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VERSITA

MIP-based Immunoassays: State of the Art, Limitations and Perspectives

Abstract
Immunoassay is one of the most popular analytical methods with widespread applications. However, it presents several drawbacks because of the proteic nature of the antibodies. Molecular imprinting technology has shown a growing ability to prepare artificial molecular recognition systems, with binding properties very similar to those of natural antibodies. This review deals with the application of molecular imprinting technology to immunoassay, with an attention for the state of the art, the current limitations and the possible solutions to these issues.

Keywords
Molecularly imprinted polymers • Plastibodies • Molecularly imprinted sorbent assay • Immunoassay • Radioimmunoassay • Enzyme immunoassay • Fluoroimmunoassay

Claudio Baggiani*,
Laura Anfossi,
Cristina Giovannoli

Department of Chemistry,
University of Torino,
Torino, Italy

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1. Introduction

People who work in the field of molecular imprinting often describe these materials as “plastibodies”, a sort of artificial antibodies which share with natural antibodies the same binding behaviour [1]. In fact, molecularly imprinted polymers (MIPs) show a set of binding properties apparently very similar to those of natural antibodies (immunoglobulins): a marked selectivity towards the related ligands (antigens for antibodies, template molecules for MIPs) and binding properties raising form multiple reversible non-covalent interactions, characterized by well-defined thermodynamics and kinetics. These similarities (Table 1) are remarkable as one considers the fact that the structure and the genesis of natural antibodies and MIPs are completely different. Antibodies are natural biomacromolecules of proteic nature produced by animals in response to an external immune stimulus [2]. Through a mechanism of clonal selection, not related to any conceivable imprinting mechanism, the cells of the immune system delegated to the production of antibodies are able to generate with high efficiency antibodies against virtually any foreign antigen, ranging from toxins and xenobiotic molecules to viruses and large bacteria. On the contrary, molecularly imprinted polymers are man-made artificial receptors obtained through a real imprinting mechanism during a polymerization process, where the presence of a template molecule inside the emerging cross-linked polymeric structure is able to induce the formation of stable binding sites with molecular recognition properties towards the same template or strictly related molecules.

It is therefore not surprising that many investigators have thought of using polymers as artificial receptors in immunoassay-like analytical applications. From the well-known seminal work of Mosbach and co-workers about a radiotracer assay for theophylline and diazepam based on very simple imprinted polymers [3], this idea has been subsequently reissued in a number of experimental works all based on the same principle: to replace natural antibodies with artificial receptors in so-called “molecularly imprinted sorbent assay”. This review wants to give a general overview of the most of the available literature, discussing which are the potential benefits and drawbacks of replacing natural antibodies with molecularly imprinted polymers.

2. What is immunoassay?

The immunoassay techniques have been introduced for the first time about fifty years ago simultaneously by Ekins for thyroid hormones [4] and Berson and Yalow for insulin analysis [5]. Both described competitive radioimmunoassays, and the main difference was that Berson and Yalow’s insulin assay used an antibody as the receptor whilst Ekins’ assay for thyroxin used the naturally occurring thyroxin binding globulin as the receptor. The development of immunoassay is perhaps one of the most important discovery in modern analytical biochemistry. It has made possible the easy quantification of substances hitherto either immeasurable or measurable using only lengthy and labour-intensive bioassays. Subsequently, it has pervaded most, if not, all branches of the biological sciences and has

* *E-mail: claudio.baggiani@unito.it*

Table 1. A comparison between natural antibodies and molecularly imprinted polymers.

	Natural antibodies	Molecularly imprinted polymers
low-mass molecules (< 5000 Da) as immunogen / template	yes, but necessity of a covalent linker between the immunogenic carrier protein and the low-mass antigen could affect the bindig selectivity of resulting antibodies	yes, with exception for poorly functionalized or very low-mass molecules. Difficult for very polar templates
high-mass molecules (>5000 Da) as immunogen / template	yes	yes, but with marked experimental difficulties for large proteins (difficult template release, poor selectivity)
binding mechanism	well known	known, but some aspects under debate
binding affinity spectrum	discrete and narrow for monoclonal antibodies, continuous and broad for polyclonal antibodies	discrete and narrow for covalent imprinting, continuous and broad for not-covalent imprinting
mean affinity constant	frequently above 10^9 M^{-1}	rarely exceeds 10^7 M^{-1} for bulk-imprinted polymers
binding site density	low, μM	high, mmoles/g
binding kinetics	fast association, slow dissociation	slow association and dissociation
binding selectivity	high, fine tuning for monoclonals feasible. Difficult to be obtained for classes of ligands	high, fine tuning difficult when non-covalent approach is used
reproducibility	limited from batch-to-batch	very high
non-specific binding	negligible	depending from experimental conditions, rarely negligible
resistance to extreme experimental conditions (pH, cold, heat, sonication, organic solvents, denaturing agents)	no	yes
resistance to biological agents	no	yes (can be autoclaved)
needs of a solid phase as support	yes, this frequently involves the use of complex covalent coupling reactions	no, the polymer itself can be the support
reuse	very difficult	yes
cost for single batch	low for polyclonals, medium to high for monoclonals	very low (except for expensive templates)
commercial availability	high, frequently produced on demand	limited
in-house feasibility	no, a stabularium, trained people and a dedicated laboratory (monoclonals only) are necessary	yes, simple to make, with exceptions for advanced polymerization methods or mimic template approaches
health risks	not significative	sub-micrometric particles can be dangerous if inhaled. Some polymerization reagents (acrylamide, styrene, vinylpyridine) are toxic
literature	very large	very large and rapidly growing
state of the art	mature	in continuous evolution

become a vital tool in the clinical sciences. Nowadays, not only these techniques cover a pivotal function in clinical chemistry, but, under many formats, they play a growing role in different analytical fields of interests, involving analytes of environmental, food or forensic origin [6].

Immunoassay techniques are essentially based on the antibody-antigen reversible reaction and the subsequent quantitative measurement of the extant chemical species at the equilibrium. The specific interaction between antibodies and antigens provides the required analytical selectivity, while the high equilibrium constant provides high sensitivity for the assay. In consequence, the use of antibodies as highly selective analytical reagents in immunoassay is particularly appealing as it allows the direct quantitative analysis of sub-trace level analytes in extremely complex samples, with minimum pre-treatment of the matrix.

