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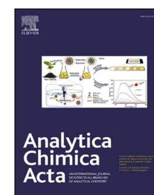


MIP-based immunoassays: A critical review

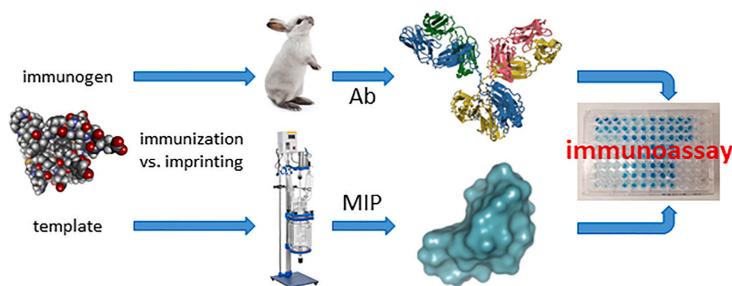
Fabio Di Nardo, Laura Anfossi, Claudio Baggiani *

Department of Chemistry, University of Torino, via Giuria 7, 10125, Torino, Italy

HIGHLIGHTS

- MIPs can mimic natural antibodies in immunoassay.
- Innovative approaches have improved the binding properties of MIPs.
- MIP-based immunoassays have passed by the proof-of-the-concept level to practical applications.
- Several issues related to the development of robust assays still remain to be explored.

GRAPHICAL ABSTRACT



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ABSTRACT

Molecularly imprinted polymers, MIPs, are man-made receptors mimicking the thermodynamic and kinetic binding behaviour of natural antibodies. Therefore, it is not surprising that many researchers have thought about MIPs as artificial receptors in immunoassay-like analytical applications, where the general machinery of the assay is maintained, but the molecular recognition is no longer assured by an antibody but by an artificial receptor. However, the number of papers devoted explicitly to applications of MIPs in the immunoassay field is quite limited if compared to the huge number of papers covering the multifaceted molecular imprinting technology. For this reason, this critical review wants to give a general view of MIP-based immunoassays, trying to highlight the critical points that have so far prevented a wider application of molecular imprinting technology in the immunoassay field and, possibly, try to suggest strategies to overcome them.

1. Introduction

Since Dickey's pioneering work [1], molecularly imprinted polymers (MIPs) have been seen as artificial molecular structures capable of mimicking the binding properties of antibodies. In fact, MIPs and antibodies exhibit similar binding properties, based on multiple reversible non-covalent interactions and characterized by selectivity towards the corresponding ligands (template molecules for MIPs, antigens for antibodies) and a well-defined thermodynamic and kinetic binding behaviour. Therefore, it is hardly surprising that many researchers have thought about MIPs as artificial receptors in immunoassay-like analytical applications. From the well-known seminal work of Mosbach and co-workers about radiotracer-based assays for theophylline and diazepam based on bulk-imprinted polymers [2], this idea has been reissued in experimental works all based on the same approach: the general machinery of the assay is maintained, but the molecular recognition is no longer assured by an antibody – as in "classical" immunoassay – but by a man-made imprinted polymer. However, despite the entire

* Corresponding author.
E-mail address: claudio.baggiani@unito.it (C. Baggiani).

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bibliography concerning the molecular imprinting technology covers more than 15,000 papers between 1993 and 2022 [3], in the same interval, as reported in Fig. 1, the number of papers devoted explicitly to applications in the immunoassay field is far more limited, amounting to about 100 works (excluding reviews). Furthermore, although exactly thirty years have passed since the Mosbach paper, and although the feasibility of MIP-based immunoassays has been demonstrated repeatedly since the end of the 1990s [4–6], to date no commercial assays are still available. For this reason, this critical review wants to give a general view of MIP-based immunoassays (called also “molecularly imprinted sorbent assays” or “pseudo-immunoassays”), trying to highlight the critical points that have so far prevented a wider application of molecular imprinting technology in the immunoassay field and, possibly, try to suggest strategies to overcome them.

2. Differences and similarities between MIPs and antibodies

The structure and the genesis of antibodies and MIPs are completely different. Antibodies used in immunoassay are, for the most part, immunoglobulins G (IgGs), the most abundant protein showing immunological activity. They are glycoproteins composed of four polypeptide chains (two identical 50 kDa heavy chains and two identical 25 kDa light chains), linked together by inter-chain disulphide bonds to compose a structure showing two identical antigen binding sites localized at the top of the short arm of the Y-shape [7]. Antibodies are produced by animals in response to an external immune stimulus, through a mechanism of clonal selection, not related to any conceivable imprinting mechanism. Conversely, MIPs are man-made artificial receptors obtained through a real imprinting mechanism during a polymerization process. The presence of template molecules 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 [8]. Thus, the binding properties of MIPs depend strictly from the nature and the strength of the non-covalent interactions between template molecules and binding sites. From a practical point of view, this makes possible a fine tuning of the binding properties by acting on the composition of the polymerization mixture and the polymerization process itself [9,10]. It should be noted that this constitutes a considerable advantage over classical immunoassay, as for the latter an analogous fine-tuning is conceivable only in the case of monoclonal antibodies, recombiant antibodies or nanobodies, which however require a complex and lengthy selection process in the case of the former [11], and considerable experimental efforts in the case of the latter [12,13].

