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Review

Molecularly imprinted polymer membranes and thin films for the separation and sensing of biomacromolecules

This review describes recent advances associated with the development of surface imprinting methods for the synthesis of polymeric membranes and thin films, which possess the capability to selectively and specifically recognize biomacromolecules, such as proteins and single- and double-stranded DNA, employing "epitope" or "whole molecule" approaches. Synthetic procedures to create different molecularly imprinted polymer membranes or thin films are discussed, including grafting/in situ polymerization, drop-, dip-, or spin-coating procedures, electropolymerization as well as micro-contact or stamp lithography imprinting methods. Highly sensitive techniques for surface characterization and analyte detection are described, encompassing luminescence and fluorescence spectroscopy, X-ray photoelectron spectroscopy, FTIR spectroscopy, surface-enhanced Raman spectroscopy, atomic force microscopy, quartz crystal microbalance analysis, cyclic voltammetry, and surface plasmon resonance. These developments are providing new avenues to produce bioelectronic sensors and new ways to explore through advanced separation science procedures complex phenomena associated with the origins of biorecognition in nature.

Keywords: Biomacromolecules / Membranes / Molecularly imprinted polymers / Surface imprinting / Thin films
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1 Introduction

The development of synthetic materials that can act as biomimetic receptors is a challenge that chemists and biochemical researchers have long pursued. Often, the objective has been to design and synthesize novel materials that exhibit performance equal to or greater than that manifested by naturally occurring antibodies. However, the increase in knowledge in systems biology has created the need to move closer to the requirements of single molecule recognition. Detection in these circumstances includes biological macromolecules such as proteins and DNA and also much more complex molecular assemblies like viruses, bacteria, spores, and cells [1]. Due to their chemical and physical stability, and their potential for reproducible, industrial manufacture, polymeric materials with biorecognition features afford new opportunities for use as "artificial receptors" in separation systems, sensors, bioelectronics, and as pharmaceutical and biomedical diagnostic screening tools [2–7]. Developments over the past decade in the synthesis of molecularly imprinted polymers (MIPs) have opened up new avenues of research, some of which are now beginning to find commercial opportunities and applications. MIPs are polymeric materials that can be designed and prepared with built-in molecular recognition capabilities. As a result of this fundamental

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Abbreviations: 4NPPC, *O*-(4-nitrophenylphosphoryl)choline; **ADH**, alcohol dehydrogenase; **AFM**, atomic force microscopy; **ATRP**, atom transfer radical polymerization; **bCyt *c***, bovine cytochrome c; **bHb**, bovine hemoglobin; **bMyo**, bovine myoglobin; **CEA**, carcinogenic embryonic antigen; **ConA**, concanavalin A; **CV**, cyclic voltammetry; **CRP**, C-reactive protein; **Cyt *c***, bovine cytochrome c; **dsDNA**, double-stranded deoxyribonucleic acid; **FITC**, fluorescein isothiocyanate; **Hb**, hemoglobin; **HEWL**, hen egg white lysozyme; **HRP**, horseradish peroxidase; **HRV**, human rhinovirus; **IgG**, immunoglobulin G; **ITO**, indium tin oxide; **MBAA**, *N*,*N*′-methylenebisacrylamide; **MIP**, molecularly imprinted polymer; **MIM**, molecularly imprinted membrane; **MMA**, methyl methacrylate; **Myo**, myoglobin; **NIP**, nonimprinted polymer; **NP**, nanoparticle; **oPD**, *o*-phenylenediamine; **PAA**, polyacrylic acid; **PAM**, polyacrylamide; **PCB**, printed-circuit board; **PCM**, polycarbonate membrane; **PDMS**, polydimethylsiloxane; **PEDOT**, poly-3,4-ethylenedioxythiophene; **PET**, polyethylene terephthalate; **PMMA**, poly(methyl methacrylate); **PoPD**, poly(*o*-phenylenediamine); **PP**, polypropylene; **PPy**, polypyrrole; **PS**, polystyrene; **PSS**, polystyrene sulfonate; **QCM**, quartz crystal microbalance; **RNase A**, ribonuclease A; **RP**, radical polymerization; **RU**, response unit; **SAM**, self-assembled monolayer; **SAW**, surface acoustic wave; **SERS**, surface-enhanced Raman spectroscopy; **SI-ATRP**, surface-initiated atom transfer radical polymerization; **ssDNA**, single-stranded deoxyribonucleic acid; **TMV**, tobacco mosaic virus; **TnT**, troponin T; **XPS**, X-ray photoelectron spectroscopy

attribute, interest has increased in their development as inexpensive, robust, selective, and sensitive molecular recognition materials, and the uses of MIPs now encompass a wide range of molecular targets in fields associated with separation sciences, catalysis, and monitoring/diagnostic devices for chemicals or pharmaceuticals [8].

These attributes of MIPs are achieved by exploiting molecular self-assembly processes mediated by the interaction between a chemical or biological template (imprint) molecule and chemical monomers with subsequent polymerization of the monomers with a suitable cross-linkage reagent to obtain functionalized cavities that chemically and spatially define the space occupied by the template molecule. Upon removal of the template molecules by washing and/or extraction, polymeric materials with nano-cavities of predetermined shape and complimentary-binding site features toward the template are generated. These materials afford template recognition through a combination of shape complementarity and multiple noncovalent interactions such as hydrogen bonds, hydrophobic, and electrostatic interactions. Alternatively, discrimination between analytes of different shapes and chemical functionalities can be achieved through reversible-covalent interactions. With current technologies, such polymeric materials can be made in a variety of physically and chemically robust and reusable formats, e.g. bulk polymers, beads, membranes, or thin films [9–12], with the ability to display selectivity and efficacy features approaching or in some cases exceeding those observed for relevant naturally occurring antibodies [13–15].

Due to the historical origins of their methods of preparation and the types of applications, most of the scientific literature related to MIPs and molecular imprinting procedures describe the use of the so-called 3D platforms, such as porous bulk materials, monoliths, disks, membranes, or beads. As a consequence, a large variety of so-called 3D imprinted microparticulate and monolithic porous polymeric materials have been successfully developed with interactive attributes analogous to those shown by antibodies or the catalytic sites of enzymes. The inherent selectivity and stability of MIPs prepared as 3D scaffolds meant that these imprinted polymers have become popular candidates as synthetic materials with biomimetic "receptor like" properties for analysis of low molecular mass compounds. These features have resulted in a great deal of success for selective recognition/capture, separation, or pseudo-enzymatic modification of low molecular weight molecules. However, the same level of success has yet to be fully translated to larger biomacromolecules, such as proteins [16, 17], the most frequently imprinted class of biological macromolecules, with the lack of binding site accessibility identified as one of the key challenges [18].

One of the first efforts at protein imprinting was described by the group of Mosbach in 1995 for the enzyme ribonuclease A (RNase A) [19, 20]. The interest in bulk imprinting with proteins has been stimulated mainly by their applications as affinity chromatography materials, rather than sensor development, as illustrated by the work of Hjertén et al. [21, 22]. A possible advantage of imprinting a "whole" protein is that the template structure will most accurately reflect that of the target (if target and template are identical) with applications described that use MIPs to facilitate protein crystallization [23]. However, with the "whole molecule" approach, the application of these 3D platforms for specific protein imprinting and recognition is constrained by a number of factors [24]. For example, the requirement for these biomacromolecules to remain in their native tertiary or quaternary state during the formation of the prepolymerization complex and during the radical polymerization, induced by heat or irradiation is difficult to achieve. Yet, this is essential if a cavity with the appropriate shape and functional properties is to be created [25, 26]. Consequently, there is limited choice of suitable solvents that can be employed to ensure adequate solubility of the biomolecule without disrupting any hydrogen-bonding sites between template and monomer during imprinting. Furthermore, the adsorption of proteins in the interior of porous bulk polymer networks suffers from restricted diffusion, slow-binding/release kinetics, and hindered-binding site accessibility. Difficulties associated with the removal of the (protein) template may moreover occur due to entrapment or covalent bonding between protein and polymer while the large imprinted sites generated by the protein template may perform as general nanopores, able to bind a range of smaller molecules, resulting in reduced selectivity [4].

The development of alternative approaches, based on the so-called 2D pellicular or surface imprinting strategies [18], where the imprint molecule is confined to a monolayer, has provided a range of solutions to some of the challenges posed by the large size, structural complexity, and conformational flexibility of biomacromolecular targets, such as proteins. Imprinted thin films can be made on a variety of substrates, e.g. planar substrates (silicon wafers, mica, sensor chips), inner walls of fused-silica capillaries, preformed porous structures (silica monoliths, membranes), and core–shell particles (silica, nanodots). Various methods to generate surface imprinted thin films, including in situ polymerization after drop-, dip-, or spin-coating, mini-emulsion polymerization, electropolymerization, core–shell polymerization, and micro-contact imprinting, have been introduced to extend the application utility of MIPs to specific biomacromolecular recognition. However, the application of these techniques with biomacromolecular templates still remains at an early stage of development with the literature still very much dominated by the use of low molecular weight (<1500) templates [27, 28]. Nevertheless, over the past several years, new types of MIP-based devices for biomacromolecular analysis and detection have been generated, predominately by grafting, spin-coating, electropolymerization, and micro-contact imprinting methods, drawing upon the guidance offered from studies with low molecular mass templates, such as drug substances, metabolites, or synthetic antigenic determinants (epitopes) of biomolecules. These advances are providing the tools needed to chart the road-map for the de novo design and synthesis of molecularly imprinted polymeric surfaces. These new materials can be integrated with advanced, multiplexing

detection devices to provide the next generation of biosensors and biochips, for use in the selective recognition and analysis of a broad range of targets, not only single biomacromolecules, but also biomolecular assemblies, viruses, and cells. In the sections below, the various approaches to these challenges, the methods of MIP membrane and thin film synthesis, sensitivity, and selectivity evaluation as well as the various methods used for target analyte detection are outlined and discussed.

