Protein-Imprinted Polymers: How Far Have "Plastic Antibodies" Come?

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Abstract[1]

Antibodies are highly selective and sensitive, making them the gold standard for recognition affinity tools. However, their production cost is high and their downstream processing is time-consuming. Molecularly imprinted polymers (MIPs) are tailor-made by incorporating specific molecular recognition sites in their structure, thus translating into receptor-like activity mode of action. The interest in molecular imprinting technology, applied to biomacromolecules, has increased in the past decade. MIPs, produced using biomolecules as templates, commonly referred to as "plastic antibodies" or "artificial receptors", have been considered as suitable cheaper and easy to produce alternatives to antibodies. Research on MIPs, designed to recognize proteins or peptides is particularly important, with potential contributions towards biomedical applications, namely biosensors and targeted drug delivery systems. This mini review will cover recent advances on (bio)molecular imprinting technology, where proteins or peptides are targeted or mimicked for sensing and therapeutic applications. Polymerization methods are reviewed elsewhere, being out of the scope of this review. Template selection and immobilization approaches, monomers and applications will be discussed, highlighting possible drawbacks and gaps in research.

Keywords: molecularly imprinted polymers, nanoparticles, artificial antibodies, biomimetics, biosensors, biomolecules, diagnostics, selective targeting, drug delivery

1. Introduction

The interest on molecular imprinting technology, studied since the 1970s, has grown exponentially between the 1970s and mid-2010s (Ansari and Masoum, 2019), with applications ranging from separation and purification, as selective adsorbers or membranes, to sensors (Alexander et al., 2006; Whitcombe et al., 2014).

Molecularly imprinted polymers (MIPs) are synthetic polymers that are tailor-made for specific recognition. As antibodies and enzymes, their three-dimensional structure and functional groups with a specific orientation are orchestrated to allow a selective molecular binding. Functional monomers interact with the template, or printed molecule, forming a template-monomer complex. Polymerization takes then place, in the presence the template, by reaction of a cross-linker and an initiator. After the polymer is formed, the template molecule is removed, leaving the MIP with empty cavities, that act as specific binding sites (Hui Lee and Doong, 2016; Scriba, 2016), as represented in a simplified scheme in Figure 1. This cavity will preferentially bind the template, as it matches the template's geometry, and it has affinity for its complementary functional groups. MIPs can be synthetized using virtually any molecule as a template, ranging from

[1] **Abbreviations:** AAm: acrylamide; AIBN: azo-bis isobutyronitrile; APMA: N-(3-aminopropyl) methacrylamide; APS: ammonium persulfate; BSA: bovine serum albumin; CEA: Carcinoembryonic antigen; DMAEM: 2-(dimethylamino)ethyl methacrylate; DMAPMA: N-(3-(dimethylamino)propyl)methacrylamide; EBA: N,N'-ethylenebis(acrylamide); EGDMA: ethylene glycol dimethylacrylate; EGFR: epidermal growth factor receptor; HPLC: high performance liquid chromatography; MAA – methacrylic acid; MBA: N,N'-methylenebisacrylamide; MIPs: molecularly imprinted polymers; N/A: not available; NIPAm: N-isopropylacrylamide; PBS: phosphate buffered saline; PDA: 1,4-bis(acryloyl)piperazine; PEGDMA: poly(ethylene glycol)dimethacrylate; PSMA: prostate-specific membrane antigen; QCM – quartz crystal microbalance; SDS: sodium dodecyl sulphate; TBAm: N-tert-butylacrylamide; TEMED: N,N,N′,N′-Tetramethylethylenediamine.

drugs, small molecules, amino acids, chiral enantiomers, DNA, peptides, proteins and even whole cells (Haginaka and Sakai, 2000; Hammam et al., 2018; Lin et al., 1997; Liustrovaite et al., 2023; Mohajeri and Ebrahimi, 2008; Ping Li et al., 2004; Suedee et al., 2002; Trinh et al., 2018), thus translating into a target molecule size range from a few Da (*e.g.* 126 Da melamine (Poma et al., 2013)) to several kDa (*e.g* 66.5 kDa bovine serum albumin (BSA) (Arabi et al., 2021b) and 180 kDa spike glycoprotein of SARS-CoV-2 (Ratautaite et al., 2023, 2022)).

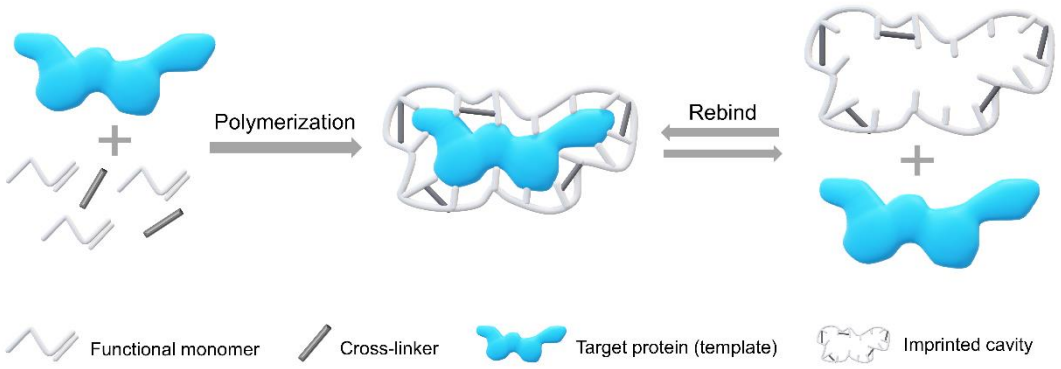

Figure 1. Schematic representation of protein MIP synthesis.

Biomolecules such as antibodies, some receptors, and enzymes are the gold standard for affinity tools, since their target recognition capacity is highly selective and sensitive. However, the use of natural biomolecules presents disadvantages, including: limited working conditions, such as mild temperature, narrow pH range and low stability in organic solvents. Antibody production in mammalian cells has been optimized over last decades, still the associate production costs are high. Recombinant expression in bacterial or yeast systems still presents limitations like endotoxin production (Arbabi-Ghahroudi, 2022; Asaadi et al., 2021; Liu and Huang, 2018; Malaquias et al., 2021; Mark et al., 2022; Thompson et al., 2016). Therefore, given that MIPs are usually cheap, easy to synthetize in a reproducible way, and have shown robust performances in a variety of solvents (Hui Lee and Doong, 2016; Wackerlig and Schirhagl, 2016), MIPs, using biomolecules as templates, have been considered as suitable alternatives for medical diagnosis and theragnostics in the biomedicine field and as replacement of enzymes in catalytic processes or even used as bioelectrodes for energy harvesting based on microbial fuel cells in more advanced MIP applications (Ostovan et al., 2022).

Due to their functional similarity to their natural counterparts, MIPs designed for biomolecules have been referred to as “plastic antibodies” or “artificial receptors”. Research developed on MIPs selective for biomacromolecules, like proteins or peptides, is particularly important, since these have the potential to contribute towards biomedical applications aiming at biosensors, toxic analyte sequestration, or drug delivery systems, among others (Canfarotta et al., 2018; Chen et al., 2016; Hui Lee and Doong, 2016; Suedee et al., 2002).

Molecularly imprinted technology has significantly contributed on the development of novel biosensors for disease detection. Namely, several cancer diagnostics sensors based on MIPs has been development, using prostate, breast, ovarian and hepatic cancer biomarkers as target molecules (Pilvenyte et al., 2023b). Polypyrrole-based electrochemical MIP sensors were developed targeting the CA-125 marker for

epithelial ovarian cancer (Rebelo et al., 2019), the CA15-3 marker for breast cancer (Santos et al., 2018), and the PSA protein marker for prostate cancer (Yazdani et al., 2019). In the latter study, it was developed a sensor for PSA with a limit of detection (LOD) so low as 2.0 pg/mL, which is below the threshold "risk" values of 4.0-10.0 ng/mL of PSA concentration in blood, thus showing the potential competitiveness of this assay. Biomarkers for neurodegenerative diseases is another relevant focus area of MIP-based biosensors with recent advances in the development of MIP-based electrochemical sensors for Alzheimer's and Parkinson's diseases (Pilvenyte et al., 2023a). For instance, a very competitive Alzheimer's disease MIP biosensor was designed for an amyloid-β peptide using a combination of polypyrrole and carbon nanotubes, reaching a LOD as low as 0.3 fg/mL (Özcan et al., 2020). A MIP-based biosensor was similarly developed for α-synuclein peptides with a LOD of 10 fg/mL, for Parkinson's disease, using aniline, the monomer of the conducting polymer polyaniline, and *m*-aminobenzenesulfonic acid (Lee et al., 2021). Overall, such studies suggest a growing tendency in developing MIP-based biosensors using such functional monomers to obtain an electroconductive polymeric structure in the MIP to enable high detection sensitivities (Canfarotta et al., 2016; Ramanavicius et al., 2022; Ramanavicius and Ramanavicius, 2022).

This mini-review is focused on current advances on molecular imprinting technology where proteins, peptides and epitopes are used as templates, and/or MIPs that are designed to mimic biological (macro)molecules. Polymerization methods are reviewed elsewhere (Haupt et al., 2020), being out of the scope of this review. Template selection and immobilization approaches, functional monomers and applications will be discussed, highlighting possible drawbacks and gaps in the literature.

2. Protein imprinting

Proteins are biomacromolecules of significant interest in research, with special focus on their detection and quantification, since they are often biomarkers of important human diseases, including viral infections (Cennamo et al., 2021; Liv et al., 2021; Raziq et al., 2021; Sukjee et al., 2022; Tai et al., 2005), hormonal and DNA regulation processes (Rachkov and Minoura, 2000; Zhang and Liu, 2018), and many cancer types (Canfarotta et al., 2018; Han et al., 2019; Tang et al., 2018; Zhang et al., 2015). However, detection and quantification of proteins requires labour and cost-intensive separation methods, often based on immunoassays.

Table 1 shows a short overview of the common functional monomers, initiators, cross-linkers and solvents used on studies for protein imprinted MIPs. Apart from the polymerization method chosen, reagents for the polymerization reaction appear to be quite similar among studies with protein imprinted MIPs, without much novelty presented in terms of functional monomers, initiators and cross-linkers selected, in agreement with what has already been reviewed elsewhere (Teixeira et al., 2021; Yang et al., 2019).

