

## Electrosynthesized molecularly imprinted polymers for protein recognition

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*Dedicated to the memory of Professor Marco Mascini.*

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## **Abstract**

Molecularly imprinted polymers (MIPs) for the recognition of proteins are expected to possess high affinity through the establishment of multiple interactions between the polymer matrix and the large number of functional groups of the target. However, while highly affine recognition sites need building blocks rich in complementary functionalities to their target, such units are likely to generate high levels of non-specific binding. This paradox, that nature solved by evolution for biological receptors, needs to be addressed by the implementation of new concepts in molecular imprinting of proteins. Additionally, the structural variability, large size and incompatibility with a range of monomers made the development of protein MIPs to take a slow start. While the majority of MIP preparation methods are variants of chemical polymerization, the polymerization of electroactive functional monomers emerged as a particularly advantageous approach for chemical sensing application. Electropolymerization can be performed from aqueous solutions to preserve the natural conformation of the protein templates, with high spatial resolution and electrochemical control of the polymerization process. This review compiles the latest results, identifying major trends and providing an outlook on the perspectives of electrosynthesised protein-imprinted MIPs for chemical sensing.

**Keywords:** molecularly imprinted polymers; proteins; surface imprinting; electropolymerization

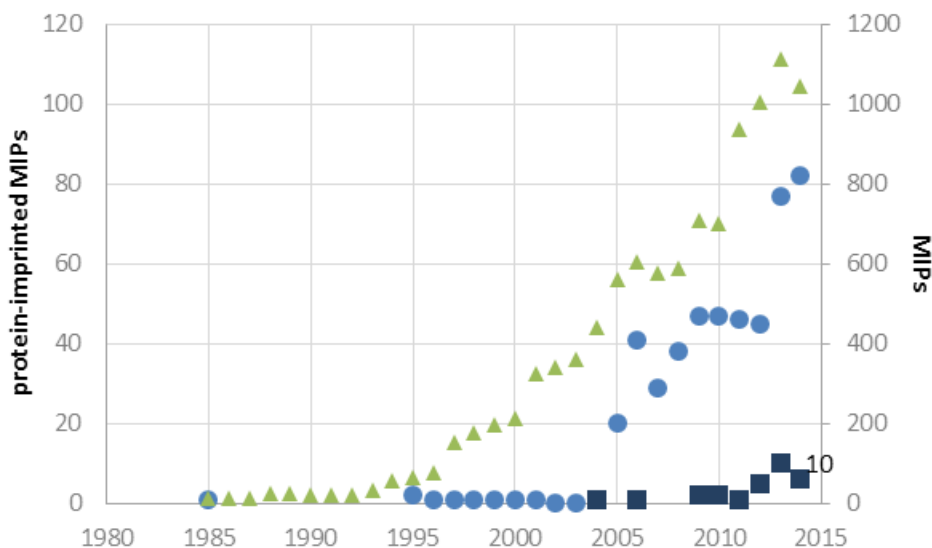
## 1. Introduction

Molecular imprinting is a universal concept to generate materials with “molecular memory” by performing a polymerization of suitable functional monomers in the presence of a target molecule acting as a template. The subsequent removal of the template creates recognition sites in the molecularly imprinted polymer (MIP) that can, further on, selectively rebind the target. Whilst nature has an arsenal of 20 amino acids, MIPs are typically prepared from one to four monomers. Nevertheless, this concept proved to be successful in preparing selective sorbents for compounds of small molecular weight (~200-1200 Da). However, one should confront several specific problems if the target is a macromolecular protein [1] as the classical bulk methodologies worked out for small molecular weight compounds generally fail to address the peculiarities of protein targets. The difficulties are largely attributed to the intrinsic properties of the proteins as detailed below.

- Due to their fragility irreversible conformational changes may occur during polymerization [2] and the rebinding of the native conformation to such imprinted sites is not favored.
- The large size of the proteins makes them difficult to remove from, or rebind to a highly cross-linked 3D polymeric network traditionally used in small molecule imprinting, i.e., during imprinting the macromolecules may become irreversibly entrapped in the polymeric material.
- The large number of potential interaction sites on the surface of proteins may lead to cross-reactivity of the imprinted polymers and nonspecific adsorption onto bulk polymeric material.