According to Ekins [7], immunoassay techniques can be classified through three basic criteria:

(1) the presence/absence of a tracer molecule able to bind the analytical antibody (labelled / unlabelled assays). In the unlabelled format, the binding of the analyte to the analytical antibody is measured directly through the monitoring of physical of bulk effects due to the formation of the analyte-antibody complex. In the labelled format a tracer molecule similar to the analyte, able to generate a strong analytical signal is used to measure the extent of the analyte-antibody complex. Usually, tracers consist of molecules of analyte or an analogue marked with radionuclides (radioimmunoassay), enzymes

(enzyme immunoassay), fluorescent or luminescent molecules (fluorescence and luminescence immunoassay);

(2) the presence/absence of a competitive equilibrium between tracer and analyte for the binding site of the analytical antibody (competitive / non-competitive assays). In the competitive format (Figure 1) a variable amount of analyte compete with a fixed amount of tracer for a fixed and limited amount of antibodies, which is insufficient to bind all of the analyte and tracer molecules. The multiple equilibria due to the competition reaction results in a decrease of the analytical signal produced by the tracer when an increasing amount of analyte is present in the sample and it is bound to the analytical antibody. In this kind of assay the analytical antibody is usually immobilized on a solid surface and the competition is in solution between the analyte and a tracer. Alternatively (Figure 2), a fixed amount of analyte is immobilized onto a solid surface and it competes with the analyte in the sample for a fixed amount of labelled analytical antibody introduced in solution.

In the non-competitive format (Figure 3) an excess of analytical antibody is bound to the analyte, and the complex is then detected with a tracer able to bind the complex but not the free, non-bounded antibody in excess. This format is typical

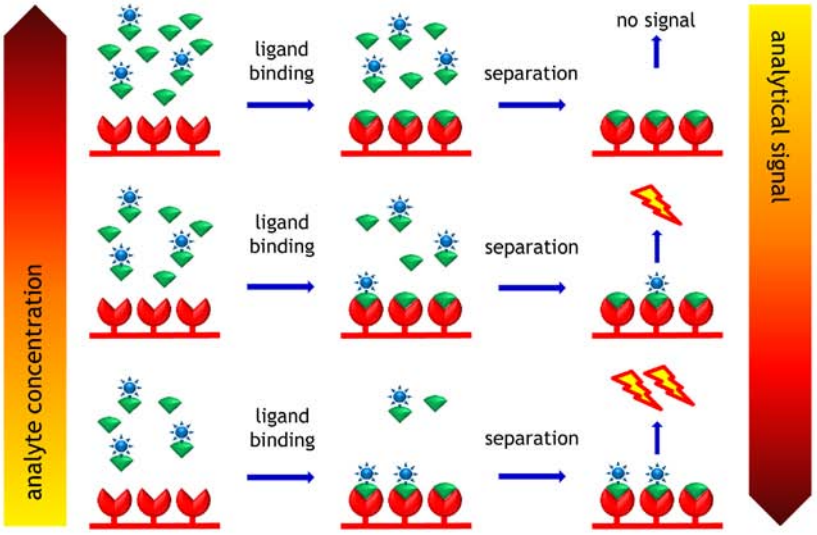


Figure 1. Schematic representation of a labelled, competitive, heterogeneous immunoassay with binding equilibria between a variable amount of analyte and a fixed amount of tracer for a fixed and limited amount of analytical antibody immobilized onto a solid surface.

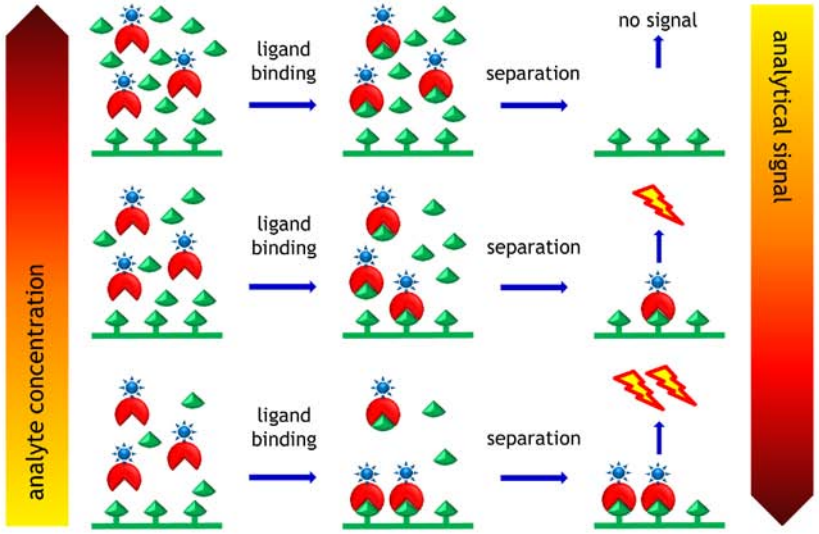


Figure 2. Schematic representation of a labelled, competitive, heterogeneous immunoassay with binding equilibria between a variable amount of analyte and a fixed amount of labelled analytical antibody for a fixed and limited amount of analyte immobilized onto a solid surface.

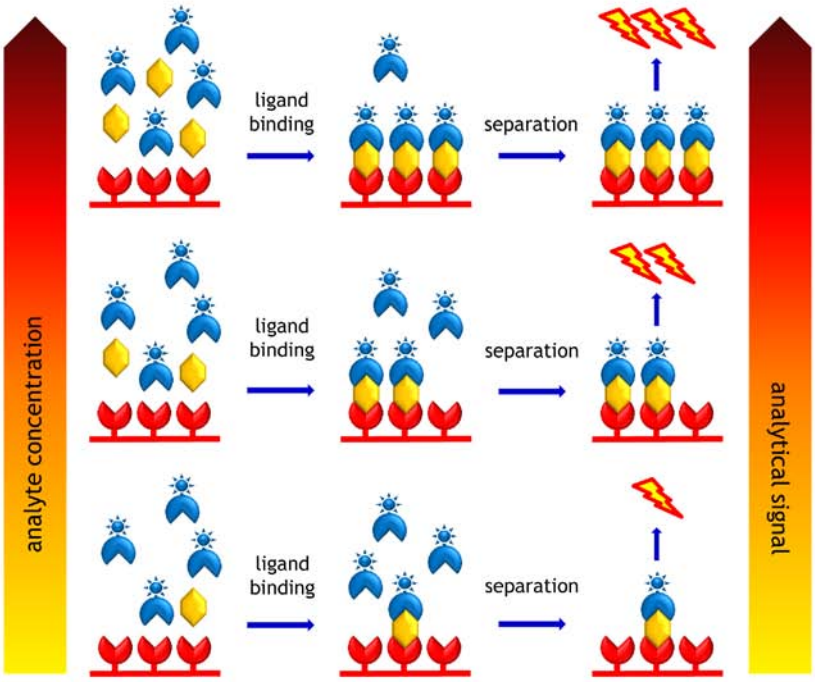


Figure 3. Schematic representation of a labelled, non-competitive, heterogeneous immunoassay (sandwich assay), where an excess of analytical antibody is bound to the analyte, and the complex is then detected with a tracer able to bind the complex but not the free, not-bounded antibody in excess.

of the so-called "sandwich assay", where the analyte is large enough to have portions of the surface (epitopes) recognized by different, non cross-reacting, antibodies without any steric impediment. In this case, one of the antibodies (capture antibody) is bound in excess to a solid surface, then the analyte is absorbed by the capture antibody and the complex is quantified with the appropriately labelled second antibody.