Table 1. Short overview of polymerization reagents (monomers, cross-linkers, initiators) and solvent for selected studies using protein imprinting technology.

Template	Monomers	Cross-linker	Initiator	Solvent	Ref.
Cytochrome c	AAm	MBA EBA PDA PEGDMA	APS TEMED	Water Tris-buffered saline	(El Kirat et al., 2009)
	AAm	MBA	APS	PBS	(Li et al., 2006)
Haemoglobin	Dopamine	N/A	APS	PBS	(Ouyang et al., 2010)
	AAm	MBA	APS	PBS	(Li et al., 2006)
BSA	DMAEM	MBA PEGDMA	Irgacure ® 2959	Potassium phosphate buffer and ethanol	(Kryscio and Peppas, 2012)
Albumin	AAm	MBA	APS	PBS	(Li et al., 2006)
DNAzyme complex	AAm NIPAm DMAPMA	MBA	APS TEMED	Aqueous buffer solution	(Zhang and Liu, 2018)
Prostate-specific membrane antigen (PSMA)	AAm DMAEM	EGDMA	AIBN	Methanol/water mixture	(Tang et al., 2018)
Epidermal growth factor receptor (EGFR)	NIPAm TBAm Acrylic acid APMA	MBA	APS TEMED	PBS	(Canfarotta et al., 2018)
Ribonuclease A	AAm	MBA	APS	PBS	(Li et al., 2006)
Human serum albumin (HSA)	3-(methacryloxy) propyltrimethoxysilane	-	-	PBS Tween 20 solution	(Guoning et al., 2020)

AAm: acrylamide; AIBN: azo-bis isobutyronitrile; APMA: N-(3-aminopropyl) methacrylamide; APS: ammonium persulfate; DMAEM: 2-(dimethylamino)ethyl methacrylate; DMAPMA: N-(3-(dimethylamino)propyl)methacrylamide; EBA: N,N'-ethylenebis(acrylamide); EGDMA: ethylene glycol dimethylacrylate; MBA: N,N'-methylenebisacrylamide; N/A: not available; NIPAm: N-isopropylacrylamide; PBS: phosphate buffered saline; PDA: 1,4-bis(acryloyl)piperazine; PEGDMA: poly(ethylene glycol)dimethacrylate; SDS: sodium dodecyl sulphate; TBAm: N-tert-butylacrylamide; TEMED: N,N,N′,N′-Tetramethylethylenediamine.

To obtain MIPs for biomacromolecules, two main protein imprinting strategies have been developed: protein imprinting, including i) non-oriented surface imprinting and ii) oriented surface imprinting, where the entire protein works as template; and epitope imprinting, where the template will be a part of the structure of the target protein. Representative examples found in literature of protein and epitope imprinting strategies discussed in this review are represented in Figure 2.

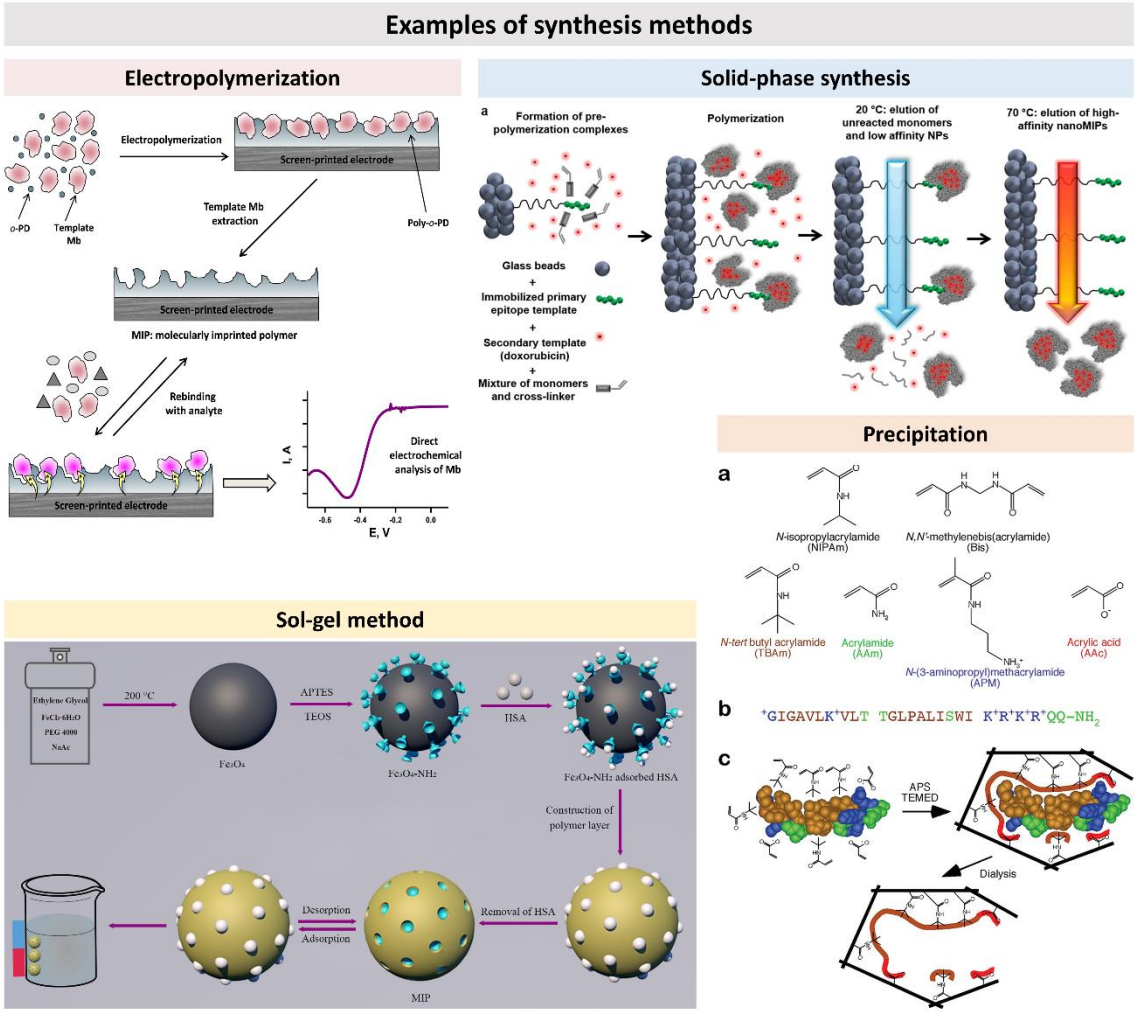

Figure 2. Representative synthesis methods: non-oriented surface imprinting – electropolymerization (reprinted from (Shumyantseva et al., 2016), Copyright 2023, with permission from Elsevier) and precipitation (reprinted with permission from (Hoshino et al., 2008), Copyright 2023 American Chemical Society); oriented surface imprinting – sol-gel (reprinted from (Guoning et al., 2020), Copyright 2023, with permission from

Elsevier); epitope imprinting: solid-phase synthesis (reprinted with permission from (Canfarotta et al., 2018), Copyright 2023 American Chemical Society).

2.1. Protein imprinting

In the protein imprinting approach, the whole protein is the template. Such strategy, following the traditional MIP concept, could be argued as the most appropriate biomimetic approach in terms of binding affinity, since it would retain the tertiary structure of the target protein, as well as, affinity groups for weak protein interactions, such as hydrogen bonds, electrostatic and van der Waals interactions (Boysen, 2019). However, epitope imprinting, which will be reviewed further ahead, may provide a recognition mechanism more similar to natural receptors. Depending on the application, it may be useful to have a binding site for only a specific peptide motif of the biomolecule. This is particularly relevant for cell membrane proteins, when considering specific biological variants detection, or to promote for cost-effective MIPs development and manufacture strategies. The literature reports MIPs targeting common proteins, such as BSA, albumin, ribonuclease A, horseradish peroxidase (HRP), or cytochrome c, using molecular imprinting methods based on different strategies for the immobilization of the target, and varied functional monomers (Kryscio and Peppas, 2012; Li et al., 2006; Wang et al., 2019). Additional examples are resumed in Table 2, including methods for removal of the template after MIP synthesis. Template removal techniques include protein denaturation steps, so that the template changes its conformation and will be released from the imprinted cavity, followed by washing steps to promote the elution of the denatured template. A drawback of these methods is that they do not allow recovery of the template molecule for reuse. Another methodology is based solely in washing with mild solvents, like aqueous solutions, thus relying on disruption of weak interactions, such as electrostatic and hydrogen bonds, and the slow diffusion of the protein through the polymer network. Additionally, the use of thermo-responsive monomers (e.g. N-isopropylacrylamide) in the structure of the MIP allows the release of the protein simply by increasing the temperature of the washing solution above the lower critical solution temperature (LCST), which leads to increase space between polymer chains, allowing the protein to be released from the MIP's cavity.

Table 2. Summary of protein templates and template removal procedures for selected studies using protein imprinting technology.

Template	Template removal	Ref.
Cytochrome c	Digestion with trypsin and washing with SDS solution	(El Kirat et al., 2009)
	Washing with ethanol, NaOH, and acetic acid with SDS solutions	(Li et al., 2006)
Haemoglobin	Washing with SDS solution	(Ouyang et al., 2010)
	Washing with ethanol, NaOH, acetic acid with SDS solutions	(Li et al., 2006)
BSA	Washing with potassium phosphate buffer	(Kryscio and Peppas, 2012)
Albumin	Washing with ethanol, NaOH, and acetic acid with SDS solutions	(Li et al., 2006)
DNAzyme complex	Washing with water	(Zhang and Liu, 2018)
Prostate-specific membrane antigen (PSMA)	Soaking in aqueous NH_3/methanol, followed by washing with water and methanol	(Tang et al., 2018)
Epidermal growth factor receptor (EGFR)	Washing with water in solid phase extraction and centrifugal dialysis	(Canfarotta et al., 2018)
Ribonuclease A	Washing with ethanol, NaOH, acetic acid with SDS	(Li et al., 2006)
Listeria monocytogenes	Acetic acid and trypsin	(Liustrovaite et al., 2023)

Some studies report the development of MIPS for the whole tertiary protein structure in liquid solution, by addition of the target biomolecule to the reaction mixture (Wang et al., 2014; Yang et al., 2023). Some studies have even reported the use of whole cells, like bacteria, to develop MIP-based sensors reaching relevant LOD values, a MIP-based electrochemical sensor developed for Listeria monocytogenes has a LOD of 70 CFU/mL (Liustrovaite et al., 2023). However, the most common approach reported resorts to solid phase synthesis, in which the template protein is first immobilized in a solid support such as silica beads (Canfarotta et al., 2016; Zhang et al., 2009), glass surfaces (El Kirat et al., 2009; Kryscio and Peppas, 2012) and other silica moulds (Dabrowski et al., 2019; Li et al., 2006) and an affinity chromatography step is used for the synthesis and purification of the molecularly imprinted nanoparticles (MIP-NPs) (Figure 2 and Figure 3).