All these difficulties caused a considerable lag in the development of protein imprinted MIPs compared to small molecule imprinting. Thus while the first research paper on a protein-imprinted MIP appeared already in 1985 [3] a steady increase in the publications can only be observed since 2005 (Figure 1). However, even in 2014 less than 1 % of the papers published on MIPs involved protein targets. Additionally, the range of implemented protein targets is very narrow and rather restricted to templates having properties that facilitates the imprinting process. In this respect proteins with good conformational stability, and distinct physical-chemical properties (e.g. high isoelectric point, and glycosylation) were generally preferred. Such properties facilitates the formation of strong and/or selective interactions such as electrostatic interaction between the positively charged proteins (e.g., lysozyme,[4] avidin[5]) and negatively charged polymers as well as between glycan moieties and aminophenyl boronic acid (APBA) monomers [6]. This is a strong indication that the field of

macromolecular imprinting is still very much at the proof of concept level and an enhancement in selectivity and affinity is required.



**Figure 1.** Number of publications on all (circles) and electrosynthesized (squares) protein-imprinted polymers and on all MIPs (triangles, right axis), until the end of 2014 (ref. [www.mipdatabase.com](http://www.mipdatabase.com))

Despite of the difficulties, imprinting methodologies custom tailored for protein targets are worthwhile to pursue beyond the inherent importance of protein analysis for several reasons.

- The prospects of preparing “plastic antibodies” are in general better for proteins than for small molecular weight targets as proteins are expected to generate higher affinity MIPs through the establishment of multiple interactions with their large number of functional groups.
- Molecularly imprinted polymers are expected to outperform their biological counterparts in terms of robustness and shelf-life.
- MIPs are more robust to environmental conditions and cost effective as compared to antibodies and such their application in affinity assays is resourceful.

### *1.1. Main concepts to overcome the difficulties of imprinting protein targets*

During the past decade different strategies have been introduced to overcome the barriers of protein imprinting and these were reviewed in several monographies [1, 7-20]. To avoid denaturation of protein templates polymerization in aqueous media using water soluble monomers and initiators was an evident

choice, although there have been some concerns about the deteriorating role of water on H-bonding and dipole-dipole interactions between the functional monomers and the template.

The problem of the restricted diffusion of the bulky macromolecular template in the highly cross-linked polymer network initiated fundamentally different strategies. The first approach, using lightly cross-linked hydrogels similar to the ones used in gel electrophoresis, was initiated by Hjertén and his coworkers [21, 22]. Though not many direct evidences, the formation of highly permeable polymer structures or gels seem to not fulfil expectations with respect to the quality and stability of the imprints owing to excessive chain flexibility [20]. Another important approach is the “*epitope imprinting*”, whereby a small, representative peptide sequence is used as the template instead of the native protein. This approach enables the implementation of non-aqueous polymerization media and complete template removal [23, 24]. However, the rebinding of the targeted, much larger native protein may be still hindered in the MIP monolith. Therefore *surface imprinting* emerged as the main strategy for macromolecular imprinting. This approach restricts the formation of imprinted binding sites to the surface of a polymer or to a very thin polymer layer the thickness of which is comparable to the size of the protein template. In extreme cases proteins are captured by 2D monomolecular layers of suitable functional monomers anchored to an existing surface [25, 26]. To maximize the binding capacity of the respective MIPs, generation of polymeric nanostructures with high area/volume ratio comes as a natural necessity to take full advantage of the surface imprinted sites. Creative methods have been devised to confine the templated sites exclusively to the polymer surface such as micro-contact imprinting techniques [4, 27-30] and sacrificial template support methods [5, 31]. Imprinted soluble nanogels, having dimensions comparable to that of protein clusters were also shown to allow facile template exchange between the polymer and the solution [32]. The so-called solid-phase synthesis approach made feasible the reproducible, controlled and large scale production of such imprinted nanogels [33-35]. Interfacial polymerization on planar surfaces or supporting beads [36-38] can create ultra-thin polymer films that only partially bury the protein template without completely encapsulating it. Adherent MIP films can be polymerized *in-situ* by free-radical polymerization for example by spin coating [39] or drop-casting the pre-polymerization mixture onto the substrate and inducing polymerization [40]. Better control over the thickness of the sensing layer can be achieved with surface confined polymerization methods, where either the initiator [41, 42] or a pendant polymerizable group [43] is attached to the surface. Not only the thickness, but the area of the polymer film can be kept very small by the spatial confinement of the initiation process using light [44] or heat [45] supplied by a laser.

