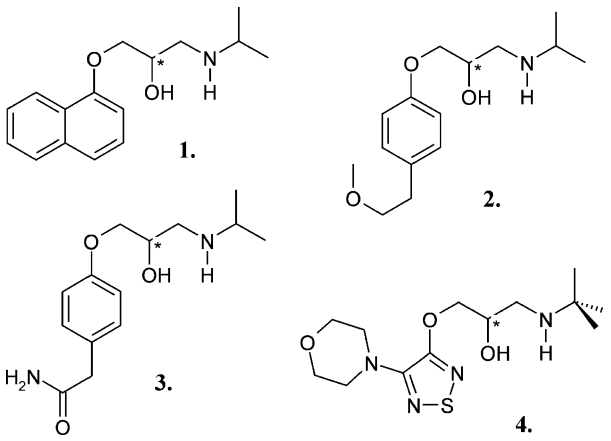

Fig. 6. Propranolol and related β-blockers studied in MIAs: (1) propranolol, (2) metoprolol, (3) atenolol, (4) timolol.

assay were usually achieved. The most thorough investigation of an aqueous MIA was made for *S*-propranolol [29]. Optimum conditions for the aqueous assay were found at 25 mM sodium citrate pH6.0 containing 2% (v/v) ethanol. The IC_{50} and limit of detection values were similar as in toluene/acetic acid, but interestingly a different pattern of selectivity was observed (Table 2). Compared to the organic MIA, lower enantioselectivity but increased species selectivity is observed (i.e. *R*-propranolol cross-reacts more but the related atenolol, metoprolol and timolol cross-react less) (Fig. 6). This can be understood because the recognition sites which bind the probe strongest are likely to be different in the different solvents: in organic solvents, the polar interactions are most responsible for recognition whilst in aqueous solvents hydrophobic contacts are also likely to be important. Consequently, the 'recognised features' change. Similar changed patterns of selectivity have been observed in MIAs for morphine [28] and bupivacaine [39]. Thus, the selectivity of a MIA may be tailored by judicious choice of the solvent.

The *S*-propranolol MIA was also applied to real blood plasma and urine samples. Samples were diluted with ethanol and phosphate buffer, and MIP and ^{3}H-*S*-propranolol added directly. The assays measured plasma and urine concentrations in the range 20–1000 nM with accuracies of 89–107 and 91–125% and precisions of 3–13 and 1–7%, respectively [15].

3.3. Binding site heterogeneity

In an optimised MIA the 'bad' binding sites, which have the lowest affinity for the template/probe, are essentially ignored. However, since the template is often expensive, it is desirable to optimise the polymer such that the 'good' sites constitute a significant proportion of the total sites present. In such a case, the MIA solvent can be optimised so a relatively small amount of MIP is used and yet the situation described as ideal in Fig. 3 still pertains.

For instance in Andersson's *S*-propranolol MIA when the aqueous system was optimised only 50 μg of polymer was required per assay [29]. When Andersson calculated the binding site dissociation constants and populations for the *S*-propranolol MIP based on a bis-Langmuir model, the strongest binding sites were found to have $d_1 = 0.63\,\mu\text{mol g}^{-1}$ and $K_{a1} = 2.5 \times 10^8\,\text{M}^{-1}$, thus the strong sites were similarly numerous but much stronger than, for instance, in the caffeine MIP described in Section 2.5.

Strategies to increase the yield and homogeneity of binding sites in MIPs (to achieve 'stoichiometric imprinting' or 'monoclonal MIPs') are a subject of much attention. 'Good' binding sites arise from template-multiple monomer complexes in the prepolymerisation mixture, these can be increased in number by using stronger template-monomer interactions. However, when non-covalent MIPs are synthesised for use in MIA another strategy can be used to make the template go further. Simply, a much lower template:monomer ratio is used, e.g. 1:1000 instead of the 1:4 or 1:10 commonly employed. This strategy may yield fewer 'good' (template-multiple monomer) complexes in the prepolymerisation mixture, and so fewer 'good' binding sites in the MIP, but these will be a much higher proportion of the total template used. The 'bad' binding sites which arise from the excess of free monomer present during polymerisation are ignored in a well set-up MIA.