Immobilization of the protein to the solid support could be achieved using an affinity ligand of the protein (Ambrosini et al., 2013), or by chemical functionalization of the surface of the solid support (Canfarotta et al., 2016). The use of an affinity ligand enables the orientation of the immobilized protein, meaning that all binding sites are constructed with similar orientation, thus improving binding site homogeneity.

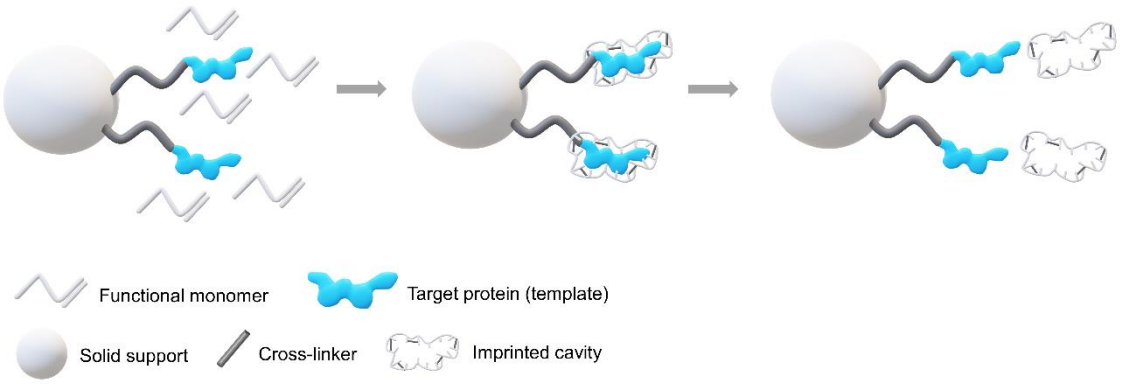

Figure 3. Schematic representation of protein imprinting with MIP synthesis with the target protein immobilized on the surface of a silica bead.

Ambrosini and co-workers reported the use of solid-phase synthesis of MIP-NPs for protein recognition using the model protein trypsin (23 kDa) (Ambrosini et al., 2013), adapting the solid-phase synthesis method previously developed for synthesis of a MIP for melamine, a small molecule (<1 kDa) (Poma et al., 2013). Trypsin was immobilized on the surface of glass beads, according with the approach reported for melamine, and the beads were then packed into a column, where the functional monomers (NIPAM and EBA) were added, and the reaction took place. Finally, several washing steps were performed for purification of the MIPs. Despite the difference in size of the target molecules, the immobilization on the solid support appears to depend mostly on the functional groups present in the target molecule, thus rendering solid-phase synthesis a versatile method for MIP synthesis. Still, one has to consider that large biomolecules are more complex and they often have similar reactive groups (e.g. amines or carboxylic acids) on different locations, which makes more challenging to obtain specificity on immobilization of the template biomolecule with uniform orientations. One advantage of the solid-phase synthesis strategy is the decrease of the cost of the process due to the solid phase being reused several times for MIP synthesis, thereby saving template molecules. One study reported the maintenance of the size and K_D of the MIPs for over 30 batches of template reuse (Poma et al., 2013), that value being inferior to the standard protein A chromatography performance for antibody production, which can be reused for 100 cycles. However, stability of proteins under reaction conditions for several cycles should be assessed, as literature is scarce concerning this information and there is a severe lack of studies presenting a process design and model along with realistic economic analyses.

To selectively separate lysozyme from a mixture of proteins in aqueous solution, acrylamide and acryloyl-β-cyclodextrin were used as functional monomers (Zhang et al., 2009). Here, the target protein was immobilized on the surface of silica beads and the polymerization reaction took place around the immobilized lysozyme. After removal of the template and consequent detachment from the beads, the MIPs were packed in a column. A successful high performance liquid chromatography (HPLC) separation was achieved, with lysozyme selectively separated from cytochrome c, BSA and avidin, with a maximum adsorption capacity for lysozyme of 44.6 mg/g, being 4 times higher than for the remaining proteins (Zhang et al., 2009). In a different study, instead of using solid phase synthesis, the same acrylamide and methacrylic acid were also used as functional monomers to obtain a lysozyme imprinted MIP. Impressively, the obtained MIP was

successfully used to purify this protein from egg white using a chromatographic column, with maximum adsorption capacity reaching 94.8 mg/g, purity close to 100% and mass recovery of 98.2% (Wang et al., 2014). Overall, these studies highlight the high values of maximum adsorption capacities achieved through solid-phase synthesis of MIPs for proteins, thus suggesting that immobilization and consequent orientation of the template molecule might contribute to higher selectivity of the MIPs.

A different approach that also does not require the immobilization of the template is MIP electrosynthesis, where cyclic voltammetry is used for electropolymerisation of the MIP. Here, a pre-polymerisation mixture of template protein and monomer is prepared, which is then deposited onto an electrode surface by applying cyclic potential sweeps. A study on the electrochemical quantification of troponin T (37 kDa), a biomarker of myocardial injury, used o-phenylenediamine as a monomer and a gold electrode for deposition of the MIP to build a biosensor based on a redox probe (Karimian et al., 2014). A similar study for recognition and electrochemical detection of myoglobin (18 kDa) used screen-printed electrodes where the pre-polymerisation mixture of myoglobin and o-phenylenediamine was electrodeposited (Shumyantseva et al., 2016). Even so, it is necessary to consider the difficulty to scale-up electrosynthesis processes due to potential difficulties to increase the electrode active area and electronic transport in the bulk of the reaction mixture. Furthermore, recyclability of the template is not considered, thus limiting the applicability of this method to less expensive targets, or resulting in process with prohibitive costs.

To the best of our knowledge, only one molecular imprinting methodology was reported with the objective to synthetize a replica of the protein, which is based on a two-step imprinting process. The strategy follows the approach to: i) firstly, to obtain a molecular cast of the target antibody, synthetized as a MIP particle, and then ii) to perform the second imprinting stage, analogous to a stamping method, in which polymerization occurs by compression of the pre-synthetized MIPs particles onto a pre-polymerized layer placed on the surface of a quartz crystal microbalance (QCM) electrode. Therefore, the polymer layer will be covered with molecularly imprinted antibody replicas after removing the stamp (Hussain et al., 2013; Jenik et al., 2009; Latif et al., 2014; Schirhagl et al., 2010). This methodology limits the range of applications to those that are usually based on immobilized antibodies on surfaces, such as biosensors or immunoassays that are often performed on chips. However, it is difficult to gather if the production of such immunoassay platforms could be improved with this strategy instead of using actual antibodies. Again, literature is found lacking on an actual economic analysis of the cost of production.

Although direct imprinting for detection and separation of proteins seems to be fairly well explored in the literature, there is still a call for designing MIPs for other challenging proteins with biomedical interest, particularly disease biomarkers, like surface membrane proteins expressed in cancer. One point of concern is the fact that cross-selectivity between similar proteins may impair a MIP performance, resulting on false positives, as several studies look at the selectivity of the MIPs against proteins that are not the target, but share similar structural characteristics. Indeed, in a competitive assay, using the previously mentioned lysozyme MIPs, no statistical difference was found in adsorption capacity for lysozyme, trypsin and cytochrome c, three proteins of high isoelectric point. While such MIP bound preferentially to lysozyme, the maximum adsorption capacity was close to 800 mg/g for the three proteins (Culver et al., 2016). This result raises a concern over the fact that imprinting alone may not account for the selectivity of certain classes of proteins.

2.2. Epitope imprinting

When entire proteins are used as templates for MIPs preparation, their efficient removal after polymerization is impaired, due to difficult diffusion through the MIP network. Additionally, proteins' tertiary conformations, which depend on conditions, such as pH, solvent and temperature (Kryscio et al., 2012), are unstable, contributing to lack of MIPs selectivity. To overcome such drawbacks, using only a part of the protein as template has been purposed as an imprinting strategy. Indeed, in the epitope imprinting approach, short linear peptides are used as the target molecules for the MIP. Therefore, in this case, the selective recognition neglects the protein 3D conformational specificity and relies on amino acid recognition, since imprinting is based only on the amino acid sequence, the primary structure of the peptide, instead of secondary and tertiary structures of proteins. Epitope design and selection strategies have been extensively discussed elsewhere (Tse Sum Bui et al., 2023), covering computational tools for selection of appropriate amino acids sequences and peptide length to maximize affinity of the MIP developed.

A possible strategy for proteins that have their C- or N-terminus exposed, such extremity is used as the site around which the MIP will be formed and the protein selectively captured. Nonapeptides, peptides with nine amino acid sequence length, as target molecules have been selected the length large enough to allow the unique identification of a particular protein (Nishino et al., 2006). Such strategy was used for the cases of MIPs development for cytochrome c, BSA and alcohol dehydrogenase (ADH), in which as nine amino acid sequence peptides of the C-terminus of these proteins were successfully used as templates in the synthesis of molecular imprinted films (Nishino et al., 2006). In these examples, the authors also used solid phase synthesis to facilitate the polymerization reaction around the template peptides.

