



## Review

## Critical review and perspective of macromolecularly imprinted polymers

David R. Kryscio<sup>a</sup>, Nicholas A. Peppas<sup>a,b,\*</sup><sup>a</sup>The University of Texas at Austin, Cockrell School of Engineering, Department of Chemical Engineering, Austin, TX 78712, USA<sup>b</sup>The University of Texas at Austin, Cockrell School of Engineering, Department of Biomedical Engineering, Austin, TX 78712, USA

## ARTICLE INFO

## Article history:

Received 5 August 2011

Received in revised form 25 October 2011

Accepted 3 November 2011

Available online 11 November 2011

## Keywords:

Molecular recognition

Molecularly imprinted polymers

Protein imprinting

Monomer–protein interaction

## ABSTRACT

Molecular recognition is a fundamental and ubiquitous process that is the driving force behind life. Natural recognition elements – including antibodies, enzymes, nucleic acids, and cells – exploit non-covalent interactions to bind to their targets with exceptionally strong affinities. Due to this unparalleled proficiency, scientists have long sought to mimic natural recognition pathways. One promising approach is molecularly imprinted polymers (MIPs), which are fully synthetic systems formed via the crosslinking of organic polymers in the presence of a template molecule, which results in stereo-specific binding sites for this analyte of interest. Macromolecularly imprinted polymers, those synthesized in the presence of macromolecule templates (>1500 Da), are of particular importance because they open up the field for a whole new set of robust diagnostic tools. Although the specific recognition of small-molecular-weight analytes is now considered routine, extension of these efficacious procedures to the protein regime has, thus far, proved challenging. This paper reviews the main approaches employed, highlights studies of interest with an emphasis on recent work, and offers suggestions for future success in the field of macromolecularly imprinted polymers.

© 2011 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.

## 1. Introduction

Molecular recognition is a fundamental biological mechanism ubiquitous in nature. This elegant, yet simple, mechanism is found in a variety of biological processes, including antibody/antigen recognition in the immune system, enzymatic catalysis, signal transduction, and nucleic acid interactions such as replication, transcription, and translation. Natural recognition is driven largely by non-covalent forces, including ionic interactions, hydrogen bonding, van der Waals forces, pi interactions, and entropic considerations such as the hydrophobic effect [1]. Molecular recognition relies on the complex between receptor and substrate, which Emil Fischer first described as the “lock and key model” over a century ago. This elegant concept asserts that the formation of the complex is the result of intermolecular interactions between complementary functional groups on the lock or receptor (protein/enzyme) and the desired key or substrate (analyte) [2]. In other words, the two molecules must correspond both spatially and chemically.

The main receptors responsible for specific recognition in nature are antibodies (immunoglobulins), enzymes, nucleic acids, and cells. Antibodies, the most widely studied, are large Y-shaped glycoproteins (or groups of proteins), 10–40 nm in diameter,

produced and used in immune systems of higher-order organisms to identify and neutralize foreign substances (antigens). Antibodies consist of two identical heavy polypeptide chains (55 kDa) linked via disulfide bridges with two identical light polypeptide chains (25 kDa) which form the characteristic Y-structure [3]. Each tip of the Y-structure contains a short variable sequence of amino acids, called the paratope, which is specific for one particular moiety of the antigen, termed the epitope [4]. In addition to the residue sequence at the N-terminus, specific recognition is also due to the spatial orientation of the binding pocket defined by the heavy and light chains. Despite the complex milieu of biological fluids, antibodies bind to their target with exceptionally strong affinities, with typical dissociation constants on the order of  $10^{-8}$ – $10^{-11}$  M [5], which is superior to most  $K_d$  values of other natural receptors. As a result, antibodies are the current gold standard and are employed extensively in a variety of bioassay and biosensor applications.

## 2. Biosensors

The field of biosensor technology has exploded since its origins from the seminal papers in the 1960s in which enzymes were used to detect biological compounds [6–8]. More recently, the combination of a greater understanding of biological recognition processes and integrated circuit technology has led to increased interest in commercializing highly specific, accurate, and reliable microsensor devices for a variety of applications in many disparate areas. Fields

\* Corresponding author at: The University of Texas at Austin, Cockrell School of Engineering, Department of Chemical Engineering, Austin, TX 78712, USA. Tel.: +1 512 471 6644; fax: +1 512 471 8227.

E-mail address: [peppas@che.utexas.edu](mailto:peppas@che.utexas.edu) (N.A. Peppas).

where biosensor devices have been pursued and developed include agriculture, food safety, national security, environmental protection, chemical production, and biomedical diagnostics [9].

A biosensor is characterized by two main components: a recognition or sensing element, which specifically interacts with a target analyte, and a transducing element, which converts the interaction into a quantifiable effect. Key requirements of an effective biosensor are specificity for its desired target and detection capabilities over the entire relevant concentration range [10]. This necessitates that the recognition element be able to selectively bind the analyte with high affinity and selectivity.

In biomedical applications, the most common forms of recognition elements are based on antibody/antigen, enzymatic, nucleic acid/DNA, cellular, and biomimetic (synthetic bioreceptor) interactions [11,12]. Even analytes which do not have a natural receptor can be recognized by creating suitable recognition elements using recombinant antibodies [13] or phage display antibody libraries [14].

Although a variety of transducers have been studied, the most widespread include electrochemical, optical, and piezoelectric [15]. Electrochemical sensors measure the changes that result from the interaction between the analyte and sensing surface of the detecting electrode. Electrochemical sensors can be separated into subgroups dependent on the measurement mode and include amperometric (current), potentiometric (voltage), conductometric (conductance), impedimetric (impedance), and field effect transistors (voltage) [15].

More than half of the reported literature on biosensors is based on electrochemical transducers due to their sensitivity, simplicity, and low cost [16]. Optical biosensors transduce a biological event using an optical signal such as absorbance, fluorescence, chemiluminescence, surface plasmon resonance (to probe refractive index), or changes in light reflectivity [17]. Optical biosensors are advantageous for screening a large number of samples simultaneously, but cannot easily be miniaturized for *in situ* applications [9]. Lastly, piezoelectric-based sensors are operated by applying an oscillating voltage at the resonance frequency of the piezoelectric crystal and measuring the change in this frequency when the desired analyte interacts with the crystal surface. The most common piezoelectric sensors are those which include surface acoustic wave (SAW), bulk acoustic wave (BAW), and quartz crystal microbalance (QCM)-based components. Several excellent reviews have recently been written on biosensors based on these transducer platforms [9,17–20].

### 3. Molecularly imprinted polymers (MIPs)

Despite the successes of systems based on natural recognition elements and enzyme amplification, their inherent disadvantages – which include poor chemical, physical, and long-term stability; batch-to-batch variability; skilled-labor intensive; as well as relatively high cost [10,21] – have led researchers to investigate alternative synthetic receptor systems which can overcome these weaknesses. One such technique that has gained significant interest recently is molecularly imprinted polymers (MIPs).

Molecularly imprinted materials have been called “antibody mimics” because these systems attempt to mimic the interactions of their natural counterparts and have achieved affinities and selectivities that approach those of the current gold standard. In a recent study, Lotierzo et al. [22] reported that their MIP system was able to outperform monoclonal antibodies under the same conditions with a broader detection range and better long term stability. Seminal work by Polyakov [23] in the 1930s, using silica matrices, was the first report in which selectivity effects were explained in terms of a template effect, although the additives

were included after polymerization. The inspiration for the field of MIPs, however, evolved from Linus Pauling's hypothesis on antibody formation in the immune system [24]. Pauling proposed that the primary structure of any antibody was identical but that the free chains could surround an antigen and memorize its shape, with a term called “complementariness” [24]. Later work in Pauling's lab showed distinct selectivity for a small molecule, methyl orange, in a silica-based system, similar to Polyakov's approach, except that the template molecule was present during synthesis [25]. Twenty years later, Mosbach [26] laid the foundation for the current field with the first report of organic polymers in this arena. This study employed polyacrylamide gel networks with acrylic functionalities for the entrapment of enzymes and cells [26]. Interestingly, acrylamide and acrylic-based monomers remain important still today as backbone components in many MIP systems.

Molecular imprinting is a promising field in which a polymer network is formed with specific recognition for a desired template molecule. Briefly, functional monomers are chosen which exhibit chemical structures designed to interact with the desired template molecule via covalent or non-covalent chemistry. The monomers are then polymerized in the presence of the desired template; the template is subsequently removed; and the product is a polymer with binding sites specific to the template molecule of interest (Fig. 1). This technique has been successfully applied to small molecule templates in the areas of separations, artificial enzymes, chemical sensors, and pharmaceuticals [27]. The imprinting of small molecules is well developed, and tailor-made molecular imprints are now available commercially. Companies that produce products based on imprinted molecules include MIP Technologies AB (Lund, Sweden – now a subsidiary of Biotage AB), POLYIntell (Val de Reuil, France), MIP Solutions Inc. (Kettle Falls, Washington, USA), and Semorex (Fenwood, NJ, USA). For more in-depth background information on small-molecular-weight MIPs, refer to a recently published review article [28].

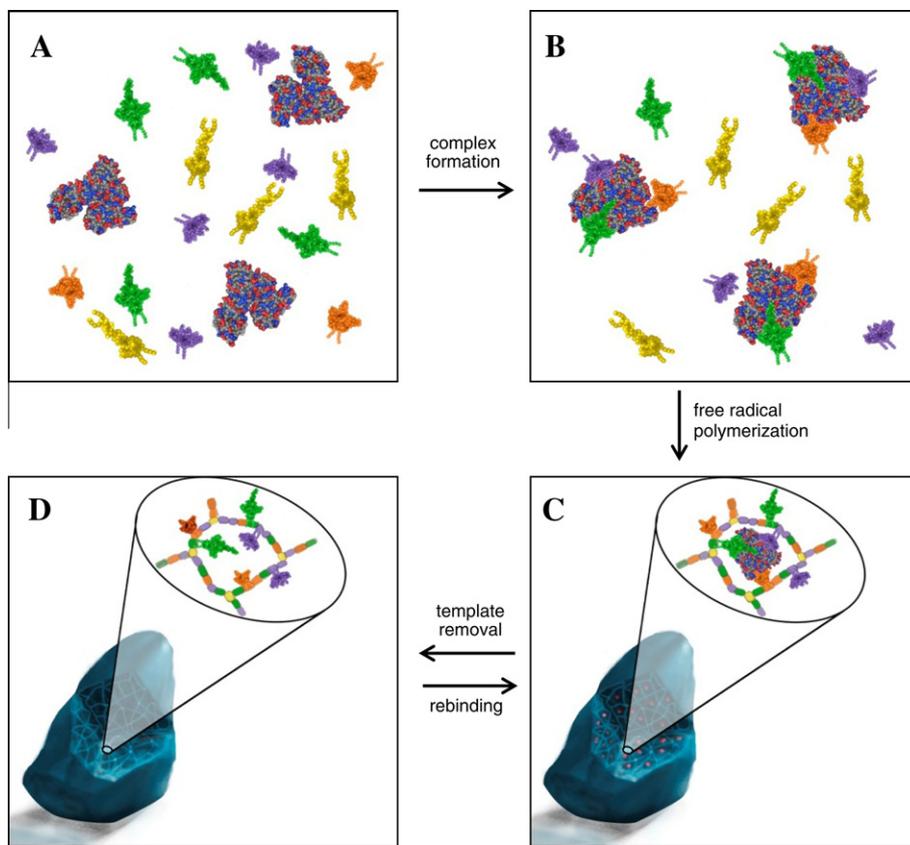
### 4. Imprinted polymers with macromolecular templates

Macromolecular imprinted polymers, MIPs synthesized in the presence of macromolecular templates (>1500 Da), have received a significant amount of interest from the scientific community over the past several years, especially since 2005 (Fig. 2). Due to their importance, proteins are the most extensively studied template in macromolecular imprinting. However, less than 2% of published work in the area of MIPs uses proteins as templates.