(3) the presence/absence of a separation step between the free and the antibody-bound tracer molecule (homogeneous / heterogeneous assays). In the homogenous assay the analyte bound to the analytical antibody does not need to be separated from the remaining free analyte as the analytical signal depends only by the presence of the immuno complex. On the contrary, in the heterogeneous assay the analyte bound to the analytical antibody must to be separated from the remaining free analyte before the development of the analytical signal.

3. Molecularly imprinted sorbent assays

From the seminal work of Mosbach and co-workers [3], several papers concerning the design and the development of molecularly imprinted sorbent assays have been published. However, it is remarkable that of the eight possible formats resulting from the classification seen in the previous section, only assays based on labelled (except some fluorescence-based assays, that are unlabelled, see section 3.3), competitive, homogenous or heterogeneous formats have been reported. There may be many justifications to this fact, but mostly the absence of unlabelled assays can be explained by reasons of their poor sensitivity due to the usually low analytical signal generated by the ligand, while the absence of non-competitive assays may also be explained by the fact that, currently, it is quite difficult to obtain efficient MIPs with such characteristics, i.e., nanosized polymeric structures and molecular recognition properties focused on different epitopes present in the same target molecule. In consequence, it seems more appropriate to review the available literature in terms of the nature of the used tracer, and taking into the account that the overwhelming majority of the MIP-based immunoassays are labelled and competitive by default.

3.1. Radiochemical tracers

Immunoassay based on radiochemical tracers (radioimmunoassay), typically ^{3}H ^{14}C or ^{125}I-labeled molecules, has been a technique of enormous success, as the labelled ligand show the same binding behaviour of the target analyte, thus avoiding unwanted binding differences between tracer and analyte in the equilibrium reaction with the antibodies. In spite of the success of this technology, expensive costs to prepare labelled compounds not available in the market and growing concerns about the hazards of manipulate radiochemicals, united to legislative restrictions to detention, use and disposal of radionuclides caused the progressive decrease of popularity of this technique, and the concomitant raise of alternative, less troublesome, tracers.

The first molecularly imprinted sorbent assays reported in literature was a heterogeneous assay involving the use of

radiochemical tracers [3]. In this work molecularly imprinted ethylene dimethacrylate-co-methacrylic acid (EDMA-co-MAA) polymers for theophylline and diazepam were prepared by bulk polymerization and used to detect the corresponding analytes by competition with tritiated tracers. Even if selectivity in aqueous buffer and blood samples was not observed, the assays performed in organic extracts showed a good level of sensitivity (detection limits: 3.5 μM for theophylline, 0.2 μM for diazepam) and very good selectivity (Tables 2 and 3). It is remarkable that figure of merits resulted comparable with several corresponding commercially available immunoassays, demonstrating for the first time the feasibility of MIP-based immunoassays.

The feasibility of molecularly imprinted sorbent assays performed in organic solvents has been further demonstrated in the years immediately prior to 2000 by the same research group, describing assays based on EDMA-co-MAA imprinted polymers for various target analytes: corticosteroids [8], morphine and [Leu^5]encephalin [9], cyclosporine A [10] and atrazine [11]. When

Table 2. Cross-reactivity of template analogs to the binding of [^{3}H]-theophylline in toluene to a theophylline-imprinted MIP compared with correspondent commercial radioimmunoassay [3].

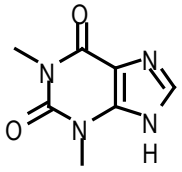
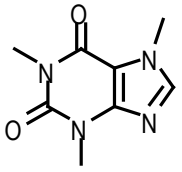
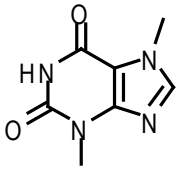
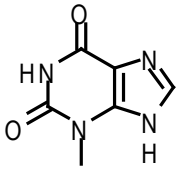
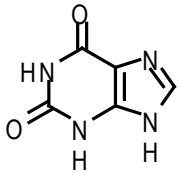
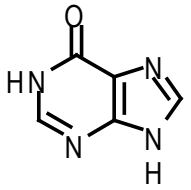
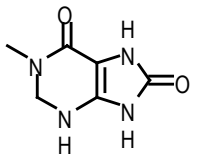
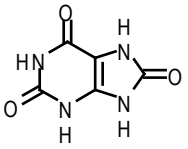
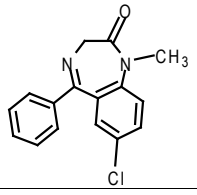
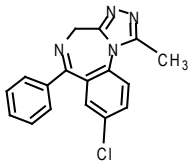
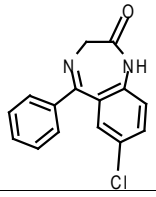
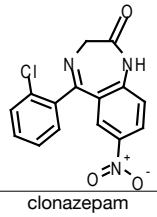
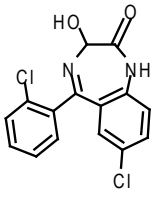
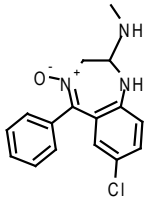
			
theophylline CR_{50}MIP: 100 CR_{50}Ab: 100	caffeine CR_{50}MIP: <1 CR_{50}Ab: <1	theobromine CR_{50}MIP: <1 CR_{50}Ab: <1	3-methylxanthine CR_{50}MIP: 7 CR_{50}Ab: 2
			
xanthine CR_{50}MIP: <1 CR_{50}Ab: <1	ipoxanthine CR_{50}MIP: <1 CR_{50}Ab: <1	1-methyluric acid CR_{50}MIP: <1 CR_{50}Ab: <1	uric acid CR_{50}MIP: <1 CR_{50}Ab: <1

Table 3. Cross-reactivity of template analogs to the binding of [^{3}H]-diazepam in toluene to a diazepam-imprinted MIP compared with correspondent commercial radioimmunoassay [3].

		
diazepam CR_{50}MIP: 100 CR_{50}Ab: 100	alprazolam CR_{50}MIP: 40 CR_{50}Ab: 44	desmethyldiazepam CR_{50}MIP: 27 CR_{50}Ab: 32
		
clonazepam CR_{50}MIP: 9 CR_{50}Ab: 5	lorazepam CR_{50}MIP: 4 CR_{50}Ab: 1	chlordiazepoxide CR_{50}MIP: 2 CR_{50}Ab: <1

corticosterone and cortisol were used, the assays performed in tetrahydrofuran showed detection limits in the range 10-100 nM for both the analytes, while selectivity towards several related corticosteroids was very good, with cross-reactivity under 10% level and in accordance with selectivity measured for several commercial polyclonal antiserums.