MIP-NPs were also synthetized for an exposed C-terminus peptide of green fluorescent protein (GFP) by inverse microemulsion polymerization, using AAm and MBA as monomers and APS and TEMED as initiators (Zeng et al., 2011). Surfactants were also added so that polymerization occurred in inverse microemulsion, where the peptide was correctly oriented at the interface of water and oil domains, without the need for previous template surface immobilization. In another study an exposed antigenic domain of Lpp20, an outer membrane lipoprotein antigen specifically expressed by all the *H. pylori* strains, was used as a template in inverse mini-emulsion polymerization, using AAm as functional monomer. The obtained MIP was successfully assessed to capture the bacteria *H. pylori* (Han et al., 2015). While examples of epitope imprinting by inverse emulsion polymerization are still scarcely reported, possibly due to poor stability of epitopes on the water/oil interface, this is an innovative and apparent simple method for MIPs preparation, as it skips the steps of template immobilization required on solid phase synthesis, *i.e.* avoids the steps of activation and functionalization of the solid support, immobilization of template and several washing steps.

It is worth noting that most studies overviewed here use acrylate-based functional monomers, without discussion further progresses in the literature in the selection and use of alternatives types of monomers. The broader use of different functional monomers is reviewed elsewhere (Teixeira et al., 2021). Again, for the most studies reviewed, MBA and APS are the recurrent choices for cross-linker and initiator, respectively. Such selection has several advantages in terms of polymerization techniques efficiency and obtained MIP performance. However, considering today's concerns on promoting the development of sustainable and greener processes in MIP design (Arabi et al., 2021a),

alternative reagents could be procured, such as, for example, itaconic acid obtained from fermentation of Aspergillus species.

Overall, epitope imprinting has the advantage of relying only on the use of short peptides as templates instead of the whole protein, which are potentially easy to synthetize, and thus more available than their protein counterparts. However, not all proteins meet the criteria set for this approach.

2.2.1. Conformational epitope imprinting

Epitope imprinting based on the primary structure of an exposed peptide sequence, although efficient, is limited, as not all proteins meet the required criteria, such as the target protein to include an exposed C-terminus or an amino acid sequence both specific and short enough. A possible solution for these limitations is the use of conformational epitope imprinting, this approach uses as template the primary, secondary and tertiary structure of the selected epitope, thus, incorporating the recognition of the specific 3D conformation of the epitope into the MIP.

This strategy was applied in the design of a MIP for the p32 protein, by inverse microemulsion polymerization (Zhang et al., 2015). p32, a membrane protein that is overexpressed on the surface of varied tumour cell types, has the potential to target and mediate drug delivery and thus the developed MIP has the potential to actively targeting tumours. This protein has an N-terminal α-helix in the extracellular domain, which was set as the target site for the MIP. The peptide apamin mimics the extracellular domain of p32, as it has a sequence of seven aa residues identical to the one present on this domain, and it was successfully used as the imprinting template (Zhang et al., 2015).

2.2.2. Peptide imprinting

Peptide imprinting has not only been used for protein recognition, but also for recognition of the peptides themselves. In this strategy, a short peptide sequence is again used as template. Synthesis of peptide-selective MIPs has been reported for varied peptides. A MIP for the recognition of the hormone oxytocin was synthetized by bulk polymerization, using as template a tetrapeptide with the same three amino-acid C-terminal section of the structure of oxytocin, and using methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as crosslinker (Rachkov and Minoura, 2000).

A MIP for the recognition of the hormone angiotensin II, with a detection limit of 8 pM, was achieved by free radical polymerization using as functional monomer sodium acrylate, and as cross-linker poly(ethylene glycol) diacrylate. In this case, the whole peptide was used as template since the it has a short eight aa sequence (Rachkov et al., 2004).

The synthesis of MIP-NPs for the specific binding of melittin, which is a bee venom biotoxin, was achieved by precipitation polymerization (Hoshino et al., 2008). Melittin has 26 amino acids, of which 6 are positively charged, 6 residues at the C-terminus are hydrophilic, and the remainder is mostly composed of non-polar amino acid residues. In this study, the authors established a rationale for monomer selection considering the overall polarity of the peptide and the individual charge of each amino acid residue of the peptide sequence. Hence, the optimum monomer combination should comprise a mix of hydrophobic and negatively charged monomers to bind to the opposing charges of the residues of the target peptide. The two most successful monomer combinations contain 40% of hydrophobic monomers (TBAm) and 5% of negatively

charged functional monomers (acrylic acid, AAc), or 5% hydrogen bonding monomer (AAm), 5% negatively charged monomers (AAc) and 40% of hydrophobic monomers (TBAm). Dissociation constants (K_D) obtained by nonlinear fitting of Langmuir isotherms for these MIPs were in the range of 7.3-25 pM, which are comparable to the dissociation constant of a natural antibody (17 pM). The values of K_D of the MIPs are of the same order of magnitude as the ones found for antibodies, which range from 10 pM to 5 nM depending on antibody (Friguet et al., 1985; Landry et al., 2015; Pan et al., 2016), thus suggesting that the same affinities can be achieved for the MIP as the natural counterpart, showing the importance of functional monomer selection for successful biomacromolecule MIP synthesis. These strategies illustrate a more holistic approach where the global charge and hydrophobic/hydrophilic balance of molecules is considered on MIP design, to potentiating more probable success on tailoring host site formation to template specific structural properties.

3. Applications

MIPs for biomacromolecules have the potential for numerous applications, from bioseparation and purification processes in biotechnology and pharmaceutical industries to biomedical applications. Many of the studies described in this section target selective recognition of proteins aiming at the potential application of MIPs in separation and purification of proteins within pharma or food industry processes. In this context, the use of MIPs has been explored in affinity chromatography as it has been demonstrated for lysozyme, BSA, haemoglobin, and cytochrome c (Li et al., 2006; Ouyang et al., 2010; Wang et al., 2019, 2014; Zhang et al., 2009).

The biomedical field covers a broad spectrum of potential applications for biomacromolecule MIPs, spanning from biosensors and targeted drug delivery. MIP-based biosensors have been extensively explored in the literature, with protein quantification method being the prime target for MIP application, as the current technology relies on expensive immunoassays. An especially motivating application of MIP biosensors is their use as diagnostic tools. Figure 4 and Table 3 resume some examples of recent studies using MIPs for proteins, coupled to varied sensing units, as biosensor diagnostic tests with clinical relevance. As previously mentioned, MIP-based biosensors for detection of biomarkers of cancer and neurodegenerative diseases, often using electrochemical detection, have been a focus of recent research. Nevertheless, MIP-based electrochemical biosensors have also been developed for other clinically relevant biomarkers such as troponin T (Karimian et al., 2014) or myoglobin (Shumyantseva et al., 2016) in cardiac disease. Several studies have also explored MIPs for detection of viral detection systems, for example for poliovirus (Wang et al., 2010), bovine leukaemia virus (Ramanaviciene and Ramanavicius, 2004) and dengue virus (Tai et al., 2005).

Of particular relevance in recent years are MIP-based sensors for detection of SARS-CoV-2, for instance using a disposable electrochemical chip with high sensitivity (Raziq et al., 2021), or a surface plasmon resonance optical sensor (Cennamo et al., 2021), capable of detecting SARS-CoV-2 in nasopharyngeal swab samples of COVID-19 positive patients (Figure 4). Further works using MIP-based electrochemical sensors for SARS-CoV-2 have been developed using electroconductive polymers, namely polypyrrole, with significant sensitivity of detection with calibration curves with protein concentrations ranging 0-25 µg/mL (Ratautaite et al., 2023, 2022). A study worth noting is the design of an electrochemical sensor using two alkane thiols (11-mercaptoundecanoic acid and 6-mercapto-1-hexanol) to form a self-assembled monolayer MIP for the spike protein of SARS-CoV-2 with a reported LOD as low as 0.34

nM and a limit of quantification around 1 nM (Zukauskas et al., 2023). Overall, electrochemical detection coupled to MIP for proteins appears to be a promising method to reach low LODs for relevant clinical targets.