## 2. Electrosynthesis of protein-imprinted MIPs

While the great majority of MIP preparation methods are variants of chemical polymerization, the electropolymerization of functional monomers emerged as particularly attractive approach to prepare MIPs for chemical sensing applications [46, 47]. This is mainly due to the effortless *in-situ* spatial confinement of polymer layers by electropolymerization onto the surface of a suitable electrode resulting in a proliferation of chemically-modified electrodes and electrochemical sensors that utilizes MIPs to confer selectivity [48, 49]. Moreover, there are a number of other well-established transducers compatible with *in situ* electrosynthesis of MIPs such as quartz crystal resonators, surface plasmon resonance and acoustic wave sensors. Additionally, electrosynthesis is especially well-suited for the synthesis of protein-imprinted MIPs. Most of the electropolymerizable monomers can be deposited from aqueous solutions where protein molecules preserve their natural conformation. Buffers that enhance the stability of proteins can serve as supporting electrolytes during the electrochemical polymerization process. Compared to free radical polymerization initiated generally either thermally or by UV light, which may cause structural changes in the protein template, [50] electropolymerization most often requires no external initiator. The lack of initiators is also a major advantage with respect to chemically initiated polymerization. For instance the commonly used persulfate initiators, that are strong oxidizing agents, may alter the structure of proteins that are susceptible to oxidation [51]. The precise control over electrosynthesis enables the fine tuning of the polymer layer thickness, which is particularly important for the surface imprinting of proteins. Electrosynthesis can be used straightforwardly to create several nanometer thick polymer films [52, 53] and with the aid of sacrificial materials, micro and nanostructures with surface confined binding sites [5, 54-56]. It must be noted that beside direct electrooxidation of suitable monomers the versatility of electrochemical synthesis enables surface confined polymer deposition also by electrochemically generating the “active” initiator [57] or electrochemically changing the local pH in the close vicinity of the electrode [58].

These indisputable benefits motivated us to compile the literature and evaluate the state-of-the art of electrosynthesized protein MIPs. The paper follows the work flow of MIP preparation and testing:

- Functional monomers for the electrosynthesis of protein MIPs
- Strategies for the electrosynthesis of protein-imprinted polymers
- Template removal
- Detection of the template binding

### *2.1. Functional monomers for the electrosynthesis of protein MIPs*

Electrochemical polymerization of monomers (Fig. 2) onto conducting substrates leads to the formation of either conducting or non-conducting polymer films depending on the choice of the monomer and the electropolymerization conditions. Electrically conducting polymers can be grown in thicker films, which is beneficial if the goal is to prepare complex 3D micro- or nanostructured MIPs. In contrast, the growth of insulating polymer films is self-limiting in terms of thickness, i.e., the insulating polymer layer blocks the electron transfer between the electrode and the monomer after its thickness exceeds the range of electron transfer (generally in the lower nanometer range)[59] and/or a sufficiently compact film is formed to halt the permeation of the monomer to the electrode.