Assays for morphine and [Leu^5]encephalin were performed in mixed organic-aqueous medium, obtaining selectivity comparable with several commercially available monoclonal antibodies (Table 4). Interestingly, the assay for [Leu^5]encephalin was developed by using the corresponding anilide as template mimic to enhance its solubility in the porogenic solvent during the polymerization process.

The assay for the immunosuppressant drug cyclosporine A was performed in diisopropyl ether after direct extraction of haemolysed blood samples. It showed good sensitivity at 100 ng/ml level not only for the template, but also for several related and clinically relevant metabolites.

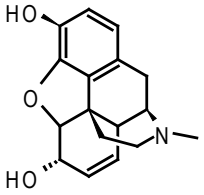
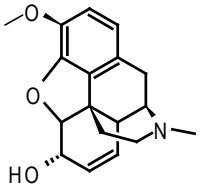
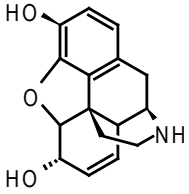
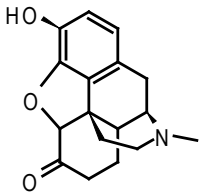
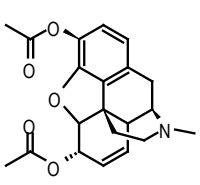
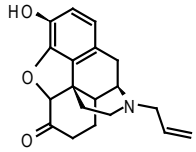
Atrazine was used to develop an assay working in toluene with a resulting limit of detection of about 0.1 μM. The authors evaluated the selectivity with a quite large library of potentially interfering substances, finding a negligible cross-reactivity for most of these, except for terbutylazine and desisopropylatrazine molecules very similar to the templating agent whose cross-reactivity was found to be significant (CR_{50}: 44% for terbutylazine and 53% for desisopropylatrazine). It is emphasized that also in this case the pattern of cross-reactivity is comparable to that reported in an aqueous environment in the case of mono- and polyclonal antibodies. A very similar assay for atrazine was independently developed by Moldoon and Stanker [12]. The assay, developed using ^{14}C-atrazine as radiotracer, was optimized in acetonitrile, resulting with a dynamic range of 1-100 μg/ml. Selectivity was good for ametryne and deethylatrazine, but not for propazine, which was recognized with a CR_{50} of 75%.

In the same period, development of MIP-based assays working in aqueous buffers were described for yohimbine [13], (S)-propranolol [14,15] and 2,4-dichlorophenoxyacetic acid (2,4-D) [16]. When performed in phosphate buffer at pH 5, the assay for the α2-adrenoreceptor antagonist yohimbine showed a very good selectivity with respect to the very similar analog corinanthine, with IC_{50} of 1.8 μM (corinanthine IC_{50} = 340 μM) and an estimated limit of detection at level of 0.2 μM.

The assay for the β-blocker (S)-propranolol optimized in citrate buffer at pH 6 showed high template selectivity with respect to several structurally-related molecules ((S)-propranolol IC_{50} = 0.52 μM, (R)-propranolol IC_{50} = 3.0 μM, (R,S)-atenolol IC_{50} = 76 μM, (R,S)-metoprolol IC_{50} = 170 μM, and (R,S)-timolol IC_{50} = 770 μM) and a detection limit for the target analyte of 6 nM. When toluene was substituted to the aqueous buffer polymer in the assay, different selectivity profiles were obtained and the values for IC_{50} decreased of about one order of magnitude for all the ligands considered. The authors explained this fact as due to a different balance between hydrophobic and polar interactions in toluene and water, since polar interactions, such as hydrogen bonds, are strong in non-polar solvents and hydrophobic interactions are strong in water. The same effects were observed when the polymer formulation was modified by changing the cross-linker from ethylene dimethacrylate to trimethylolpropane trimethacrylate.

A EDMA-co-4VP (ethylene dimethacrylate-co-4-vinylpyridine) polymer was used to develop an assay for the herbicide 2,4-dichlorophenoxyacetic acid. The effect of the buffer pH was studied in the range 3-9, finding a significant

Table 4. Effect of medium on the cross-reactivity of template analogs to the binding of [^{3}H]-morphine to a morphine-imprinted polymer [9].

		
morphine CR_{50}(buffer): 100 CR_{50}(toluene): 100	codeine CR_{50}(buffer): 25 CR_{50}(toluene): 4.7	normorphine CR_{50}(buffer): 9.9 CR_{50}(toluene): 8.3
		
hydromorphone CR_{50}(buffer): 15 CR_{50}(toluene): 6.0	heroin CR_{50}(buffer): 8.3 CR_{50}(toluene): 2.3	naloxone CR_{50}(buffer): 0.4 CR_{50}(toluene): <0.1

radiotracer rebinding in acidic conditions. When phosphate buffer pH 7 was used, a working range of 0.135-45 μM with a detection limit of about 0.1 μM was obtained. Selectivity was good with respect to several structurally-related herbicides (CR_{50}: 24% for 4-chlorophenoxyacetic acid, 15% for 2,4-dichlorophenylacetic acid, 10% for 4-chlorophenylacetic acid, 7% for 2,4-dichlorophenoxyacetic acid methylester, 2% for phenoxyacetic acid and <0.1% for phenoxyethanol). Interestingly, the long chain analog 2,4-dichlorophenoxybutyric acid was recognized at the same level of the template (CR_{50}: 95%).

(S)-Propranolol was the target analyte considered for the development of a molecularly imprinted sorbent assay based on imprinted–magnetic iron oxide composite beads to separate the bound radiotracer from the unbound fraction [17]. Nanoparticles of magnetic iron oxide were incorporated using a suspension polymerisation methodology with a perfluorocarbon liquid as the dispersing phase for the preparation of imprinted TRIM-co-MAA acrylic acid superparamagnetic microbeads. The assay optimized in citrate buffer at pH 6 showed high template selectivity with respect to some structurally-related molecules: (S)-propranolol (IC_{50}=0.19 μM), (R)-propranolol (IC_{50}=1.0 μM) and (R,S)-metoprolol (IC_{50} = 26.5 μM).