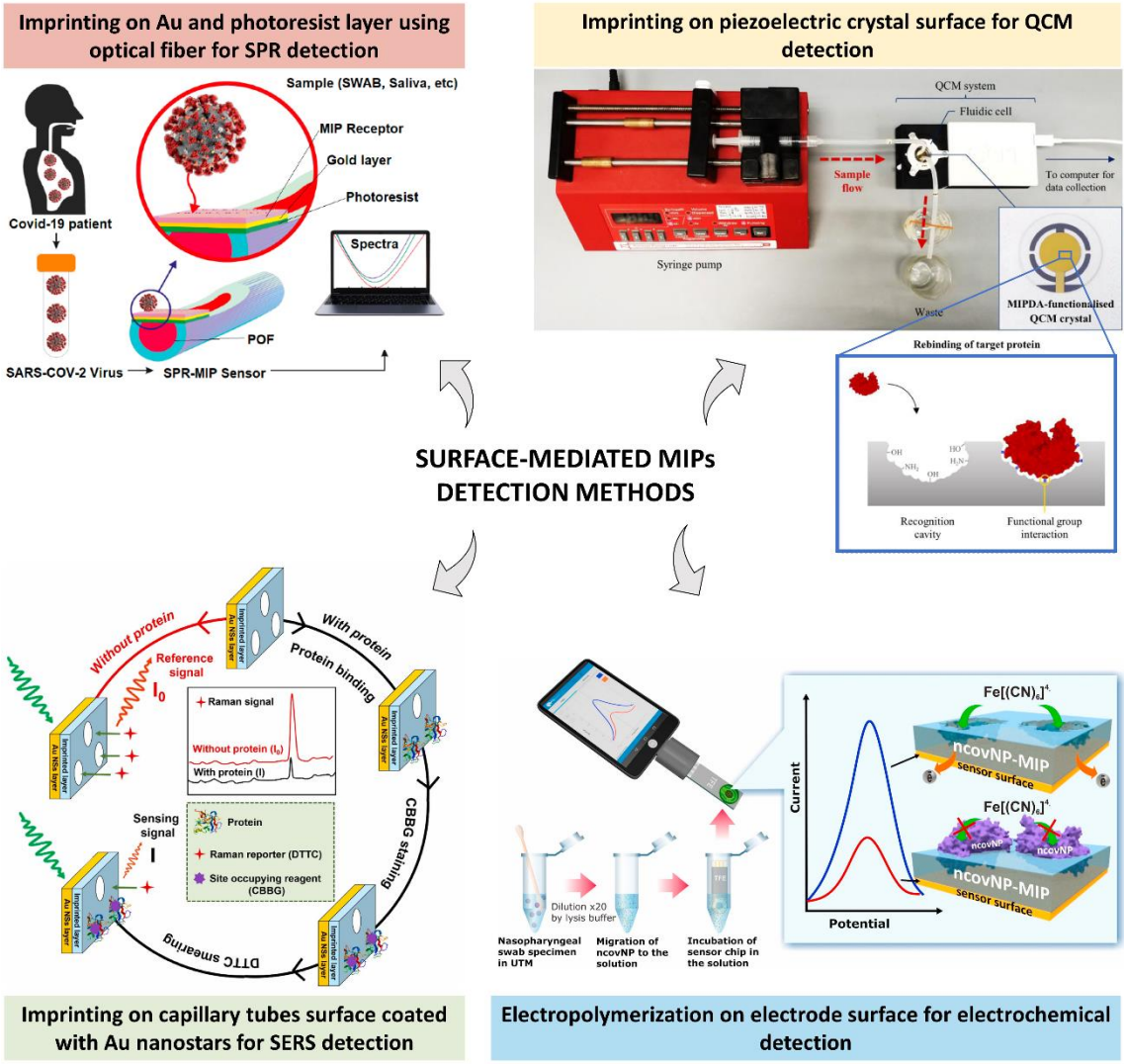

Figure 4. Representative surface mediated MIPs detection methods, including surface plasmon resonance (SPR) using a plastic optical fiber (POF) (reprinted with permission from (Cennamo et al., 2021) under Creative Commons License – Attribution 4.0 International – CC BY 4.0 – https://creativecommons.org/licenses/by/4.0/legalcode), QCM (reprinted from (Lim et al., 2023), Copyright 2023, with permission from Elsevier), surface-enhanced Raman scattering (SERS) (reprinted from (Arabi et al., 2021b), Copyright 2023, with permission from Elsevier), and an electrochemical method (reprinted from (Raziq et al., 2021), Copyright 2023, with permission from Elsevier).

Table 3. MIPs for proteins with clinical relevance and detection units as biosensor diagnostic tools.

Target protein	Clinical relevance	Detection	Ref.
Troponin T	Cardiac biomarker for early cardiac disease diagnosis	Electrochemical	(Karimian et al., 2014)
Myoglobin	Very early cardiac biomarker of acute myocardial infarction	Square wave and differential pulse voltammetry	(Shumyantseva et al., 2016)
Albumin	Indicator of kidney or liver disfunction	QCM	(Lin et al., 2004)
Butyrylcholinesterase	Acute and chronic liver damage indicator; prognostic indicator in cancer	Electrochemical	(Ozcelikay et al., 2019)
Human chorionic gonadotropin	Marker of ectopic pregnancy or trophoblastic tumours; screen for fetal congenital abnormalities	Chemosensing by extended-gate field-effect transistors and capacitive impedimetry	(Dabrowski et al., 2019)
PSMA	Prostate cancer biomarker	Love wave sensor	(Tang et al., 2018)
Carcinoembryonic antigen (CEA) and poliovirus	CEA: Colon cancer biomarker; Poliovirus: causative agent of poliomyelitis	Potentiometry	(Wang et al., 2010)
Bovine leukaemia virus glycoprotein	Bovine leukaemia diagnostic	Electrochemical	(Ramanaviciene and Ramanaviciu s, 2004)
Dengue virus NS1 protein	Indicator of dengue virus infection	QCM	(Tai et al., 2005)
Human interleukin-1	Indicator of varied inflammatory diseases	Luminescence	(Tao et al., 2006)
SARS-CoV-2 nucleoprotein	COVID-19 diagnostic	Electrochemical	(Raziq et al., 2021)
Subunit 1 of the SARS-CoV-2 Spike protein	COVID-19 diagnostic	Surface plasmon resonance	(Cennamo et al., 2021)
SARS-CoV-2 Spike protein	COVID-19 diagnostic	Electrochemical	(Ratautaite et al., 2023, 2022; Zukauskas et al., 2023)

MIPs may also be explored as targeting agents, for example, of a tumour, by binding to the membrane surface protein of the tumour cells, namely of a protein that is overexpressed on the surface of cancer cells. In this line of work, MIP-NPs have been

developed to target the extracellular α-helix domain of the p32 protein in *in vitro* and *in vivo* models (Zhang et al., 2015). Such type of MIPs, loaded with fluorescent probe (IR-783 dye), were assessed for tumour imaging in mice, and when encapsulating a photosensitizer compound, such as methylene blue, that was used for photodynamic treatment of tumours. The encapsulation process took place during polymerization by simply adding the desired compounds (IR-783 and methylene blue) to the aqueous phase before initiating the inverse microemulsion polymerization, where AAm and MBA were used, respectively, as functional monomer and cross-linker (Zhang et al., 2015). This study demonstrated that MIP-NPs can simultaneously function as targeting tools and nanocarriers for drugs.

In another study, the C-terminal linear peptide of EGFR (amino acids 418−435: SLNITSLGLRSLKEISDG), an overexpressed receptor on the cell surface of many tumours, was used as the template for production of MIP-NPs by solid phase synthesis. This MIP was assessed for targeted drug delivery to MDA-MB-468 breast cancer cells (Canfarotta et al., 2018). In this case, a dual imprinting strategy was followed, in which the chosen drug (doxorubicin) was used as a secondary template present in solution with the monomers (NIPAm, TBAm and APMA) and the cross-linker (MBA) (Canfarotta et al., 2018), as represented in Figure **5**5. After binding of the MIP to the EGFR receptor, the anticancer drug would be released by diffusion and accumulate around cancer cells, promoting cancer cell death. This study illustrates a strategy where the MIP presents binding sites for the drug and for the membrane receptor, demonstrating that MIP-NPs can selectively deliver a drug, by response to the tumour microenvironment, to specific cell targets.

The potential of using MIPs for therapies in humans is an extremely important feature that need to be addressed. Some studies have demonstrated efficacy in cell culture and some have used *in vivo* models, such as mice. For instance, a MIP targeting the folate receptor in cancer cells have been tested in mice bearing tumours, showing both the safety of using the MIPs in an organism, while showing the effectiveness of the targeting of the tumour and drug delivery (Liu et al., 2017).

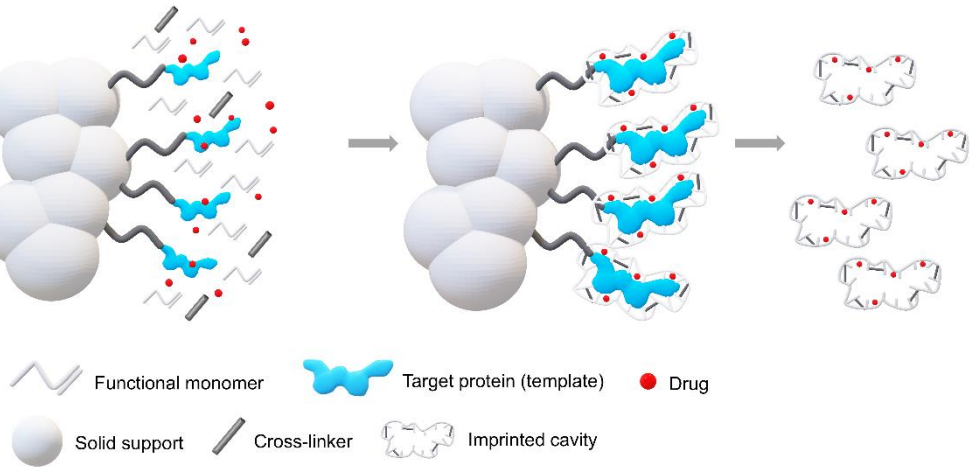

Figure 5. Schematic representation of dual imprinting strategy using solid-phase synthesis.

An important feature in clinical applications is the successful detection of a therapeutic targeting agent by imaging techniques. A synergistic chemo- and photo-dynamic cancer therapy with a dual imaging agent relying on dual-template MIP-NPs was recently demonstrated in *in vitro* and *in vivo* models. In this study, the MIPs were

synthetized for the CD59 epitope, as this protein is overexpressed in solid tumours, and the secondary template was doxorubicin as the chemotherapy agent. For the dual fluorescent/magnetic resonance imaging, gadolinium-doped silicon quantum dots were first prepared and used as the core of the MIP-NPs, and photosensitizer chlorin e6, the photo-dynamic cancer therapeutic, was embedded in the silica core. The polymerization took place on the surface using NIPAm, Aam and TBAm as functional monomers and MBA as cross-linker (Peng et al., 2020). The synthesis process complexity increases the number of variables to be considered over the synthesis, *i.e.* functional monomers that will match functional groups in both peptide and drug templates, cross-linkers, initiators and solvents compatible for both molecules. This work successfully showed that the already evidenced targeting ability of MIPs for biomarkers can be further explored to comprise several therapeutic options. Furthermore, it also evidences the potential of protein MIP-NPs to be coupled to imaging tools, allowing a more precise guided cancer treatment.

4. Conclusions

The field of molecular imprinting of biomacromolecules has seen significant growth over the past decade despite the inherent challenges, such as the need of their synthesis to be compatible with the use of aqueous media and allow for the immobilization of the target molecule, to ensure that proteins and peptides maintain their native conformations during polymerization.

In light of the advantages of MIPs, such as being cheap, easy to synthetize in a reproducible way, and showing robust performances in a variety of solvents, it is not surprising that MIPs for biomolecules have been considered as suitable for medical diagnosis and therapeutics, but also as replacement of enzymes in catalytic processes or even used as bioelectrodes for energy harvesting.