The most widespread electrochemical polymerization technique for preparing protein MIPs is cyclic voltammetry. By varying the number of scan cycles and the scan rate, the thickness and the compactness of the deposited polymer film can be controlled, respectively. Too small scan rates result in tight films entrapping the template, while too large scan rates lead to a loose polymer network with low recognition ability [56],[85]. Potentiostatic deposition lacks the control over film compactness, but the film thickness can be precisely adjusted by the charge consumed during electropolymerization [48],[49]. Furthermore, the applied potential can be pulsed in order to create films with enhanced adhesion to the electrode surface [26]. The application of pulsed potential aids also the replenishment of the depleted solution layer adjacent to the electrode by the low diffusivity macromolecular template [79], [83], and thus to enhance the incorporation of the protein template into the growing polymer.

MIPs based on electrically conducting polymers primarily involve the electrochemical oxidation of pyrrole and ethylenedioxythiophene (EDOT) monomers (Fig. 2 f, g) to form protein-imprinted polypyrrole (PPy) [60] and poly(3,4-ethylenedioxythiophene) (PEDOT) [5] polymers. The removal of oxygen from the monomer solution before polymerization might be crucial for the reproducible deposition of stable conducting films as reported explicitly for PPy [61]. Polypyrrole can be additionally overoxidized by applying high potentials (e.g. 1.0 V vs. Ag/AgCl) in buffer [62] or in alkaline solutions in the presence of oxygen. Such overoxidation induces the formation of additional functionalities, e.g. carboxyl groups, with the respective polymers becoming electrically insulating. The use of PEDOT-PSS (PEDOT doped with polystyrene sulfonate) for creating different protein-imprinted nanostructures was pioneered by Gyurcsányi's group. The functional groups of this polymer offer various interactions, including hydrogen-bond, electrostatic and  $\pi$ - $\pi$  interactions and yet it has been found to show very low nonspecific adsorption of proteins [5]. These properties make PEDOT-PSS an excellent candidate for



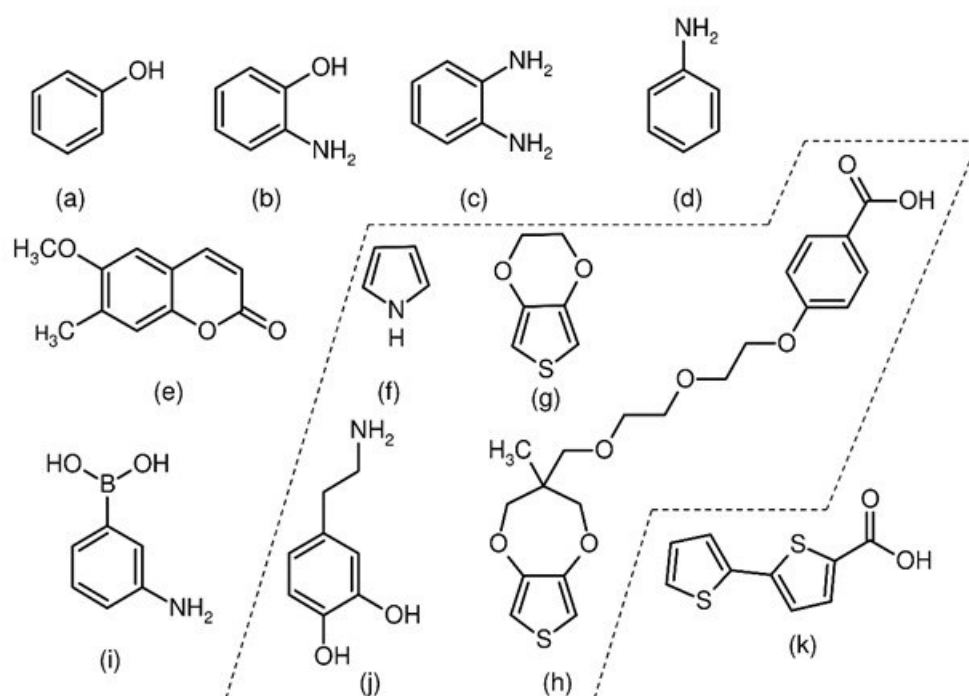
protein imprinting. However, electrosynthesized PEDOT MIPs have only been prepared to date for a single model template, avidin [56, 63, 64].