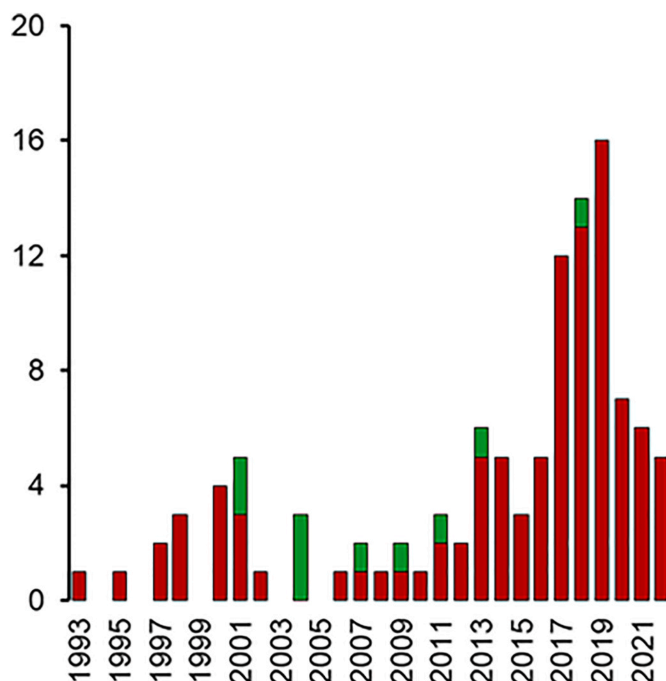

Fig. 1. Number of papers published between 1993 and 2022 devoted explicitly to applications of MIPs in the immunoassay field (immunosensor-like system excluded). Red bars: research papers, green bars: reviews. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Apart from the profound structural differences, however it is universally recognized that antibodies and MIPs share the same behaviour in terms of binding properties. Indeed, in both situations, it is possible to quantitatively describe the receptor-ligand equilibrium with formally equivalent langmuirian binding isotherm models [14]. This correspondence is further confirmed by the fact that both MIPs and polyclonal antibodies present a continuous distribution of affinity, due to the presence of multiple classes of binding sites characterized by molecular recognition towards the same ligand but with affinity of different magnitude [15,16]. In the case of polyclonal antibodies, this distribution is due to the simultaneous presence of antibodies produced by multiple clonal lines, while in the case of MIPs it can be attributed to the amorphous and intrinsically disordered nature of the polymeric structure around the individual binding sites. The fact that antibodies and MIPs share the same binding behaviour has an immediate and profound consequence on the use of MIPs in immunoassay: since the equilibria governing the assay are the same, it is possible in principle to replace antibodies with MIPs without fear of altering the assay basic behaviour [17,18]. Consequently, there is no fundamental obstacle to the development of MIP-based immunoassays.

3. MIP-based immunoassay at large

In according with the Ekins’s general classification of immunoassays [19], the most part of the literature about MIP-based immunoassays reports a *heterogeneous and competitive* format, typically with radionuclide-based tracers in the early works of the 90s [20], and enzyme- or fluorescence-based tracers in more recent works [21,22].

Heterogeneous means that these assays require the presence of a separation step between the free and the MIP-bound tracer molecule. The absence from literature of MIP-based immunoassays in the homogeneous format is not surprising, as it does not depend on the presence of a MIP in the assay machinery but rather on the general difficulty of making effective assays that do not include a free-bound separation step regardless of the type of receptor used.

Competitive means that it requires the presence of a competitive equilibrium between the tracer and the analyte for the binding site of the MIP. In this format (Fig. 2) a variable amount of analyte compete with a fixed amount of tracer for a fixed amount of binding sites. The multiple equilibria due to the competition reaction results in a decrease of the analytical signal produced by the bound tracer when an increasing amount of analyte is present in the sample and it is bound to the MIP. In this kind of assay, the MIP is usually immobilized on a solid surface (or is the solid surface itself) and the competition happens at the interface between the solid surface and the bulk of the solution containing the analyte and tracer molecules.

The sensitivity (expressed as IC_{50}, the concentration of analyte which decreases the analytical signal, i.e. the bound tracer, to 50% of the signal measured in the absence of analyte) is inversely proportional to the binding affinity of the antibody [23,24], approximately inversely proportional to the amount of available binding sites and inversely proportional to the equilibrium constant of the MIP for the analyte and to the specific activity of the tracer.

It must be noted that the competitive assay can exist in an alternative format (Fig. 3), where a fixed amount of analyte is immobilized onto a solid surface and it competes with the analyte for a fixed amount of labelled MIP introduced in solution. Nevertheless, at the present in