2 Epitope imprinting

One strategy adopted to circumvent some of the challenges posed by the use of a "whole protein" approach in biomacromolecular imprinting in the 3D mode has been to use a synthetic epitope template, usually a small peptide fragment of a protein, typically 4–15 amino acid units in size. Similar to the "sequence-recognition" approach, where a peptide with the same amino acid sequence as that of an exposed N- or C-terminus of a target protein is used as the template the "epitope" approach employs a small peptide, selected with an amino acid composition and sequence that replicate a similar conformational arrangement as found for the same amino acid residues on the surface of the protein (the epitope), as a surrogate template for the whole protein. Although this approach, originally proposed by Rachkov and Minoura [29], facilitates the removal of the template after the polymerization, the access of macromolecules is still hindered in highly cross-linked bulk MIPs, unless, of course, the epitope and the original molecule are of comparable size.

Nevertheless, the epitope molecularly imprinted polymer can then interact as an "artificial antibody" with the appropriate protein, provided the same (or similar) epitopic peptide structure is accessible on the surface of the protein, thereby mimicking nature's method of achieving antigen/antibody biorecognition [30]. This process has the advantage of completely avoiding the use of costly high-purity biomacromolecular template molecules, and reduces the impact of incomplete template removal. Additionally, the need to retain the template in the same conformational state as that found for the native state of the biomacromolecule, may not have to be as stringently controlled, since the biomacromolecular target molecule is not involved in the MIP preparation and only needs to bind to the imprinted cavity with sufficient affinity as dictated by the application requirements. A summary of thin films made using the epitope approach is shown in Table 1.

Much of the early research described in the scientific literature related to the development of MIP-based "artificial antibodies" was aimed at exploiting the use of smaller biomimetic structures. These studies often employed linear peptide sequences of only a few amino acid units in length, which as imprint molecules mimicked the antigenic linear epitopes of larger peptides, polypeptides, or proteins. For example, a tetrapeptide, Tyr-Pro-Leu-Gly-NH_2 (YPLG-NH_2), was employed to prepare MIP particles for the recognition of the cyclic nonapeptide hormone

Table 1. Epitope/peptide imprinting of thin films

Imprinting technique	Template	Substrate	Characterization	Application	References
In situ polymerization	15-mer peptide epitope of Dengue virus NS1 protein target	Gold QCM chip	QCM	QCM sensor for the serological detection of dengue virus	[32]
In situ polymerization	9-mer peptide epitope corresponding to the C-terminal domains of (Cyt *c*, ADH, and BSA targets	Glass and silica	Film incubation followed by SDS-PAGE and MALDI-TOF MS	Fundamental investigation	[33]
In situ polymerization	9- to 14-mer peptide epitope of anthrax protective antigen PA83	QCM chip	QCM	QCM sensor for protective antigen from *Bacillus anthracis* (anthrax)	[34]
In situ polymerization	Human HIV type 1 gp 41 fragments	QCM chip	AFM, FTIR, QCM, XPS	QCM sensor for HIV-1 related peptides/proteins	[35]
Electro-polymerization	Fluorescence-labeled C-terminus of Cyt c, residues 96–104	SPR gold chip	AFM, ellipsometry, fluorescence imaging, SPR, zeta potential measurements	Fundamental investigation	[36]
Sol–gel reaction	C-terminal 9-mer peptide of BSA	CdTe QDs	FTIR, TEM, XPS	Quantification of BSA with fluorescence spectroscopy	[37]
Incubation	HIgG exhibiting the same Fc region but different Fab region to the target anti-HIV-1	Fe_3O_4@SiO_2 NPs	AFM, SEM, TEM, XRD	Sandwich ECL immunosensor for the detection of anti-HIV-1	[38]

ECL, electro-chemiluminescence; HIgG, human immunoglobulin G; QD, quantum dots.

oxytocin, $\overbrace{\text{CYS-Tyr-Ile-Gln-Asn-Cys}}$-Pro-Leu-Gly-$NH_2$, in which the two cysteine residues form a disulfide bond [29,30]. The resulting MIP demonstrated high affinity for the template YPLG-NH_2 in addition to oxytocin and several related derivatives. A similar approach was used to prepare a monolithic capillary electrochromatographic column to separate oxytocin from a mixture of proteins [31]. Thus, the use of small synthetic peptides as template molecules appears an attractive option for the subsequent molecular recognition of larger molecules. The more obvious approach, however, namely the use of much larger polypeptides as templates, has yet to generally prove successful when employed in the 3D format with microparticulate beads or monoliths, where insufficient access to the binding sites within the polymer matrix continues to be problematic.

In contrast, however, the development of epitope imprinted thin films by the 2D format approach has documented the capability for selective protein recognition [32,33]. For example, the selective recognition of the Dengue virus NS1 protein using an imprinted thin film supported on the modified surface of a gold quartz crystal microbalance (QCM) disk has been based on the use of a randomly orientated 15-mer linear peptide epitope template [32]. As no attempt was made in this case to control the film thickness or placement of the cavities within the polymer matrix, some embedded peptide template remained. Nevertheless, the resultant QCM sensor was able to bind not only the 15-mer template and the purified NS1 protein but also to bind other proteins containing the same 15-mer epitopic structure, which was present in the unpurified supernatant of the cultured Dengue virus. Indeed, the MIP-binding performance compared favorably to the corresponding monoclonal antibodies immobilized onto a QCM chip surface with dissociation constants of 0.04 and 0.05 nM, respectively, and as such documented the potential for development into an early Dengue fever diagnostic technique by the detection of the associated viral protein.

The imprinting of nonapeptide epitopes of the exposed C-termini for the MIP recognition of proteins bovine cytochrome *c* (bCyt *c*), alcohol dehydrogenase (ADH), and BSA has also been reported [33]. To produce these imprinted thin films, epitope templates were immobilized through their N-termini onto a glass or silicon surface by a covalent tether, and a monomer solution containing a free radical initiator then deposited and exposed to UV irradiation. After polymerization, the glass substrate and/or silicon wafer was separated from the MIP film. Each MIP film was interrogated using a buffer solution containing a mixture of five proteins (bCyt *c*, ADH, BSA, carbonic anhydrase, and trypsin inhibitor) resulting in both the bCyt *c*-MIP and BSA-MIP demonstrating a distinct selectivity for their respective target proteins with the ADH-MIP also binding to ADH, albeit with a greatly reduced affinity. Notably, the nonimprinted polymer (NIP) control films, which were prepared in the absence of the epitope templates, displayed negligible protein affinity in competitive-binding assays, thus establishing the usefulness of the epitope imprinting approach for protein recognition when combined with orientational constraints for the template.

The epitope approach has also been employed in the in situ polymerization of 9- to 14-mer peptides related to the anthrax protective antigen PA83 on a QCM chip that was then used as a QCM sensor for the detection of the protective antigen from *Bacillus anthracis* (anthrax) [34]. A similar approach has been used for the imprinting of human HIV virus type 1 glycoprotein 41 (gp41) fragments on a QCM chip for subsequent use as a QCM sensor for HIV-1 related peptides/proteins [35]. QCM measurements demonstrated that the resulting MIP film had a high affinity toward the template peptide and did bind the corresponding gp41 target protein with high specificity. The dissociation constant (K_d) of the MIP for the template peptide was calculated to be 3.17 nM through Scatchard plot analysis, which is similar to those for monoclonal antibodies. Direct detection of the gp41 protein was achieved quantitatively using the resulting MIP-based biomimetic sensor. The detection limit of gp41 was 2 ng/mL, which was comparable to a reported ELISA method.

A novel strategy to prepare a selective ultrathin MIP film directly on a gold-based transducer surface for peptide and protein detection in aqueous solution was demonstrated using a combination of epitope- and electrochemical surface imprinting approaches [36] using a synthetic, fluorescence-labeled peptide template derived from the surface-exposed C-terminus of Cyt c (residues 96–104). The labeled peptide template was chemisorbed onto the gold surface as an oriented monolayer through an additional C-terminal cysteine. After formation of a nonconductive hydrophilic poly(scopoletin) film through electropolymerization, the template was electrochemically stripped off. Recognition capabilities of the films demonstrated that the resulting MIP films were able to selectively capture the template peptide and the corresponding fluorescence-labeled target protein. In case of the peptide recognition, the MIP film was shown to discriminate between the template peptide and peptide analogues with single amino acid mismatched sequences. Further, 2D nonapeptide epitope imprinted polymers have been applied as thin film coatings onto CdTe quantum dots, wherein the specific recognition and direct fluorescence quantification of the target protein BSA was achieved based on fluorescence quenching [37].

The versatility of the epitope imprinting approach has been demonstrated through grafting of a hydrophilic MIP film onto silicate-coated magnetic iron oxide nanoparticles (Fe_3O_4@SiO_2 nanoparticles (NPs)) [38]. A human immunoglobulin G was used as the template, exhibiting the same Fc region but different Fab region to the target immunodeficiency virus type 1 antibody (anti-HIV-1). The derived magnetic molecularly imprinted polymer particles were used as capture probes and antigen conjugated with horseradish peroxidase (HRP-HIV-1) as a label in a sandwich electrochemiluminescence immunosensor for the sensitive detection of anti-HIV-1.

While these successes are encouraging, the identification and synthesis of an appropriate epitope to mimic the

recognition behavior of a particular protein may not always be straightforward or even feasible due to these epitope's thermodynamic marginal stability. The epitope's conformations are sensitive to their solvational environment, pH, and temperature, often further complicated by the lack of information regarding the protein structure or its binding domains. To address these issues, in the subsequent sections of this review, emphasis has been placed on several alternative approaches for the deployment of surface imprinting techniques based on knowledge gained from these earlier imprinting procedures with low molecular weight (≤1500 Da) chemical or epitope templates.

3 Imprinting of entire biomacromolecules

To achieve high adsorptive capacities with classically prepared porous beaded or monolithic MIPs, the synthetic strategy usually has to be tailored to ensure that the template molecule can be embedded throughout the porous polymer network. Such 3D molecular imprinting approaches have been used to prepare bulk MIPs (and through grinding particles), beads, monoliths, membranes, and films. Subsequent removal of the template in principle affords a high number of selective-binding cavities. With these porous materials, the efficacy of analyte recognition is largely driven by the intraparticulate mass transfer processes and kinetics. In many cases, however, the extent of template embedding is so extensive that extraction of the template is incomplete leading to hindered, poorly accessible-binding sites. In contrast, 2D surface imprinting approaches, have been used to prepare planar coatings in the form of thin films, electrodes, inner capillary walls, core–shell particle, and postsynthetic coatings on membranes, create template cavities directly at the liquid-solid film interface of a polymer, thereby permitting more efficient template removal, faster binding kinetics, and improved-binding site accessibility (Fig. 1).