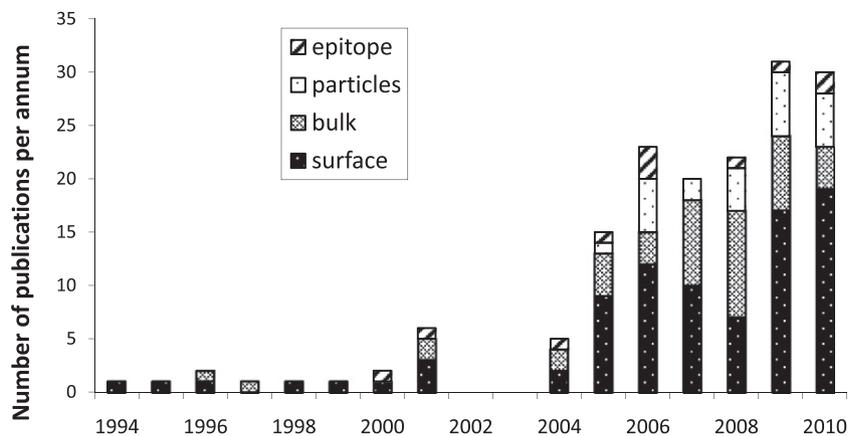
A summary of the comparison of MIPs to natural recognition elements is shown in Table 1. Of note is the fact that MIPs have many advantages over antibodies in terms of their overall stability, ease of synthesis and use, as well as facile integration with transducers. However, at this point MIPs are not able to directly compete with the binding affinity and selectivity demonstrated by natural recognition elements, especially for current applications where antibodies are used in their soluble form.

Nevertheless, synthetic systems exhibiting the ability to selectively recognize specific macromolecules in a complex milieu would be advantageous over natural counterparts for a variety of applications. In the laboratory setting, the low cost, reusability and overall robustness would be useful in the isolation, extraction, or purification of proteins in assays. Specifically, techniques which currently utilize antibodies bound to a solid support – immunoassays, immunoaffinity chromatography, and immunosensors – would benefit from robust synthetic recognition elements.

Potential applications of macromolecular MIPs beyond the laboratory include in biosensing, removal/neutralization of toxic biomacromolecules in the body as well as targeted therapeutic



**Fig. 1.** General MIP procedure. (A) Solution mixture of protein template, cross-linking monomer (yellow), and functional monomers (green, purple, orange); (B) complex formation between functional monomers and template via covalent or non-covalent chemistry; (C) the formation of the polymer network typically via free radical polymerization; and (D) template removal step which leaves binding sites specific to the original template.



**Fig. 2.** The number of macromolecular imprinting articles published by year and approach for period of 1994–2010.

**Table 1**

Comparison of natural recognition elements with MIPs.

	Natural recognition elements	MIPs
Binding affinity	High affinity/specificity	Varies (especially for macromolecular templates)
Generality	One receptor per analyte	MIPs can be developed for any template
Robustness	Limited stability (each element has own operational requirements)	Stable in variety of conditions (pH, T, ionic strength, solvents)
Cost	Expensive synthesis but cost-effective	Inexpensive
Storage	Days at room temperature	Long term storage without loss in performance (several months to years)
Synthesis/preparation	Time-intensive	Facile
Sensor integration	Poor compatibility with transducer surfaces	Fully compatible
Infrastructure required	Expensive analytical instruments/skilled labor	Label-free detection

delivery in feedback-controlled devices. Protein MIPs are particularly well suited to be employed as diagnostic tools in settings where medical infrastructure is lacking, such as in the detection of communicable diseases prevalent in developing countries or after natural disasters.

## 5. Critical barriers to the success of protein-recognitive MIPs

In spite of the interest and large potential impact of these systems, relatively little progress has been made in the field towards realizing the potential of these synthetic antibodies for a variety of reasons. Most notably, the inherent properties of proteins – size, complexity, and conformational instability – have been the main hindrances for macromolecular MIPs as they prevent the direct extension from the far more successful field of small-molecular-weight MIPs.

Traditional imprinted polymers tend to be relatively dense networks (small pore sizes for diffusion of the template into and out of the matrix) in order to retain the binding sites created during polymerization. This is problematic for large templates such as proteins as they can become entrapped in the network after polymerization and cannot easily diffuse back into the network to find binding sites subsequently. Network diffusion limitations, in both directions, lead to inadequate recognition properties. Additionally, slow leakage of the template is sometimes observed, which can significantly impact recognition results, especially in the testing of trace substances.

Unlike smaller templates, proteins are complex biopolymers composed of linear sequences of amino acids that present a large number of potential recognition sites. Different portions of a protein exhibit distinct chemical functionality. Because of the large number of potential heterogeneous binding sites, proteins lend themselves to having multiple weak interactions, which favors nonspecific binding. To achieve a relatively easy on/off binding event where the protein template can be removed with minimal damage to the three-dimensional (3-D) cavities, a non-covalent recognition process is favored. Therefore, the same supramolecular interactions that occur in nature are exploited to achieve recognition. However, these non-covalent interactions are relatively weak; thus, specificity against competitor proteins remains a challenge.

In most cases, the polymerization conditions employed during traditional imprinting procedures are non-physiological. Changes in protein template structure would lead to conformations different than those found in their natural environment, causing the binding sites formed during polymerization to be specific to this alternate state. Therefore, when re-binding is attempted later under physiological conditions, specific recognition of the template is not observed. Indeed, recent work in our lab [29,30] clearly shows the detrimental effects protein MIP constituents have on macromolecules in aqueous polymerization conditions. Specifically, systematic circular dichroism studies were performed with the three most frequently used protein templates (bovine serum albumin, lysozyme, and bovine hemoglobin) in the presence of several common monomers (acrylamide, methacrylic acid, aminophenylboronic acid, acrylic acid, and N-isopropylacrylamide) and crosslinkers (N,N' methylenebisacrylamide and ethylene glycol dimethacrylate) at relevant polymerization concentrations. These reactants were found to induce significant changes in the secondary structure of all three protein templates at concentrations far below what are used in the literature. This is obviously a cause for concern and potentially a large reason for the lack of success in the protein MIP field to date.

Another major obstacle is the solvent, as one must be chosen that does not interfere with the monomer-template interaction while still allowing complete miscibility between all the

constituents. Proteins are often insoluble and/or unstable in the aprotic organic solvents typically used in small-molecular-weight imprinting. And, while proteins are completely miscible in aqueous solutions, it is far from the ideal solvent as water will compete for and potentially disrupt hydrogen bonding between the monomer and template, the interaction upon which many systems rely for recognition.

## 6. General approaches to macromolecular imprinting

Despite these obstacles, many groups have undertaken the task of developing protein-recognitive polymers over the past decade through a variety of approaches – commonly categorized as bulk, particle, surface, and epitope. Fig. 2 shows that interest in the field of macromolecular imprinting has increased considerably since 2005. It is also clear that the field has been dominated by surface imprinting, with approximately 60% of all papers published describing this approach.

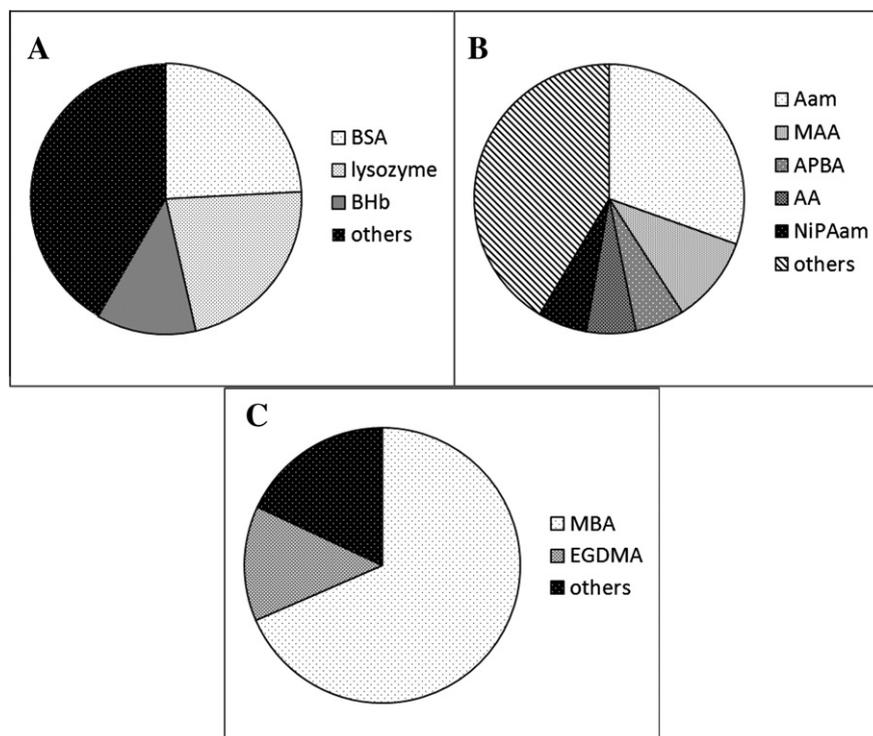
An exhaustive analysis of the literature reveals a few obvious trends. First, with few exceptions [31–36], almost all of the literature to date employs model proteins as templates. Of those, bovine serum albumin (BSA), lysozyme, and bovine hemoglobin (Bhb) are the most common (Fig. 3A). The overwhelming majority of studies employ acrylamide (AAm) and N,N' methylenebisacrylamide (MBA) as a functional monomer and crosslinker, respectively (Fig. 3B and C). The five most common monomers – acrylamide (AAm), methacrylic acid (MAA), aminophenylboronic acid (APBA), acrylic acid (AA), and N-isopropylacrylamide (NiPAAM) – account for nearly 60% of the monomers used in the literature (Fig. 3B). And as a whole, MBA and ethylene glycol dimethacrylate (EGDMA) are crosslinkers in over 80% of the published reports (Fig. 3C). Consequently, it seems that despite the large number of studies to date (around 175 in total), researchers tend to employ the same components in their work. The following discussion overviews each of the four general approaches and highlights studies of interest from the literature. Other recent reports are summarized for each approach in Tables 2–5.