Insulating polymers offer benefits in terms of preparing ultrathin films in the nanometer range as well as enabling the use of certain electrochemical detection schemes. These are based on the modulation of MIP film permeability for redox markers by protein binding or on direct electrochemical detection of proteins. In the latter respect, polyscopoletin has been introduced by Scheller's group for the selective capture of cytochrome c and its detection by direct electron transfer [6]. Polymerization of the natural coumarin-derivative scopoletin is robust, does not depend on deoxygenation of the monomer solution, and yields a conformal, hydrophilic film on different electrode materials [65]. Imprinting of polyscopoletin with a wide range of proteins, e.g., cytochrome c [6],[66], concanavalin A [67], and ferritin [53] has been already demonstrated. At present, the insulating MIP films prevail also in terms of binding affinity as highly conformal polyphenol nanocoatings imprinted with ferritin were reported to have affinity in the fM concentration region [68].

Aniline [69] and related compounds such as *o*-phenylenediamine [70],[71] and *o*-aminophenol [72] were also favored for protein imprinting since they bear functionalities that can participate in hydrogen-bonding,  $\pi$ - $\pi$  and other types of interactions with the template, depending on the nature of the substituents. A monomer leading to more specific target-MIP interactions is the 3-aminophenylboronic acid, which is suited for the recognition of glycoproteins through the ability of the  $-\text{B}(\text{OH})_2$  moiety to form under alkaline conditions reversible covalent bonds with vicinal diols that are commonly found in carbohydrates [73] and glycoproteins [74]. This opportunity, although well exploited in case of chemically synthesized pAPBA MIPs [75],[76], has not been employed with electropolymerized films, which have only been prepared to date for the recognition of BSA [77], lysozyme and cytochrome c [78]; all non-glycated proteins. Wang et al. imprinted pAPBA with hemoglobin, a protein that exists to a minor extent in glycated form as well, but there is no mention on the exact form of the protein [79]. Future works with electrosynthesized pAPBA MIPs for glycoprotein recognition should consider prior immobilization of the template glycoprotein, which was found essential in obtaining satisfactory selectivity against other glycoproteins [76]. Beside biocompatibility, a wide variety of functional groups and extensive knowledge about the formed polymers motivated the use of dopamine [80, 81] and acrylamide [57, 82] for protein MIPs.

In addition to the monomer and template, the pre-polymerization mixture can contain additional compounds that are also incorporated in the polymer. This option is especially obvious in case of conducting polymers where the formation of the charged polymer film requires the incorporation of

charge compensating ions, e.g., oppositely charged small ions or polyelectrolytes from the supporting solution. However, this mechanism enables also the incorporation of nanoparticles such as polystyrene sulfonate (PSS) modified superparamagnetic nanoparticles into PEDOT microrods as shown by Ceolin et al.. Such nanoparticles may confer further functionality to the MIPs like the opportunity to handle MIP particles by an external magnetic field [55], which in turn enables their application for separation and pre-concentration.