Thus, Mayes and Lowe showed that a MIA for morphine worked equally well when the MIP was synthesised using a morphine:MAA ratio of 1:500 as 1:5 [16], while Yilmaz et al. showed a MIA for theophylline worked equally well with a theophylline:MAA ratio of 1:1000 as 1:4 [17]: theophylline-imprinted polymers with only 2.5 μmol template per gram of monomers (compared with 151 $\mu\text{mol g}^{-1}$ in Vlatakis et al.'s MIA [5]) were employed, and the MIA functioned with caffeine cross-reacting less than 0.1%. These works demonstrate that the oft-quoted drawback of MIPs, the cost of the template required for their preparation, may be overcome: MIA is applicable even to expensive templates.

Because MIA probes only the sites of the MIP with strongest affinity for the probe, significant binding site heterogeneity is not detrimental. Thus, at higher concentrations, the MIP employed for the caffeine MIA described in Sections 2.4–5 actually binds significantly more theophylline than caffeine, owing to the greater basicity of theophylline and a large number of non-selective acidic binding sites with only weak affinity for caffeine. However, because the sites with high affinity for caffeine are selective, the polymer can still be employed in a selective assay for caffeine. Similarly, Andersson showed that a MIP imprinted with racemic *R,S*-propranolol could be employed in MIA assays for *S*-propranolol [29]. Although the polymer must contain an equal number of sites of high affinity for each enantiomer, *R*-propranolol competes only weakly with ^{3}H-*S*-propranolol for the strongest *S*-binding sites and in the assay cross-reacted only 1.4%. This implies that impure

analyte could be used as a template in imprinting, and the resulting MIP used successfully in a MIA, provided that the probe employed in the assay is pure.

4. Variations

Progress has been made on the development of new MIP compositions and morphologies, both 'traditional' MIPs and alternative imprinted materials, on alternatives to radiolabelled probes which may be more readily accepted by the analytical community, and on assays in different formats.

4.1. Novel polymer formats

Although the majority of MIA assays reported have employed 'traditional' non-covalent vinyl/acrylate-based MIPs, fabricated in organic solvents as macroporous monoliths then ground and sieved into useful particles, there have been many innovations in both the composition of MIPs and their macromorphology.

4.1.1. Functional monomers

MAA remains often the monomer of first choice due to its ability to interact with templates as a hydrogen bond donor or acceptor, and form ion pairs. However, the more acidic trifluoromethylacrylic acid (TFMAA) may be superior for some templates [17,18]. Haupt et al. employed the basic monomer 4-vinylpyridine (4-VPy) to imprint the acidic template 2,4-dichloro phenoxyacetic acid (2,4-D) [36]. They also employed an aqueous-methanolic solvent in the MIP fabrication, reasoning that 2,4-D should also interact with the monomer via hydrophobic interactions. The resulting MIA was highly successful, in particular its ability to discriminate 2,4-D methyl ester, which cross-reacts approximately 100% with most biological anti-2,4-D antibodies [41]. This reflects the need to couple 2,4-D to a carrier protein in order to raise antibodies, which is most readily achieved via formation of an ester, and demonstrates one advantage of using MIPs in assays for small molecules.

4.1.2. Magnetic MIP beads

Suspension polymerization in perfluorocarbon liquids was developed to produce regular spherical MIP beads, initially for chromatography [42]. Ansell and Mosbach found that magnetic iron oxide could be incorporated in the synthesis, producing hybrid superparamagnetic MIPs [30]. Magnetic MIPs imprinted with *R,S*-propranolol were employed in a MIA in aqueous phase for *S*-propranolol, exhibiting a similar pattern of selectivity to the bulk MIP-based MIA developed by Andersson [29]. However, the polymer could be removed from solution by a magnet, obviating the need for centrifugation, which could be useful for assays involving very large sample numbers, and for enabling automation.

4.1.3. Fine (<1 μm) particles

Haupt et al. for their several MIA designs for 2,4-D [36,43,44], prepared a 2,4-D-imprinted MIP monolith which was ground in the traditional manner, but rather than selecting the intermediate-sized (∼25 μm) particles as usually done, they took the fine particles. These exhibited the same selectivity as larger particles, but the incubation times were reduced due to shorter diffusion distances, and handling was easier since the particles stayed in suspension so could be pipetted more accurately.