With the aim to facilitate the assay equilibration and the mass transfer process during the ligand rebinding process, competitive radiotracer assays have been developed for theophylline [18], 17α-estradiol [18] and 17β-estradiol [19] by using submicron-sized beads prepared by precipitation polymerization. The assays for theophylline and 17α-estradiol resulted highly selective for the template analytes when performed in acetonitrile. The assay for 17β-estradiol was not tested on real samples, but it was used to successfully screen for endocrine disrupting chemicals, showing high selectivity for the template molecule and a limited recognition for related 17α-estradiol, estrone, and 17α-ethynylestradiol, with a cross-selectivity of 14%, 5%, and 0.7%, respectively.

Beside heterogeneous radiotracer-based assays, an example of homogenous assay format has been applied for (S)-propranolol using the scintillation proximity approach [20]. In this approach a scintillation reporter monomer, 4-(hydroxymethyl)-2,5-diphenyloxazole acrylate was covalently embedded in imprinted microspheres. When tritium-labelled analyte was rebounded to the beads, the β-emission from the radiotracer caused the reporter monomers near the binding site to fluoresce proportionally. If used in competitive format, the fluorescence signal decreases in effect of the presence of unlabelled analyte that competes with the radiotracer molecules for the occupation of the binding sites. The assay, performed in mixed acetonitrile-citrate buffer solution, resulted selective for (S)-propranolol with respect to the (R)-enantiomer. It was optimized to detect (S)-propranolol in a working range of 1-1000 ng/ml, but no application on real samples was evaluated.

In conclusion of this section, it should be noted that, despite the undeniable advantage provided by the possibility of using tracers with molecular structure identical to the target analyte, the number of papers dealing with molecularly imprinted sorbent assays based on radiochemical tracers is largely decreased after the year 2000. As for antibody-based radioimmunoassay, the causes of this decline can be identified as unavailability in the market of isotopic-labelled tracers for many analytes of interests and to the legislative restrictions to detention, use and disposal of radionuclides.

3.2. Enzymatic tracers

The use of enzyme-labelled antigens has been described as early as 1968 and it has become of the most popular labels for immunoassays, largely superseding radioimmunoassay [21,22]. Its popularity is mainly due to the absence of drawbacks typical of radiotracers, the commercial availability of many enzymes at low cost and high degree of purity and the easiness of conjugation with small- and large-mass antigens. Moreover, many enzyme labels, notably horseradish peroxidase and alkaline phosphatase, can be assayed using simple colorimetric/fluorimetric reactions, thus not requiring more complex and expensive detection devices than an ordinary multichannel colorimetric/fluorimetric reader (microplate reader) [6].

The first enzyme-labelled molecularly imprinted sorbent assay reported in literature is a heterogeneous assay for the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) [23]. The tracer was prepared by direct labelling of tobacco peroxidase with 2,4-D, and the TRIM-co-4VP imprinted polymer was obtained by precipitation polymerization. When performed in phosphate buffer at pH 7, a preliminary assay performed with ^{3}H-labeled 2,4-D showed a very good selectivity with respect to some analogues of the template molecule (CR_{50}: 25% for 4-chlorophenoxybutyric acid, 10% for 4-chlorophenoxyacetic acid and <0.1% for phenoxyethanol). Calibration curves obtained from sampling the unbound enzyme tracer after the end of the competition reaction resulted in a dynamic range of detection from 40 to 600 μg/ml in colorimetric detection mode (o-phenylenediamine/H_2O_2) and from 1 to 200 μg/ml in chemiluminiscence mode (luminol/H_2O_2).

In a following work, the same authors describing a microplate-based assay based on the same 2,4-D-imprinted microbeads as described previously [24]. In this work, the microbeads were glued in microplate wells by using polyvinyl alcohol, and the amount of bound tracer was measured in chemiluminiscence mode (luminol/H_2O_2) in a high-throughput imaging format with a CCD camera. The calibration curve resulted in a dynamic range from 0.01 to 100 μg/ml. This assay was successfully applied to tap water spiked with the target analyte. In a further development, a flow-injection competitive assay with chemiluminiscence detection analogous to the enzyme assays seen previously was developed using a 2,4-D-imprinted polymer grafted onto the inner surface of a glass capillary [25]. In this assay the 2,4-D-enzyme conjugate was fluxed together with the analyte through the capillary. After a washing step, the chemiluminescent substrate was injected and the bound fraction was quantified. The calibration curve resulted in a dynamic range from 0.5 ng/ml to 50 μg/ml in continuous mode and 5 to 100 pg/ml in stopped-flow mode.

The development of imprinted microplates has been described by Piletsky and co-workers for an enzyme-labelled

assay for epinephrine [26,27]. In this approach, the bottom of the wells was grafted with a thin layer of imprinted poly-3-aminophenylboronic acid obtained by oxidation with ammonium persulphate. Interestingly, the enzymatic tracer was obtained by the conjugation between horseradish peroxidase and norepinephrine, an analogue of the target analyte. The calibration curve performed on the optimized assay in phosphate buffer at pH 6 resulted in a dynamic range from 1 to 100 μM when measured in colorimetric mode (ABTS/H_2O_2). The assay was found to be selective for epinephrine with respect to phenylephrine, (-)-isoproterenol, (+)-isoproterenol, norepinephrine and catechol. The same approach was used to prepare protein-imprinted microtitration plates with binding properties towards horseradish peroxidase, lactoperoxidase, microperoxidase and haemoglobin [28]. Rebinding studies and the determination of the affinity dissociation constant showed that the size and the charge of the template protein greatly affected the binding performances of the assay as negatively charged small proteins imprinted poly-3-aminophenylboronic acid was better than positively charged large proteins. Competition studies performed in phosphate buffer at pH 7 on haemoglobin-imprinted microplates showed a certain degree of selectivity with respect to proteins like bovine serum albumin penicillin G acylase.