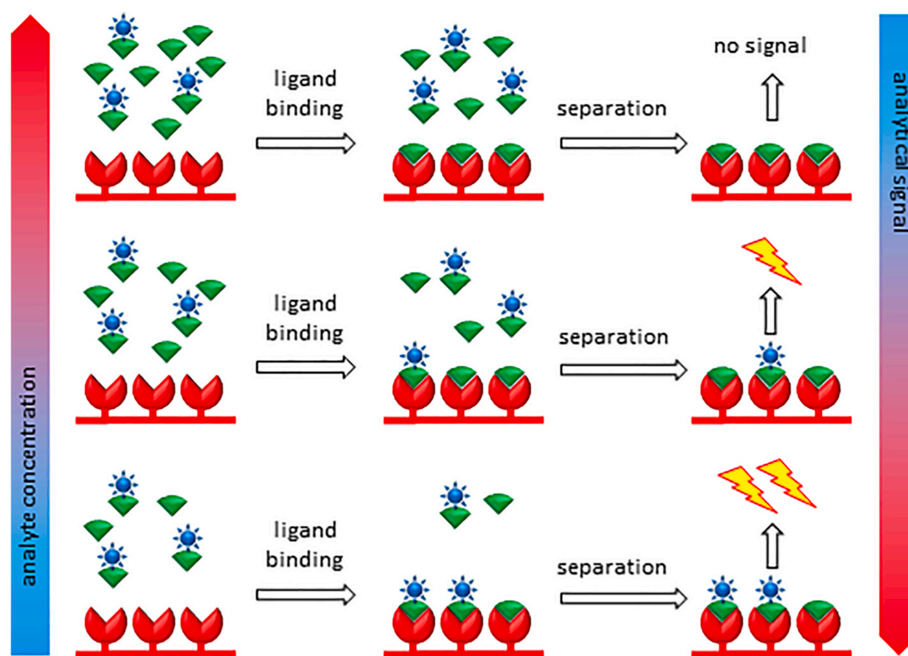

Fig. 2. Scheme of a competitive, heterogeneous immunoassay with binding equilibria between a variable amount of analyte and a fixed amount of tracer for a fixed and limited amount of receptor immobilized onto the solid surface. Red symbols: receptor onto solid phase; green symbols: analyte; blue symbol: tracer. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

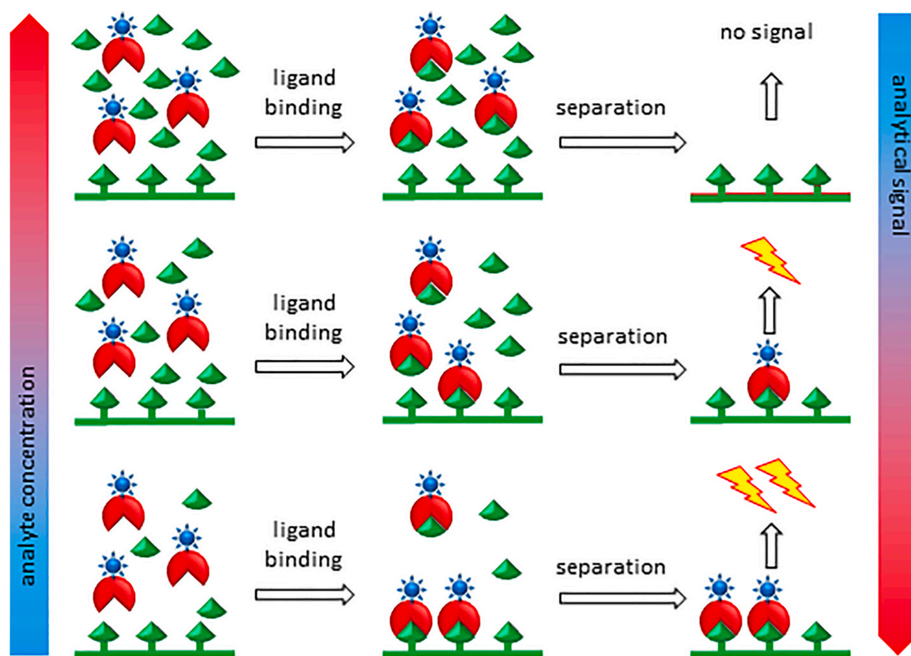

Fig. 3. Scheme of a competitive, heterogeneous immunoassay with binding equilibria between a variable amount of analyte and a fixed amount of labelled receptor for a fixed and limited amount of analyte immobilized onto the solid surface (At the present, in the literature this format is not yet described for MIPs). Red symbols: receptor conjugated with tracer; green symbols: analyte and analyte grafted onto solid phase; blue symbol: tracer. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

literature there are no available examples of this format, presumably due to the greater technical difficulty of having MIPs micro-sized particles capable of remaining in solution during the competition reaction with the solid phase.

There are also some examples of *non-competitive format* (Fig. 4) [25–29], where an excess of MIP 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 MIP in excess. This format is typical of the so-called “sandwich assay”, where the analyte is a macromolecule, typically a protein, large enough to have portions of the surface (epitopes) recognized by different, not cross-reacting, binding sites without any steric impediment. In this case, one of the binding sites (capture binding site) is present in excess onto a solid surface, then the analyte is bound and the complex is quantified with a labelled second binding site. Thus, the analytical signal is directly proportional to the amount of analyte present in the sample.

4. Critical points in MIP-based immunoassays

Apart the different nature of MIPs and antibodies, a general comparison of the features relevant to MIP-based immunoassays is given in Table 1, the main aspects of which will be discussed below.