4.1.4. Microspheres

Mosbach's group demonstrated that uniform microsphere MIPs (<1 μm) could be produced by precipitation polymerization under very dilute conditions [20]. Microsphere MIPs have subsequently been applied in MIAs for theophylline, 17-β-estradiol, caffeine, *S*-propranolol (all MAA-based) [21,22,31], and 2,4-D (4-VPy-based) [13]. Microspheres exhibit the same handling advantages as 'fine' particles. They are non-porous so that accessible recognition sites are considered to be limited to the surface, yet the best recognition sites in the theophylline-MIP microspheres were found to be of similar affinity to those of bulk MIPs, and more numerous. The selectivity of the MIA developed with these microspheres also appeared to be similar to that with bulk MIP. Because the microspheres' recognition sites are at the surface they could be adapted to MIA for 2,4-D with enzyme labels (Section 4.2), and because they form stable suspensions they could be used in a proximity-scintillation based MIA (Section 4.3).

4.1.5. Immobilised templates

Yilmaz et al. demonstrated a MIA for theophylline based on a MIP produced by polymerising TFMAA/DVB in the pores of a silica gel matrix on which theophylline had been immobilised [22]. The silica gel was dissolved and the resulting polymer processed to yield particles with surface recognition sites for theophylline. More polymer was required for the MIA than when a conventional MIP imprinted with free theophylline molecules was used, suggesting a lower density of good recognition sites, but the selectivity of the assay was similar. The approach could be used to imprint larger molecules including proteins or compounds which are poorly soluble.

4.1.6. Surface-imprinted silica

The first MIA not using vinyl or acrylate-based imprinted polymers was demonstrated for *N*-acetylated μ-conotoxin GIIIB (NacGIIIB) and used surface-imprinted silica, generated by treatment of silica gel with a mixture of amino-functionalised and non-functionalised silanes in the presence of NacGIIIB [37]. Unlabelled NacGIIIB could displace labelled ^{14}C-NacGIIIB from the material more effectively than the related peptide NacGIIIA or the non-acetylated forms GIIIA or GIIIB. However, a non-imprinted polymer was not studied and since the

competitors studied are all more basic than the probe it is unclear to what extent selective recognition sites are responsible for the results.

4.1.7. Thin polymer films on glass

Thin *S*-propranolol imprinted films were employed in MIAs by Marx and Liron [32]. Silane-based films were found to exhibit higher specific binding in phosphate buffer pH 7.6 than acrylate-based ones. In a buffer-based competitive MIA, *S*-propranolol displaced ^{3}H-*S*-propranolol more effectively than *R*-propranolol, metoprolol or timolol. Although films made in this way are of interest in developing sensors, it is unclear whether they offer any advantages for MIA.

4.1.8. Thin layers on microtiter plate wells

The microtiter plate format is universally employed in biological assays such as ELISA and enables a very high throughput, in particular when combined with automation. Thus, thin layers of MIPs in microtiter plate wells are of immense interest for MIAs involving colorimetric or fluorescence detection. Two approaches have been described: the entrapment of 2,4-D imprinted microspheres in films of polyvinylalcohol hydrogel [45] and the preparation of imprinted poly-3-aminophenylboronic acid and related conjugated polymers which precipitate as films on the microwell surface [14,46] (Section 4.2).

4.1.9. Capillaries

Danielsson et al. have used imprinted polymer coatings on the inner surface of capillaries in a flow-injection based assay for 2,4-D using an enzyme-labelled probe (Section 4.2) [47,48]. The inner surface of a 0.9 mm diameter glass capillary was treated with 3-methacryloxypropyl trimethoxysilane, and an acrylate-based imprinting mixture was introduced and polymerised at 60 °C.

4.2. Novel probes

Most reported MIAs have employed radiolabelled probes. However, since safety and regulatory incentives exist to avoid radioactivity, a number of non-radiolabel MIAs have been developed (Table 3) [13,14,43–56]. The advantage of using radiolabelled probes (besides the inherent sensitivity) is that the imprint molecule, probe and target analyte may be chemically identical. Thus, MIP recognition sites which bind the probe most strongly are likely to be highly selective for the analyte. When using fluorescent, electroactive or enzyme-labelled probes there is no guarantee that the sites interrogated by the probe have the best selectivity for the analyte. Four approaches may be envisaged:

1. The native analyte is imprinted, a labelled form of the analyte is used as the probe. In this case the best

Table 3
Reported MIAs employing non-radiolabelled probes

Template	Probe	Assay solvent	Competitors	Reference
Chromophore-labelled probes				
Chloramphenicol	Chloramphenicol-methyl red	MeCN	Chloramphenicol, chloramphenicol diacetate, thiamphenicol	[49,50]
Biotin methyl ester	Biotin nitrophenyl ester	MeCN	Biotin methyl ester	[51]
Fluorophore-labelled probes				
Triazine	5-(4,6-Dichlorotriazinyl)aminofluoresceine	EtOH	Triazine, atrazine, simazine	[52]
Chloramphenicol	Dansylated chloramphenicol	MeCN	Chloramphenicol, chloramphenicol diacetate, thiamphenicol	[53]
Atrazine	5-(4,6-Dichlorotriazinyl)aminofluoresceine	Water	Atrazine, atraton-D, metribuzin	[46]
Enzyme-labelled probes				
2,4-D	2,4-D-tobacco peroxidase	Phosphate pH7/Triton X-100 (999:1)	2,4-D, related acids and esters	[13,45,47,48]
Epinephrine	Norepinephrine-horseradish peroxidase	Phosphate pH6	Epinephrine, related catechols	[14,46]
Microcystin-*L*,*R*	Microcystin-horseradish peroxidase	Phosphate pH7	Microcystin-*L*,*R*, related peptides	[54]
Unrelated chromophore/fluorophore probes				
2,4-D	7-Carboxy-methoxy-4-methylcoumarin	Phosphate pH7/Triton X-100 (999:1)	2,4-D, related aromatics	[43,55]
Electroactive probes				
2,4-D	Homogentisic acid	Phosphate pH7/MeOH (9:1)	2,4-D	[44,55]
2-C-4-H	2-C-4-H	Phosphate pH7.4/EtOH (9:1)	2,4-D	[56]

recognition sites for the analyte may be unable to accommodate the larger labelled molecule. The probe may bind at less precise sites, and the selectivity of the assay will be compromised. Nevertheless, MIAs have been developed following this principle using colorimetric [49–51], fluorescent [46,52,53], and enzyme labels [13,14,45–48,54] (Table 3). MIPs with recognition sites mostly at the surface, rather than within the polymer network, may be most useful in this strategy.

2. The labelled analyte is imprinted, and used as the probe. The recognition sites in the resulting MIP may however be complementary not just to features of the analyte moiety, but also to those of the label moiety. Thus, competitors resembling the label may compete successfully in the MIA and appear as 'false positives'. No successful MIA following this approach has been reported.
3. The native analyte is imprinted, an unrelated molecule is used as the probe. This strategy relies on the probe binding at the best imprinted sites. The ideal probe should have similar functionalities to the analyte, and not be larger than the analyte for the reasons in (1) above. It may bind quite weakly to the well-imprinted sites, but it must bind even more weakly to the poorly-imprinted sites. With a judicious choice of probe good results have been obtained [43,44,55].
4. An unrelated molecule is imprinted and employed as the probe. This strategy may suffer from similar disadvantages to (2), depending on the extent to which probe and analyte differ. In practice, only one example has been reported, in which the molecules differ only very slightly [56]. Finding an electroactive or fluorescent molecule with sufficiently high degree of structural similarity to an analyte may not always be possible.

4.2.1. Chromophore/fluorophore-labelled probes

The Karube group reported the first non-radiolabel MIA [52]. 1,3,5-Triazine was imprinted in a traditional acrylate polymer, and the fluorescent probe was 5-(4,6-dichlorotriazinyl)aminofluorescein (DCTAF) (Fig. 7). Triazine displaced more probe than did the substituted triazines simazine or atrazine, but the structure of the probe raises several questions: given the bulk of the fluorophore and of the two chlorine atoms attached to the triazine ring it seems surprising that it binds to many of the binding sites in a conventional triazine-imprinted MIP at all, in particular that it should bind to the most selective ones. The acidic groups on the fluorescein moiety might also be expected to give strong non-specific interactions with the basic MIP, whilst the chloro groups may react with any nucleophile present.