A brief synopsis of the different types of protein and biomacromolecular surface imprinted membranes and thin films is shown in Tables 2 and 3, including various surface imprinting strategies and characterization techniques that have been utilized.

3.1 Biomacromolecular imprinted membranes

Molecularly imprinted membranes (MIMs) can be made by 3D imprinting methods (as bulk polymers or flat sheet polymers, e.g. composed of fibres) or by 2D imprinting methods, where a membrane scaffold is coated postsynthetically with a MIP thin film [39–41]. Surface biomacromolecular imprinted membranes can be prepared by a variety of methods such as membrane deposition, in situ polymerization, phase inversion precipitation, and grafting to or from a solid support [42].

3.1.1 Phase inversion

The preparation of α-amylase imprinted membranes by a phase inversion process using the polysaccharide dextran and poly(ethylene-*co*-vinyl alcohol) (EVAL) has been reported [43]. The imprinted membranes, containing varying dextran/EVAL ratios, were examined for their binding affinity and selectivity by permeating solutions of α-amylase, albumin, pepsin, and amyloglucosidase through the membrane, after which the permeated solutions were analyzed by UV spectroscopy. Comparable affinity for α-amylase (51 kDa) and pepsin (34.5 kDa) was observed, although the imprinted membranes, in particular the imprinted membrane containing a smaller proportion of dextran, demonstrated higher preferential binding of the target with respect to the nonimprinted reference membranes. The larger proteins, albumin (69 kDa) and amyloglucosidase (118 kDa) on the other hand displayed relatively low affinity (less than half that of the target or pepsin) suggesting that the larger molecular mass may have limited cavity accessibility.

3.1.2 In situ polymerization

MIMs can be derived as porous imprinted thin films or as thin films that are prepared or deposited onto a preformed membrane substrate. These functional films can then sequester

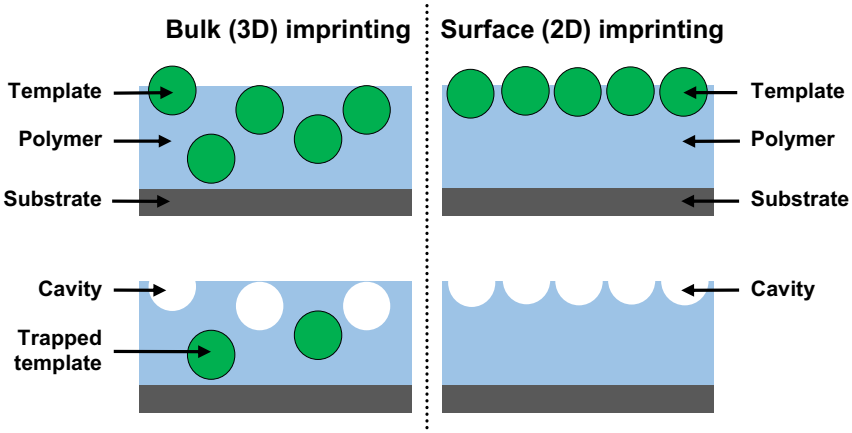


Figure 1. Illustration showing the difference between a typical 3D bulk (left) and a 2D surface (right) imprinting method before (top) and after (bottom) template removal showing the position of cavities and the related likelihood of the template becoming trapped within the polymer matrix.

Table 2. Biomacromolecule imprinting of membranes

Imprinting technique	Template	Substrate	Characterization	Application	References
Phase inversion	α-amylase	Poly(EVAL)/dextran membrane	HPLC, permeability apparatus	Filtration or adsorption of target enzyme	[43]
In situ polymerization	Albumin, Hb, and cyt c	Nanoporous alumina membrane	SEM, UV-Vis spectroscopy	Affinity chromatography of proteins	[124]
In situ polymerization	BSA and tetrameric bHb	Agarose gel membrane	Bradford test, FTIR-, and UV spectroscopy	Chromatographic protein analysis	[44]
In situ photo-lithography	CRP	Cyclic olefin copolymer	AFM, SEM, optical microscopy	Electronic microfluidic biochips as point-of-care protein sensing platform	[125]
Surface grafting	IgG	Track-etched PET membrane	Static-binding capacity measurements of single proteins	Affinity chromatography of proteins	[45]
Surface grafting	BSA	PP	DSC, FTIR, SEM, TGA	Affinity chromatography of proteins	[46]
Electropolymerization	BSA and bHb	Sacrificial nanofibers	SEM	Affinity chromatography of proteins	[47]

DSC, differential scanning calorimetry; TGA, thermogravimetric analysis.

a particular analyte or facilitate its selective transport across the membrane. One reported example of an imprinted membrane involved an aqueous protein template solution mixed with an aqueous solution of 1% agarose at 50°C [44]. Subsequent cooling, drying, treatment of the film with proteinases and extensive washing, resulted in an agarose gel membrane containing a random distribution of protein recognition sites. This method was performed using BSA and tetrameric bovine hemoglobin (bHb) as the protein templates and resulted in MIP membranes with adsorption capacities of 25.9 and 117.5 mg/g for BSA and bHb, respectively. The corresponding nonimprinted membranes adsorbed up to 6.51 mg/g BSA and 53.92 mg/g bHb, respectively, indicating that the higher level of the bHb adsorption was also due to the significant participation of nonspecific interactions. This outcome was consistent, in part, with bHb having a smaller molecular diameter and larger diffusion coefficient ($\sigma = 6.5$ nm and $1.5 \pm 0.1 \times 10^{-5}$ cm^2s^{-1} compared to 15 nm and $6.0 \pm 0.1 \times 10^{-7}$ cm^2s^{-1} for BSA), thus resulting in a greater access of bHb to the interior porous zones of the membrane. The free energy of interaction of the protein with the polymer backbone itself may have also contributed to this higher capacity.

3.1.3 Surface grafting

Macroporous membrane adsorbents are an attractive format for the application of affinity materials because they enable the fast separations of larger molecules. One of the preferred methods to prepare such membranes is by surface grafting of functional (imprinted) hydrogel layers to the base membrane's pore surface. An example of such surface grafting approach was reported for the synthesis of an antibody-imprinted membrane by a two-step surface grafting process [45]. In this work, a two-step imprinting strategy, combining surface imprinting and scaffold imprinting, was applied to prepare a MIP adsorbent for immunoglobulin G (IgG). Track-etched polyethylene terephthalate (PET) membranes with previously introduced aliphatic C—Br groups as initiator on the pore surface were used to prepare first a functional polymer scaffold, grafted poly(methacrylic acid), by surface-initiated atom transfer radical polymerization (SI-ATRP). After template protein (IgG) binding to the scaffold, UV-initiated cross-linking copolymerization of acrylamide and *N,N′*-methylenebisacrylamide (MBAA) led to a grafted MIP hydrogel layer. The influence of the three independent parameters, scaffold chain length by SI-ATRP reaction time, degree of cross-linking of the MIP layer by MBAA content, and grafted MIP layer thickness by UV irradiation time, were studied to optimize protein-binding capacity and selectivity. The best IgG MIP membrane adsorbent was then used to separate IgG from mixtures with HSA, demonstrating IgG-binding capacities and eluted IgG purities, which were almost independent of the excess of HSA. The transfer of the approach from the model PET to other base membranes with higher specific surface area appears to be straightforward, and the resulting affinity materials would be suited for the capturing of an antibody from a complex mixture.

The preparation of a protein-imprinted polyacrylamide (PAM) hydrogel membrane used nonwoven polypropylene (PP) fiber as matrix, BSA as template molecule, acrylamide (AM) as functional monomer, and MBAA as cross-linker by UV radiation-reduced polymerization [46]. Factors that influence the adsorption capacity of such MIPs were investigated, such as monomer concentration, cross-linker concentration, template molecule amount, and pH values of the BSA solution. The rebinding and recognition properties of the PP-grafted imprinting PAM hydrogel membrane were evaluated. The results showed that the PP-*g*-PAM MIP

Table 3. Biomacromolecule imprinting of thin films

Imprinting technique	Template	Substrate	Characterization	Application	References
In situ polymerization and grafting	Microperoxidase, cyt c, HRP, and HbA_0	Polystyrene microtiter plates	UV spectroscopy with microplate reader	Fundamental investigation	[51]
In situ polymerization	HEWL	Gold-coated SPR sensor chip	SPR	SPR sensor	[52]
In situ polymerization	Myo	Porous alumina surface	Confocal scanning light microscopy, fluorescence microscopy, SCA, SEM	Fundamental investigation	[53]
In situ polymerization	Cyt *c*	Microscope cover glass	AFM, fluorescence analysis	Fundamental investigation	[54]
In situ polymerization	FITC-albumin, TRITC-lectin, and geletin, fibroblasts	PDMS mold from silicone master	Cell culture, FTIR, fluorescence microscopy, SCA, XPS	Fundamental investigation	[107]
In situ polymerization with ATRP and RP	RNase	Gold-coated SPR sensor chip	QCM, SPR	Fundamental investigation	[55]
Drop-coating	ds DNA (verotoxin 32 amino acid sequence)	Modified glass slide and gold sensor chip	Circular dichroism (CD), fluorescence spectroscopy, SPR, SCA	SPR sensor for native dsDNA	[56]
Spin-coating	HEWL and bCyt *c*	Gold surfaces of QCM electrodes	QCM	QCM sensor	[57]
Spin-coating	Parapox ovis virus (ORF)	QCM chip	AFM, QCM,	QCM sensor	[58]
Electrochemical polymerization	Bovine leukaemia virus gp51	Platinum-black electrode	Pulsed amperometric detection (PAD)	PAD sensor	[65]
Electro chemical-polymerization	HEWL or bCyt *c*	Screen-printed platinum supports	CV	CV sensor	[67]
Electropolymerization	Avidin	Porous, sacrificial membrane, gold electrode	Fluorescence microscopy	Fundamental investigation	[68]
Electropolymerization	BSA	Carbon electrode	Differential pulse voltammetry (DPV), FTIR, SEM	CV sensor	[70]
Electropolymerization	bHb	Gold electrode	DPV, EIS, SEM	CV sensor	[72]
Electropolymerization	bHb	Sacrificial silica microspheres, gold electrode	DPV, EIS, SEM	Electrochemical sensor	[69]
Electrochemical polymerization	ssDNA	ITO-coated glass substrate	CV, FTIR, and SEM	CV sensor	[79]
Electrochemical synthesis by potential pulses /potential cycling	Fish sperm dsDNA	PGE	AFM, CV	CV sensor	[80]
Electrochemical polymerization	Cyt c	Gold electrode	CV	Fundamental investigation	[73]
Electropolymerization	BSA	SPR sensor chip	SEM, SPR	SPR sensor	[71]
Cyclic voltammetric polymerization	Cardiac TnT	Gold electrode	AFM, CV	Fundamental investigation	[74]
Electropolymerization	ConA	Gold-coated QCM sensor chip	QCM	QCM sensor	[75]
Electropolymerization through micro-electrospotting	Ferritin	Gold SPR imaging chip	AFM, SPR	SPR sensor	[77]
Electropolymerization	Cancer marker CEA	Screen-printed electrode	Confocal microscopy, CV, FTIR, SWV, Raman	Biosensor	[76]
Electropolymerization	IgG	SAW chip	Ellipsometry, SAW	SAW sensor	[78]
Micro-contact imprinting	HEWL, TMV, and erythrocyte	QCM chip	AFM, QCM	Fundamental investigation	[90]
Micro-contact imprinting	C reactive protein (CRP)	Microscope cover glass	AFM, ELISA	Fundamental investigation	[83]