The use of 3-aminophenylboronic acid oxidation to prepare film of controlled thickness in the bottom of microplate wells has been reported by Wang and co-workers in an assay for estrone [29]. The use of an ionic liquid in the polymerization liquid mixture allowed a reduction of the cracking and shrinking of the imprinted films, facilitating the access of the horseradish peroxidase-labelled conjugate to the binding sites. The calibration curve performed on the optimized assay in phosphate buffer at pH 7 resulted in a detection limit of 8 ng/ml with a dynamic range from 5 ng/ml to 20 μg/ml when measured in colorimetric mode (TMB/H_2O_2). The assay was found to be moderately selective for estrone (CR_{50}: 47% 17β-estradiol, 43% estriol, 30% progesterone and 40 diethylstilbestriol). The assay was applied to the analysis of estrone-spiked lake and river water at three concentration levels (100, 200, and 400 ng/ml) and validated by HPLC. The same authors have subsequently published some works about enzyme-tracer molecularly imprinted sorbent assays for the drugs ractopamine [30] and methimazole [31], and the pesticide trichlorfon [32]. The assay for ractopamin (IC_{50}: 15.8 ng/ml, detection limit 10 ng/ml) was applied to fortified urine and pork samples with recoveries ranging from 77.7% to 108.9% (urine) and from 93.5% to 101.1% (pork). The assay for methimazole (IC_{50}: 70 ng/ml, detection limit 1 ng/ml) was successfully applied to fortified urine samples with recoveries ranging from 90% to 95%. The assay for trichlorfon (IC_{50}: 6.8 μg/ml, detection limit 6.8 ng/ml) was successfully applied to fortified leek samples with recoveries ranging from 106% to 110.5%.

In conclusion of this section, the use of enzyme labels in molecularly imprinted sorbent assays is less problematic than the use of radiochemical tracers. However, some drawbacks are clearly present in enzymatic tracers. Imprinted polymers work well in organic or mixed aqueous/organic solutions but enzymes are sensible to inactivation in organic solvents than radiochemical or fluorescent tracers. Enzymes are biomacromolecules characterized by slow diffusion in nanometre-sized pores typical of imprinted polymers, thus assay kinetics can be impractically slow. Last but not least, imprinted polymers have moderately hydrophobic surfaces, prone to irreversibly adsorb biomacromolecules like enzymes, in this manner increasing the analytical signal due to non-specific interactions.

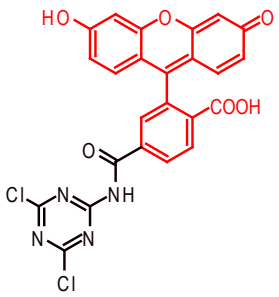
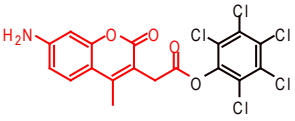
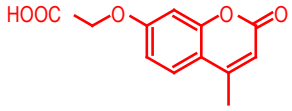
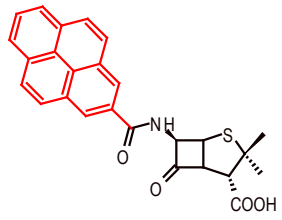
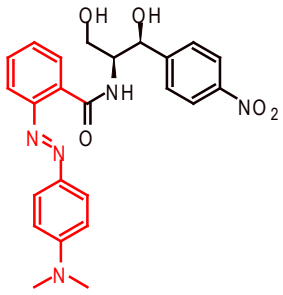
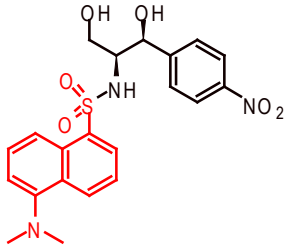
3.3. Fluorescent tracers

Usually, in molecularly imprinted sorbent assays based on fluorescent tracers the target analyte is not fluorescent, and a fluorescent tracer can be used in a competitive approach (Table 5). This approach solves the difficulty of obtain proper analytical signals from poorly or completely non-fluorescent targets, and it is strongly competitive to the approach consisting in the cumbersome derivatisation of the target analyte with a fluorescent tag before of a non-competitive assay. Even though, the main limit of this approach frequently is the difficulty to obtain an imprinted polymer able to bind in the same manner both the target and the fluorescent tracer [33].

The first fluorescent molecularly imprinted sorbent assay was reported in literature by Piletsky and co-workers. It describes a competitive approach for triazine [34]. In this work the competing label was a fluorescent analog of the analyte, 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein, and the analytical signal was given by the fluorescence of the free tracer at the equilibrium. The assay showed a dynamic range from 10 μM to 100 mM, and resulted selective for triazine with respect to atrazine and simazine. The same tracer was used in a microplate-based assay [27]. In this approach, the bottom of the wells was grafted with a thin layer of imprinted poly-3-aminophenylboronic acid obtained by oxidation with ammonium persulphate. The assay performed in phosphate buffer at pH 6 resulted in a detection limit of 8 μM.

A fluorescent competitive assay is described by Piletsky and co-workers for the determination of chlorophenols in water [35]. The assay was based on the competition between pentachlorophenol-aminomethylcoumarin acetate as tracer and chlorophenols for the binding to a pentachlorophenol-imprinted TRIM-co-urocanic acid polymer. The competition reaction was performed on microfiltration plates in phosphate buffer at pH 7. The amount of unbound tracer was measured by HPLC analysis of the fluorescent product of the enzymatic reaction. The optimized conditions resulted in a dynamic range from 0.1 to 25 μg/ml with a detection limit of 0.1 μg/ml). The assay was found to well recognize chlorophenols other than pentachlorophenol (CR_{50}: >95% 2,4,6-trichlorophenol, >95% 2,4-dichlorophenol, >95% 4-cyano-2,6-dibromophenol) but also several unrelated pesticides (CR_{50}: 62% γ-hexachlorocyclohexane, 61% mecoprop, 58% diuron, 57% isoproturon, 56% chlorpyriphos, 50% simazine, 45% glyphosate, 43% 2,4-dichlorophenoxyacetic acid, 37% hexachlorobenzene, 35% 2,4,6-trichlorobenzoic acid). The assay was applied to the analysis of pentachlorophenol in water and packaging materials.

Table 5. Examples of fluorescent tracers used in molecularly imprinted sorbent assays for analytes reported in brakets.

		
5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein (triazines) [27,34]	aminomethylcoumarin-PCP (pentachlorophenol) [35]	7-carboxymethoxy-4-methylcoumarin (2,4-dichlorophenoxyacetic acid) [35-37,40,41]
		
6-pyrenylamidopenicillanic acid (penicillins) [38,39]	chloramphenicol-methyl red conjugate (chloramphenicol) [42,43]	dansylchloramphenicol (chloramphenicol) [44]

A fluorescent competitive assay for the herbicide 2,4-dichlorophenoxyacetic acid in organic and aqueous solvents has been described by Haupt and co-workers [35,37]. In this approach the tracer is represented by 7-carboxymethoxy-4-methylcoumarin, a molecule with limited similarity with the analyte. Notwithstanding the poor molecular recognition of the tracer by the imprinted polymer, in phosphate buffer at pH 7, the assay showed a dynamic range from 0.1 to 50 μM, with a detection limit of 0.1 μM and a good selectivity towards the target template with respect to related herbicides.