A chromophore-labelled probe was used for detection of chloramphenicol [49] but in this case the chloramphenicol-imprinted MIP was used in HPLC mode with the probe, methyl red-labelled chloramphenicol, incorporated in the mobile phase (Fig. 8) (Section 4.3). The approach was later refined [50] and another assay developed using a fluorophore-labelled probe, dansylated chloramphenicol,

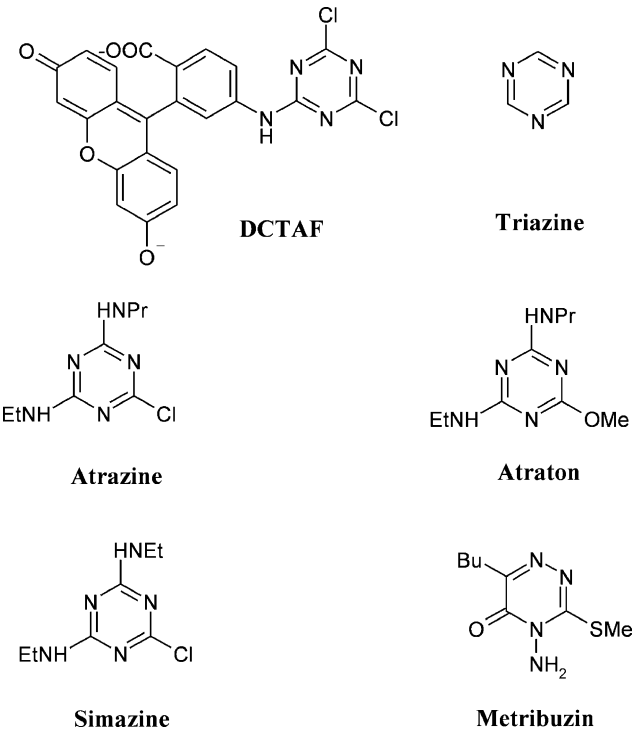

Fig. 7. Molecules used in fluorescent probe MIAs for triazine and atrazine [46,52].

which should exhibit better sensitivity and selectivity since it may be used at lower concentrations and hence to probe fewer (better) binding sites [53] (Fig. 8). The approach was also investigated for β-estradiol, but no competitive binding was observed [57]. A related approach using a colorimetric label was employed by Takeuchi et al. for biotin methyl

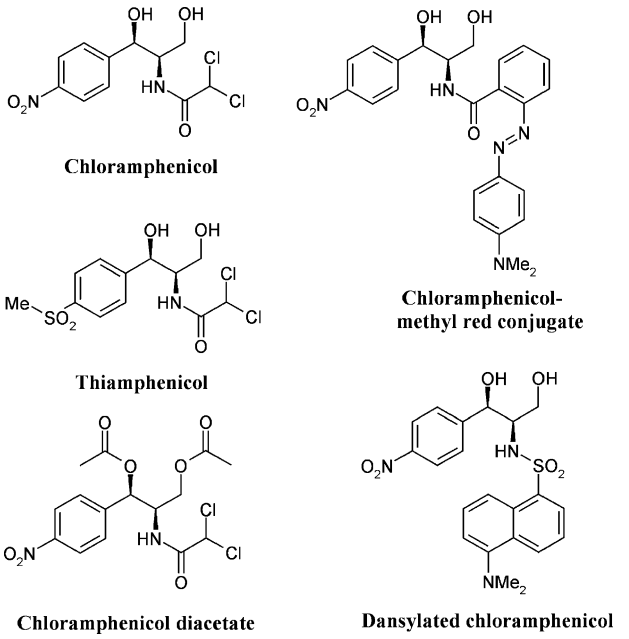

Fig. 8. Molecules used in flow-through MIAs of chloramphenicol [49,50,53].

ester but although the analyte successfully displaced the probe no competitors were studied [51].

DCTAF was also employed in a MIA for atrazine based on atrazine-imprinted MIPs formed in the wells of microtitre plates [46]. The MIPs were formed by a novel imprinting approach: the oxidative polymerisation of 3-thiophenylboronic acid and 3-aminophenylboronic acid in ethanol/aqueous potassium dichromate in the presence of atrazine, which yielded thin, coloured films. It is unclear how imprinting proceeds in this system. The results suggest that in the MIA, atrazine is more successful at displacing the probe than are the interferents Atraton (which differs from atrazine by just a methoxy/chlorine substitution) or Metribuzin (which is a molecule of very different structure, Fig. 7). It is unclear from the data presented how much of the probe is actually displaced and in combination with the concerns about the reactivity of this probe, and its structural difference to the analyte, more evidence is required to demonstrate that this system can be a useful MIA.