Concerning the template molecule, most proof-of-concept studies used as templates the readily available and "low-cost" proteins, such as BSA or cytochrome c. This could be somewhat expected as more interesting protein targets are expensive, as is the case of human disease biomarkers, such as receptors that are overexpressed on the surface of tumour cells. However, the impact of using MIPs, instead of antibodies, is based on the cost effectiveness of the process to obtain MIPs, therefore it is important to maintain low costs when expensive templates are used. A possible strategy could be the reuse of the template in several polymerization reactions, when it is immobilized on a solid surface, similarly to what is seen for catalytic enzymes in bioprocesses, that are reused throughout batches until they lose enzymatic activity. Unfortunately, the literature is severely lacking on studies reporting for how many batches, can the templates be reused in MIPs synthesis, with only one available study reporting the maintenance of the MIPs properties (size and K_D) for over 30 batches (Poma et al., 2013). Therefore, there is the need of additional studies on templates recyclability, scaling up of synthesis and purification of MIPs for proteins. It is fundamental that process design and model accompanied by economic analyses to be performed for such systems in order to assess the applicability of MIPs for proteins in industry, concerning MIPs economic competitiveness with the use of antibodies.

Epitope imprinting appears to be a promising approach for the near future as it not only minimizes the complications of dealing with very large and condition-sensitive templates during polymerization, but also decreases the difficulty in template removal after syntheses, due to diffusion constraints in the polymer network. This strategy could contribute to decrease the cost associated with the template as well. However, this also

comes with limitations as these epitopes need to comply with certain characteristics, namely an appropriate length to ensure a specific recognition mechanism, and loss of the tertiary structure contributions to recognition specificity.

In terms of applications, the potential of using MIPs as biosensors seems to be a consensus in the literature, with most studies focusing on diagnostics, ranging from cardiovascular and neurodegenerative diseases to cancer. There is a continuous call for new MIP development as new pathogens are identified, and more reliable disease biomarkers are being assessed in biology and medicine. The targeting potential for directed drug delivery has also been reported showing that MIPs for biomarkers can target tumours and function as drug carriers, for example. However, it was not until recently that MIPs with dual affinity sites (biomarker and drug) were synthetized and tested in *in vitro* and *in vivo* models. Indeed, this adds complexity to the synthesis process and possible cross interference in binding site formation for the protein should be further assessed. So far, studies have relied on MIP-NP endocytosis by the cells for drug delivery or laser incidence for photodynamic therapy. Thus, it would be interesting to assess the incorporation of stimuli responsive characteristics on MIPs, such as pH-responsive or electro-responsive, for enhanced controlled targeted drug delivery.

Furthermore, it is extremely important to ensure the safety of use of MIPs in human patients. However, very few studies have been performed using animal models in pre-clinical settings, to show the safety and efficacy of MIPs for therapeutic applications.

Overall, moving forward to clinical approval and commercialization of MIPs for proteins and peptides for biomedical applications, it would be interesting to see further studies reporting biocompatibility, safety and immunogenic responses using in vivo models to ease bench to bedside transition. Additionally, for studies claiming reusability of the template and downsizing of production costs, it will be important to have experimental data and economic analysis supporting claims that would ease the path to commercial success of MIPs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work is financed by national funds from FCT - Fundação para a Ciência e a Tecnologia, I.P., with dedicated funds from the project eOnco (2022.07252.PTDC) and the PhD scholarship (SFRH/BD/145057/2019), iBB (UIDB/04565/2020 and UIDP/04565/2020), i4HB (LA/P/0140/2020). This publication is part of the I+D+i project PID2021-125767OB-I00 funded by MCIN/AEI/ 10.13039/501100011033 and, as appropriate, by “ERDF A way of making Europe”, by the European Union. Authors are thankful to the Agència de Gestió d'Ajuts Universitaris i de Recerca (2021 SGR 00387) for financial support. Support for the research of C.A. was also received through the prize “ICREA Academia” for excellence in research funded by the Generalitat de Catalunya.

References

Alexander, C., Andersson, H.S., Andersson, L.I., Ansell, R.J., Kirsch, N., Nicholls, I.A., O’Mahony, J., Whitcombe, M.J., 2006. Molecular imprinting science and technology: A survey of the literature for the years up to and including 2003. J.

644 Mol. Recognit. 19, 106–180. https://doi.org/10.1002/jmr.760
645 Ambrosini, S., Beyazit, S., Haupt, K., Tse Sum Bui, B., 2013. Solid-phase synthesis of
646 molecularly imprinted nanoparticles for protein recognition. Chem. Commun. 49,
647 6746–6748. https://doi.org/10.1039/c3cc41701h
648 Ansari, S., Masoum, S., 2019. Molecularly imprinted polymers for capturing and
649 sensing proteins: Current progress and future implications. Trends Anal. Chem.
650 114, 29–47. https://doi.org/10.1016/j.trac.2019.02.008
651 Arabi, M., Ostovan, A., Li, J., Wang, X., Zhang, Z., Choo, J., Chen, L., 2021a.
652 Molecular Imprinting: Green Perspectives and Strategies. Adv. Mater. 33.
653 https://doi.org/10.1002/adma.202100543
654 Arabi, M., Ostovan, A., Zhang, Z., Wang, Y., Mei, R., Fu, L., Wang, X., Ma, J., Chen,
655 L., 2021b. Label-free SERS detection of Raman-Inactive protein biomarkers by
656 Raman reporter indicator: Toward ultrasensitivity and universality. Biosens.
657 Bioelectron. 174, 112825. https://doi.org/10.1016/J.BIOS.2020.112825
658 Arbabi-Ghahroudi, M., 2022. Camelid Single-Domain Antibodies: Promises and
659 Challenges as Lifesaving Treatments. Int. J. Mol. Sci. 23.
660 https://doi.org/10.3390/ijms23095009
661 Asaadi, Y., Jouneghani, F.F., Janani, S., Rahbarizadeh, F., 2021. A comprehensive
662 comparison between camelid nanobodies and single chain variable fragments.
663 Biomark. Res. 9. https://doi.org/10.1186/s40364-021-00332-6
664 Boysen, R.I., 2019. Advances in the development of molecularly imprinted polymers
665 for the separation and analysis of proteins with liquid chromatography. J. Sep. Sci.
666 42, 51–71. https://doi.org/10.1002/jssc.201800945
667 Canfarotta, F., Lezina, L., Guerreiro, A., Czulak, J., Petukhov, A., Daks, A., Smolinska-
668 Kempisty, K., Poma, A., Piletsky, S., Barlev, N.A., 2018. Specific Drug Delivery
669 to Cancer Cells with Double-Imprinted Nanoparticles against Epidermal Growth
670 Factor Receptor. Nano Lett. 18, 4641–4646.
671 https://doi.org/10.1021/acs.nanolett.7b03206
672 Canfarotta, F., Poma, A., Guerreiro, A., Piletsky, S., 2016. Solid-phase synthesis of
673 molecularly imprinted nanoparticles. Nat. Protoc. 11, 443–455.
674 https://doi.org/10.1038/nprot.2016.030
675 Cennamo, N., D'agostino, G., Perri, C., Arcadio, F., Chiaretti, G., Parisio, E.M.,
676 Camarlinghi, G., Vettori, C., Di Marzo, F., Cennamo, R., Porto, G., Zeni, L., 2021.
677 Proof of concept for a quick and highly sensitive on-site detection of sars-cov-2 by
678 plasmonic optical fibers and molecularly imprinted polymers. Sensors 21, 1–17.
679 https://doi.org/10.3390/s21051681
680 Chen, W., Meng, Z., Xue, M., Shea, K.J., 2016. Molecular imprinted photonic crystal
681 for sensing of biomolecules. Mol. Imprinting 4, 1–12.
682 https://doi.org/10.1515/molim-2016-0001
683 Culver, H.R., Steichen, S.D., Peppas, N.A., 2016. A Closer Look at the Impact of
684 Molecular Imprinting on Adsorption Capacity and Selectivity for Protein
685 Templates. Biomacromolecules 17, 4045–4053.
686 https://doi.org/10.1021/acs.biomac.6b01482
687 Dabrowski, M., Zimińska, A., Kalecki, J., Cieplak, M., Lisowski, W., Maksym, R.,
688 Shao, S., D'Souza, F., Kuhn, A., Sharma, P.S., 2019. Facile Fabrication of
689 Surface-Imprinted Macroporous Films for Chemosensing of Human Chorionic
690 Gonadotropin Hormone. ACS Appl. Mater. Interfaces 11, 9265–9276.
691 https://doi.org/10.1021/acsami.8b17951
692 El Kirat, K., Bartkowski, M., Haupt, K., 2009. Probing the recognition specificity of a
693 protein molecularly imprinted polymer using force spectroscopy. Biosens.