### 6.1. Bulk imprinting

Bulk imprinting, the standard technique which has been so successful for small-molecular-weight MIPs, is the most straightforward approach to macromolecular imprinting. The general bulk imprinting procedure was outlined in Section 3 and illustrated in Fig. 1. The advantages to this approach are that 3-D binding sites are formed for the entire protein and that there are a multitude of facile procedures already present in the literature.

In a recent bulk protein imprinting strategy, Ou et al. [37] used equimolar amounts of methacrylic acid (MAA), acrylamide (AAm), and 2-(dimethylamino)ethyl methacrylate (DMAEMA) as the functional monomers and N,N'-methylenebisacrylamide (MBA) as the crosslinker to imprint for lysozyme. The charged monomers were used to exploit electrostatic interactions with the oppositely charged amino acid residues of lysozyme. Up to 27% (w/w) of the original template remained entrapped in the polymer after the template removal process. After lyophilizing, moderate affinities were demonstrated (1.3–3.4 times more lysozyme absorbed with the imprinted polymer than the control polymer); however, the rebinding was not specific as the MIPs absorbed essentially the same amount of BSA (13.3% w/w) as lysozyme (15% w/w).

In another bulk imprinting study, Hawkins et al. [38] took an interesting approach to quantify the recognition of the protein template, bovine hemoglobin (Bhb), using AAm as the functional monomer and MBA as the crosslinker. In contrast to the majority of imprinting literature, this procedure accounts for non-specific



**Fig. 3.** Relative frequency of common components in macromolecular MIP literature for period of 1994–2010. (A) Macromolecular templates – bovine serum albumin (BSA), bovine hemoglobin (BHb); (B) functional monomers – acrylamide (AAm), methacrylic acid (MAA), aminophenylboronic acid (APBA), acrylic acid (AA), and N-isopropylacrylamide (NiPAAm); (C) crosslinkers – N,N' methylenebisacrylamide (MBA) and ethyleneglycol dimethacrylate (EGDMA).

**Table 2**

Summary of recent macromolecular imprinting research using bulk approach.

Ref(s).	Template(s)	Components	X-linker mol.%, mon:temp molar ratio	Comments
[36]	Interleukin-1	TEOS/APTES/C8-TMOS/HAPTS	n/a	<ul style="list-style-type: none"> <li>sol-gel polymerization, luminescence with xerogels for quantification</li> <li>~2 pg ml<sup>-1</sup> detect limit, &lt;2 min response t, &gt;95% reversibility after 25 cycles</li> <li>compared well with ELISA</li> </ul>
[134]	Lysozyme	MAH, HEMA, EGDMA	650:1 mol MAH:Lys	<ul style="list-style-type: none"> <li>UV polymerization in acetonitrile (metal-ion imprinting)</li> <li>IF: 1.5–4 depending on Lys concentration selectivity: ~3.6–4.1</li> <li>reusable for 25 cycles up to 95% of original</li> <li>thermal polymerization in MOPS buffer (pH = 7.4)</li> <li>able to detect and remove HSA from cerebrospinal fluid and serum of patients with ALS</li> </ul>
[33,135]	HSA	AAm, MBA	2.4 mol.% MBA, 371,000:1 mon:temp	<ul style="list-style-type: none"> <li>thermal polymerization in Tris-HCl buffer</li> <li>stimuli response polymerization volume decrease of MIP (not NIP) as concentration of Lys increase</li> <li>IF ~ 1–2.4 (depending on NaCl concentration), selectivity shown as well</li> </ul>
[71]	Lysozyme, Cyt C	NiPAAm, MAA, AAm, MBA	1.3 mol.% MBA, 250:1 mol mons:temp	<ul style="list-style-type: none"> <li>thermal polymerization in Tris-HCl, temperature responsive polymer network</li> <li>max IF (~2.6) at 40 °C, selectivity (1.8–21)</li> <li>showed ability to purify BSA from bovine calf serum, reusable up to six cycles</li> </ul>
[74]	BSA	DMAPMA/Aam/NiPAAm, MBA as X-linker	3 mol.% MBA, 2450:1 mons:temp	<ul style="list-style-type: none"> <li>thermal polymerization in DI at T = 38 °C</li> <li>adsorption ↓ as polymerization time ↑ from 24 to 60 h</li> <li>IF = 1.7, selectivity against myoglobin = 14.5 (but NIP selectivity ~5)</li> <li>TEGDMA (linear X-linker) gave better results overall than PETTA (branched)</li> </ul>
[136]	BSA	DMAPMA, TEGDMA/PETTA	9 mol.% X-linker, 7970:1 DMAPMA:BSA	<ul style="list-style-type: none"> <li>thermal polymerization in Tris-HCl buffer (10 mM, pH = 7.4), metal ion imprinting</li> <li>IF ~ 1.2–2.5 (much higher absorption with Cu(II) in solution)</li> <li>max # of binding sites on BSA is 16; 96% of original absorption after six uses</li> </ul>
[80]	BSA	4-VP, NiPAAm, AAm, MBA, Cu(OAc) <sub>2</sub>	1.3 mol.% MBA; 934 mol mons:BSA	

4-VP: 4-vinyl pyridine; APTES: aminopropyltriethoxysilane; C8-TMOS: n-octyltrimethoxysilane; Cyt C: cytochrome C; DMAPMA: 3-dimethylaminopropyl methacrylamide; HAPTS: bis(2-hydroxy-ethyl)aminopropyltriethoxysilane; HEMA: 2-hydroxyethyl methacrylate; IF: imprinting factor; PETTA: pentaerythritol tetraacrylate; TEOS: tetraethoxysiloxane.

**Table 3**  
Summary of recent macromolecular imprinting research using particle-based approach.

Ref(s).	Template(s)	Components	X-linker mol.%, temp:mon molar ratio	Comments
[105,106,108,137,138]	BSA	Sodium alginate, CaCl <sub>2</sub>	n/a	<ul style="list-style-type: none"> <li>• ionic X-linking with alginate and CaCl<sub>2</sub></li> <li>• IF ~ 3.5, selectivity ~ 2.4–3.5</li> <li>• Langmuir isotherm behavior</li> </ul>
[59,61]	Rnase A, BSA, Lysozyme	MMA, EGDMA, surfactant (SDS, PVA)	4000:1 mol MMA:prot, 75 mol.% EGDMA	<ul style="list-style-type: none"> <li>• looked at effect of rxn type, surfactant, homogenization, electrolytes on the protein structure</li> <li>• emulsion polymerization (thermal initiated much more successful than UV init.)</li> <li>• IF with Rnase (~1.2–7), BSA (~1), lysozyme (~1)</li> <li>• Selectivity adequate (max. of 2)</li> </ul>
[35]	Staphylococcus aureus protein	AAm, MBA, ethyl cellulose	~5 mol.% X-linking, 1.2E6:1 AAm:SpA	<ul style="list-style-type: none"> <li>• thermal polymerization (emulsion and bulk)</li> <li>• IF (~3.5–5.3) and selectivity (5.2 vs. ovalbumin, 3.5 vs. <i>E. coli</i>)</li> <li>• docking studies used to look at AAm and SpA interaction</li> </ul>
[139]	Trypsin	Methacrylamide, EBA, methacryloylaminobenzamide	60% X-linker	<ul style="list-style-type: none"> <li>• large particles (<math>d \sim 1 \mu\text{m}</math>) synthesized via UV polymerization</li> <li>• IF ~ 2.5–2.8, selectivity ~ 2–5</li> <li>• three orders of magnitude better enzyme inhibition than low MW competitor inhibitor</li> </ul>
[34,140]	Amylase, lipase, lysozyme, BSA	EVAL, quantum dots	n/a	<ul style="list-style-type: none"> <li>• quantum dot composite nanoparticle MIPs (dispersion polymerization)</li> <li>• dissolved proteins in DMSO during polymerization synthesis</li> <li>• IF for amylase (~3.8), lipase (~2.6), lysozyme (~2.1), albumin (~6.1)</li> <li>• &gt;94% accuracy in concentrations of proteins with real saliva samples (compared to commercial system)</li> </ul>

DMSO: dimethyl sulfoxide; EBA: N,N-ethylenebis(acrylamide); EVAL: poly(ethylene-co-vinyl alcohol); IF: imprinting factor; MMA: methyl methacrylate; PVA: poly(vinyl alcohol).

binding via load, wash, and elution phases. Briefly, the protein is incubated with the polymer particles (load), rinsed five times in water (wash), and then eluted with a sodium dodecyl sulfate (SDS)/acetic acid aqueous solution (elution). Detection of the protein template in the elution phase subsequent to load and wash steps indicates that specific recognition has occurred. SDS is a surfactant that has been shown to denature proteins by destroying the secondary structure [39], thus causing a protein molecule that is chemically attached to the MIP to alter its conformation, cleaving non-covalent bonds, and allowing subsequent diffusion out of the network. Excellent affinities (~16–23 times more BHB seen in MIP elution than control elution phases) and selectivities (3.8 vs. myoglobin and 5.1 vs. cytochrome C) were demonstrated [38].

In spite of these great results, there are several unanswered questions for this particular study. First, after optimization, only ~48% of the initial template was removed after polymerization, which is well below typical values of ~70–90% seen in the literature [40]. Secondly, more BHB was recovered in the recognition studies than was initially added to the load phase. This may be a result of the fact that there was still quite a bit of template left in the polymer particles before the rebinding studies were performed.

It is worth noting here that template removal is a crucial step in protein imprinting, especially in the bulk approach, which is often not properly addressed. The most common methods include aqueous SDS/acetic acid [32,38,41–62], SDS/NaOH [63–69], aqueous NaCl [37,70–84], or just simply water/buffer [36,42,85–95]. Despite the importance, relatively few studies have focused on optimizing the template removal process [37,38,49,96]. Additionally, the use of SDS/acetic acid for template removal, while highly successful, has led to experimental artifacts in subsequent recognition studies by several groups [38,44,50,51,59,60,97]. This problem, attributed to the inability to completely remove SDS from the polymer network, was highlighted quite effectively in a recent review article [98]. As a result, one must be mindful of the compro-

mise between effective template removal and integrity of the binding sites as well as ensuring that the washing compounds are completely removed prior to recognition studies.

The results obtained in the bulk imprinting studies mentioned above are representative of those typically seen in the literature. A few inherent obstacles have prevented this strategy from being successful, including diffusional limitations, solubility concerns of the template in organic solvents often used in small molecule imprinting, and conformational changes in the protein template caused by the non-physiological conditions employed. As a result, alternative approaches have received increased interest in the past few years.