A non-related fluorescent tracers has been used by Moreno-Bondi and co-workers to develop fluorescent assays with shared selectivity towards penicillins [38]. The fluorescent tracers were synthesized conjugating the 6-aminopenicillanic acid structure typical of all the penicillins with pyrene or dansyl fluorescent tags. A library of six imprinted polymers prepared with penicillin G as template was screened for the efficient rebinding of penicillin G and fluorescent tracer. Pyrene-labelled penicillin G and the TRIM-co-MAA imprinted polymer provided the best assay for penicillin G in HEPES buffer at pH 7.5, with a dynamic range from 3 to 390 μM and a limit of detection of 0.32 μM for penicillin G. Good recognition was seen for penicillin G-related antibiotics amoxicillin, ampicillin and penicillin V, but not for oxacillin, cloxacillin, dicloxacillin and nafcillin. Other antibiotics, such as chloramphenicol, tetracycline, or cephapirin were not recognized at all. An automated fluorescent assay for penicillins was subsequently developed by the authors using the same fluorescent tracer [39]. The detection limit was decreased to 0.197 μM with a dynamic range from 0.680 to 7.21 μM. Selectivity was observed for ampicillin, oxacillin, penicillin V, amoxicillin and nafcillin. The assay was successfully applied to spiked urine samples with excellent recoveries.

The use of imprinted core-shell microbeads prepared by controlled/living polymerization has been described by Lu and co-workers for a fluorescent competitive assay for the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) [40]. By using as tracer the structurally related fluorophore 7-carboxymethoxy-4-methylcoumarin, the authors obtained an assay in phosphate buffer at pH 7 with a dynamic range from 50 nM to 20 μM and a limit of detection of 10 nM. The use of surface-imprinted microbeads improved the assay performances with respect to bulk polymer but not the binding selectivity [36].

The same tracer has been used to develop an homogenous assay for 2,4-D based on the measurement of the decreasing of fluorescence polarization as a result of the binding events between the tracer, the analyte and imprinted nanospheres in phosphate buffer at pH 7 [41]. The limit of detection of the assay was 10 μM for 2,4-D, while selectivity was shown for the template molecule and the related herbicides 3,4-dichlorophenoxy acid and 2,4-dichlorophenoxybutyric acid.

In conclusion of this section, as in the case for enzyme tracers, the use of fluorescent tracers in molecularly imprinted sorbent assays is much less problematic than the use of radiochemical tracers. Moreover, fluorimetric detection can be

superior to colorimetric in terms of sensitivity of the assay and fluorescent tracers are more stable and solvent-compatible than enzymes. However , drawbacks may arise from the preparation of efficient fluorescent tracers as conjugates between fluorescent tags and analytes can be of difficult synthesis, products need to be isolated and purified, and the fluorescence properties (quantum yield, λmax of excitation/emission, etc.) of conjugates can be quite different from the same properties of the parent fluorophoric molecule.

4. Molecularly imprinted displacement assays

In the so-called "displacement assay", the antibody is covalently grafted onto a macroporous stationary phase packed in a capillary or in a chromatographic column. The binding sites of the antibody are saturated with a labelled antigen. Then, the analytical sample containing the unlabelled analyte is injected into the column, resulting in quantitative displacement of the bound tracer that is eluted and detected at the outlet of the column (Figure 4).

This assay has been applied using imprinted polymer instead of antibodies, using colorimetric or fluorimetric detection of the labelled antigens (Table 5). The first example is by Karube and co-workers [42]. A chloramphenicol-methyl red conjugate was used as colorimetric tracer in a competitive displacement assay on chloramphenicol-imprinted EDMA-co-DEAEM (N,N-diethylaminoethyl methacrylate) polymer packed in a HPLC column. Injections of chloramphenicol produced a concentration dependent displacement of the conjugate within a dynamic range from 5 to 1000 µg/ml with a detection limit of 5 µg/ml. The assay was successfully applied to serum samples spiked with 10-20 µg/ml of chloramphenicol or its analogue thiamphenicol. In a further work [43], the authors used a monolithic imprinted polymer prepared *in situ*, slightly decreasing the detection limit to 3 µg/ml and increasing the selectivity for the couple chloramphenicol/thiamphenicol. The same assay was described by Suarez-Rodriguez [44], where the tracer was the fluorescent conjugate between chloramphenicol and dansylchloride. The assay, optimized for a packed photometric flow-cell, was found to be strongly dependent form of the concentration of the tracer in the mobile phase and the sample volume. The detection limit was 8 µg/ml and the working range was up to 100 µg /ml.

The same approach was used by Piletsky and co-workers for the detection of several amino acids [45]. Rhodamine B was used as non-related fluorescent tracer in a competitive displacement assay on a L-phenylalaninamide-imprinted EDMA-co-MAA polymer packed in a HPLC column. Injections of template, D-phenylalaninamide, L-phenylalanine or L-tryptophan produced concentration dependent displacement of Rhodamine B from the polymer.

5. Current limitations and perspectives

The feasibility of sorbent assays based on molecularly imprinted polymers as artificial substitutes of antibodies has been demonstrated by the experimental studies reported in the previous sections of this review. However, with respect to other research fields typical of molecular imprinting technology (solid phase extraction, sensoristics etc.), molecularly imprinted sorbent assays seem to find some difficulties to spread in the scientific community. Furthermore, unlike imprinted polymers for solid phase extraction applications, immunoassay kits based on this kind of materials are completely absent from the multimillionaire commercial immunoassay market.