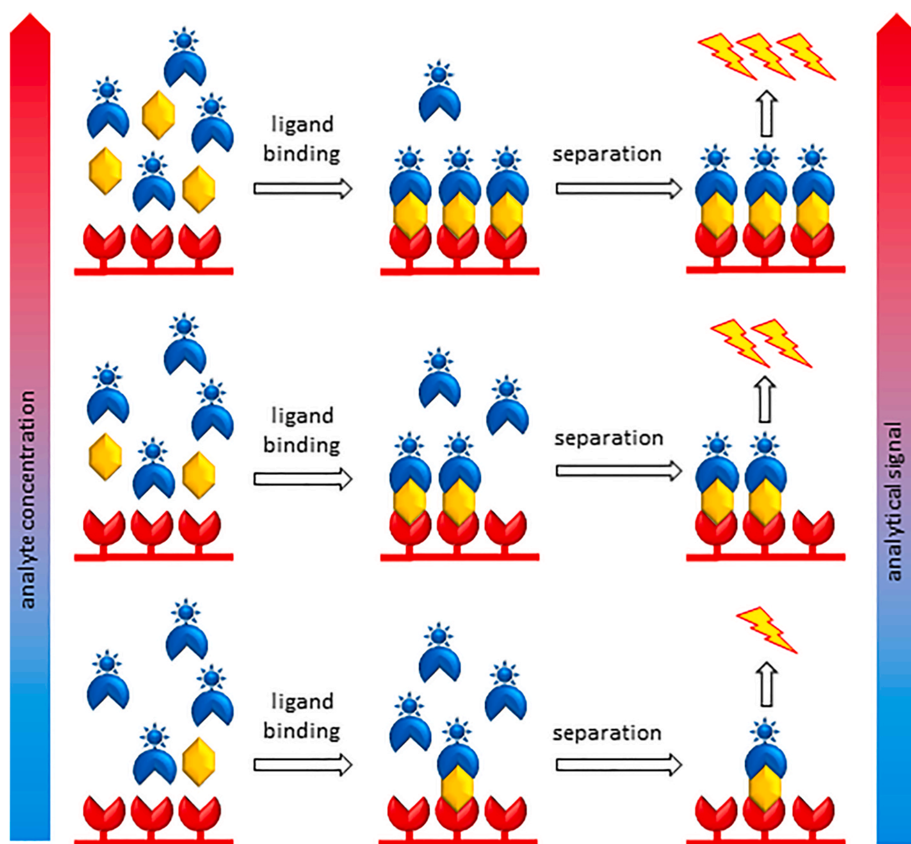

Fig. 4. Scheme of a non-competitive, heterogeneous immunoassay (sandwich assay), where the analyte is bound to the analytical receptor (in excess of the analyte and immobilized onto the solid surface) and to the secondary receptor conjugated with a tracer, able to bind the complex but not the free, analytical receptor. Red symbols: analytical receptor on solid phase; yellow symbols: analyte; blue symbol: secondary receptor conjugated with tracer. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4.1. Assay sensitivity

As previously seen, in competitive immunoassay, sensitivity[1] is inversely proportional to the binding affinity of the antibody. Since the equilibria governing competitive assays are the same both in the case of antibodies and of MIPs, it is obvious that also in the latter case it is important to have high affinity MIPs. Because traditional polymerization techniques (bulk, precipitation, emulsion, etc.) typically produce MIPs with affinity rarely higher than 10^6–10^7 L mol^{-1}, most of the assays show sensitivity in the order of magnitude of 0.1–1 μmol L^{-1}, whereas higher sensitivities are rarely reported [30,31].

A remarkable exception, consisting in assays characterized by high sensitivity in nmol L^{-1}-range or less, is represented by chemiluminescence-based enzyme-immunoassays reported for phenothiazines and benzodiazepines in pork meat [32] and amantadine and rimantadine in pork and chicken meat [33], respectively. In this case the use of a luminol-H_2O_2 system characterized by a very low unspecific signal, allowed to obtain assay sensitivities comparable to commercial immunoassays when performed on meat samples.

The recent introduction of catecholamines as self-polymerizing functional monomers in weakly basic aqueous solutions [34], allowed to set up very sensitive and selective assays based on imprinted thin layers directly prepared in 96-wells microplates. Significant examples are polydopamine films for the dye malachite green in water and fish samples [35], and poly-norepinephrine films for the hormones procalcitonin in plasma and gonadorelin in urine [36,37]. Another example of high sensitivity due to the use of catecholamines-derived thin layers is represented by the assay for chloramphenicol described by Chen et al. [38]. This approach is unique among MIP-based immunoassays, since, despite the low mass of the target molecule, it is a non-competitive assay that exploits the recognition of two distinct parts of the target molecule by two completely different receptors, one of which is a MIP. The authors prepared the capture receptor by imprinting dopamine in presence of 2,2-dichloroacetamide, while the labelled receptor binding the nitrophenyl structure of chloramphenicol, was prepared by conjugating β-cyclodextrin with the enzyme invertase, achieving in this way high selectivity and sensitivity up to 50 nmol L^{-1}.

Imprinted nanoparticles present several advantages compared with bulk polymers: due to larger surface/mass ratio, they form stable dispersions in aqueous buffers, show a limited binding heterogeneity, reduced non-specific binding and improved mass transfer and binding kinetics [39,40]. An innovative approach to imprinted nanoparticles is represented by the solid-phase synthesis (Fig. 5), where the polymerization takes place in the interstitial space between loosely packed beads grafted with template molecules, and the growth of cross-linked polymeric chains takes place in proximity of the beads surface. Once the polymerization process is stopped, the non-covalent interaction between imprinted nanoparticles (nanoMIPs) and template molecules is strong enough to allow any residual monomers, polymerization by-products and low affinity polymer to be washed away, while the high affinity nanoMIPs can be can be retrieved later [41,42]. Among the various advantages, because of the affinity separation step performed at the end of the polymerization process, the solid phase synthesis produces nanoparticles with a significantly higher affinity if compared with MIPs prepared by solution synthesis [43]. It is therefore no coincidence that one of the most relevant applications of nanoMIPs is in the so-called molecularly imprinted nanoparticle assay (MINA) [44], where competitive, nanoparticle-based enzyme-immunoassays show sensitivities markedly higher than the other MIPs, generally approaching the order of magnitude of 1–10 nmol L^{-1}. This is the case with assays for vancomycin [45], leukotrienes and insulin [46] and cocaine and its metabolites in

[1] intended as "minimum sensitivity", not be confounded with the "maximum sensitivity" as defined in according to IUPAC guidelines (https://doi.org/10.1351/goldbook).