## 6.2. Particle-based imprinting

The majority of bulk imprinting involves wet sieving or crushing the polymer after polymerization and before template removal procedures to minimize diffusional limitations. However, this produces irregularly shaped and polydisperse particles and may destroy potential binding sites [99,100]. As a result, various studies have explored the use of emulsion or suspension polymerizations to directly synthesize micro-/nanoparticles [55–57,59–61]. The main differences between bulk and particle platforms are the addition of stabilizers/surfactants and that the monomer/template are at a much lower concentration in the pre-polymerization solution. Drawbacks to this method are that residual amounts of stabilizers have remained in the polymer particles even after extensive washing as well as the potential disruption of the monomer–template complex due to the presence of surfactants.

In a few recent studies by the same group, Pang et al. [55–57] synthesized polyacrylamide particles after optimizing temperature and crosslinking density using an inverse suspension polymerization with bovine serum albumin (BSA) as the model protein template. Ethyl cellulose, the stabilizer, was dissolved in toluene to form the continuous phase into which an aqueous monomer

**Table 4**  
Summary of recent macromolecular imprinting research using surface approach.

Ref(s).	Template(s)	Components	X-linker mol.%, mon:temp molar ratio	Comments
[141]	Albumin	DMAPMA; TEGDMA/TMPTMA/PETTA as X-linker, Au electrode	90 mol.% X-linker, 205:1 mon:albumin	<ul style="list-style-type: none"> <li>UV polymerization (no solvent), spin coat onto QCM with various electrodes, thiol SAM</li> <li>IF (~8), selectivity (~10, but competitor proteins much smaller)</li> </ul>
[63–69,142]	Rnase A, Lysozyme, Myoglobin, Ovalbumin, CRP	Styrene, MMA, MAA, DMAEMA, 4-VP, HEMA; various PEG(n)DMA X-linkers	30–75 mol.% X-linker	<ul style="list-style-type: none"> <li>microcontact imprinting (adsorp of protein onto glass slide, sandwich onto mon solution, UV polymerization)</li> <li>optimized functional monomer and X-linker using ITC</li> <li>varying IF (1.5–20) and selectivity (3–35) depending on template</li> </ul>
[143]	BSA	MAA, EGDMA, silica beads, saccharose (sugar coating)	54 mol.% EGDMA	<ul style="list-style-type: none"> <li>thermal polymerization in EtOH (ice bath), surface of silica beads</li> <li>able to see shift (<math>\Delta</math> color) down to 1 ng ml<sup>-1</sup> of BSA (up to 10 mg ml<sup>-1</sup>)</li> <li>IF ~ 7–20, selectivity ~ 5–20 (various concentrations and competitors)</li> </ul>
[144]	Cyt. C	AAm, mica, MBA/EBA/PDA or PEGDMA as X-linker	3.3 mol.% MBA	<ul style="list-style-type: none"> <li>thermal polymerization in DI water, BSA attached to mica surface</li> <li>used AFM for first time to measure force of interact of protein w/surface MIP</li> <li>IF (~1.1–4.1)</li> </ul>
[60,97]	BSA	MMA, EGDMA, Fe <sub>3</sub> O <sub>4</sub> particles	80 mol.% EGDMA, BSA surf immobilized	<ul style="list-style-type: none"> <li>thermal polymerization, BSA immobilized on Fe<sub>3</sub>O<sub>4</sub> particles, core-shell emulsion (lengthy method)</li> <li>higher BSA adsorption than lit (up to 56 mg g<sup>-1</sup>)</li> <li>IF (~6.5), selectivity (~3 vs. lysozyme)</li> </ul>
[145,146]	Avidin	PSS/PEDOT, Au electrode, in porous PC membrane	n/a	<ul style="list-style-type: none"> <li>electrochemical polymerization, of electrically conducting polymers in PBS</li> <li>BSA adsorption onto PCM membrane, precise microrods formed in PCM pores</li> <li>IF ~ 5, selectivity not clearly shown</li> </ul>
[58,62]	Lysozyme, BHB	Acryloyl- $\beta$ -cyclodextrin, AAm, MBA	~2 mol.% MBA, 1100:1 mons:Lys, 4800:1 mons:BHB	<ul style="list-style-type: none"> <li>thermal polymerization in PBS (10 mM, pH = 7) on silica beads</li> <li>dynamic and isotherm adsorption studies (IF ~ 3–5)</li> <li>BHB: IF ~ 4.9, selectivity ~ 3.5–4.7 (vs. BSA, lysozyme, Cyt C)</li> <li>Lysozyme: selectivity (~4.2, vs. variety of competitors)</li> </ul>
[94]	CEA	11-mercapto-1-undecanol	19:1 vol thiol:template	<ul style="list-style-type: none"> <li>novel SAM surface MIP (in aqueous acetic acid) for variety of cancer biomarkers</li> <li>IF ~ 25, selectivity ~ 30 (vs. hemoglobin) at relevant conc (ng ml<sup>-1</sup>)</li> <li>2–10 min response time</li> </ul>
[147]	BHB	Dopamine, silanol-modified superparamagnetic nanospheres	3225:1 mol DA:BHB	<ul style="list-style-type: none"> <li>thermal polymerization in PBS on core-shell superparamagnetic NP</li> <li>thorough optimization &amp; facile separation with external magnetic field</li> <li>IF (~3–3.5), selectivity (~3–4, for variety of competitors)</li> </ul>
[82]	Lysozyme	NiPAAm/AAm/MAA/MBA	1.4 mol.% MBA, 270 mol mons:Lys	<ul style="list-style-type: none"> <li>UV polymerization with iniferter modified PS beads in Tris-HCl</li> <li>Template removal up to 88% w/w</li> <li>IF (~0.8–2.6), selectivity (~4–9 for various competitors and temperature)</li> </ul>
[84]	Lysozyme	MMA/TMPTMA, CaCO <sub>3</sub> as porogen, Au QCM electrode	41 mol.% TRIM (X-linker), 54,000 mol MMA:Lys	<ul style="list-style-type: none"> <li>UV polymerization on surface of QCM transducer</li> <li>~10 min response time, IF (~3.7), selectivity (~4)</li> </ul>

4-VP: 4-vinyl pyridine; APTES: aminopropyltriethoxysilane; CEA: carcinoembryonic antigen; CRP: C-reactive protein; Cyt C: cytochrome C; DA: dopamine; DMAPMA: 3-dimethylaminopropyl methacrylamide; EBA: N,N-ethylenebis(acrylamide); HEMA: 2-hydroxyethyl methacrylate; IF: imprinting factor; ITC: isothermal titration calorimetry; MMA: methyl methacrylate; PC: polycarbonate; PDA: 1,4-bis(acryloyl) piperazine; PEDOT: poly-3,4-ethylenedioxythiophene; PEG(n)DMA: poly(ethylene glycol) (n) dimethacrylate; PETTA: pentaerythritol tetraacrylate; PS: polystyrene; PSS: polystyrene sulphonate; SAM: self-assembling monolayer; TMPTMA: trimethylolpropane trimethacrylate; TEOS: tetraethoxysiloxane.

solution containing AAm and MBA was added. Free radical polymerization yielded relatively polydisperse particles ranging in size from 150 to 280  $\mu$ m which were rinsed in SDS/acetic acid for template removal. Rebinding studies on the resultant microparticles showed affinities of ~4 (amount loaded into MIPs vs. control polymers) and adequate selectivities (~3.8 and ~5.4) over hemoglobin and ovalbumin, respectively.

In a novel approach recently published from our group [101], BSA-imprinted particles were synthesized via the ionic crosslinking of sodium alginate (SA) in calcium chloride (CaCl<sub>2</sub>). To do so, an aqueous solution of BSA/SA was added dropwise from a syringe into a solution of CaCl<sub>2</sub>. Alginate is a hydrophilic natural linear polysaccharide which is both biocompatible and biodegradable

[102]. This rapid and facile procedure does not require the addition of organic solvents or surfactants, which has been the case for the majority of previous protein imprinting studies with alginate particles [103–107] as well as other particle-based imprinting studies.

While this study produced particles in the size range of 2–3  $\mu$ m, the diameter is easily tunable by changing the viscosity of the BSA/SA solution or using a nitrogen gas stream. For example, Ying et al. [108] report a similar protein imprinting procedure in which alginate particles down to 300  $\mu$ m were produced by the addition of a nitrogen gas stream aimed at the tip of the syringe to decrease the droplet size. Standard pharmaceutical spray technologies have also been shown to produce ionically crosslinked alginate particles below 50  $\mu$ m with a narrow particle size

**Table 5**  
Summary of recent macromolecular imprinting research using epitope approach.

Ref(s).	Template(s)	Components	X-linker mol.%, mon:temp molar ratio	Comments
[117,118]	Angiotensin II, SA (octapeptide)	Na acrylate, PEGDA; and MAA,EGDMA	86–96 mol.% PEGDA, 8:1–32:1 mon:temp	<ul style="list-style-type: none"> <li>• thermal polymerization of bulk monolith</li> <li>• moderate IF for SA (octapeptide), none for angiotensin</li> <li>• IF affected by pH, ionic strength of PBS, and% acetonitrile in aqueous loading solution</li> </ul>
[121,133]	15-mer peptide for dengue virus protein	AA/AAm/N-benzylacrylamide	73:1 mons:peptide template	<ul style="list-style-type: none"> <li>• UV polymerization of thin film on surface of QCM transducer</li> <li>• IF and selectivity shown (for 15-mer peptide and parent protein in patient samples)</li> </ul>
[88]	Epitopes for Cyt c, ADH, and BSA	AAm, MBA, PEG(200)DA	33% X-linker	<ul style="list-style-type: none"> <li>• UV polymerization, complex protocol (each nonapeptide was chemically synthesized)</li> <li>• IF up to 5 with Cyt. C MIPs; selectivity shown in all MIPs</li> <li>• specific binding in BSA MIPs – changed one amino acid and no binding</li> </ul>

Cyt C: cytochrome C; EBA: N,N-ethylenebis(acrylamide); IF: imprinting factor; PEG(200)DA: polyethylene glycol (200) diacrylate.

distribution [109]. Additionally, alginate acid-based protein-imprinted thin films were recently reported by our group which demonstrated successful imprinting of BSA [110]. Although further study is needed, alginate acid-based systems appear promising going forward as a platform technology for the production of protein MIPs without the need for harsh synthesis conditions or surfactants, thus eliminating concerns of protein conformational instability.

### 6.3. Surface imprinting

In the most common protein MIP strategy, surface imprinting, the imprinted binding sites are located at or very near the surface of the polymer. This is achieved by either synthesizing a thin polymer film using similar approaches to those in bulk imprinting or by attaching the protein template on the surface of a substrate (flat or spherical) with subsequent polymerization. This method facilitates diffusion of the large macromolecule into and out of the network, thereby minimizing template size concerns. Additionally, surface-imprinted MIPs tend to be more physically robust due to the presence of the support and allow for easier integration with sensor platforms. However, the trade-off is a decrease in specificity as only a portion of the protein is imprinted, thus later recognized. Many excellent studies have been published using this approach, some of which are highlighted below and in Table 4.