694 Bioelectron. 24, 2618–2624. https://doi.org/10.1016/j.bios.2009.01.018
695 Friguet, B., Chaffotte, A.F., Djavadi-Ohaniance, L., Goldberg, M.E., 1985.
696 Measurements of the true affinity constant in solution of antigen-antibody
697 complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods 77,
698 305–319. https://doi.org/10.1016/0022-1759(85)90044-4
699 Guoning, C., Hua, S., Wang, L., Qianqian, H., Xia, C., Hongge, Z., Zhimin, L., Chun,
700 C., Qiang, F., 2020. A surfactant-mediated sol-gel method for the preparation of
701 molecularly imprinted polymers and its application in a biomimetic immunoassay
702 for the detection of protein. J. Pharm. Biomed. Anal. 190, 113511.
703 https://doi.org/10.1016/J.JPBA.2020.113511
704 Haginaka, J., Sakai, Y., 2000. Uniform-sized molecularly imprinted polymer material
705 for (S)-propranolol. J. Pharm. Biomed. Anal. 22, 899–907.
706 https://doi.org/10.1016/S0731-7085(00)00293-4
707 Hammam, M.A., Wagdy, H.A., El Nashar, R.M., 2018. Moxifloxacin hydrochloride
708 electrochemical detection based on newly designed molecularly imprinted
709 polymer. Sensors Actuators, B Chem. 275, 127–136.
710 https://doi.org/10.1016/j.snb.2018.08.041
711 Han, J., Sun, Y., Hou, J., Wang, Y., Liu, Y., Xie, C., Lu, W., Pan, J., 2015. Preliminary
712 investigations into surface molecularly imprinted nanoparticles for Helicobacter
713 pylori eradication. Acta Pharm. Sin. B 5, 577–582.
714 https://doi.org/10.1016/j.apsb.2015.09.003
715 Han, S., Su, L., Zhai, M., Ma, L., Liu, S., Teng, Y., 2019. A molecularly imprinted
716 composite based on graphene oxide for targeted drug delivery to tumor cells. J.
717 Mater. Sci. 54, 3331–3341. https://doi.org/10.1007/s10853-018-3023-8
718 Haupt, K., Medina Rangel, P.X., Bui, B.T.S., 2020. Molecularly imprinted polymers:
719 Antibody mimics for bioimaging and therapy. Chem. Rev. 120, 9554–9582.
720 https://doi.org/10.1021/acs.chemrev.0c00428
721 Hoshino, Y., Kodama, T., Okahata, Y., Shea, K.J., 2008. Peptide imprinted polymer
722 nanoparticles: A plastic antibody. J. Am. Chem. Soc. 130, 15242–15243.
723 https://doi.org/10.1021/ja8062875
724 Hui Lee, S., Doong, R.A., 2016. Design of Size-Tunable Molecularly Imprinted
725 Polymer for Selective Adsorption of Pharmaceuticals and Biomolecules. J.
726 Biosens. Bioelectron. 07. https://doi.org/10.4172/2155-6210.1000228
727 Hussain, M., Wackerlig, J., Lieberzeit, P.A., 2013. Biomimetic strategies for sensing
728 biological species. Biosensors 3, 89–107. https://doi.org/10.3390/bios3010089
729 Jenik, M., Schirhagl, R., Schirk, C., Hayden, O., Lieberzeit, P., Blaas, D., Paul, G.,
730 Dickert, F.L., 2009. Sensing picornaviruses using molecular imprinting techniques
731 on a quartz crystal microbalance. Anal. Chem. 81, 5320–5326.
732 https://doi.org/10.1021/ac8019569
733 Karimian, N., Turner, A.P.F., Tiwari, A., 2014. Electrochemical evaluation of troponin
734 T imprinted polymer receptor. Biosens. Bioelectron. 59, 160–165.
735 https://doi.org/10.1016/j.bios.2014.03.013
736 Kryscio, D.R., Fleming, M.Q., Peppas, N.A., 2012. Conformational studies of common
737 protein templates in macromolecularly imprinted polymers. Biomed. Microdevices
738 14, 679–687. https://doi.org/10.1007/s10544-012-9648-5
739 Kryscio, D.R., Peppas, N.A., 2012. Surface imprinted thin polymer film systems with
740 selective recognition for bovine serum albumin. Anal. Chim. Acta 718, 109–115.
741 https://doi.org/10.1016/j.aca.2012.01.006
742 Landry, J.P., Ke, Y., Yu, G.L., Zhu, X.D., 2015. Measuring Affinity Constants of 1,450
743 Monoclonal Antibodies to Peptide Targets with a Microarray-based Label-Free

Assay Platform. J. Immunol. Methods 417, 86. https://doi.org/10.1016/J.JIM.2014.12.011
Latif, U., Qian, J., Can, S., Dickert, F.L., 2014. Biomimetic receptors for bioanalyte detection by quartz crystal microbalances — from molecules to cells. Sensors (Switzerland) 14, 23419–23438. https://doi.org/10.3390/s141223419
Lee, M.-H., Thomas, J.L., Su, Z.-L., Yeh, W.-K., Monzel, A.S., Bolognin, S., Schwamborn, J.C., Yang, C.-H., Lin, H.-Y., 2021. Epitope imprinting of alpha-synuclein for sensing in Parkinson's brain organoid culture medium. Biosens. Bioelectron. 175.
Li, Y., Yang, H.H., You, Q.H., Zhuang, Z.X., Wang, X.R., 2006. Protein recognition via surface molecularly imprinted polymer nanowires. Anal. Chem. 78, 317–320. https://doi.org/10.1021/ac050802i
Lim, H.J., Saha, T., Tey, B.T., Lal, S.K., Ooi, C.W., 2023. Quartz crystal microbalance-based biosensing of proteins using molecularly imprinted polydopamine sensing films: Interplay between protein characteristics and molecular imprinting effect. Surfaces and Interfaces 39, 102904. https://doi.org/10.1016/J.SURFIN.2023.102904
Lin, J.M., Nakagama, T., Uchiyama, K., Hobo, T., 1997. Capillary electrochromatographic separation of amino acid enantiomers using on-column prepared molecularly imprinted polymer. J. Pharm. Biomed. Anal. 15, 1351–1358. https://doi.org/10.1016/S0731-7085(96)02013-4
Lin, T.-Y., Hu, C.H., Chou, T.C., 2004. Determination of albumin concentration by MIP-QCM sensor. Biosens. Bioelectron. 20, 75–81. https://doi.org/10.1016/j.bios.2004.01.028
Liu, S., Bi, Q., Long, Y., Li, Z., Bhattacharyya, S., Li, C., 2017. Inducible epitope imprinting: "Generating" the required binding site in membrane receptors for targeted drug delivery. Nanoscale 9, 5394–5397. https://doi.org/10.1039/c6nr09449j
Liu, Y., Huang, H., 2018. Expression of single-domain antibody in different systems. Appl. Microbiol. Biotechnol. 102, 539–551. https://doi.org/10.1007/s00253-017-8644-3
Liustrovaite, V., Pogorielov, M., Boguzaite, R., Ratautaite, V., Ramanaviciene, A., Pilvenyte, G., Holubnycha, V., Korniienko, V., Diedkova, K., Viter, R., Ramanavicius, A., 2023. Towards Electrochemical Sensor Based on Molecularly Imprinted Polypyrrole for the Detection of Bacteria—Listeria monocytogenes. Polymers (Basel). 15. https://doi.org/10.3390/polym15071597
Liv, L., Çoban, G., Nakiboğlu, N., Kocagöz, T., 2021. A rapid, ultrasensitive voltammetric biosensor for determining SARS-CoV-2 spike protein in real samples. Biosens. Bioelectron. 192. https://doi.org/10.1016/j.bios.2021.113497
Malaquias, A.D.M., Marques, L.E.C., Pereira, S.S., de Freitas Fernandes, C., Maranhão, A.Q., Stabeli, R.G., Florean, E.O.P.T., Guedes, M.I.F., Fernandes, C.F.C., 2021. A review of plant-based expression systems as a platform for single-domain recombinant antibody production. Int. J. Biol. Macromol. 193, 1130–1137. https://doi.org/10.1016/j.ijbiomac.2021.10.126
Mark, J.K.K., Lim, C.S.Y., Nordin, F., Tye, G.J., 2022. Expression of mammalian proteins for diagnostics and therapeutics: a review. Mol. Biol. Rep. 49, 10593–10608. https://doi.org/10.1007/s11033-022-07651-3
Mohajeri, S.A., Ebrahimi, S.A., 2008. Preparation and characterization of a lamotrigine imprinted polymer and its application for drug assay in human serum. J. Sep. Sci. 31, 3595–3602. https://doi.org/10.1002/jssc.200800377

Nishino, H., Huang, C.S., Shea, K.J., 2006. Selective protein capture by epitope imprinting. Angew. Chemie - Int. Ed. 45, 2393–2396. https://doi.org/10.1002/anie.200503760

Ostovan, A., Arabi, M., Wang, Y., Li, J., Li, B., Wang, X., Chen, L., 2022. Greenificated Molecularly Imprinted Materials for Advanced Applications. Adv. Mater. 34. https://doi.org/10.1002/adma.202203154

Ouyang, R., Lei, J., Ju, H., 2010. Artificial receptor-functionalized nanoshell: Facile preparation, fast separation and specific protein recognition. Nanotechnology 21. https://doi.org/10.1088/0957-4484/21/18/185502

Özcan, N., Medetalibeyoglu, H., Akyildirim, O., Atar, N., Yola, M.L., 2020. Electrochemical detection of amyloid-beta protein by delaminated titanium carbide MXene/multi-walled carbon nanotubes composite with molecularly imprinted polymer. Mater. Today Commun. 23.

Ozcelikay, G., Kurbanoglu, S., Zhang, X., Soz, C.K., Wollenberger, U., Ozkan, S.A., Yarman, A., Scheller, F.W., 2019. Electrochemical MIP sensor for butyrylcholinesterase. Polymers (Basel). 11, 1–11. https://doi.org/10.3390/polym11121970

Pan, Y., Sackmann, E.K., Wypisniak, K., Hornsby, M., Datwani, S.S., Herr, A.E., 2016. Determination of equilibrium dissociation constants for recombinant antibodies by high-throughput affinity electrophoresis. Sci. Reports 2016 61 6, 1–11. https://doi.org/10.1038/srep39774

Peng, H., Qin, Y.-T., He, X.-W., Li, W.-Y., Zhang, Y., 2020. Epitope Molecularly Imprinted Polymer Nanoparticles for Chemo-/Photodynamic Synergistic Cancer Therapy Guided by Targeted Fluorescence Imaging. ACS Appl. Mater. Interfaces. https://doi.org/10.1021/acsami.0c00468

Pilvenyte, G., Ratautaite, V., Boguzaite, R., Plausinaitis, D., Ramanaviciene, A., Bechelany, M., Ramanavicius, A., 2023a. Molecularly Imprinted Polymers for the Recognition of Biomarkers for Some Neurodegenerative Diseases. J. Pharm. Biomed. Anal. 228.

Pilvenyte, G., Ratautaite, V., Boguzaite, R., Ramanavicius, A., Viter, R., Ramanavicius, S., 2023b. Molecularly Imprinted Polymers for the Determination of Cancer Biomarkers. Int. J. Mol. Sci. 24. https://doi.org/10.3390/ijms24044105

Ping Li, Fei Rong, Yibing Xie, Van Hu, Chunwei Yuan, 2004. Study on the binding characteristic of S-naproxen imprinted polymer and the interactions between templates and monomers. Zhurnal Anal. Khimii 59, 1043–1048.