Table 1
Comparison of key features between natural antibodies and molecularly imprinted polymers.

	antibodies	molecularly imprinted polymers
low-mass molecules (<5 kDa) as immunogen/ template	yes (the linker between the immunogenic carrier protein and the low-mass antigen affects the binding properties of resulting antibodies)	yes (for nanoMIPs the linker between the solid phase and the template can affect the binding properties of the polymer)
high-mass molecules (>5 kDa) as immunogen/ template	yes	yes, (efficient imprinting attained by surface or epitope-imprinting approach)
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	continuous and broad
mean affinity constant	frequently above 10^8 L mol^{-1}	rarely exceeds 10^6 L mol^{-1}, but nanoMIPs prepared by solid phase synthesis can reach 10^9 L mol^{-1}
binding kinetics	k_{ass}: from 10^5 to 10^6 L mol^{-1} s^{-1} k_{dis}: from 10^{-3} to 10^{-7} s^{-1}	k_{ass}: from 10^1 to 10^5 L mol^{-1} s^{-1} k_{dis}: from 10^{-1} to 10^{-3} s^{-1}
binding selectivity	high, group-selectivity difficult to obtain	high, group-selectivity possible
reproducibility	limited from batch-to-batch	very high
non-specific binding	negligible	strongly depending from experimental conditions
resistance to chemical & biological agents	very limited	yes
cost for single batch	up to 1 K€ for polyclonals, up to 10 K€ for monoclonals	<10 € (with exception for expensive templates)
commercial availability	high, frequently produced on demand	very limited
feasibility in a chemical laboratory	no, a stabularium, trained people and a dedicated laboratory (monoclonals only) are necessary	yes

serum [47], octopamine in urine [48], gentamicin in milk [49], fumonisin B1 in maize [51,51], biotin [52], microcystin-LR [53], florfenicol in milk and fish samples [54], domoic acid in water [55] and trypsin in serum [56].

4.2. Assay selectivity

In competitive immunoassay selectivity is usually expressed by the cross-reactivity (CR_{50}), that is the ratio of the respective IC_{50} values for the interfering molecule and the analyte (Fig. 6) [57]. As the value of IC_{50} is inversely proportional to the binding affinity of the antibody, selectivity can be seen as the ratio of the respective binding affinity for the interfering molecule and the analyte. Thus, since the absolute value of the compared affinities is irrelevant with respect to the value of their numerical ratio, even MIP-based immunoassays characterized by a not particularly high sensitivity can show a high selectivity, comparable to that of classical immunoassays. However, most of the literature reports selectivity data referring to a few most probable interfering substances, while the number of works reporting more extensive selectivity studies is quite limited. As example of selectivity characterization is given by the work of Andersson et al. [58], where the selectivity of assays for morphine and Leu-enkephalin was studied by competition with several structural analogs. About morphine, the assay resulted selective for morphine, but analogs with small structural differences were partially recognized (codeine, nor-morphine, hydromorphone, heroin), while more structurally different analogs (naloxone, nalotrexone) were recognized only marginally. About the peptide Leu-enkephalin, the results showed that in the case of a template of peptidic nature, thus more structurally complex than a simple organic molecule, the selectivity of the assay resulted more troublesome, as peptides structurally related to the template but different by one or two amino acids showed significant cross-reactivity, as did α-endorphin, a much longer peptide containing Leu-enkephalin in its sequence, while only a truncated Leu-enkephalin was recognized marginally.

The fact that often a template and closely similar molecules are recognized almost quite well by the MIP finds interesting applications when it is intended to determine a set of structurally related analytes excluding any other possible interfering substances. In fact, if antibodies are used as recognition element, it is necessary to carefully design the molecular structure of the hapten in order to preserve the common

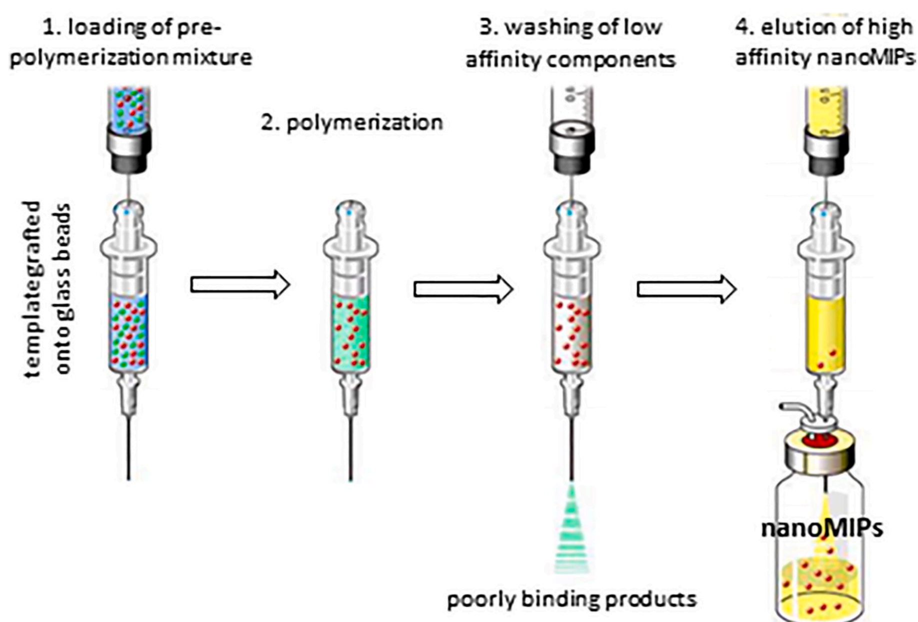

Fig. 5. Scheme of the preparation of nanoMIPs by solid-phase synthesis method.