(*Continued*)

Table 3. Continued

Imprinting technique	Template	Substrate	Characterization	Application	References
Spin-coating, micro-contact imprinting	TMV, HRV serotypes HRV-1a, and HRV-16	QCM chip	QCM	QCM sensor	[26]
Micro-contact imprinting	Trypsin	QCM chip	AFM, QCM	QCM sensor	[25]
Micro-contact imprinting	HEWL, RNase A, and Myo	Microscope cover glass	AFM, ELISA, isothermal titration calorimetry (ITC)	Fundamental investigation	[86]
Micro-contact imprinting	RNase A	Microscope cover glass	AFM, ELISA, ITC	Fundamental investigation	[87]
Spin-coating, micro-contact imprinting	Insulin	QCM chip	AFM, QCM	QCM sensor for insulin	[122]
Micro-contact imprinting	Myo	Microscope cover glass	AFM, fluorescence/luminescence detection, FTIR	Fundamental investigation	[84]
Phase inversion micro-contact printing	BSA	Glass disc		Fundamental investigation	[85]
Micro-contact imprinting with protein silica beads	FN	Polyethylene	SEM, XPS	Fundamental investigation	[109]
Micro-contact imprinting	Ova	Microscope cover glass	AFM, ELISA, microcalorimetry, UV-spectroscopy	Fundamental investigation	[101]
Micro-contact imprinting	*Synechococcus* and *Synechocystis* cyanobacteria	Microfluidic chip	AFM	Fundamental investigation	[96]
Micro-contact imprinting	BSA	Glass microscope slide	Fluorescence microscopy	Fundamental investigation	[88]
Lithography, electropolymerization	Avidin	Gold-coated quartz crystal	AFM, epifluorescence microscopy, QCM	QCM sensor	[89]

EIS, electrochemical impedance spectroscopy; Ova, ovalbumin; PAD, pulsed amperometric detection; PGE, pencil graphite electrode; SCA, static contact angle; SWV, square wave voltammetry; TRITC,tetramethylrhodamine.

exhibited an obvious improvement in terms of adsorption capacity for BSA as compared with nonimprinted membranes. PP-*g*-PAM MIPs could recognize the template proteins of hen egg white lysozyme (HEWL), ovalbumin, bHb, and γ-globulin, whereby the selectivity factor (β) was above 2.0. The imprinting efficiency of PP-*g*-PAM MIP tended to be stable after three cycles and maintained 76% of the initial value of the imprinting efficiency after five repetitions.

3.1.4 Electropolymerization

The synthesis of surface molecularly imprinted electrospun affinity membranes with multimodal pore structures for efficient separation of proteins has also been reported [47]. In this approach, template molecules were immobilized onto the surface of electrospun fibers, and then collectively used as sacrificial templates to prepare surface imprinted nanofiber membranes with the fibers containing tubule channels. BSA and bHb were chosen as template molecules. Imprinted electrospun affinity membranes with multimodal pore structures were successfully fabricated. Surface molecularly imprinted fibrous membranes with bi-, tri-, or tetramodal pore structures have been fabricated in the absence or presence of silica nanoparticles in the molecular imprinting precursor. Compared with membranes with a bi- or trimodal pore structure, tetramodal pore membranes exhibited favorable recognition properties and efficient separations toward the target protein molecules in aqueous media due to the combination of efficient mass transport, high surface area, and good accessibility of target molecules to recognition sites.

3.2 Biomacromolecular imprinted thin films

Surface-imprinted MIP thin films can be prepared by a variety of methods such as in situ polymerization, grafting, drop-, dip-, or spin-coating procedures of the prepolymerization solution followed by polymerization, electrochemical polymerization (electropolymerization), lithography, including micro-contact printing whereby a macromolecular stamp (e.g. protein) is employed to create an imprint of the biomolecule in a polymer surface. One of the advantages of surface imprinting methods with thin films is the opportunity to better control-binding kinetics.

MIP thin films can be directly deposited onto the surface of transducers for the manufacture of biological sensors. Such integrations then allow various types of transductions, such as voltammetry, piezoelectric microgravimetry, capacitive impedimetry, fluorescence and chemiluminescence

spectroscopy, surface-plasmon resonance (SPR), or surface acoustic wave (SAW) sensing. One important aspect of MIP thin film-based sensors manufacture is the ability to control the surface area-to-volume ratio [48]. A high ratio will increase the sensitivity of a sensor. This can be achieved with a variety of different approaches, which include introducing porosity to the thin film using a porogen or a sacrificial substrate.

Some of the earliest reports of protein imprinted planar thin films have involved the coating of proteins, that were deposited on an atomically flat mica surface, with disaccharides onto which a plasma film was formed through a radio-frequency glow-discharge plasma deposition, covalently binding the disaccharides, which was subsequently glued to a solid support [49, 50]. The "sugar shell" around the protein molecule was thought to inhibit drying-induced or plasma-induced denaturation. The mica substrate was then peeled off and the protein removed leaving complimentary polysaccharide-like nanocavities, or nanopits, functionalized with hydroxyl groups, which were assumed to be appropriately positioned and orientated to maximize interaction with the protein template. However, only a slight increase in protein adsorption was observed for such imprinted surfaces compared to the nonimprinted mica control surfaces with a considerable extent of nonspecific protein adsorption apparent when crude protein preparations were employed. In contrast, the protein-imprinted surfaces displayed some preferential selectivity toward the template protein when subjected to competitive adsorption studies using a binary protein mixture.

3.2.1 Grafting and in situ polymerization

An elegant technique, which imparts a high level of control over the thickness of the film, is polymer grafting to or from a surface. Grafting involves immobilization of initiators on the surface followed by surface-initiated polymerization to generate the tethered polymer chains ("grafting from") or the attachment of an end-functionalized polymer by an appropriate chemical reaction ("grafting to").

A MIP thin film with an average thickness of 100 nm has been prepared [51] with 3-aminophenylboronic acid grafted onto the surface of polystyrene microtiter plates in the presence of microperoxidase, cytochrome c (Cyt c), HRP, lactoperoxidase, and hemoglobin HbA_0. However, complete template removal could not be achieved with small amounts (<10%) of these proteins remaining trapped within the polymer matrix. Even so, each MIP displayed selective template recognition compared to the NIP controls with most MIPs displaying dissociation constants in the nanomolar range, indicating strong polymer-template interactions. Notwithstanding this, the size, shape, and charge of the template proteins were observed to significantly influence the imprinting efficacy with the smaller template microperoxidase resulting in a relatively high dissociation constant (1.5 μM) reflecting weaker polymer–template interactions than observed for the larger protein templates.

A HEWL MIP film was grafted onto a SPR gold sensor chip by radical copolymerization with acrylic acid and MBAA for the label-free detection of HEWL in the presence of NaCl in the prepolymerization solution [52]. The highest selectivity and imprinting effect were observed when a rebinding buffer containing 20 mM NaCl was used.

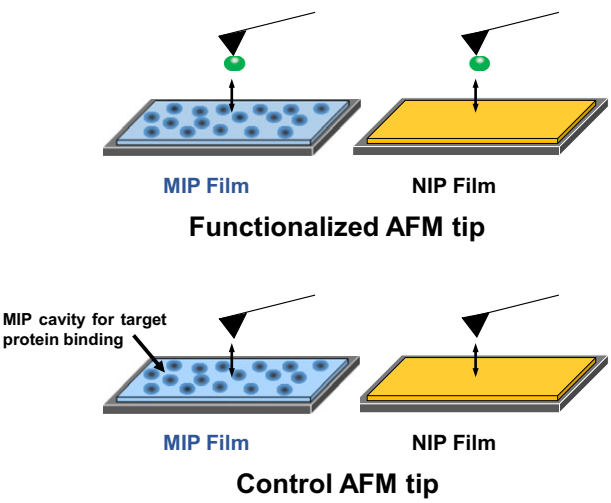
Polymer films composed of surface-bound, high-aspect ratio MIP nanofilaments, molecularly imprinted with myoglobin (Myo) were made using a porous alumina template whereby the template was immobilized by a glutaraldehyde linker [53]. This method led to a hierarchically, nanostructured material, capable of rebinding fluorescein isothiocyanate (FITC)-labeled Myo. Macroscopic contact angle measurements showed that the wetting properties of a particular surface, e.g. a nanostructured, hydrophobic, divinylbenzene-based surface or a more hydrophilic ethyleneglycol dimethacrylate-based surface could be modified through a change of the aspect ratio of the filaments.