Poma, A., Guerreiro, A., Whitcombe, M.J., Elena, V., 2013. Solid-Phase Synthesis of Molecularly Imprinted Polymer Nanoparticles with a Reusable Template – " Plastic Antibodies ." Adv. Funct. Mater. 23, 2821–2827. https://doi.org/10.1002/adfm.201202397

Rachkov, A., Hu, M., Bulgarevich, E., Matsumoto, T., Minoura, N., 2004. Molecularly imprinted polymers prepared in aqueous solution selective for [Sar1,Ala8]angiotensin II. Anal. Chim. Acta 504, 191–197. https://doi.org/10.1016/S0003-2670(03)00764-5

Rachkov, A., Minoura, N., 2000. Recognition of oxytocin and oxytocin-related peptides in aqueous media using a molecularly imprinted polymer synthesized by the epitope approach. J. Chromatogr. A 889, 111–118. https://doi.org/10.1016/S0021-9673(00)00568-9

Ramanaviciene, A., Ramanavicius, A., 2004. Molecularly imprinted polypyrrole-based synthetic receptor for direct detection of bovine leukemia virus glycoproteins. Biosens. Bioelectron. 20, 1076–1082. https://doi.org/10.1016/j.bios.2004.05.014

Ramanavicius, S., Ramanavicius, A., 2022. Development of molecularly imprinted polymer based phase boundaries for sensors design (review). Adv. Colloid Interface Sci. 305. https://doi.org/10.1016/j.cis.2022.102693
Ramanavicius, S., Samukaite-Bubniene, U., Ratautaite, V., Bechelany, M., Ramanavicius, A., 2022. Electrochemical molecularly imprinted polymer based sensors for pharmaceutical and biomedical applications (review). J. Pharm. Biomed. Anal. 215. https://doi.org/10.1016/j.jpba.2022.114739
Ratautaite, V., Boguzaite, R., Brazys, E., Plausinaitis, D., Ramanavicius, S., Samukaite-Bubniene, U., Bechelany, M., Ramanavicius, A., 2023. Evaluation of the interaction between SARS-CoV-2 spike glycoproteins and the molecularly imprinted polypyrrole. Talanta 253.
Ratautaite, V., Boguzaite, R., Brazys, E., Ramanaviciene, A., Ciplys, E., Juozapaitis, M., Slibinskas, R., Bechelany, M., Ramanavicius, A., 2022. Molecularly imprinted polypyrrole based sensor for the detection of SARS-CoV-2 spike glycoprotein. Electrochim. Acta 403. https://doi.org/10.1016/j.electacta.2021.139581
Raziq, A., Kidakova, A., Boroznjak, R., Reut, J., Öpik, A., Syritski, V., 2021. Development of a portable MIP-based electrochemical sensor for detection of SARS-CoV-2 antigen. Biosens. Bioelectron. 178. https://doi.org/10.1016/j.bios.2021.113029
Rebelo, T.S.C.R., Costa, R., Brandão, A.T.S.C., Silva, A.F., Sales, M.G.F., Pereira, C.M., 2019. Molecularly imprinted polymer SPE sensor for analysis of CA-125 on serum. Anal. Chim. Acta 1082, 126–135. https://doi.org/10.1016/j.aca.2019.07.050
Santos, A.R.T., Moreira, F.T.C., Helguero, L.A., Sales, M.G.F., 2018. Antibody biomimetic material made of pyrrole for CA 15-3 and its application as sensing material in ion-selective electrodes for potentiometric detection. Biosensors 8. https://doi.org/10.3390/bios8010008
Schirhagl, R., Lieberzeit, P.A., Dickert, F.L., 2010. Chemosensors for Viruses Based on Artificial Immunoglobulin Copies. Adv. Mater. 22, 2078–2081. https://doi.org/10.1002/adma.200903517
Scriba, G.K.E., 2016. Chiral recognition in separation science – an update. J. Chromatogr. A 1467, 56–78. https://doi.org/10.1016/j.chroma.2016.05.061
Shumyantseva, V. V., Bulko, T. V., Sigolaeva, L. V., Kuzikov, A. V., Archakov, A.I., 2016. Electrosynthesis and binding properties of molecularly imprinted poly-o-phenylenediamine for selective recognition and direct electrochemical detection of myoglobin. Biosens. Bioelectron. 86, 330–336. https://doi.org/10.1016/j.bios.2016.05.101
Suedee, R., Srichana, T., Rattananont, T., 2002. Enantioselective release of controlled delivery granules based on molecularly imprinted polymers. Drug Deliv. J. Deliv. Target. Ther. Agents 9, 19–30. https://doi.org/10.1080/107175402753413145
Sukjee, W., Thitithanyanont, A., Manopwisedjaroen, S., Seetaha, S., Thepparit, C., Sangma, C., 2022. Virus MIP-composites for SARS-CoV-2 detection in the aquatic environment. Mater. Lett. 315, 131973. https://doi.org/10.1016/j.matlet.2022.131973
Tai, D.F., Lin, C.Y., Wu, T.Z., Chen, L.K., 2005. Recognition of dengue virus protein using epitope-mediated molecularly imprinted film. Anal. Chem. 77, 5140–5143. https://doi.org/10.1021/ac0504060
Tang, P., Wang, Y., Huo, J., Lin, X., 2018. Love wave sensor for prostate-specific membrane antigen detection based on hydrophilic molecularly-imprinted polymer. Polymers (Basel). 10. https://doi.org/10.3390/polym10050563
Tao, Z., Tehan, E.C., Bukowski, R.M., Tang, Y., Shughart, E.L., Holthoff, W.G.,

Cartwright, A.N., Titus, A.H., Bright, F. V., 2006. Templated xerogels as platforms for biomolecule-less biomolecule sensors. Anal. Chim. Acta 564, 59–65. https://doi.org/10.1016/j.aca.2006.01.076

Teixeira, S.P.B., Reis, R.L., Peppas, N.A., Gomes, M.E., A Domingues, R.M., 2021. Epitope-imprinted polymers: Design principles of synthetic binding partners for natural biomacromolecules. Sci. Adv. 7.

Thompson, M.K., Fridy, P.C., Keegan, S., Chait, B.T., Fenyö, D., Rout, M.P., 2016. Optimizing selection of large animals for antibody production by screening immune response to standard vaccines. J. Immunol. Methods 430, 56–60. https://doi.org/10.1016/j.jim.2016.01.006

Trinh, T., Liao, C., Toader, V., Barlóg, M., Bazzi, H.S., Li, J., Sleiman, H.F., 2018. DNA-imprinted polymer nanoparticles with monodispersity and prescribed DNA-strand patterns. Nat. Chem. 10, 184–192. https://doi.org/10.1038/NCHEM.2893

Tse Sum Bui, B., Mier, A., Haupt, K., 2023. Molecularly Imprinted Polymers as Synthetic Antibodies for Protein Recognition: The Next Generation. Small 19. https://doi.org/10.1002/smll.202206453

Wackerlig, J., Schirhagl, R., 2016. Applications of Molecularly Imprinted Polymer Nanoparticles and Their Advances toward Industrial Use: A Review. Anal. Chem. 88, 250–261. https://doi.org/10.1021/acs.analchem.5b03804

Wang, P., Zhu, H., Liu, J., Ma, Y., Yao, J., Dai, X., Pan, J., 2019. Double affinity integrated MIPs nanoparticles for specific separation of glycoproteins: A combination of synergistic multiple bindings and imprinting effect. Chem. Eng. J. 358, 143–152. https://doi.org/10.1016/j.cej.2018.09.168

Wang, X., Dong, S., Bai, Q., 2014. Preparation of lysozyme molecularly imprinted polymers and purification of lysozyme from egg white. Biomed. Chromatogr. 28, 907–912. https://doi.org/10.1002/bmc.3207

Wang, Y., Zhang, Z., Jain, V., Yi, J., Mueller, S., Sokolov, J., Liu, Z., Levon, K., Rigas, B., Rafailovich, M.H., 2010. Potentiometric sensors based on surface molecular imprinting: Detection of cancer biomarkers and viruses. Sensors Actuators, B Chem. 146, 381–387. https://doi.org/10.1016/j.snb.2010.02.032

Whitcombe, M.J., Kirsch, N., Nicholls, I.A., 2014. Molecular imprinting science and technology: A survey of the literature for the years 2004-2011. J. Mol. Recognit. 27, 297–401. https://doi.org/10.1002/jmr.2347

Yang, K., Li, S., Liu, L., Chen, Y., Zhou, W., Pei, J., Liang, Z., Zhang, L., Zhang, Y., 2019. Epitope Imprinting Technology: Progress, Applications, and Perspectives toward Artificial Antibodies. Adv. Mater. 31. https://doi.org/10.1002/adma.201902048

Yang, M., Dong, Q., Guan, Y., Zhang, Y., 2023. Molecularly Imprinted Polymers with Shape-Memorable Imprint Cavities for Efficient Separation of Hemoglobin from Blood. Biomacromolecules. https://doi.org/10.1021/acs.biomac.2c01285

Yazdani, Z., Yadegari, H., Heli, H., 2019. A molecularly imprinted electrochemical nanobiosensor for prostate specific antigen determination. Anal. Biochem. 566, 116–125.

Zeng, Z., Hoshino, Y., Rodriguez, A., Yoo, H., Shea, K.J., 2011. Synthetic Polymer Nanoparticles with Antibody-Like Affinity for a Hydrophilic Peptide. ACS Nano 4(1), 1–12. https://doi.org/doi:10.1021/nn901256s

Zhang, W., Qin, L., He, X.-W., Li, W.-Y., Zhang, Y.-K., 2009. Novel surface modified molecularly imprinted polymer using acrylol-beta-cyclodextrin and acrylamide as monomers for selective recognition of lysozyme in aqueous solution. J. Chromatogr. A 1216, 4560–4567.

Zhang, Y., Deng, C., Liu, S., Wu, J., Chen, Z., Li, C., Lu, W., 2015. Active targeting of tumors through conformational epitope imprinting. Angew. Chemie - Int. Ed. 54, 5157–5160. https://doi.org/10.1002/anie.201412114

Zhang, Z., Liu, J., 2018. Intracellular delivery of a molecularly imprinted peroxidase mimicking DNAzyme for selective oxidation. Mater. Horizons 5, 738–744. https://doi.org/10.1039/c8mh00453f

Zukauskas, S., Rucinskiene, A., Ratautaite, V., Ramanaviciene, A., Pilvenyte, G., Bechelany, M., Ramanavicius, A., 2023. Electrochemical Biosensor for the Determination of Specific Antibodies against SARS-CoV-2 Spike Protein. Int. J. Mol. Sci. 24. https://doi.org/10.3390/ijms24010718