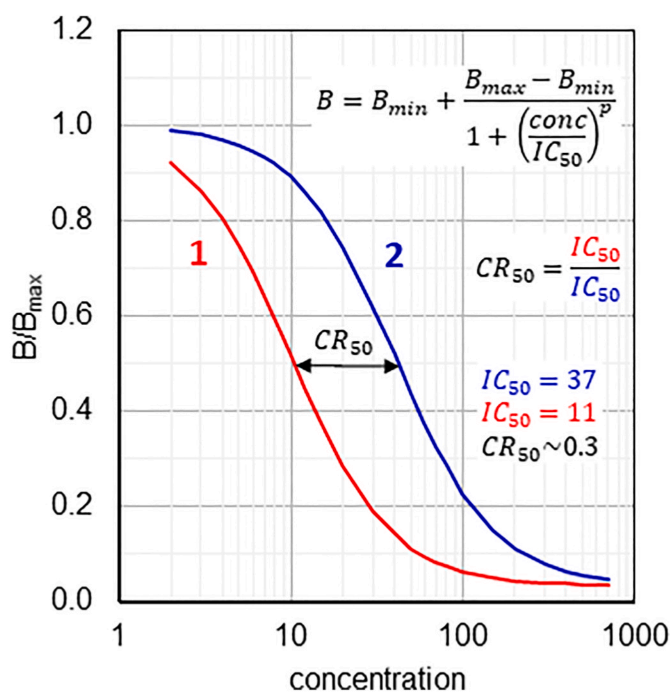

Fig. 6. Cross-reactivity in immunoassay: 4-parameters sigmoidal curves calculated for two analytes in competitive and heterogeneous conditions. B_{max}: analytical response in absence of ligand, B_0: analytical response at ligand infinite concentration (tracer non specific binding for $B_0 > 0$), IC_{50}: mid-range ligand concentration; p: curve slope at mid-range ligand concentration. Analyte 1: $B_{max} = 3$, $B_0 = 0.1$, $IC_{50} = 11$; $p = 1.5$; analyte 2: $B_{max} = 3$, $B_0 = 0.1$, $IC_{50} = 37$; $p = 1.5$.

structural characteristics, excluding as much as possible the uncommon ones and maintaining at least one distinct functionality for conjugation with the carrier protein. Of consequence, this approach is complex and not always successful [59–61]. Conversely, the use of MIPs has a significant advantage, as it is not necessary to extensively modify the template to achieve a polymer with group-selectivity. As example, Mattsson et al. synthetized histamine-imprinted microparticles by precipitation polymerization, and used them to set up a competitive fluorescence-based assay for biogenic amines in fish extracts [62]. The resulting assay quantified histamine in a broad range (1−430 μmol L^{-1}), recognized also structurally similar biogenic amines like tyramine, tryptamine, spermine and spermidine, providing simultaneous analysis and assessment of the total amount of biogenic amines.

The solid phase polymerization synthesis represents an interesting approach to class-selective MIPs. As example, Garcia-Cruz et al. used benzoylecgonine as solid phase-supported template to set up a class-selective nanoparticle-based enzyme-immunoassay for opioids in serum [47]. The assay detected cocaine, benzoylecgonine and norcocaine without cross-reactivity towards common coexisting interfering substances like paracetamol and caffeine, and with a pM-level limit of detection, which was almost three orders of magnitude lower than the limit of detection expected from commercial antibody-based assays.

Alternatively, when a single template produces a MIP without class-selectivity, it can be attained using a multiple-template approach, where a MIP is prepared by simultaneous imprinting of two or more different templates, with the only limitation that templates should be mutually compatible, *i.e.* share the same functional monomers and polymerization conditions [63]. Xia et al. prepared two chemiluminiscence-based enzyme-immunoassays based on a double-imprinted polymer obtained using simultaneously chlorophenothiazine and clonazepam as templates [32]. The assays detected simultaneously 4 phenothiazines (acepromazine, promethazine, chlorpromazine and perphenazine) and 5 benzodiazepines (clonazepam, diazepam, nitrazepam, oxazepam and estazolam) in pork meat with sensitivity in the nmol L^{-1} range.

4.3. Nature of the target analyte

It is known that antibodies can be obtained against molecular targets that can range from small organic molecules to large biomacromolecules and cellular components, with the only limitation that in the case of organic molecules with a molecular mass lower than 3–5 kDa it is necessary to stimulate the immune response by the use of immunogenic proteins on which the antigen, suitably modified (hapten), is covalently conjugated. Consequently, resulting antibodies will preferentially recognize the hapten, with possible repercussions on the selectivity of the immunoassay [64,65]. Conversely, while it is generally easy to prepare MIPs with excellent molecular recognition properties towards small organic molecules, proteins and peptides have long been considered "difficult" templates due to the poor compatibility with the polymerization conditions normally used and problems of steric hindrance in the binding site due to the rigidity of the polymer structure caused by the high degree of crosslinking. As consequence, binding kinetics can be very slow and unfavourable to the development of an assay [66,67]. Nevertheless, in recent years, considerable progress has been made in identifying strategies suitable for efficient imprinting of these templates [68,69].