In one of the seminal papers in protein imprinting, Shi et al. [111] used radio-frequency glow discharge (RFGD) plasma deposition to form thin fluoropolymer films around the protein template coated with disaccharides in a surface imprinting strategy. The protein template (including BSA, immunoglobulin G, or lysozyme) was adsorbed onto a mica surface and a disaccharide solution was added, forming hydrogen bonds between the hydroxyl groups on the sugars and the exposed polar amino acid residues on the protein during subsequent dehydration. After RFGD plasma deposition, which created covalent bonds between the disaccharide and polymer, and subsequent removal of the mica and protein, cavities specific to the protein template were present in the polysaccharide. While the imprinting factors (ratio of template absorption of MIP to that of non-imprinted) of these surface-imprinted systems were not great, selectivities (ratio of template absorption of MIP to competitor protein absorption of MIP) demonstrated were excellent. Specifically, the selectivity of the BSA MIP for BSA over IgG (immunoglobulin G) was 5–10, IgG MIP for IgG over BSA was 4–7, and more impressively lysozyme MIP absorption for lysozyme over RNase A was 20 especially due to their similar size and isoelectric point.

In general, living radical polymerizations – iniferter, reversible addition-fragmentation chain transfer (RAFT), atom-transfer

radical polymerization (ATRP), and nitroxide-mediated polymerization (NMP) – control chain growth and termination via a chain transfer agent to yield monodisperse polymers. Despite the obvious benefits that these types of polymerizations have over traditional free-radical polymerizations due to their potential ability to minimize heterogeneity of inner morphology and binding site affinities, only a few have been reported to date for small-molecular-weight MIPs [112]. In the first study that combines a controlled polymerization with protein MIPs, Qin et al. [58] modified the surface of polystyrene beads with a dithiocarbamate iniferter and synthesized an AAm/MBA-based thin film for the recognition of lysozyme. Superior imprinting factors (12.7 vs. 2.6) and selectivities (>12 vs. ~1) were demonstrated for lysozyme with the iniferter-based films relative to films synthesized with traditional thermal free radical polymerization. Additionally, the iniferter allowed for greater control of film thickness. Similar results were obtained in a more recent publication by the same group, which used an iniferter-based controlled polymerization to produce a temperature-responsive protein MIP [82]. While the compatibility of controlled polymerization ingredients with those of typical MIP recipes is sometimes limited, controlled/living free radical polymerization is a promising new area in macromolecular MIPs that merits further investigation.

In another recent study, Jing et al. [77] developed a novel method for the rapid, low-cost, and selective detection of lysozyme in human urine samples. Fe<sub>3</sub>O<sub>4</sub> nanoparticles were silanized to promote covalent attachment of the MIP layer synthesized via thermal free radical polymerization which consisted of AAm and MAA as functional monomers and MBA as crosslinker. Chemiluminescence was used to quantify the selective recognition of lysozyme after magnetic separation of the MIPs without the need for elution from a column or centrifugation, which is the case for traditional solid-phase extraction. Under optimal conditions the entire analytical procedure was achieved in less than 12 min and the limit of detection was 5 ng ml<sup>-1</sup>. Imprinting factor (~9), selectivity (up to 4), and capacity (110 mg lysozyme per g polymer) values compare quite favorably with previous surface imprinting literature [40,113]. Additionally, lysozyme concentrations in human urine samples were determined using a commercial detection kit and with the much simpler, faster, and cheaper MIP-based system. Comparison of the two sets of readings showed a nice correlation ( $r = 0.9595$ ). Therefore this Fe<sub>3</sub>O<sub>4</sub>-MIP-based system shows promise as a high-throughput analysis system for the detection of elevated lysozyme levels in urine for the diagnosis of renal diseases.

Cai et al. [32] recently prepared arrays of carbon nanotube tips coated with non-conducting polymers for the subpicogram detection of human ferritin and human papillomavirus (HPV) derived E7 protein via impedance spectroscopy. Vertically aligned nanotube

arrays were grown on a titanium layered glass substrate, a support polymer (SU-8) was spin coated onto this assembly, the exposed portion of the nanotubes were polished, and finally the MIP coating on the nanotubes was achieved by electropolymerization of polyphenol in the presence of the protein template. Highly selective recognition was achieved for both templates as a variety of similar competitor proteins for each did not produce significant impedance responses. Also, the sensitivity level reached ( $\sim 10 \text{ pg l}^{-1}$  for human ferritin) surpasses that of conventional MIP sensors and is comparable to nanosensors based on natural recognition elements. As a result, this novel label-free electrochemical surface-imprinted approach appears promising for the clinical detection of various biomarkers as well as other proteomic applications in lieu of those based on biomolecular recognition.

#### 6.4. Epitope imprinting

Combining the concepts of surface and bulk imprinting, the epitope approach employs a short polypeptide as the template during polymerization to represent a moiety of a larger polypeptide or protein ultimately desired to be recognized [88,114–121]. This technique attempts to more closely mimic the specific interaction between an antibody and antigen described earlier.

In one early study, Rachkov et al. [119] used conventional small MW components – MAA (functional monomer), EGDMA (crosslinker), and solvent (acetonitrile). A highly crosslinked polymer network was formed in the presence of a tetrapeptide (YPLG) for the subsequent recognition of the nonapeptide oxytocin (CYIQCLG) with the same C-terminus. Adequate imprinting factors ( $\sim 1.5$ – $3.5$  times more absorption of the template with the MIP than the control, depending on crosslinker composition) were obtained. Selectivity was observed for both the template and oxytocin; however, attempting to recognize the parent molecule angiotensin II (an octapeptide) using a tripeptide template was unsuccessful in another study [118].

In a milestone study, Hoshino and Shea [115] detailed the first in vivo studies for MIPs. Specifically, sub-100 nm NiPAAm-based nanoparticles (NPs) were synthesized with positive (3-aminopropylmethacrylamide), negative (AA), hydrophobic (t-butyl acrylamide), and hydrophilic (AAm) functional monomers in the presence of the template, mellitin, a 26 unit peptide present in bee venom. Preliminary in vivo studies showed no detectable toxicity as well as no significant change in body weight between NP injected and control mice 2 weeks after injection. These NPs were also highly successful in reducing mortality, as survival rates for mice injected with the MIP NPs ( $\sim 60\%$ ) 20 s after mellitin injection was significantly higher ( $p = 0.03$ ) than that of the control mice (no NP injection, 0%). Additionally, studies with the MIP NPs showed binding affinities comparable to those for antibodies to mellitin (apparent binding constant of  $\sim 10^{11} \text{ M}^{-1}$ ). Although this study imprints and recognizes a polypeptide (not for a parent macromolecule), it is a landmark achievement for the field as it is a proof-of-principle for specific template recognition in complex biological fluids with no apparent toxicity.

The epitope approach is advantageous over bulk and surface protein imprinting as it addresses, at least to some extent, all three major obstacles to successful protein imprinting – size, complexity, and conformational instability. First, the small template allows for high crosslinking and structured binding sites without concerns of template removal. Obviously, since the ultimate goal is the recognition of the parent molecule, diffusional limitations in the macromolecule rebinding studies still must be addressed. Second, analogous to natural recognition pathways as well as small-molecular-weight imprinting, the complexity of the template is minimized, thereby limiting non-specific interactions. Consequently, binding affinity and selectivity of the MIP should, in theory,

increase. Third, polypeptides are far less sensitive to their environments as secondary and tertiary structures are not present. Additionally, aprotic organic solvents can be used for dissolution without solubility or conformational change concerns. Additionally, protein biomarkers are expensive, so the ability to use short peptides of a protein would certainly be more cost-effective, although the synthesis and/or purification of functionalized peptides can be difficult. Overall, epitope imprinting allows for the employment of common small molecule MIP procedures without typical concerns of macromolecular imprinting. However, it remains to be seen whether successful epitope imprinting translates into specific recognition of its parent macromolecule as few studies using this approach have been reported to date. Nevertheless, epitope imprinting appears to be a promising approach going forward and should be explored accordingly.

#### 7. Rational design of protein MIPs

Success of the protein-imprinted polymer lies with the monomer–template complex (Fig. 1B). This complex must be thermodynamically favorable and stable under reaction conditions, but at the same time the bonds must be easily broken for subsequent template removal such that the polymer network and the binding sites are not disturbed. Macromolecular MIPs typically rely upon non-covalent interactions for recognition, with H-bonding, electrostatic, and hydrophobic interactions the most prevalent.

In theory, the ability to optimize the pre-polymerization solution by selecting monomers with high affinity for the template should lead to a polymer able to more selectively recognize the specific protein template. This will allow for a much more rapid investigation of possible compositions rather than the typical process of polymerization, template removal, and recognition studies which, in total, can take several days to weeks. Despite the importance of this interaction in the pre-polymerization solution, very few experimental studies have looked at this complex in an attempt to optimize the resultant recognitive polymer, especially with protein templates [64,67,69,122].

Wang et al. [122] used fluorescence quenching to investigate the interaction between the functional monomer, aminophenylboronic acid (APBA), and protein template, BSA. Fluorescence quenching was defined, in this case, as the shift in fluorescence intensity of BSA at 343 nm caused by the presence of APBA. Using the optimal molar ratio of APBA-to-BSA from this analysis, the protein-imprinted polymers were polymerized on the surface of activated glass spheres. Subsequent recognition studies demonstrated excellent imprinting factors (6.1 times more BSA adsorbed for the MIP than the control polymer) and selectivity (6.2 times more BSA adsorbed than competitor bovine hemoglobin). However, protein fluorescence shifts are commonly used to measure changes in conformation of the macromolecule due to unfolding of the protein which exposes tryptophan residues previously buried in the hydrophobic core [123,124]. As a result, it is not obvious whether the fluorescence shift seen in this study was due to protein–ligand binding or simply protein unfolding.

The Chou group [64,67,69] employed isothermal titration calorimetry (ITC) to determine the optimal monomer and crosslinker for several protein templates. ITC is a calorimetric technique commonly used in biochemistry to study the thermodynamics of protein–ligand interactions by monitoring the amount of heat released upon addition of discrete amounts of ligand [125]. Various protein templates were adsorbed onto glass slides and titrations were individually performed from a set of common monomers and crosslinkers. Synthesizing surface-imprinted MIPs with the best functional monomer and crosslinker pair for each template yielded films that were highly successful in subsequent recognition

studies. Imprinting factor and selectivity values as high as 15 each were achieved with this methodology. These results are promising, although the procedure is limited. First, the best monomers and crosslinkers were selected only from a set of 5–6 ligands; therefore, it is quite possible that a monomer or crosslinker not investigated could produce an even more desirable heat response. Secondly, this procedure does not give the optimal ratio of monomer to template, which means that the authors still were required to synthesize polymers with various ratios.

It is evident from the paucity of literature that there is a need for systematic and thorough optimization of the type and relative amount of monomer and crosslinker from a large set of targets. Factorial design would be useful to determine interactions between these variables and others, which are likely numerous. Such a study would provide the first real rational design of a protein MIP system.