4.2.2. Enzyme labelled probes

Enzyme labels are popular in immunoassay because a small quantity of probe can easily be detected through amplification, so assays may achieve similar sensitivity to those using radiolabels. Enzymes even more than fluophores or chromophores present the problem of size and demand accessible recognition sites at the surface of the MIP: the Mosbach/Haupt/Danielsson group used 2,4-D imprinted TRIM-4-VPy microspheres, either in suspension [13] or trapped in hydrogel films in microtiter plate wells [45]. The microspheres were first employed in a conventional radiolabel MIA for 2,4-D. Fig. 9 illustrates some of the structural analogues investigated as competitors. Table 4 shows that relative to the fine particles used by Haupt et al. the microspheres led to a 20-fold increase in IC_{50} to about 10 μg ml^{-1} (so loss in sensitivity), but exhibited similar selectivity. Then, tobacco peroxidase-labelled 2,4-D was employed as probe in phosphate buffer, and the unbound probe was quantified using either a colorimetric

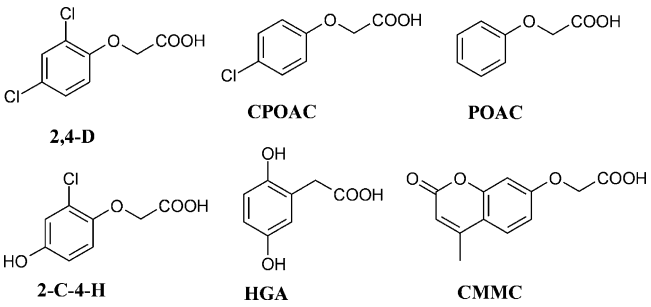

Fig. 9. 2,4-D, analogues and non-related probes studied in MIAs [13,36,43–45,47,48,55,56]. 2,4-D: 2,4-dichlorophenoxyacetic acid; CPOAC: 4-chlorophenoxyacetic acid; POAC: phenoxyacetic acid; CMMC: 7-carboxy-methoxy-4-methylcoumarin; HGA: homogentisic acid (2,5-dihydroxyphenylacetic acid); 2-C-4-H: 2-chloro-4-hydroxyphenoxyacetic acid.

assay (1,4-phenylenediamine substrate) or a chemiluminescence assay (luminol substrate). 2,4-D displaced probe from the MIP: the chemiluminescence assay was more sensitive, less conjugate was required and the IC_{50} value was about 20 μg ml^{-1}. Selectivity was excellent in both cases. Subsequently, the chemiluminescence assay was adapted to microtiter plates with detection of the bound probe via an imaging CCD camera [45]. Further, the enzyme MIA was adapted to a capillary flow-injection format [47,48] as described in Section 4.3. The IC_{50} for 2,4-D using this approach was similar to the original radiolabel MIA whilst selectivity was even better (Table 4).

Piletsky et al. employed an enzyme-labelled probe in a MIA with their novel microtitre plate polymers. Poly-3-aminophenylboronic acid films were prepared in the presence of epinephrine [14,46]. Horseradish peroxidase-labelled norepinephrine was employed as probe in phosphate buffer. Epinephrine exhibited an IC_{50} value about 10 μM with other benzenediols competing less. It remains unclear how recognition sites are formed in such polymers and the stability of the polymer is also unproven. Recently, Piletsky et al. have published an enzyme-MIA for the cyanobacteria toxin microcystin-*L*,*R*, employing

Table 4
Results obtained in various MIAs for the herbicide 2,4-D

MIA	IC50 (2,4-D) (ng ml^{-1})	Cross-reactivities (%)	
		CPOAC	POAC
Radiometric, fine particles [36]	~0.5	24	2
Radiometric, microspheres [13]	~10	25	nd
Enzyme-linked colorimetric, microspheres [13]	~200	<1	<1
Enzyme-linked chemiluminescence, microspheres [13]	~20	<1	<1
Enzyme-linked microplate, immobilised microspheres [45]	~10	32	3
Enzyme-linked flow-injection, coated capillary [47,48]	~1	<1	<1
Fluorescent probe (CMMC), fines [43,55]	~1000	42	9
Fluorescent probe (CMMC), fines, MeCN [43,55]	~400	50	14
Electroactive probe (HGA), fines [44,55]	~5000	nd	nd
Electroactive probe (2-C-4-H), fines [56]	~4000	nd	nd