The problem of steric hindrance has been addressed introducing the surface imprinting. In this approach, various polymerization techniques can be used to imprint template proteins onto solid supports, limiting the formation of binding sites onto the surface only. Thus, contrary to bulk MIPs, the binding sites are easily accessible, making easier template extraction and speeding up the binding kinetics [70,71]. This approach seems to be particularly suitable to prepare 96-wells microplates for MIP-based immunoassay of proteins. As example, Ali et al. covalently grafted microplates with a thin layer of silica sol-gel imprinted with for recombinant human erythropoietin-alpha (rhEPO), showing very high affinity for the template, good selectivity and moderately fast rebinding kinetics [72]. A carefully optimized organogel composed of acrylic acid and N-vinylpyrrolidone, cross-linked with methylene-bis-acrylamide was used by Boonsriwong et al. to graft microplates with human serum albumin, demonstrating the feasibility of an assay for it [73]. Bi et al. obtained surface-imprinted thin films with glycoproteins using as functional monomer 4-vinylphenylboronic acid, which shown to be capable of orienting the proteins in an orderly manner on the film surface, allowing for the development of efficient assays [74].

One of the most promising methods for the imprinting of large biomacromolecules is the "epitope imprinting" approach (Fig. 7) [75,76]. In this case, once a short amino acid sequence (6–8 amino acids) characteristic of the target protein has been selected, a MIP directed towards the corresponding peptide is prepared. The resulting polymer therefore shows binding properties towards not only the template peptide, but also the protein from which it derives. This approach allows in principle to circumvent most of the problems characteristic of the biomacromolecule template, not least the difficulty of having adequate quantities of rare or expensive proteins and the incompatibility of many biomacromolecules with the typical polymerization conditions.

In the case of immunochemical assays, it should be considered that the epitope imprinting potentially allows the development of "sandwich" type assays, which are very difficult to implement with other approaches when MIPs are the binding component of the assay. This approach has been used by Xing et al. to set up a sandwich assay for the human neuron-specific enolase (hNSE) [26]. The glycated C-terminal peptide was used as template to prepare the capture element, while the N-terminal was imprinted onto Ag@SiO_2 nanoparticles functionalized with *p*-aminothiophenol to obtain a surface-enhanced Raman scattering probe element. The assay resulted selective and sensitive in the range 2–8 pg mL^{-1}, demonstrating the effective feasibility of this approach. It must be noted that, most frequently, the two molecular recognition elements of the MIP-based sandwich assays do not consist in polymers

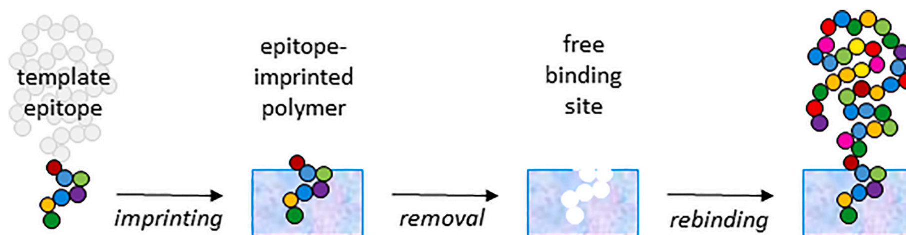

Fig. 7. Schematic depiction of the surface imprinting approach.

imprinted against different epitopes, but, respectively of a MIP imprinted against an epitope (or a whole protein), and of nanobeads or a thin layer containing 4-vinylphenylboronic acid. Although this approach works only if the analyte is a glycoprotein, the efficiency of the assay is ensured by the combined effect of the selectivity of MIP and the ability to strongly bind the oligosaccharides typical of 4-vinylphenylboronic acid [25,56,77–79].

5. Open issues in MIP-based immunoassays

In the previous sections we have highlighted how the similarity in the binding behaviour between antibodies and MIPs allows to easily develop immunochemical assays based on the latter. However, at present, there are some less considered issues which could have an influence on the assay development and optimization.

5.1. Effect of binding affinity distribution on selectivity

As seen in Section 2, MIPs show a continuous distribution of binding affinities ranging from very low to very high values. It does not represent in itself a major issue, since polyclonal antisera with similar affinity spectra have long been used in the development of immunoassays. Polyclonal antisera separated by affinity chromatography in fractions differing for binding affinity frequently show different selectivity [80,81], but in the case of MIPs it is not known whether selectivity may be influenced by the distribution of affinity. *i.e.* if distinct classes of binding sites characterized by different binding affinity are also characterized by a different selectivity pattern. Few cases of separation by affinity chromatography of nanoparticles have been reported to date, but in no case the selectivity of individual fractions has been studied [82,83]. If the possibility of separate MIPs in fractions of different selectivity were confirmed experimentally, such technique would be greatly useful to control assay selectivity without necessarily having to embark on lengthy and difficult optimization of the polymerization protocol and/or assay set up.

5.2. Tracer binding heterology

As seen in Section 3, in competitive assay antibodies are immobilized on a solid surface and competition happens at the interface between the solid surface and the bulk of the solution containing the analyte and tracer molecules, where the tracer is, in most cases, an enzyme covalently conjugated with one or more molecules of analyte through a spacer arm. Usually it is assumed that analyte and tracer have the same binding affinity for the antibody, but in reality, due to the effect of the covalent bond between analyte and enzyme this affinity is almost never the same. In classical immunoassay, this fact is well known to affect sensitivity and selectivity [84], and several approaches have been introduced to minimize it through the use of spacer arms of different position, chemical structure or length for the tracer and the conjugate used to elicit the antiserum [85–87].