Surface-bound MIP films with specific binding sites for Cyt *c* have been made with both, the 2D (surface imprinting using immobilized Cyt *c*) and 3D (bulk imprinted) approaches, using copolymers of acrylamide and different acrylic acid-based cross-linkers [54]. The binding specificity of the polymers was then investigated at the macroscale by equilibrium-binding experiments with fluorescein-labeled Cyt *c*. Imprinting factors of up to 4.1 were obtained, dependent on the cross-linker used and the degree of cross-linking. Atomic force microscopy (AFM) using cantilevers with covalently attached Cyt *c* molecules was then used to directly measure the force of interaction of the protein with the synthetic receptor sites obtained by molecular imprinting (see Fig. 2) as will be described in the section on techniques for the surface characterization of MIP thin films further below.

A RNase A MIP thin film synthesis on a gold-coated SPR sensor chip has been described using atom transfer radical polymerization (ATRP) of acrylic acid, acrylamide, and MBAA [55] as well as with conventional radical polymerization (RP) for comparison. These thin films had thicknesses of 20–60 nm. The selectivity of the MIP films was evaluated using Cyt *c*, Myo, and α-lactalbumin as reference proteins. The results from this study suggested that the parameters affecting the target protein binding by the MIPs were not just limited to the selection of reactant species (functional monomers, comonomers, and cross-linkers). These parameters also included polymer film thickness, an attribute that is controllable in activators generated by electron transfer ATRP by varying polymerization time and the density of the initiator on the substrates. For the MIP thin films produced by ATRP, the optimum film thickness determined experimentally was 15–30 nm.

3.2.2 Drop-, dip-, or spin-coating procedures and in situ polymerization

Drop-coating is a fast approach for MIP film formation. Planarity of the film can be achieved if the polymerization solution is dropped onto a silanized glass slide, immediately



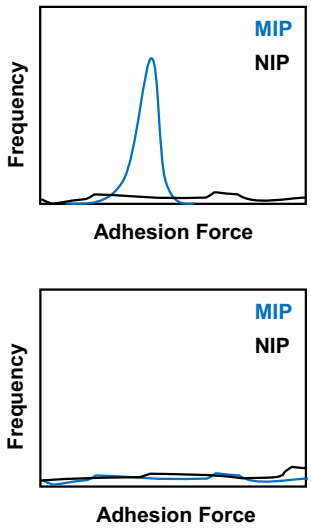


Figure 2. Schematic representation of the AFM characterization technique analogous to that employed by El Kirat et al. [54]. Interaction between the protein-functionalized AFM tip and the synthetic receptor site on the MIP film with respect to the nonimprinted control (NIP film) results in a distinct adhesion force peak in the force-distance curves.

covered with a coverslip that is then removed postpolymerization [56]. However, drop-coating of a preprepared polymer from solution can also result in nonhomogeneity of the MIP film thickness and film instability. This drawback can be overcome by procedures where a prepolymerization solution of a functional monomer–template complex is deposited by dip-coating or vertical deposition that involves slow, continuous vertical withdrawal of a solid substrate from a solution, or by spin-coating to form a thin film of defined thickness, followed by an in situ polymerization [57, 58]. Film thickness is an important consideration with respect to sensor performance, as response times and the sensitivity are inversely related to the film thickness. Spin-coating is a simple method that imparts a level of control over film thickness, wherein a monomer or polymer solution can be deposited onto a substrate material, such as a silicon wafer before spinning. Since the film thickness [59–61] and roughness [62] are inversely proportional to the spinning speed, this method provides a straightforward way to control many of the physical properties of the resultant thin film.

3.2.3 Electropolymerization

Electropolymerization of a prepolymerization complex of a template with electro-active functional monomers, in the absence of any cross-linker, is a convenient technique that allows thin polymeric films to be formed on the electrode or transducer surfaces, taking advantage of the properties of conducting polymers and inherently nonconductive proteins. Electrochemical MIP preparation is a green synthesis process, since it can be carried out without using any type of free radical formation agent. In the insulating acrylic or vinylic MIP films as recognition elements of electrochemical sensors, there is no direct path for electron transport between the MIP-recognition sites and the electrode/transducer. In contrast, MIP films prepared by electropolymerization of electro-active monomers like pyrrole or aniline are conducting and offer advantages in terms of film adherence to the transducer surface, control over the film thickness and ease of preparation [63]. The unique electronic properties of conducting polymers are the result of delocalized π-bonded electrons over the polymeric backbone. These polymers can be synthesized under mild conditions that is ideal for the embedding or immobilization of biomolecules into a polymer structure and can easily be deposited electrochemically onto substrates [64]. In such affinity sensors, the differences in capacitance and/or resistance arising in the electrochemical system during the interaction of analytes with the conducting polymer can then be converted into signals that are easily monitored [65]. The advantages of electropolymerization are the abilities to fine-tune the film thickness by controlling the charge consumed during polymerization by adjusting the time or the number of cycles and to grow the film directly at a precise area on the transducer surface [66]. Due to these features, electropolymers have become increasingly popular for the preparation of MIP films as sensing elements.

The use of cyclic voltammetry (CV), firstly to deposit or electropolymerize, either HEWL or bCyt *c* conductive MIP films upon screen-printed platinum electrodes, and subsequently as a detection method to determine the level of protein binding, has been reported [67]. To improve the response and sensitivity of the electrode, a thin polypyrrole (PPy) substrate layer was employed, involving two polymerized aminophenylboronic acid layers, the outermost of which was deposited either with the template (MIP) or without (NIP). As a result, the electrodes coated with either HEWL or bCyt *c* MIP films (or their respective NIP control films), upon interrogation with a 1 ppm protein template solution, resulted in a measureable decline in the conductivity (30.3 and 66.2% for the HEWL or bCyt *c* MIP films, respectively). The conductivity of electrodes coated with NIP films exhibited significantly less effect due to protein interaction (4.5 and 29.9% for HEWL or bCyt *c* NIP films, respectively).

One strategy for fabricating MIP films with high surface-area-to-volume ratios involves the use of a sacrificial substrate material with cylindrical pores. To generate a large

imprinted surface area on a polymer surface, the avidin template molecules were physically adsorbed onto the inner walls of the pores of sacrificial materials, such as alumina or a track-etch polycarbonate membrane (PCM) [68] that was positioned on the surface of a gold electrode. A conducting polymer of poly-3,4-ethylenedioxythiophene (PEDOT) doped with polystyrene sulfonate (PSS) was allowed to electrochemically grow inside these pores. This combination of template self-assembly and polymerization resulted in surface-imprinted nano-wire/nanotube arrays inside the porous sacrificial membrane. After confining the polymer nanorods to the nanopores, the removal of the sacrificial PCM with chloroform resulted in an array of PEDOT/PSS surface-imprinted polymer nanowires/nanotubes. Another approach using a sacrificial material was reported for the fabrication of a bisosensor for bHb [69]. Here, aldehyde group-functionalized SiO_2 microspheres were deposited onto a gold electrode, reacted with bHb, followed by electropolymerization of pyrrole into the gaps between the silica microspheres. The SiO_2 microspheres were then removed with HF etching and bHb extracted, resulting in a macroporous-structured MIP-based electrochemical sensor. Under the optimized conditions, this sensor showed fast rebinding dynamics and an excellent recognition capability for bHb.

Other fundamental investigations explored the electropolymerization of various proteins, including BSA [70,71], bHb [72], and Cyt *c* [73]. Other investigations moved away from model proteins closer to applications endpoints, e.g. that of clinical diagnostics. For example, Karimian et al. [74] fabricated a MIP sensor for cardiac troponin T (TnT), a highly sensitive cardiac biomarker for myocardial infarction. Cardiac TnT was used as template molecule for electrochemical polymerization of *o*-phenylenediamine (oPD). The MIP sensor was shown to have a high affinity to cardiac TnT in comparison with NIP electrodes in both buffer and blood serum.

A novel strategy to prepare a surface confined MIP film for protein sensing directly on a transducer surface has been reported that includes carbohydrate–carbohydrate binding protein interactions and the noncovalent interactions of the target within the MIP cavities [75]. The authors have synthesized a hybrid material for protein sensing by electrosynthesizing a MIP thin film on a mannose terminated self-assembled monolayer (SAM), containing the natural-binding partner of the target protein. Then the carbohydrate-binding protein, concanavalin A (ConA), was immobilized in an orientation-specific manner as a submonolayer on the underlying mannose modified surface. Afterwards, an ultrathin polyscopoletin film with the thickness comparable to that of the protein was electrodeposited on the top. The protein template was removed by digestion with proteinase K in combination with washing steps leaving the free imprinted sites confined to the film surface. The resulting functional material showed an approximately 20-fold higher affinity than that obtained from the mannose SAM. Recognition capability of the film was characterized by a real-time measurement using QCM. In comparison to the NIP film, the imprinted film revealed an 8.6 times higher binding capacity toward ConA. The high discrimination toward the homologs of the target protein confirmed the size and shape specificity of the imprint.

The fabrication of a screen-printed electrode produced by printed-circuit board (PCB) technology with a MIP thin film as sensing material and the application to cancer biomarker detection has been reported [76]. The selected biomarker was carcinogenic embryonic antigen (CEA) protein, routinely used to follow-up the progression of specific cancer diseases. The biomimetic material consisted of an imprinted matrix of PPy, assembled by electropolymerizing on the working electrode area, in the presence of CEA, followed by protein removal through proteolysis. A NIP film was prepared in parallel to assess the contribution of the imprinted sites to the overall analytical response. The analytical performance of the resulting device was evaluated by different electrochemical techniques, namely CV, electrochemical impedance spectroscopy, and square wave voltammetry techniques. The linear response of the normalized current density (in A cm^2) ranged from 0.05 to 1.25 pg/mL CEA concentration. Overall, producing screen-printed electrodes by means of conventional PCB technology showed promising features, mostly regarding cost and availability.

Micro-electrospotting as a new method for electrosynthesis of surface-imprinted polymer microarrays for protein recognition with gold SPR imaging chips has also been reported [77]. During electrospotting, both the gold chip and the spotting tip were electrically connected to a potentiostat as working and counter electrodes, respectively. The spotting pin enclosed the monomer-template protein solution that upon contacting the gold surface was in situ electropolymerized resulting in surface confined polymer spots of approximately 500 μm diameter. The removal and rebinding kinetics of the template and various potential interferences to such microarrays can be monitored in real-time and multiplexed manner by SPR imaging. The proof of principle for micro-electrospotting of electrically insulating surface-imprinted films was made by using scopoletin as monomer and ferritin as protein template. Micro-electrospotting in combination with SPR imaging was thus shown to offer a versatile platform for label-free and enhanced throughput optimization of the MIPs for protein recognition and for their analytical application.