## 8. Conclusions and future outlook

Despite the increasing amount of interest and numerous promising studies reported over the past several years, significant challenges still face macromolecular imprinted polymers. In reviewing the literature, a few recommendations can be made. First, the fundamental mechanisms behind template recognition have received very little attention, thus remain largely unknown. Success of the subsequent MIP depends on the stability and strength of the monomer–template complex prior to polymerization. It is clear that the dominant recognitive forces in protein MIPs are hydrogen bonding, electrostatic, and hydrophobic interactions; however, the relative importance of each of these is still in question. Elucidating this exact recognition mechanism computationally or via other fundamental approaches can go a long way towards progressing the field beyond its current state.

Second, somewhat surprisingly, true rational design of protein MIP systems is absent from the literature. As previously mentioned, the design of MIP systems after systematic optimization of the type and amount of functional monomer relative to template (among other variables) should prove useful in efficacious recognition. To do so, one could use computational modeling or conduct more thorough analyses using the aforementioned analytical techniques with a larger pool of ligands. Optimizing this interaction could have the added benefit of shedding light into the mechanisms behind recognition in macromolecular MIPs.

Third, it is obvious that more attention needs to be given to the development of robust procedures that set the standard for future work. Two of the main advantages of MIPs compared to antibodies are the ease of synthesis and that these artificial matrices can, in principle, be used to recognize any biomolecule of interest. Far too often though, it seems that the protocols reported are complex and labor-intensive [88,97,111,126–131] as well as lacking generic applicability to other protein templates. As a result, many studies are proof-of-concept for a specific approach rather than attempts to develop robust frameworks that provide convincing evidence of an imprinting effect for various templates.

Lastly, it is now evident that the general design principles of small-molecular-weight MIPs do not apply to the macromolecular regime. In recent work from our lab [29,30], we clearly show that monomers commonly employed in MIPs significantly alter the template conformation prior to polymerization. This is a significant finding as it provides insight into a potential major reason for the lack of success to date. If a template is forced into a different conformation, then the binding sites will be specific to this alternative state. Subsequent studies with the protein template in its native state will not result in specific recognition.

Based on this result, it seems that epitope and surface imprinting are promising approaches going forward as they obviate monomer contact with the sensitive macromolecular template. The field has naturally progressed in the direction of surface imprinting (Fig. 2); however, only a few epitope imprinting studies have been reported [88,117–121,132,133]. Also, living radical polymerization techniques which allow for better control of binding site morphology as well as procedures that employ biocompatible monomers, such as alginate, merit further study. Taking these things into consideration, we remain positive about the future potential of macromolecular MIPs as label-free recognition elements for ultra-sensitive biosensors as well as a wide variety of other high impact bioassay applications.

## Acknowledgements

Part of the work described in this review was supported by Grants from the National Science Foundation (BES-9706538, DGE-9972770, CTS-03-29317 and DGE-0333080). D.K. was a National Science Foundation Graduate Research Fellow.

## Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Fig.1 is difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.11.005.

## References

- [1] Chen BN, Piletsky S, Turner APF. Molecular recognition: design of “keys”. *Comb Chem High T Scr* 2002;5(6):409–27.
- [2] Jozefowicz M, Jozefonvicz J. Randomness and biospecificity: random copolymers are capable of biospecific molecular recognition in living systems. *Biomaterials* 1997;18(24):1633–44.
- [3] Glick BR, Pasternek JJ. *Molecular biotechnology: principles and applications of recombinant DNA*. Washington, DC: ASM Press; 1998.
- [4] Wang LF, Yu M. Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. *Curr Drug Targets* 2004;5(1):1–15.
- [5] Roost H, Bachmann MF, Haag A, Kalinke U, Pliska V, Hengartner H, et al. Early high-affinity neutralizing anti-viral IgG responses without further overall improvements of affinity. *Proc Natl Acad Sci USA* 1995;92(5):1257–61.
- [6] Clark LC, Lyons C. Electrode systems for continuous monitoring in cardiovascular surgery. *Ann N Y Acad Sci* 1962;102(1):29–45.
- [7] Guilbault GG, Montalvo JG. A urea-specific enzyme electrode. *J Am Chem Soc* 1969;91(8):2164–5.
- [8] Urdike SJ, Hicks GP. Enzyme electrode. *Nature* 1967;214(5092):986–8.
- [9] Luong JHT, Male KB, Glennon JD. Biosensor technology: technology push versus market pull. *Biotechnol Adv* 2008;26(5):492–500.
- [10] Sapsford KE, Bradburne C, Detchanty JB, Medintz IL. Sensors for detecting biological agents. *Mater Today* 2008;11(3):38–49.
- [11] Vo-Dinh T, Cullum B. Biosensors and biochips: advances in biological and medical diagnostics. *Fresenius J Anal Chem* 2000;366(6–7):540–51.
- [12] Byfield MP, Abuknesha RA. Biochemical aspects of biosensors. *Biosens Bioelectron* 1994;9(4–5):373–400.
- [13] Hudson PJ. Recombinant antibody fragments. *Curr Opin Biotechnol* 1998;9(4):395–402.
- [14] Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR. Making antibodies by phage display technology. *Annu Rev Immunol* 1994;12:433–55.
- [15] Sethi RS. Transducer aspects of biosensors. *Biosens Bioelectron* 1994;9(3):243–64.
- [16] Meadows D. Recent developments with biosensing technology and applications in the pharmaceutical industry. *Adv Drug Deliv Rev* 1996;21(3):179–89.
- [17] Fan XD, White IM, Shopoua SI, Zhu HY, Suter JD, Sun YZ. Sensitive optical biosensors for unlabeled targets: a review. *Anal Chim Acta* 2008;620(1–2):8–26.
- [18] Privett BJ, Shin JH, Schoenfish MH. Electrochemical sensors. *Anal Chem* 2010;82(12):4723–41.
- [19] Trojanowicz M, Wcislo M. Electrochemical and piezoelectric enantioselective sensors and biosensors. *Anal Lett* 2005;38(4):523–47.
- [20] Weetall HH. Chemical sensors and biosensors, update, what, where, when and how. *Biosens Bioelectron* 1999;14(2):237–42.
- [21] Haupt K, Mosbach K. Molecularly imprinted polymers and their use in biomimetic sensors. *Chem Rev* 2000;100(7):2495–504.
- [22] Lotierzo M et al. Surface plasmon resonance sensor for domoic acid based on grafted imprinted polymer. *Biosens Bioelectron* 2004;20(2):145–52.