In MIP-based immunoassays binding heterology is practically unavoidable, and in addition to the introduction of a spacer arm on the template molecule to obtain the enzymatic tracer, it may also be due to the hypothetical use of a template different from the target molecule in the so-called mimic template approach [88]. Moreover, as previously seen in Section 4.1, nanoMIP used in several assays are prepared by solid phase polymerization synthesis. This approach necessarily implies the use of a molecule modified with a spacer arm as a template. In this case, from the point of view of binding heterology, there is a situation very similar to that of antibodies, where the immune response is not elicited against the target molecule, but rather towards the same molecule but conjugated to a carrier protein through a spacer arm. Although therefore binding heterology is definitely present in all MIP-based immunoassays and, thus, effects on sensitivity and selectivity must be expected, at the present this issue is completely neglected by literature.

5.3. Non-specific binding

It is known from a long time that the loss of sensitivity due to non-specific binding of tracer and analyte to the solid phase represents a critical bottleneck in the development of immunoassays [28,89]. In typical 96-well microplates the solid phase is usually a polystyrene surface to which antibodies are physically adsorbed. To ensure assay sensitivity, surface coverage by antibodies must be taken low, leaving hydrophobic patches prone to non-specific binding. An efficient and mandatory way to minimize this non-specific adsorption is to use blocking agents – typically proteins such as albumin, casein or gelatin – to plug these patches, combined with the use of non-ionic surfactants in the assay buffers [90,91].

In these terms, the use of MIPs as an element of molecular recognition in an assay presents a significant difficulty. In fact, basically MIPs are cross-linked polymethacrylates, characterized by a moderately hydrophobic surface. Thus, it is quite difficult to use traditional blocking agents without also coating the MIPs and, of course, the binding sites as well, compromising the efficiency of the assay. A possible solution to this issue could be the synthesis of MIP whose surface has been made more hydrophilic by changing the composition of the polymer [92] or modifying its structure through some post-imprinting process [93,94]. Until today, there are only few papers reporting the validity of these approaches. As example, Meng et al. prepared an imprinted hydrophilic thin layer of polymer directly within the wells of a polystyrene microtiter plate. In this case the use of blocking agents was no longer necessary, as the entire surface was uniformly covered by the hydrophilic polymer, allowing the set-up of a μmol L^{-1}-level sensitive enzyme-linked immunosorbent assay for the organophosphate pest-control agent trichlorfon [95]. Moreover, as previously seen in Section 4.1, the validity of thin-layer imprinted surfaces is also confirmed by the successful use of self-polymerizing catecholamines as binding substrates in MIP-based immunoassays without needs of any blocking agent [27,35–37].

It must be noted that, thanks to the different composition of the monomer mixtures richer in polar functionalities, nanoMIPs prepared by solid phase polymerization synthesis show a more hydrophilic character which makes them more similar to natural proteins. Consequently, once physically grafted in the bottom of the wells using a highly viscous polymer – typically polyvinylalcohol – to glue the nanoparticles onto the polystyrene surface, blocking agents can be used without affecting the

binding sites in analogy to natural antibodies [47,49–51,54,55].

6. Conclusions

Despite the feasibility of MIP-based immunoassays has been shown from long time, over the past twenty years such assays developed slowly and only in the last ten years there has been a significant transition from proof-of-concept to applications on real matrices. The fact that antibodies and MIPs share the same binding behaviour implies that it is possible to replace antibodies with MIPs without fear of altering the assay basic behaviour and that, consequently, there is no fundamental obstacle to the development of MIP-based immunoassays. However, this review shows that the development of these assays has shown several relevant critical points and issues that to be resolved had to wait new advances in molecular imprinting technology. At the present, the progressive shift from micron-sized to nano-sized imprinted particles, the improved ability to prepare imprinted thin layers and innovative approaches like the solid phase polymerization technique show that there appear to be no insurmountable obstacles to the development of robust MIP-based immunoassays with sensitivity, selectivity and real matrix compatibility comparable to those of natural antibody-based immunoassays.

Declaration of competing interest

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Data availability

No data was used for the research described in the article.

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She received the M.S. degree in Chemistry with full marks and honors and the Ph.D. degree in Biochemical Sciences at University of Torino in 1997 and 2001, respectively. Since 2022 she is Full Professor of Analytical Chemistry at the Department of Chemistry (University of Torino). She carries out his activity within the Laboratory of Bioanalytical Chemistry where her main research interest is the development of innovative immunodiagnostic methods and immunosensors, based on natural antibodies and synthetic ligands, such as molecularly imprinted polymers and aptamers. She is author or co-author of more than 140 peer-reviewed research papers and reviews and 6 book chapter

Full Professor in Analytical Chemistry at the Department of Chemistry (University of Torino), he carries out his activity within the Laboratory of Bioanalytical Chemistry where his main research interest concerns the development of molecularly imprinted polymers with selective molecular recognition properties towards ligands of analytical concern. Beside the study of the kinetic and thermodynamic properties of the imprinted polymers, this line of research has led to the development of highly selective systems for the capture and pre-concentration of analytes from complex matrices, finding practical application in the field of solid phase extraction. This line of research is currently evolving towards the development of intelligent nanomaterials for applications in the immunochemical field. He is author or co-author of more than 140 peer-reviewed research papers and reviews and 6 book chapter.

Assistant Professor in Analytical Chemistry at the Department of Chemistry (University of Torino), he carries out his activity within the Laboratory of Bioanalytical Chemistry where his main research interest concerns the development of (bio) analytical methods and devices for point-of-care and point-of-need applications. He has experience in developing lateral flow immunoassay and has been involved in the technology transfer for the commercialization of the developed devices. In 2019 he was awarded the Young Bioanalytical Researcher Award by the Bioanalytical Group of the Analytical Chemistry Division of the Italian Chemical Society. He is author or co-author of more than 50 peer-reviewed research papers and reviews and 1 book chapter.