In 2014, a MIP film interfaced with SAW technology as a sensing platform for label-free protein detection was developed [78]. Ultrathin polymeric films with surface imprints of IgG were fabricated onto the multiplexed SAW chips using an electrosynthesis approach. The films were characterized by analyzing the binding kinetics recorded by the SAW system. It was shown that the capability of IgG-MIP to specifically recognize the target protein was greatly influenced by the polymer film thickness that could be easily optimized by the amount of the electrical charge consumed during the electro-deposition. The thickness-optimized IgG-MIPs demonstrated imprinting factors toward IgG in the range of 2.8–4, while their recognition efficiencies were about 4 and 10 times lower

toward the interfering proteins, IgA and HSA, respectively. The IgG-MIP preserved its capability to recognize selectively the template after up to four regeneration cycles. The approach of integration of the protein-MIP sensing layer with SAW technology allowed observing the real-time binding events of the target protein at relevant sensitivity levels and can be potentially suitable for cost-effective fabrication of a biosensor for analysis of biological samples.

The high selectivity and affinity of MIPs for the imprint molecule have also been recognized as keys behind a promising approach to develop sequence-specific DNA biosensors. Recently, the synthesis of a sequence-specific molecularly imprinted amperometric biosensor for a single-stranded deoxyribonucleic acid (ssDNA) has been reported [79]. This was achieved by using a ssDNA related to the human prostate cancer susceptibility gene p53 as the template and oPD as the functional monomer. The DNA biosensor was fabricated by an electropolymerization process on an indium-tin oxide (ITO) coated glass substrate. The resulting ssDNA imprinted poly(*o*-phenylenediamine) (PoPD)/ITO electrode was characterized using FTIR spectroscopy, SEM, and CV. The amperometric responses, i.e. steady-state Δ current (Δi) as a function of the target ssDNA concentration, were studied. The biosensor using ssDNA imprinted PoPD/ITO as the working electrode showed a linear current response to the target ssDNA concentration within the range of 0.01–300 fM. The biosensor showed a sensitivity of 0.62 A/fM, with a response time of 14 s.

The manufacture of a MIP sensor for double-stranded deoxyribonucleic acid (dsDNA) has also been reported [80]. The MIP thin film was formed by electrochemical polymerization of pyrrole with template DNA on a pencil graphite electrode. Two electrochemical methods, CV and potential pulse sequences, were used for the polymerization. Since the N–H group of the pyrrole unit can form a hydrogen bond with the C=O groups of DNA nucleobases, the nano-cavities formed are geometrically and functionally complementary to the structure of the target DNA.

3.2.4 Micro-contact or stamp lithography imprinting

Micro-contact or stamp lithography imprinting is a versatile surface imprinting technique that is rapidly gaining popularity [81, 82]. Traditional micro-contact printing involves the preparation of a polydimethylsiloxane (PDMS) stamp on a patterned master by soft lithography methods. A concentrated solution of macromolecule/protein is then applied to the patterned PDMS stamp. The macromolecule/protein is allowed to nonspecifically adsorb to the surface, after which the stamp is pressed onto a substrate (such as a gold surface) and the macromolecule/protein transferred, resulting in a patterned printed protein surface. An adaptation of this technique is micro-contact imprinting, whereby a stamp containing the template molecule, such as a protein is pressed onto the surface of a glass or polymeric substrate containing a layer of functional monomer, imprinting mixture, or preformed polymer (Fig. 3). After polymerization, the stamp and template are removed leaving an impression of the template, with the appropriate complementary functionalities orientated such that selective interactions with the template molecule can occur upon reincubation with the template.

A similar micro-contact printing approach to elegantly engineer a MIP thin film with good affinity for the C-reactive protein (CRP) by using *O*-(4-nitrophenylphosphoryl)choline (4NPPC) as the functional monomer, a commercially available analog of the known ligand phosphorylcholine, thereby mimicking the naturally occurring binding interactions with CRP has also been reported [83]. The micro-contact imprinted films were formed between two glass surfaces. The CRP template, a pentameric protein with 206 amino acid residues, was first adhered to the surface of a glass cover slip by hydrophobic interactions. The functional 4NPPC monomer was

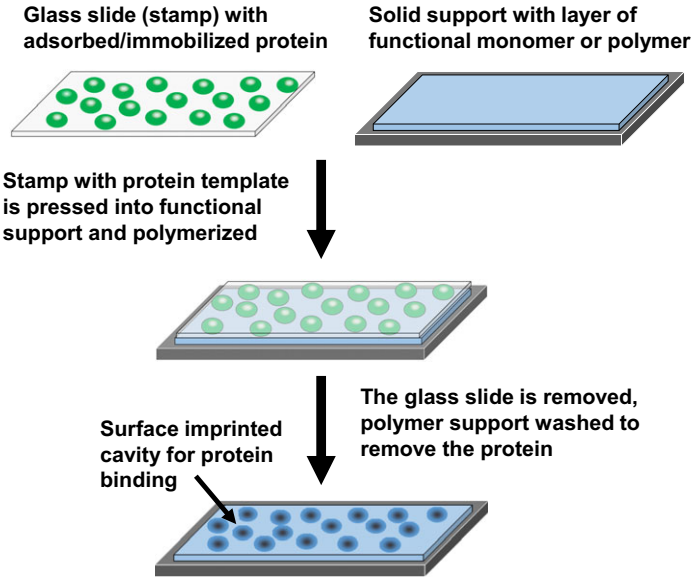


Figure 3. Diagram depicting a micro-contact method for the molecular imprinting of a protein template. After removal of the glass slide with the immobilized template proteins the imprinted polymer film contains surface cavities related to the protein.

then allowed to self-assemble with the protein monolayer before this cover slip being flipped upside down and contacted with a second glass support substrate that was functionalized with a mixture containing cross-linker and initiator. After UV polymerization, the cover slip was removed, and the polymer film on the glass support (approximately 10 μm in thickness) was washed at elevated temperature to remove the protein template. The CRP imprinted thin film was interrogated with CRP and extraneous control proteins HSA and HEWL.

The inclusion of HSA and HEWL, with smaller hydrodynamic molecular diameters of $\sigma = 6.4$ and 3.2 nm based on their partial-specific volumes respectively, in contrast to the $\sigma = 19$ nm of CRP, allowed the question to be resolved whether size exclusion was the determining factor in MIP protein recognition. The resultant MIP thin film displayed an affinity for CRP four times that of HSA and almost four hundred times that of HEWL. When examined under competitive-binding conditions with HSA, a significant reduction in the amount of CRP that could be rebound was observed, a finding interpreted as being due to protein–protein interactions rather than competitive exclusion [83]. The same research group developed in a similar manner a molecularly imprinted thin film using a micro-contact approach for the protein bovine myoglobin (bMyo) [84]. Since the bMyo template had no obvious natural ligand counterpart, a systematic screening approach was required for the cross-linker and functional monomer selection. This was achieved by examining the binding affinity of bMyo for a range of nonimprinted cross-linked polymer films to minimize nonspecific binding. The cross-linker that exhibited the lowest affinity was then used for the bMyo MIP thin film preparation with an assortment of functional monomers. Subsequent examination of these MIP films under noncompetitive conditions revealed that the functional monomer methyl methacrylate (MMA) resulted in a thin film that displayed higher selectivity for the template bMyo with negligible selectivity for bHb, HSA, and IgG proteins. Under competitive conditions using a mixture of two proteins, the bMyo-imprinted thin film exhibited a small reduction in binding affinity and selectivity for bMyo when competing with HSA and IgG, while the competition with bHb resulted in an approximate threefold reduction in both affinity and selectivity toward bMyo. Importantly however, the bMyo-imprinted thin film was able to bind bMyo from (spiked) "realistic natural matrices" of human serum and urine with imprinting factors ranging from 3.15 to 4.77 for serum diluted with phosphate buffer and 37.4 for urine, the latter finding due to extremely low nonspecific binding to the NIP material [84].

The use of a phase inversion micro-contact printing technique has been reported for the production of a surface imprinted BSA thin film [85]. In this case, BSA was immobilized onto a glass disc, onto which was cast a solution of EVAL in DMSO (EVAL/DMSO 25 wt%), which was subsequently solidified by a phase inversion procedure. The immobilized proteins were thus trapped at the interface of the glass surface and the polymer thin film. Removal of the glass disc and the protein template afforded an imprinted thin film of approximately 10–20 μm thickness, possessing BSA moulded, surface-located cavities. Avoidance of the use of a functional monomer was influenced by the possibility that strong binding interactions between monomer and template could hinder template removal. This choice also negated the need to use an initiator, thereby reducing the risk of sample contamination from both monomeric and initiator reactants. Examination of the BSA binding to imprinted and NIP films containing 27, 32, 38, and 44 mol % EVAL revealed that the use of 32 mol % EVAL resulted in maximum imprinting effectiveness with an imprinting factor of 6.8 that was over threefold greater than other preparations. The imprinting factor of 6.8 was comparable to those observed by the same research group in associated studies with MIP films templated with HEWL (6.06) [86] and RNase A (6.15) [87].

A surface imprinted thin polymer film systems for the selective recognition of BSA has been also prepared by micro-contact surface imprinting procedures by UV free-RP based on 2-(dimethylamino)ethyl methacrylate as the functional monomer and varying amounts of either MBAA or poly(ethylene glycol) (400) dimethacrylate (PEG400DMA) as the cross-linking agent [88].

A versatile approach for the synthesis of surface-imprinted polymers for selective protein recognition was proposed that combines nanosphere lithography and electropolymerization [89]. A layer of 750 nm diameter latex bead-avidin conjugate was deposited onto the surface of gold-coated quartz crystals followed by the electrosynthesis of a PEDOT/PSS film with thicknesses of the order of the bead radius. In contrast to free protein imprinting, the conjugation of the target protein to a solid surface makes possible its oriented immobilization. The removal of the polymer bead-protein conjugates, facilitated by using a cleavable protein-nanosphere linkage was shown to result in 2D arrays of periodic complementary sized wells. It was shown that selective recognition sites for avidin were generated on the surface of the PEDOT/PSS film. The binding capacity of such surface-imprinted polymer films was approximately 6.5 times higher than that of films imprinted with unmodified beads. This methodology, if coupled with properly oriented conjugation of the macromolecular template to the NPs, offers the possibility of site-directed imprinting.