IC50 values were estimated by this author from graphs of signal vs. concentration presented in each work. All MIAs were performed in phosphate buffer pH 7 with ethanol or surfactant added, except the fluorescent probe (CMMC) assay performed in acetonitrile. nd: not determined.

conventional EDMA cross-linked MIP particles with diameter 45–63 μm [54]. The MIA is conducted in phosphate buffer pH 7, using microcystin-*L*,*R*-horseradish peroxidase conjugate as a probe. It seems unlikely that such a large probe can successfully interrogate many of the good binding sites in such a polymer. Although the results suggest the probe is displaced by the analyte, and related cyclic peptides displace less, the data presented are incomplete.

4.2.3. Non-related probes

A MIA using a non-related fluorescent probe was demonstrated by Haupt and co-workers [43,55] again for 2,4-D. 7-Carboxy-methoxy-4-methylcoumarin (CMMC) was used as a probe (Fig. 9) with the 2,4-D MIP fine particles previously applied in a radiolabel MIA. Comparing the structure of the probe with the template, it may be seen that many features are shared, in particular the oxyacetic acid side chain which probably interacts with pyridine groups in the recognition sites. MIAs were developed in phosphate buffer pH7 and in acetonitrile, nonbound CMMC being measured via fluorimetry. Relative to the radiolabel MIA, the IC_{50} for 2,4-D increased greatly but the selectivity was only slightly compromised (Table 4). A non-related electroactive probe, homogentisic acid (HGA) (Fig. 9) was then studied in work aiming toward the development of a 2,4-D sensor [44]. The probe is less similar to 2,4-D than CMMC, and in preliminary MIA studies with electrochemical detection a higher concentration of 2,4-D was required to displace 50% of the probe, and selectivity was not studied (Table 4). A related study was reported by Schollhorn et al. using the electroactive analogue 2-chloro-4-hydroxyphenoxyacetic acid (2-C-4-H) as both imprint molecule and probe [56]. The IC_{50} value for 2,4-D was again high and no competitors were studied.

4.3. Novel assay formats

4.3.1. Flow-through MIA

In the first flow-through MIA, developed by Levi et al. for chloramphenicol [49], the probe was present at constant concentration in the constant flowing mobile phase, so a constant baseline absorbance was detected. When samples containing analyte or interferent (plus the probe, added at the same concentration as in the mobile phase) were injected, the probe was displaced, and detected eluting from the column as a peak of increased absorbance. Chloramphenicol displaced more probe than thiamphenicol, with chloramphenicol diacetate cross-reacting minimally. Parameters such as the probe concentration and flow rate were investigated. Chloramphenicol in bovine serum was quantified after extraction, with a limit of detection about 5 $\mu g\,ml^{-1}$. Similar approaches were used in the works of McNiven et al. [50], Suarez-Rodriguez et al. [53], Rachkov et al. [57] and Takeuchi et al. [51]. Unfortunately, in none of these cases were the results compared with static, equilibrium MIAs using the same reagents, which would have provided a useful indicator of the relative qualities of the two approaches. The limit of detection for chloramphenicol quoted above is relatively average compared with most conventional equilibrium-based MIAs. The flow-through MIA certainly has an advantage in terms of handling, although the necessity of running samples in series rather than parallel is a disadvantage.

4.3.2. Flow-injection MIA

In the capillary enzyme MIA developed by Surugiu et al. [47,48] the probe is not present at constant concentration but simply injected together with the analyte. 2,4-D imprinted MIP was formed on the inner wall of a capillary as described in Section 4.1. After extracting template, 2,4-D-peroxidase conjugate and free analyte in phosphate buffer were passed through the capillary. Binding of the probe was sufficiently strong that after excess probe and analyte were eluted the bound probe remained on the MIP. After a washing step, the luminol substrate was injected and the bound probe quantified from the emitted light. The capillary could be regenerated by flushing with glycine buffer. The results using this approach were excellent (Table 4), though the stability of the capillary and reproducibility remain to be proven.