To gain some insights about this difficulty, it is necessary to consider the "main competitor" of molecularly imprinted sorbent assay: the immunoassay. It is a quite old analytical technique, largely diffused in the scientific community, and its popularity is due to several strong points given below:

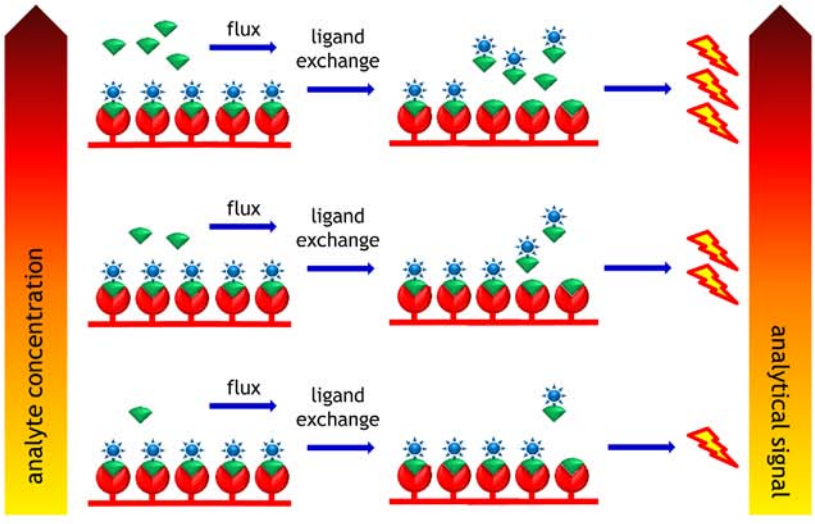


Figure 4. schematic representation of a displacement assay, where the antibody is immobilized onto a solid surface and its binding sites are saturated with a labelled antigen. The analytical sample containing the unlabelled analyte is added, resulting in a quantitative displacement of the bound tracer that is separated and quantified.

(1) high selectivity for target analyte also in very complex samples;

(2) no complex pre-treatment of the analytical samples required;

(3) high sensitivity (as a rule of thumb, in a competitive heterogeneous assay sensitivity a rough estimate of the possible detection limit is the inverse of the affinity constant);

(4) the methodological flexibility (competitive/non-competitive, labelled/unlabelled, homogeneous/heterogeneous);

(5) the variability in the nature of possible tracers, going from the most common radiochemicals, enzymes or fluorophores to rare spin labels [46] or marker-filled liposomes [47];

(6) mono- and polyclonal antisera against virtually any possible target analyte are commercially available or, alternatively, they can be produced with limited efforts;

(7) antibodies can be chemically engineered, expressed as binding peptides in phage-display technology or otherwise modified to encounter the analytical necessities of modern immunoassay [2,48];

(8) immunoassay is cheap, the cost-per-sample (comprehensive of costs of instrumentation and personnel) is low.

(9) assays developed and optimized at research laboratory level can easily be converted in robust and stable commercial-grade kits.

Today, by comparing features and performances of imprinted polymer and antibody-based assays (see Table 6), it is easy to see that molecularly imprinted sorbent assay can cope with several of these features, but many others issues seem yet to be problematic:

(1) the most important drawback is related to the low sensitivity and specificity of the imprinted polymers if compared with immunoassay-grade antisera. Assay sensitivity can be related to the binding affinity of the analytical antibody used in the assay. Unfortunately, it seems that the present state–of-the-art is not able to give us imprinted polymer characterized by very high binding affinity and enhanced selectivity. As recently observed [49], MIP/NIP pairs show a strong proportionality in the values for the binding affinities towards template molecules; thus, increasing the affinity of a MIP for a target molecule means to increase the affinity of the related NIP and, consequently, the probability that an interfering molecule could be bound by the MIP increase proportionally. At present, it is not clear if a recently proposed affinity technique which authors claim for able to isolate fractions of polymers with enhanced binding properties from pools of imprinted nanobeads will be useful to solve this problem [50,51];

(2) “Polyclonality” in MIPs, the presence of a continuous distribution of binding affinities ranging from very low to very high values, does not represent in itself a major issue, since polyclonal antisera with similar affinity spectra are always used in the development of immunoassays. The only necessary condition is obviously that the mean affinity value will be high. However, while in the case of polyclonal antisera selectivity remains the same for antibody fractions obtained by affinity chromatography and characterized by different mean affinity [52], in the case of the MIP it is not at all clear whether the selectivity may be influenced by the distribution of affinity, i.e., if classes of binding sites characterized by different mean affinity are also characterized by a different selectivity pattern. In this case, it is

Table 6. Key features and performances of antibody- and MIP-based assays.

	antibody-based	MIP-based
low-mass analytes	yes	yes
high-mass analytes	yes	yes, but with marked experimental difficulties for large proteins
assay selectivity	high, fine tuning feasible	high, but fine tuning can be difficult
assay sensitivity	very high (up to pg/ml)	high (up to ng/ml)
non-specific tracer binding	negligible	depending from experimental conditions, rarely negligible
sample pretreatment	very limited	very limited
reproducibility	high	high
robustness	moderate	moderate
speed of execution	fast	fast
complexity of esecution	moderate	moderate
automatization	yes	possible
reusability	no	dubious
cost for single analysis	low	low
in-house feasibility	yes	yes
commercial availability	yes	no
literature	very large	limited
state of the art	mature	in evolution

therefore clear that any technique capable of isolating MIPs with controlled selectivity would be of big help to the development of highly selective molecularly imprinted sorbent assays;

(3) high non-specific binding, polymer surface fouling by unwanted proteins and poor competition between enzyme-labelled tracers and analytes can be related to the hydrophobicity of the MIP surface. In the past, many efforts have been made about "water-compatible" or "hydrophilic" MIPs [32,53-58], but it must be considered that the hydrophobic properties can play a decisive role in the interaction between the ligand and the binding site. Therefore, it seems still problematic to selectively suppress hydrophobic interactions outside the binding sites fully preserving those inside;

(4) unlike binding sites of antibodies that are easily accessible and exhibit antigen-induced fit, in MIPs narrow porosity and rigidity of the polymer structure due to the high degree of crosslinking implies problems of steric hindrance at the entrance of the ligand in the binding site. Consequently, binding kinetics can be very slow and unfavourable to the development of an assay, especially when analytes or tracers are represented by biomacromolecules. Surface grafting polymerization techniques could solve partially the issue of steric hindrance [59-61], while polymer rigidity can be reduced by decreasing the relative amount of cross-linker introduced in the pre-polymerization mixture or introducing more flexible cross-linkers with the risk that polymer backbone flexibility will be achieved at the expense of molecular recognition properties [62]. At present, no definitive solution have been identified to cope with this issue, but the use of thermoresponsive co-monomers (typically N-isopropylacrylamide) seems to allow some flexibility in the imprinted binding sites without adversely affecting the molecular recognition [63,64].

6. Conclusions

In the last twenty years, molecularly imprinted sorbent assays developed slowly. Despite the feasibility of such assays has been shown for many formats, and as a proof-of-the-concept its efficacy has been demonstrated in many studies with respect to other fields of application typical of molecular imprinting, today molecularly imprinted sorbent assay is still at the developmental stage, and a certain number of relevant issues remain to be solved. However, it is reasonable that the constant advances in molecular imprinting technology in the coming years could help resolve these issues, enabling the development of more affordable and sophisticated assays and, in a more distant outlook, of commercial kits based on the molecularly imprinted sorbent assay technique.

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