- [23] Polyakov M. Adsorption properties and structure of silica gel. *Zhur Fiz Khim* 1931;2:799–805.
- [24] Pauling L. A theory of the structure and process of formation of antibodies. *J Am Chem Soc* 1940;62:2643–57.
- [25] Dickey FH. The preparation of specific absorbents. *Proc Natl Acad Sci USA* 1949;35(5):227–9.
- [26] Mosbach K, Mosbach R. Entrapment of enzymes and microorganisms in synthetic cross-linked polymers and their application in column techniques. *Acta Chem Scand* 1966;20(10):2807–10.
- [27] Cormack PAG, Mosbach K. Molecular imprinting: recent developments and the road ahead. *React Funct Polym* 1999;41(1–3):115–24.
- [28] Kryscio DR, Peppas NA. Mimicking biological delivery through feedback-controlled drug release systems based on molecular imprinting. *AIChE J* 2009;55(6):1311–24.
- [29] Kryscio DR, Fleming MQ, Peppas NA. Protein conformational studies for molecularly imprinted polymers. *J Molec Recogn*, submitted for publication.
- [30] Kryscio DR, Fleming MQ, Peppas NA. Conformational studies of common templates for macromolecularly imprinted polymers. *Biosens Bioelectron*, submitted for publication.
- [31] Bolisay LD. Molecularly imprinted polymers for tobacco mosaic virus recognition. *Biomaterials* 2006;27:4165–8.
- [32] Cai D et al. A molecular-imprint nanosensor for ultrasensitive detection of proteins. *Nat Nanotechnol* 2010;5(8):597–601.
- [33] Ghasemzadeh N, Nyberg F, Hjerten S. Highly selective artificial gel antibodies for detection and quantification of biomarkers in clinical samples. I. Spectrophotometric approach to design the calibration curve for the quantification. *J Sep Sci* 2008;31(22):3945–53.
- [34] Lee MH, Chen YC, Ho MH, Lin HY. Optical recognition of salivary proteins by use of molecularly imprinted poly(ethylene-co-vinyl alcohol)/quantum dot composite nanoparticles. *Anal Bioanal Chem* 2010;397(4):1457–66.
- [35] Pan J, Xue XH, Wang JH, Me HM, Wu ZY. Recognition property and preparation of *Staphylococcus aureus* protein A-imprinted polyacrylamide polymers by inverse-phase suspension and bulk polymerization. *Polymer* 2009;50(11):2365–72.
- [36] Tao ZY et al. Templated xerogels as platforms for biomolecule-less biomolecule sensors. *Anal Chim Acta* 2006;564(1):59–65.
- [37] Ou SH, Wu MC, Chou TC, Liu CC. Polyacrylamide gels with electrostatic functional groups for the molecular imprinting of lysozyme. *Anal Chim Acta* 2004;504:163–6.
- [38] Hawkins DM, Stevenson D, Reddy SM. Investigation of protein imprinting in hydrogel based molecularly imprinted polymers (HydroMIPs). *Anal Chim Acta* 2005;542(1):61–5.
- [39] Laemmli UK. Cleavage of structural proteins during assembly of head of bacteriophage-T4. *Nature* 1970;227(5259):680–5.
- [40] Turner NW, Jeans CW, Brain KR, Allender CJ, Hlady V, Britt DW. From 3D to 2D: a review of the molecular imprinting of proteins. *Biotechnol Prog* 2006;22:1474–89.
- [41] Chen RR, Qin L, Jia M, He XW, Li WY. Novel surface-modified molecularly imprinted membrane prepared with infiter for permselective separation of lysozyme. *J Membr Sci* 2010;363(1–2):212–20.
- [42] Demirel G, Ozcetin G, Turan E, Caykara T. PH/temperature-sensitive imprinted ionic poly (N-tert-butylacrylamide-co-acrylamide/maleic acid) hydrogels for bovine serum albumin. *Macromol Biosci* 2005;5(10):1032–7.
- [43] Fu G-Q. Imprinting effect of protein-imprinted polymers composed of chitosan and polyacrylamide: a re-examination. *Biomaterials* 2008;29:2138–42.
- [44] Fu GQ, Zhao JC, Yu H, Liu L, He BL. Bovine serum albumin-imprinted polymer gels prepared by graft copolymerization of acrylamide on chitosan. *React Funct Polym* 2007;67(5):442–50.
- [45] Gai QQ, Qu F, Liu ZJ, Dai RJ, Zhang YK. Superparamagnetic lysozyme surface-imprinted polymer prepared by atom transfer radical polymerization and its application for protein separation. *J Chromatogr A* 2010;1217(31):5035–42.
- [46] Guo TY, Xia YQ, Hao GJ, Song MD, Zhang BH. Adsorptive separation of hemoglobin by molecularly imprinted chitosan beads. *Biomaterials* 2004;25(27):5905–12.
- [47] Guo TY et al. Chemically modified chitosan beads as matrices for adsorptive separation of proteins by molecularly imprinted polymer. *Carbohydr Polym* 2005;62(3):214–21.
- [48] Guo TY, Xia YQ, Wang J, Song MD, Zhang BH. Chitosan beads as molecularly imprinted polymer matrix for selective separation of proteins. *Biomaterials* 2005;26(28):5737–45.
- [49] Hawkins DM, Trache A, Ellis EA, Stevenson D, Holzenburg A, Meininger GA, et al. Quantification and confocal imaging of protein specific molecularly imprinted polymers. *Biomacromolecules* 2006;7(9):2560–4.
- [50] Janiak DS, Ayyub OB, Kofinas P. Effects of charge density on the recognition properties of molecularly imprinted polymeric hydrogels. *Macromolecules* 2009;42(5):1703–9.
- [51] Janiak DS, Ayyub OB, Kofinas P. Effects of charge density on the recognition properties of molecularly imprinted polyampholyte hydrogels. *Polymer* 2010;51(3):665–70.
- [52] Li Y. Protein recognition via surface molecularly imprinted polymer nanowires. *Anal Chem* 2006;78:317–20.
- [53] Linares AV, Vandeveld F, Pantigny J, Falcimaigne-Cordin A, Haupt K. Polymer films composed of surface-bound nanofilaments with a high aspect ratio, molecularly imprinted with small molecules and proteins. *Adv Funct Mater* 2009;19(8):1299–303.
- [54] Lu Y, Yan CL, Wang XJ, Wang GK. Protein imprinting and recognition via forming nanofilms on microbeads surfaces in aqueous media. *Appl Surf Sci* 2009;256(5):1341–6.
- [55] Pang X. Soft-wet polyacrylamide gel beads with the imprinting of bovine serum albumin. *React Funct Polym* 2006;66:1182–8.
- [56] Pang XS, Cheng GX, Li RS, Lu SL, Zhang YH. Bovine serum albumin-imprinted polyacrylamide gel beads prepared via inverse-phase seed suspension polymerization. *Anal Chim Acta* 2005;550(1–2):13–7.
- [57] Pang XS, Cheng GX, Lu SL, Tang EJ. Synthesis of polyacrylamide gel beads with electrostatic functional groups for the molecular imprinting of bovine serum albumin. *Anal Bioanal Chem* 2006;384(1):225–30.
- [58] Qin L, He XW, Zhang W, Li WY, Zhang YK. Surface-modified polystyrene beads as photografting imprinted polymer matrix for chromatographic separation of proteins. *J Chromatogr A* 2009;1216(5):807–14.
- [59] Tan CJ, Tong YW. The effect of protein structural conformation on nanoparticle molecular imprinting of ribonuclease A using miniemulsion polymerization. *Langmuir* 2007;23(5):2722–30.
- [60] Tan CJ, Tong YW. Preparation of superparamagnetic ribonuclease A surface-imprinted submicrometer particles for protein recognition in aqueous media. *Anal Chem* 2007;79(1):299–306.
- [61] Tan CJ, Wangrangsamakul S, Bai R, Tong YW. Defining the interactions between proteins and surfactants for nanoparticle surface imprinting through miniemulsion polymerization. *Chem Mater* 2008;20(1):118–27.
- [62] Zhang W, Qin L, Chen RR, He XW, Li WY, Zhang YK. Protein imprinted polymer using acryloyl-beta-cyclodextrin and acrylamide as monomers. *Appl Surf Sci* 2010;256(9):3000–5.
- [63] Chou PC. C-reactive protein thin-film molecularly imprinted polymers formed using a micro-contact approach. *Anal Chim Acta* 2005;542:20–5.
- [64] Hsu CY, Lin HY, Thomas JL, Chou TC. Synthesis of and recognition by ribonuclease A imprinted polymers. *Nanotechnology* 2006;17:s77–83.
- [65] Hsu CY, Lin HY, Thomas JL, Wu BT, Chou TC. Incorporation of styrene enhances recognition of ribonuclease A by molecularly imprinted polymers. *Biosens Bioelectron* 2006;22(3):355–63.
- [66] Liao PC, Tyan YC, Wang CY, Hsu JF, Chou TC, Lin HY. Assessing the binding selectivity of molecularly imprinted polymer artificial antibodies by mass spectrometry based profiling system. *J Biomed Mater Res Part A* 2009;91A(2):597–604.
- [67] Lin HY. The microcontact imprinting of proteins: the effect of cross-linking monomers for lysozyme, ribonuclease A and myoglobin. *Biosens Bioelectron* 2006;22:534–43.
- [68] Lin HY, Rick J, Chou TC. Optimizing the formulation of a myoglobin molecularly imprinted thin-film polymer-formed using a micro-contact imprinting method. *Biosens Bioelectron* 2007;22(12):3293–301.
- [69] Su WX, Rick J, Chou TC. Selective recognition of ovalbumin using a molecularly imprinted polymer. *Microchem J* 2009;92(2):123–8.
- [70] Bereli N, Andac M, Baydemir G, Say R, Galaev IY, Denizli A. Protein recognition via ion-coordinated molecularly imprinted supermacroporous cryogels. *J Chromatogr A* 2008;1190(1–2):18–26.
- [71] Chen ZY, Hua ZD, Xu L, Huang Y, Zhao MP, Li YZ. Protein-responsive imprinted polymers with specific shrinking and rebinding. *J Mol Recognit* 2008;21(1):71–7.
- [72] Fu GQ, He HY, Chai ZH, Chen HC, Kong JA, Wang Y, et al. Enhanced lysozyme imprinting over nanoparticles functionalized with carboxyl groups for noncovalent template sorption. *Anal Chem* 2011;83(4):1431–6.
- [73] He HY, Fu GQ, Wang Y, Chai ZH, Jiang YZ, Chen ZL. Imprinting of protein over silica nanoparticles via surface graft copolymerization using low monomer concentration. *Biosens Bioelectron* 2010;26(2):760–5.
- [74] Hua ZD, Chen ZY, Li YZ, Zhao MP. Thermosensitive and salt-sensitive molecularly imprinted hydrogel for bovine serum albumin. *Langmuir* 2008;24(11):5773–80.
- [75] Hua ZD, Zhou S, Zhao MP. Fabrication of a surface imprinted hydrogel shell over silica microspheres using bovine serum albumin as a model protein template. *Biosens Bioelectron* 2009;25(3):615–22.
- [76] Jing T et al. Magnetic molecularly imprinted nanoparticles for recognition of lysozyme. *Biosens Bioelectron* 2010;26(2):301–6.
- [77] Jing T et al. Rapid and selective determination of urinary lysozyme based on magnetic molecularly imprinted polymers extraction followed by chemiluminescence detection. *Anal Chim Acta* 2011;692(1–2):73–9.
- [78] Kimhi O, Bianco-Peled H. Study of the interactions between protein-imprinted hydrogels and their templates. *Langmuir* 2007;23(11):6329–35.
- [79] Kublickas R. Polyacrylamide gels containing ionized functional groups for the molecular imprinting of human growth hormone. *Polym Bull* 2007;58:611–7.
- [80] Zhang W, Qin L, He XW, Li WY, Zhang YK. Novel surface modified molecularly imprinted polymer using acryloyl-beta-cyclodextrin and acrylamide as monomers for selective recognition of lysozyme in aqueous solution. *J Chromatogr A* 2009;1216(21):4560–7.
- [81] Qin L, He XW, Jia M, Li WY, Zhang YK. A thermosensitive monolithic column as an artificial antibody for the on-line selective separation of the protein. *Chem Eur J* 2011;17(5):1696–704.
- [82] Qin L, He XW, Yuan X, Li WY, Zhang YK. Molecularly imprinted beads with double thermosensitive gates for selective recognition of proteins. *Anal Bioanal Chem* 2011;399(10):3375–85.