4.3.3. Scintillation proximity assay

Homogenous binding assay formats are highly popular with biological immunoassays because there is no need for physical separation of solid phase and supernatant: reagents are simply added and measurements recorded. Such assays are therefore well-suited to automation and high throughput. The scintillation proximity MIA developed by Ye and Mosbach [31] was the first non-separation MIA. They used microsphere MIPs imprinted with *S*-propranolol and containing the scintillation monomer 4-hydroxymethyl-2,5-diphenyloxazole acrylate. Binding of the probe ^{3}H-*S*-propranolol to the microspheres enabled excitation of the scintillant and photoemission, which was suppressed when analyte was present and displaced the probe (Fig. 10). The initial MIA was performed

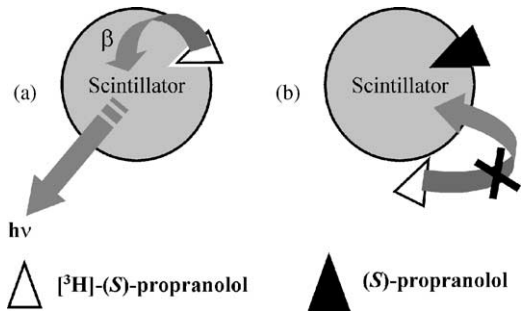

Fig. 10. Schematic of the scintillation proximity MIA for (*S*)-propranolol [31,33]: (a) the bound, tritium-labelled (*S*)-propranolol triggers the scintillator to generate the fluorescent light; (b) when the tritium-labelled (*S*)-propranolol is displaced by the unlabelled (*S*)-propranolol, it is too far from the antenna and the scintillator to efficiently transfer the radiation energy; therefore, no fluorescence can be generated.

in toluene/AcOH (99.5:0.5) and appeared to be of similar sensitivity to the bulk MIP MIA developed by Andersson [29], and similar selectivity against *R*-propranolol, although data did not extend far enough to determine IC_{50}s. Toluene was required in order to effect energy transfer between the β-emitter and the scintillant: the assay did not work in other solvents. This was addressed in later work by incorporating DVB in the MIP as cross-linker [33]: its aromatic groups fill the same relay function as the toluene. The new MIPs were used in MIAs in acetonitrile/acetic acid (99.5:0.5, v/v/v) and in acetonitrile/citrate buffer pH6 (50:50, v/v). Both MIAs employed polymer concentrations of 0.2 mg ml^{-1} and the IC_{50} values for *S*- and *R*-propranolol were approximately 10^3 and 10^5 ng ml^{-1} (in organic solvent), 10^3 and 3×10^4 ng ml^{-1} (in cosolvent mixture). The better stereoselectivity of the organic MIA corresponds to the observations of Andersson on his MIA using bulk *S*-propranolol imprinted MIPs. A concern is the relative inefficiency of imprinting in microsphere MIPs in some cases, but if a MIP can be made for an analyte in this way scintillation proximity MIA should be generally applicable and represents an exciting step forward.

5. Conclusion

MIPs provide a complementary range of reagents to antibodies for application in ligand binding assays, in particular for small molecules. Many of the perceived drawbacks of MIPs do not hinder their successful application in MIAs: many aqueous-phase MIAs have been demonstrated (but the ability to perform MIA in organic solvents may sometimes be useful), the quantity of template needed in MIP syntheses may be dramatically reduced when they are to be used in MIAs, the presence of a heterogenous distribution of binding sites is not a problem provided that the fraction of sites which bind the analyte strongest are highly selective.

Rapid advances are being made in terms of new MIP formats and new assay designs replacing radiolabels with fluorophores, electroactive groups and enzyme labels. The range of probes investigated with 2,4-D suggest that formats using alternative probes are often just as selective as radiolabel MIA. The best combination of sensitivity and selectivity has been achieved with the capillary-based enzyme MIA. Methods based on MIPs in microtitre plates, particularly in combination with enzyme-labelled probes, offer the advantages of high throughput and although the preparation of MIPs in such formats need further investigation, it is anticipated more such assays will be developed. Similarly the scintillation-proximity MIA has the advantage of requiring minimal manipulation and should be suitable for automation.

In many cases studies so far have been limited to proof-of-principle and it is expected that there will be more emphasis on demonstrating complete analytical procedures based on MIA as the field becomes more widely accepted.

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