Surface biomacromolecular imprinting by stamp lithographic techniques has not been limited to protein templates. Hayden et al. [26, 90, 91] and [92] have extended the lithographic molecular imprinting technique, pioneered by Alexander and Vulfson a decade earlier [93], to include the detection of cells and viruses in addition to protein complexes. The stamp lithographic bioimprinting technique has been successfully applied to include imprinted thin films for *Saccharomyces cerevisiae* cells [91, 94, 95], red blood cells [90], and the tobacco mosaic virus (TMV) [26, 90, 91]. In one example, a remarkable selectivity between two human rhinovirus (HRV) serotypes (HRV-1a and HRV-16) was demonstrated, indicating that selectivity is influenced not only by shape but also by the chemical functionality of the virus surface [26]. Simi-

larly, cyanobacteria cells were used as supra-macromolecular templates for the preparation of imprinted PDMS thin films that could discriminate between different strains of cyanobacteria when incorporated into microfluidic chips [96]. Cellular discrimination was further enhanced, firstly by controlling the orientation of the cell imprints such that they were parallel to the flow, and secondly by modulation of the pH to protonate the functional groups involved in recognition on both the cells surface and within the MIP cavities.

These micro-contact imprinting or stamping techniques provide the opportunity for organic solvent-free preparation of synthetic protein receptor sites, thereby increasing the probability that the imprinting will preserve the protein template in its native state. Other advantages of this procedure include (i) the use of a minimum amount of template, which is often very expensive, (ii) the formation of an homogenous monolayer, thereby negating diffusion related problems and permitting complete removal of the template protein [83], and (iii) the ability to generate patterned MIP surfaces for a variety of applications including biosensors for real-time diagnostics and lab-on-a-chip applications.

3.3 Techniques for the surface characterization of MIP thin films

The physical, chemical, and functional characterization of MIP bulk materials, particles, beads, and monoliths usually involved a variety of analytical techniques. These methods include SEM, particle size analysis, surface area, and pore size determination, NMR spectroscopy, IR spectroscopy, thermogravimetric analysis, differential scanning calorimetry, and LC–MS/MS analysis. For the functional characterization of MIP membranes and thin films a suite of different surface characterization methods can also be employed [97]. These methods include static contact angle measurements and X-ray photoelectron spectroscopy (XPS).

The evaluation of the performance of MIP-mediated surface biomacromolecular recognition materials has involved a diverse range of analytical methods. These have included techniques that are typically employed during protein purification and analysis such as SDS-PAGE [33, 98–100], ELISA [83, 87, 101], MALDI-TOF MS [33], optical spectroscopy [99, 100, 102, 103], and HPLC [104–106]. In addition, other analytical techniques have included those commonly associated with the characterization of polymer membranes and thin films and surfaces such as luminescence and fluorescence detection, FTIR, surface-enhanced Raman spectroscopy (SERS), AFM, QCM analysis, cyclic voltammetry (CV), and SPR spectroscopy. Several of these surface characterization methods, QCM, CV, and SPR analysis can be directly used for sensing, e.g. using capacitance-voltage measurements, conductimetric sensors, amperometric sensors, piezoelectric quartz sensors, electrochemical sensors, SPR sensors, and acoustic wave sensors (see Tables 1 and 3).

3.3.1 Luminescence and fluorescence detection

Luminescence and fluorescence detection has been used for examining a variety of proteins bound to MIP thin films including avidin [68], BSA [37,88], Cyt *c* [54] FITC-albumin, and tetramethylrhodamine-HIV-1 antibody [38], lectin [107], and Myo [53]. For example, Lin et al. [84] have produced a MIP film with a micro-contact imprinting method and evaluated the binding behavior of bMyo with a bMyo imprinted thin film by the detection of a luminescent secondary antibody bound to a primary anti-Myo antibody. The polymer selectivity was further evaluated using IgG and HSA, both of which were fluorescence labelled and Hb, which was in turn quantified using a fluorescence labeled anti-Hb antibody. These results confirmed a strong preference of the imprinted film for bMyo. Similarly, fluorescence detection was utilized to quantify the amount of FITC-labeled albumin (FITC-albumin) to an imprinted poly(methyl methacrylate) (PMMA) patterned scaffold [107]. The scaffold was incubated with the fluorescent protein, washed, and the fluorescence observed using fluorescence microscopy. The protein concentration was then determined by correlating the fluorescence intensity of the image with a linear calibration curve. Using this technique, it was observed that the imprinted PMMA scaffolds were able to bind up to approximately 110 fg/mm^2 of albumin [107].

3.3.2 X-ray photoelectron spectroscopy

XPS is a quantitative spectroscopic technique that allows the elemental composition of a surface to be determined. XPS (also known as electron spectroscopy for chemical analysis) uses X-rays to eject electrons from the inner-shell orbitals of atoms and can be used to detect the chemical composition of chemical surfaces within a 5–10 nm depth.

XPS has been used for the characterization of various proteins bound to MIP thin films such as avidin [108], BSA [37], and fibronectin (FN) [109]. For example, XPS was applied to detect the specific adsorption of histidine-tagged proteins onto silica surfaces modified with Ni^{2+}:NTA-derivatized poly(ethylene glycol) (PEG) [110]. Similarly, XPS methods have been applied to characterize the binding affinity of avidin to polyacrylic acid (PAA) brushes by correlating the number and distribution of observed carbon atoms with the expected distribution of carbon atoms in avidin and the PAA surface [108]. XPS has also been applied on numerous occasions for the surface characterization of MIP films wherein the changes in surface chemistry composition can be monitored during MIP film preparation such as reported by [109].

3.3.3 FTIR spectroscopy

FTIR spectroscopy has been routinely employed as a characterization technique with MIP-imprinted films [35,70,76,79] and membranes [44,46], including examples whereby the surface functionality introduced during both the polymerization process and in the final imprinted polymer were examined by FTIR. Less common, however, has been the application

of FTIR as a MIP detection technique with examples including the detection the biomacromolecule such as bMyo [84]. In this latter case, bMyo imprinted and NIP thin films were prepared on glass cover slip substrates using a micro-contact imprinting approach after which various techniques including FTIR were employed to detect the surface and cavity bound bMyo [84]. Micro-contact imprints were formed between the two glass surfaces with the protein adhered to one cover slip and the monomers onto the other, thereby allowing site-specific reorganization of the functional monomer with the template molecule. Examination of the MIP thin film revealed an absorbance band at 1627 cm^{-1}, which was assigned to amide N–H bending and was used for identification of the bMyo on the polymer surfaces. This band was visible on the MIP surface before and after rebinding of the template molecule, with the later having significantly greater intensity. The presence of this band before rebinding of the template molecule was attributed to residual template trapped within the polymer matrix, an observation consistent with the AFM image. This band was absent with the corresponding NIP surface, with the results in good agreement with AFM images, thus demonstrating the applicability of FTIR spectroscopy as a useful tool for analyte sensing and investigation of the binding mechanism in MIPs.

3.3.4 Surface-enhanced Raman spectroscopy

As Raman spectroscopy is an analytical tool capable of providing a structural "fingerprint" of a molecule by probing its vibrational behavior, this technique has been extensively employed in the study of polymerization processes and polymer characterization [111, 112]. SERS is a powerful technique for the sensitive and selective detection of low-concentration analytes. It combines modern laser spectroscopy with the optical properties of metallic nanostructures, resulting in strongly increased Raman signals when molecules are attached to nanometre-sized gold or silver structures.

A major challenge in the application of SERS to the detection of selectively adsorbed substances arises from the necessity to combine the selective MIP film layer with a SERS-active metal surface. An early use of SERS as a means of MIP characterization was reported by [113] with an *N*-benzyloxycarbonyl-L-aspartic acid imprinted thin film prepared on gold and silver surfaces. SERS bands associated with the template molecule could be unequivocally assigned and used to quantify the amount of absorbed template molecule. However, the stability of the polymer layers on these SERS surfaces was problematic. In some cases, to achieve better attachment of the polymer films, the metal surfaces (SERS-carriers) were modified by immersion in 10 mM ethanolic solution of cysteamine. This additional layer of cysteamine on the gold layer improved the stability of the polymer film but also led to weaker Raman bands. The loss of Raman intensity was also associated with the loss of SERS enhancement with increasing thickness of the supporting layer.

Recently, a molecularly imprinted plasmonic nanosensor has been fabricated for selective SERS detection of protein biomarkers [114]. The plasmonic nanosensor was prepared as an ultrathin MIP layer on the surface of gold nanorods imprinted with the target protein. This nanosensor enabled selective capture of the target protein biomarker from human serum. A sensitive SERS detection of the protein biomarkers with a strong Raman enhancement was achieved by formation of protein imprinted gold nanorods aggregates, stacking of protein imprinted gold nanorods onto a glass plate, or self-assembly of protein-imprinted gold nanorods into close-packed arrays. High specificity and sensitivity of this method were demonstrated with a detection limit of at least 10–8 mol/L for the target protein. This could provide a promising alternative for the currently used immunoassays and fluorescence detection, and offer a very sensitive, nondestructive, and label-free technique for clinical diagnosis applications.

3.3.5 Atomic force microscopy characterization and detection

AFM is a high-resolution type of scanning probe microscopy, which allows probing surfaces with a sharp tip on the free-swinging end of a cantilever in either the contact, taping or noncontact mode. Through cantilever deflection-measurements, topographic images of a surface can be constructed. AFM, which has the capacity for molecular and submolecular resolution and in situ imaging capability, is a versatile tool that can be used to quantify and visualize surface chemical properties, hydrophobicity, and local mechanical properties of surfaces. It can also probe the structure, properties, and functions of a biological specimen under physiological conditions with unprecedented nanometer resolution [115].