- [83] Tov OY, Luvitch S, Bianco-Peled H. Molecularly imprinted hydrogel displaying reduced non-specific binding and improved protein recognition. *J Sep Sci* 2010;33(11):1673–81.
- [84] Zhou DZ, Guo TY, Yang Y, Zhang ZP. Surface imprinted macroporous film for high performance protein recognition in combination with quartz crystal microbalance. *Sensor Actuat B Chem* 2011;153(1):96–102.
- [85] Burow M, Minoura N. Molecular imprinting: synthesis of polymer particles with antibody-like binding characteristics for glucose oxidase. *Biochem Biophys Res Commun* 1996;227(2):419–22.
- [86] Hirayama K, Sakai Y, Kameoka K. Synthesis of polymer particles with specific lysozyme recognition sites by a molecular imprinting technique. *J Appl Polym Sci* 2001;81:3378–87.
- [87] Hirayama K, Burow M, Morikawa Y, Minoura N. Synthesis of polymer-coated silica particles with specific recognition sites for glucose oxidase by the molecular imprinting technique. *Chem Lett* 1998;8:731–2.
- [88] Nishino H. Selective protein capture by epitope imprinting. *Angew Chem Int Edit* 2006;45:2392–6.
- [89] Okutucu B, Zihnioglu F, Telefoncu A. Shell-core imprinted polyacrylamide crosslinked chitosan for albumin removal from plasma. *J Biomed Mater Res Part A* 2008;84A(3):842–5.
- [90] Schirhagl R, Lieberzeit PA, Blaas D, Dickert FL. Chemosensors for viruses based on artificial immunoglobulin copies. *Adv Mater* 2010;22(18):2078–81.
- [91] Schirhagl R, Podlipna D, Lieberzeit PA, Dickert FL. Comparing biomimetic and biological receptors for insulin sensing. *Chem Commun* 2010;46(18):3128–30.
- [92] Turan E, Ozcetin G, Caykara T. Dependence of protein recognition of temperature-sensitive imprinted hydrogels on preparation temperature. *Macromol Biosci* 2009;9(5):421–8.
- [93] Uysal A, Demirel G, Turan E, Caykara T. Hemoglobin recognition of molecularly imprinted hydrogels prepared at different pHs. *Anal Chim Acta* 2008;625(1):110–5.
- [94] Wang YT et al. Potentiometric sensors based on surface molecular imprinting: detection of cancer biomarkers and viruses. *Sensor Actuat B Chem* 2010;146(1):381–7.
- [95] Wang YT, Zhou YX, Sokolov J, Rigas B, Levon K, Rafailovich M. A potentiometric protein sensor built with surface molecular imprinting method. *Biosens Bioelectron* 2008;24(1):162–6.
- [96] Derazshamshir A, Baydemir G, Andac M, Say R, Galaev IY, Denizli A. Molecularly imprinted PHEMA based cryogel for depletion of hemoglobin from human blood. *Macromol Chem Phys* 2010;211(6):657–68.
- [97] Tan CJ, Chua MG, Ker KH, Tong YW. Preparation of bovine serum albumin surface-imprinted submicrometer particles with magnetic susceptibility through core-shell miniemulsion polymerization. *Anal Chem* 2008;80(3):683–92.
- [98] Verheyen E, Schillemans JP, van Wijk M, Demeniex MA, Hennink WE, van Nostrum CF. Challenges for the effective molecular imprinting of proteins. *Biomaterials* 2011;32(11):3008–20.
- [99] Ciardelli G, Cioni B, Cristallini C, Barbani N, Silvestri D, Giusti P. Acrylic polymeric nanospheres for the release and recognition of molecules of clinical interest. *Biosens Bioelectron* 2004;20(6):1083–90.
- [100] Bossi A, Piletsky SA, Piletska EV, Righetti PG, Turner APF. Surface-grafted molecularly imprinted polymers for protein recognition. *Anal Chem* 2001;73:5281–6.
- [101] Herrero EP, Del Valle EMM, Peppas NA. Protein imprinting by means of alginate-based polymer microcapsules. *Ind Eng Chem Res* 2010;49(20):9811–4.
- [102] Augst AD, Kong HJ, Mooney DJ. Alginate hydrogels as biomaterials. *Macromol Biosci* 2006;6(8):623–33.
- [103] Ying XG, Cheng GX, Zhao KY. Preparation and characterization of protein imprinted agarose microspheres. *Polym Bull* 2010;65(3):245–63.
- [104] Zhang ZH, Long YM, Nie LH, Yao SZ. Molecularly imprinted thin film self-assembled on piezoelectric quartz crystal surface by the sol–gel process for protein recognition. *Biosens Bioelectron* 2006;21(7):1244–51.
- [105] Zhao KY, Cheng GX, Huang JJ, Ying XG. Rebinding and recognition properties of protein-macromolecularly imprinted calcium phosphate/alginate hybrid polymer microspheres. *React Funct Polym* 2008;68(3):732–41.
- [106] Zhao KY, Huang JJ, Ying XG, Cheng GX. Macromolecularly imprinted calcium phosphate/alginate hybrid polymer microspheres with the surface imprinting of bovine serum albumin in inverse-phase suspension. *J Appl Polym Sci* 2008;109(4):2687–93.
- [107] Zhao KY, Wei JF, Cheng GX, Yang CX, Chen L. Preparation of bovine serum albumin-imprinted calcium polyacrylate/alginate hybrid microspheres via Ca<sup>2+</sup> crosslinking. *J Appl Polym Sci* 2009;113(2):1133–40.
- [108] Ying XG, Cheng GX, Liu GP, Qu RJ, Wang YQ, Zhang LG. Specific rebinding property of protein macromolecularly imprinted polymer microspheres based on calcium alginate hydrogel via gas jetting-dropping method. *J Appl Polym Sci* 2010;117(4):2331–9.
- [109] Herrero EP, Del Valle EMM, Galan MA. Development of a new technology for the production of microcapsules based in atomization processes. *Chem Eng J* 2006;117(2):137–42.
- [110] Bayer CL, Herrero EP, Peppas NA. Alginate films as macromolecular imprinted matrices. *J Biomater Sci Polym Ed* 2011;22(11):1523–34.
- [111] Shi HQ, Tsai WB, Garrison MD, Ferrari S, Ratner BD. Template-imprinted nanostructured surfaces for protein recognition. *Nature* 1999;398:593–7.
- [112] Bompert M, Haupt K. Molecularly imprinted polymers and controlled/living radical polymerization. *Aust J Chem* 2009;62(8):751–61.
- [113] Bossi A, Bonini F, Turner APF, Piletsky SA. Molecularly imprinted polymers for the recognition of proteins: the state of the art. *Biosens Bioelectron* 2007;22:1131–7.
- [114] Hoshino Y, Kodama T, Okahata Y, Shea KJ. Peptide imprinted polymer nanoparticles: a plastic antibody. *J Am Chem Soc* 2008;130(46):15242–3.
- [115] Hoshino Y, Koide H, Urakami T, Kanazawa H, Kodama T, Oku N, et al. Recognition, neutralization, and clearance of target peptides in the bloodstream of living mice by molecularly imprinted polymer nanoparticles: a plastic antibody. *J Am Chem Soc* 2010;132(19):6644–5.
- [116] Hoshino Y, Urakami T, Kodama T, Koide H, Oku N, Okahata Y, et al. Design of synthetic polymer nanoparticles that capture and neutralize a toxic peptide. *Small* 2009;5(13):1562–8.
- [117] Rachkov A. Molecularly imprinted polymers prepared in aqueous solution selective for [Sar<sup>1</sup>, Ala<sup>8</sup>] angiotensin II. *Anal Chim Acta* 2004;504:191–7.
- [118] Rachkov A, Minoura N. Recognition of oxytocin and oxytocin-related peptides in aqueous media using a molecularly imprinted polymer synthesized by the epitope approach. *Chromatography A* 2000;889:111–8.
- [119] Rachkov A, Minoura N. Towards molecularly imprinted polymers selective to peptides and proteins. The epitope approach. *Biochim Biophys Acta* 2001;1544:255–66.
- [120] Tai DF, Jhang MH, Chen GY, Wang SC, Lu KH, Lee YD, et al. Epitope-cavities generated by molecularly imprinted films measure the coincident response to anthrax protective antigen and its segments. *Anal Chem* 2010;82(6):2290–3.
- [121] Tai DF, Lin CY, Wu TZ, Chen LK. Recognition of dengue virus protein using epitope-mediated molecularly imprinted film. *Anal Chem* 2005;77(16):5140–3.
- [122] Huafang Wang WL, Xiwen He, Langxing Chen, Yukui Zhang. M-aminophenylboronic acid as a functional monomer for fabricating molecularly imprinted polymer for the recognition of bovine serum albumin. *React Funct Polym* 2008;68:1291–6.
- [123] Hopkins TR, Spikes JD. Denaturation of proteins in 8M urea as monitored by tryptophan fluorescence – chymotrypsin chymotrypsinogen and some derivatives. *Biochem Biophys Res Commun* 1967;28(3):480–5.
- [124] Vivian JT, Callis PR. Mechanisms of tryptophan fluorescence shifts in proteins. *Biophys J* 2001;80(5):2093–109.
- [125] Jelezarov I, Bosshard HR. Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. *J Mol Recognit* 1999;12(1):3–18.
- [126] Bonini F, Piletsky S, Turner APF, Speghini A, Bossi A. Surface imprinted beads for the recognition of human serum albumin. *Biosens Bioelectron* 2007;22:2322–8.
- [127] Dhruv H, Pepalla R, Taveras M, Britt DW. Protein insertion and patterning of PEG bearing Langmuir monolayers. *Biotechnol Prog* 2006;22(1):150–5.
- [128] Du X, Hlady V, Britt D. Langmuir monolayer approaches to molecular recognition through molecular imprinting. *Biosens Bioelectron* 2005;20:2053–60.
- [129] Shi HQ, Ratner BD. Template recognition of protein-imprinted polymer surfaces. *J Biomed Mater Res* 2000;49:1–11.
- [130] Sunayama H, Ooya T, Takeuchi T. Fluorescent protein recognition polymer thin films capable of selective signal transduction of target binding events prepared by molecular imprinting with a post-imprinting treatment. *Biosens Bioelectron* 2010;26(2):458–62.
- [131] Turner NW, Wright BE, Hlady V, Britt DW. Formation of protein molecular imprints within Langmuir monolayers: a quartz crystal microbalance study. *J Colloid Interf Sci* 2007;308:71–80.
- [132] Ozcan AA, Say R, Denizli A, Ersoz A. L-histidine imprinted synthetic receptor for biochromatography applications. *Anal Chem* 2006;78(20):7253–8.
- [133] Tai DF, Lin CY, Wu TZ, Huang JH, Shu PY. Artificial receptors in serologic tests for the early diagnosis of dengue virus infection. *Clin Chem* 2006;52(8):1486–91.
- [134] Odabasi M, Say R, Denizli A. Molecularly imprinted particles for lysozyme purification. *Mater Sci Eng B* 2007;27(1):90–9.
- [135] Ghasemzadeh N, Nyberg F, Hjerten S. Highly selective artificial gel antibodies for detection and quantification of biomarkers in clinical samples. II. Albumin in body fluids of patients with neurological disorders. *J Sep Sci* 2008;31(22):3954–8.
- [136] Hu CH, Chou TC. Albumin molecularly imprinted polymer prepared with a semi-rigid crosslinker in mixed organic/aqueous media. *Microchim Acta* 2009;165(3–4):399–405.
- [137] Ying XG, Zhang FJ, Zhang LG, Cheng GX. Effect on rebinding behavior with different composition and structure of the dually imprinted alginate polymer microspheres using proteins and o/w emulsion drops as dual templates. *J Appl Polym Sci* 2010;115(6):3516–26.
- [138] Zhang FJ, Cheng GX, Ying XG. Emulsion and macromolecules templated alginate based polymer microspheres. *React Funct Polym* 2006;66(7):712–9.
- [139] Cutivet A, Schembri C, Kovensky J, Haupt K. Molecularly imprinted microgels as enzyme inhibitors. *J Am Chem Soc* 2009;131(41):14699–702.
- [140] Lin HY, Ho MS, Lee MH. Instant formation of molecularly imprinted poly(ethylene-co-vinyl alcohol)/quantum dot composite nanoparticles and their use in one-pot urinalysis. *Biosens Bioelectron* 2009;25(3):579–86.
- [141] Lin TY, Hu CH, Chou TC. Determination of albumin concentration by MIP-QCM sensor. *Biosens Bioelectron* 2004;20:75–81.
- [142] Chen YW, Rick J, Chou TC. A systematic approach to forming micro-contact imprints of creatine kinase. *Org Biomol Chem* 2009;7(3):488–94.

- [143] Hu XB, Li GT, Huang J, Zhang D, Qiu Y. Construction of self-reporting specific chemical sensors with high sensitivity. *Adv Mater* 2007;19(24):4327–32.
- [144] El Kirat K, Bartkowski M, Haupt K. Probing the recognition specificity of a protein molecularly imprinted polymer using force spectroscopy. *Biosens Bioelectron* 2009;24(8):2618–24.
- [145] Lautner G, Kaev J, Reut J, Opik A, Rappich J, Syritski V, et al. Selective artificial receptors based on micropatterned surface-imprinted polymers for label-free detection of proteins by SPR imaging. *Adv Funct Mater* 2011;21(3):591–7.
- [146] Menaker A, Syritski V, Reut J, Opik A, Horvath V, Gyurcsanyi RE. Electrosynthesized surface-imprinted conducting polymer microrods for selective protein recognition. *Adv Mater* 2009;21(22):2271–5.
- [147] Ouyang RZ, Lei JP, Ju HX. Artificial receptor-functionalized nanoshell: facile preparation, fast separation and specific protein recognition. *Nanotechnology* 2010;21(18):1–9.