AFM has been employed to pattern and subsequently study the topography of a thiol-functionalized nanoscale patterned surfaces under noncovalent protein immobilization conditions [116]. Similarly, the topography of a polystyrene-*block*-poly(methyl methacrylate) (PS-*b*-PMMA) surface was studied using AFM, thereby revealing that protein immobilization occurred at the PS regions [117]. Similarly, AFM has been used to detect surface immobilized DNA structures [118–120]. With respect to its potential as a surface characterization and imaging tool evaluation tool, AFM has predominantly been applied for the characterization of the differences between nonimprinted and imprinted surfaces, both before and after template extraction. The morphology of a CRP MIP film prepared by a micro-contact approach was examined before and after template extraction using AFM imaging [83]. The image before extraction revealed little in the way of surface features, while postextraction the image clearly demonstrated the presence of pores with a diameter of 20 nm, almost identical to that of CRP (19.6 nm).

A qualitative examination of a bMyo imprinted thin film was conducted before and after template removal in addition to postrebinding to both the MIP and NIP surfaces [84]. Before template removal several protein aggregates were clearly visible on the MIP surface, most of which were removed during the subsequent washing procedure. After rebinding, the

MIP surface closely resembled that of the initial surface with many protein aggregates visible, while the control NIP surface displayed no protein adhered to the surface at all [84]. AFM was employed to examine the surface of HEWL imprinted, TMV imprinted, and *Saccharomyces cerevisiae* (yeast) imprinted polymer films, the latter of which displayed a qualitative correlation between the strength of cell attachment to the MIP and the intensity of the AFM images [25, 26].

AFM methods have also been used to directly measure the magnitude of the interaction forces with remarkable sensitivity and positional precision thereby enabling the recognition of surface bound proteins. An elegant AFM characterization technique for bCyt *c* (13 kDa) imprinted thin films using a biofunctionalized AFM tip has been reported [54]. Imprinted films, both 2D (surface imprinted using immobilized Cyt *c*) and 3-D (bulk imprinted), were investigated initially by traditional AFM followed by AFM using bCyt *c* functionalized cantilevers (Fig. 3). Initial AFM analysis revealed the surface topography of the 2D MIP film was complimentary to that of a mica surface containing immobilized template, whereas the 3D MIP and NIP films displayed smooth surfaces without distinguishable cavities. Subsequent analysis using either bCyt *c* functionalized AFM cantilever tips or control cantilevers tips containing the SAM without the protein, enabled direct determination of the interaction force between the protein and the artificial receptor by recording the cantilever deflection as the AFM tip was pushed toward or retracted from the sample surface. The interactive (unbinding) force between the Cyt *c* tip and the MIP films obtained from the force-distance curves was found to be 85 ± 15 pN (2D MIP) and 95 ± 15 pN (3D MIP), values typical of natural ligand–receptor interactions [54]. When investigated with an unmodified control tip, nonspecific forces up to 450 pN were observed at significantly reduced frequencies and without a defined adhesion peak. Further examples of the use of AFM in the surface characterization are provided in Tables 1 and 3.

3.3.6 Quartz crystal microbalance analysis

The QCM technique allows measuring mass variations per unit area under vacuum, in the gas phase or in liquid systems, through the change in frequency of a quartz crystal resonator, taking advantage of the piezoelectric effect [121]. The deposition or coating of a MIP layer onto a QCM chip produces a MIP sensor that generates a frequency measurement, resulting from a mass change upon analyte binding.

A trypsin imprinted thin films was directly deposited onto the surface of a QCM transducer by either a stamp lithography process or by in situ polymerization of a MIP solution deposited directly onto the electrode surface with spin- or drop-coating [25]. The solution imprinted thin film produced a strong sensor response, enabling the detection of a 100 ng/mL trypsin solution with a 3:1 S/N. The stronger response for the sensor prepared by in situ polymerization was attributed to the probability that a greater number of binding sites were created compared to sensors prepared using the stamping technique. A possible reason for this outcome can be attributed to the more complete engulfing of the trypsin protein by the polymer, whereas the crystal stamp imprinted film can result in only partial engulfing of the trypsin molecule leading to a reduction in interaction energy and thus lower sensitivity. The effects of protein denaturation on recognition was also examined by conducting binding assays employing both native and denatured trypsin, with a fivefold decrease in sensitivity observed for the denatured protein.

HEWL and bCyt *c* imprinted thin films have been investigated, both individually and coimprinted, prepared directly on QCM electrode surfaces [57]. Initial studies into the coated QCM electrodes revealed fast sensor equilibration times, typically less than 3 min, and the capability for reuse due to the relative ease with which the bound protein could be removed. The HEWL-imprinted film exhibited a typical saturation curve profile with the QCM responses directly proportional to HEWL concentration until saturation was reached. However, the bCyt *c*-imprinted film resulted in more erratic QCM responses, likely due to dominant nonspecific interactions. Interestingly, it was observed that upon interrogation of the coimprinted film using only individual proteins, negligible recognition was exhibited. When interrogated with a solution containing a mixture of the template proteins in the same ratio as employed for imprinting, recognition of the protein–protein pair was observed.

Since then, QCM sensors have been produces for a variety of different proteins, including the anthrax protective antigen [34, 35], avidin [89], ConA [75], dengue virus protein [32], insulin [122], and trypsin [25]. Other applications of QCM detection using electrodes coated with biomacromolecular surface imprinted thin films have included the recognition of whole cells such as *S. cerevisiae* and TMV [90, 95]. The use of MIP-coated QCM transducers for detection is a highly sensitive and relatively simple technique to employ, however, selective analyte detection from a complex matrix or crude sample may be problematic, requiring suitable sample pretreatment or separation before analysis.

3.3.7 Cyclic voltammetry

Electrochemical techniques have also been employed to monitor biomacromolecular-binding events with MIP thin films. Rick and Chou [67] have reported the use of cyclic voltammetry (CV) as a detection method to determine the level of protein binding. CV sensors have been produced for a variety of different proteins, including BSA [70], bCyt *c* [67], bHb [69, 72], HEWL [67], as well as ssDNA [79] and dsDNA [80].

3.3.8 SPR spectroscopy

SPR is the resonant oscillation of conduction electrons at the interface between a material of negative and positive permittivity in response to incident light. SPR spectroscopy can be used to detect a mass change at the surface of a functionalized metal substrate following ligand–affinant interaction and can generate both equilibrium-binding data and

measurements of the kinetics of interactions. Protein immobilization by molecular imprinting on a sensor surface can be monitored by SPR with the response unit (RU) directly proportional to the change in surface mass. With laser-based SPR detectors, 1 RU is approximately equivalent to 1 pg mm^{-2}.

SPR was used to evaluate protein-binding ability, more specifically, BSA affinity, to a metal transducer surface coated with a thioacetal containing reactive polymer layer [123]. A SPR sensor for HEWL based on molecularly imprinted thin films was prepared for which equilibrium binding was attained after as little as 2 min and reliable measurements could be made after only 10 s [52]. The SPR MIP sensors were regenerated by washing with NaCl for 60 s thus making them a useful detection tool in the arsenal of MIP-based sensor technology.

4 Conclusions

As documented in this review, the field of biomacromolecular imprinted materials in the form of membranes and thin films has advanced considerably in the last decade. The main types of biological macromolecules that have been used as targets were proteins as well as single- or double-stranded DNA. In addition, molecular assemblies like viruses and cells have also been imprinted.

Two principal imprinting strategies, the "epitope" approach (using a synthetic template, usually a small fragment of a biomacromolecule), or "whole molecule" approach (using the entire biomacromolecule), have been pursued, each with its own merits. The 3D molecular imprinting methods so successfully employed for the imprinting of molecular targets of low mass were demonstrated to have their limitations for the preparation of polymeric materials using biomacromolecular targets due to their large size, structural complexity, and conformational flexibility. The development of alternative approaches, based on 2D or surface imprinting, where the imprint molecule is confined to a monolayer, has provided a range of solutions to some of these challenges, including overcoming some of the difficulties experienced with binding site accessibility and trapping of these large targets within the polymeric network. In 2D imprinting, an increase of surface area was shown to be achievable for membranes or thin films, in particular with introduced porosity or sacrificial substrates.

For the synthesis of molecularly imprinted polymer membranes or thin films a variety of synthesis procedures have been employed. Molecularly imprinted membranes can be made either by following a parallel approach, in which molecular imprinting and formation of the membrane are performed simultaneously for instance in phase-inversion polymerization or alternatively, by a sequential approach, were the imprinted polymeric material forms a thin membrane on a preformed porous nonimprinted structure, involving in situ polymerization, surface grafting, or electropolymerization. The application end-points of MIP membranes were mainly affinity-based filtration, separation as well as sensing. Molecularly imprinted thin films were formed either directly on the indented planar substrate (e.g. silicon wafer, mica, sensor chips), using a suite of synthesis procedures including grafting/in situ polymerization, drop-, dip-, or spin-coating procedures, electropolymerization or by micro-contact or stamp lithography imprinting procedures. A combination of procedures, e.g. grafting of a target biomacromolecule to a substrate and spin-coating of the prepolymerization mixture or grafting of the target biomacromolecule to a substrate that is then used as a stamp micro-contact printing, can result in surface imprinting thin films of defined film thickness. Because of their versatility, MIP thin films can be readily adapted to biomacromolecule sensing and have found applications as biomimetic receptors exhibiting performance akin to or exceeding their natural counterparts.

Numerous highly sensitive techniques for analyte detection can be employed, encompassing luminescence and fluorescence spectroscopy, X-ray photoelectron spectroscopy, FTIR spectroscopy, surface-enhanced Raman spectroscopy, atomic force microscopy, quartz crystal microbalance analysis, cyclic voltammetry, and SPR.

In the future, it can be confidently expected that MIP membranes will find many applications and solve challenging separation problems in the area of analytical or possibly preparative chromatography of biomacromolecules, due to their ability to interact specifically with the analytes in combination with membranes of high permeability allowing high-throughput filtrations. The application of catalytically active MIP membranes as tools for multistage syntheses is also conceivable. The advances MIP thin films also offer new opportunities to develop low cost, miniaturized lab-on-a-chip, bioelectronic sensors, or point of care diagnostic applications, which are anticipated to find their way into commercially relevant products. These developments are also expected to aid the exploration of complex phenomena associated with the origins of biorecognition.

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