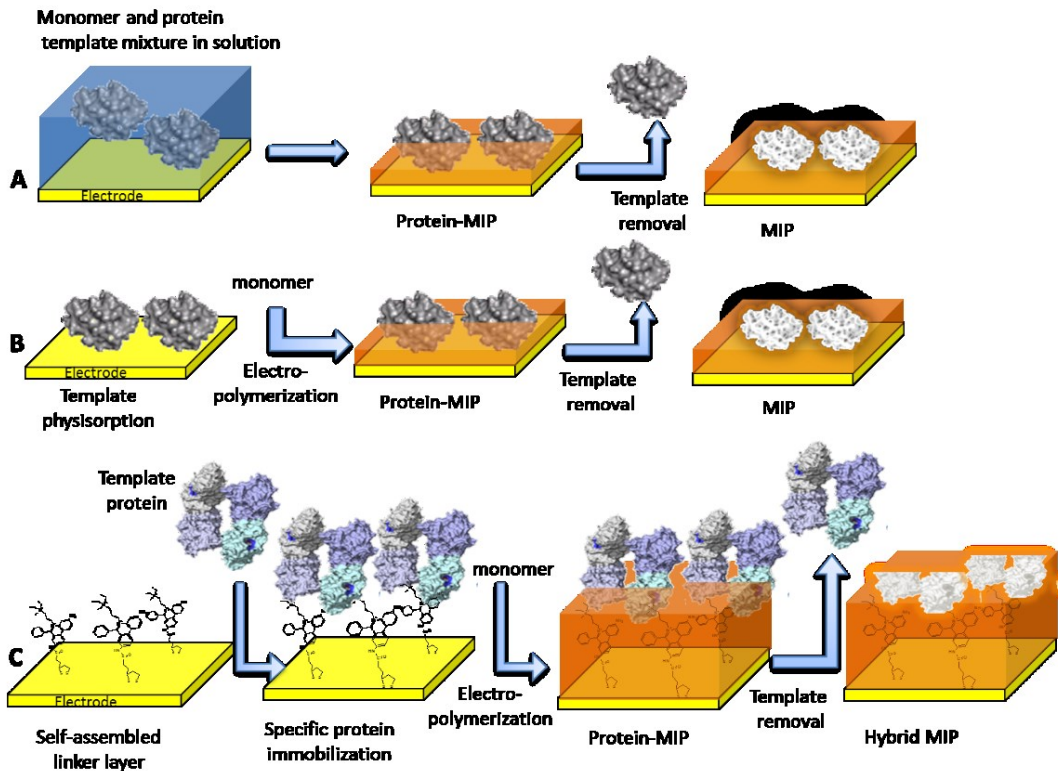


**Figure 2.** Representative monomers applied for the electro-synthesis of protein imprinted polymers: phenol (a), *o*-aminophenol (b), *o*-phenylenediamine (c), aniline (d), scopoletin (e), pyrrole (f), 3,4-ethylenedioxythiophene (g), proDOT-COOH (h), aminophenylboronic acid (i), dopamine (j) and 2,2'-bithiophene-5-carboxylic acid (k). Monomers within the dashed inset polymerize into conductive films.

## *2.2. Strategies for the electrosynthesis of protein-imprinted polymers*

Electrosynthesized MIPs have been developed for both relatively small monomeric proteins such as cytochrome c, serum albumin and troponin T, but also for oligomeric proteins like hemoglobin, avidin, lectins, and acetylcholinesterase (see Table 1 for a comprehensive compilation). The most straightforward approach, i.e. the formation of the pre-polymerization complex by simply dissolving the template in the monomer solution has already led to clear imprinting effect (Figure 3A). Karimian et al. reported that a troponin T-imprinted poly(o-phenylenediamine) film bound two orders of magnitude more troponin T than the control polymer [70],[83]; while Kan [62] and Li [57] have obtained an imprinting factor of ca. 7 for bovine hemoglobin with polypyrrole- and polyacrylamide-based MIPs, respectively.

Local enrichment of the protein at the electrode surface by spontaneous adsorption was also used to immobilize the template for enlarged binding capacities. Wang et al. incubated the electrode in the monomer-template mixture to allow for protein adsorption on the surface before starting the polymerization [79] (Fig. 3B). The adsorption of charged proteins can be promoted by applying an opposite conditioning potential to the electrode during incubation as described in the work of Cai et al. [68]. Ramanaviciene and Ramanavicius performed the polymerization of pyrrole with short potential pulses intermitted with longer resting periods to compensate for local depletion of monomer and protein template in the close vicinity of the electrode [84].



**Figure 3.** Various surface imprinting methodologies for the electro-synthesis of MIP films for selective recognition of proteins: electropolymerization of a mixture of protein and monomer from solution (A); preconcentration of the protein (peptide in case of epitope imprinting) on the surface of an electrode either by physisorption (B) or by using a self-assembled anchor layer for oriented immobilization of the protein (e.g. a weak inhibitor of an enzyme) (C).

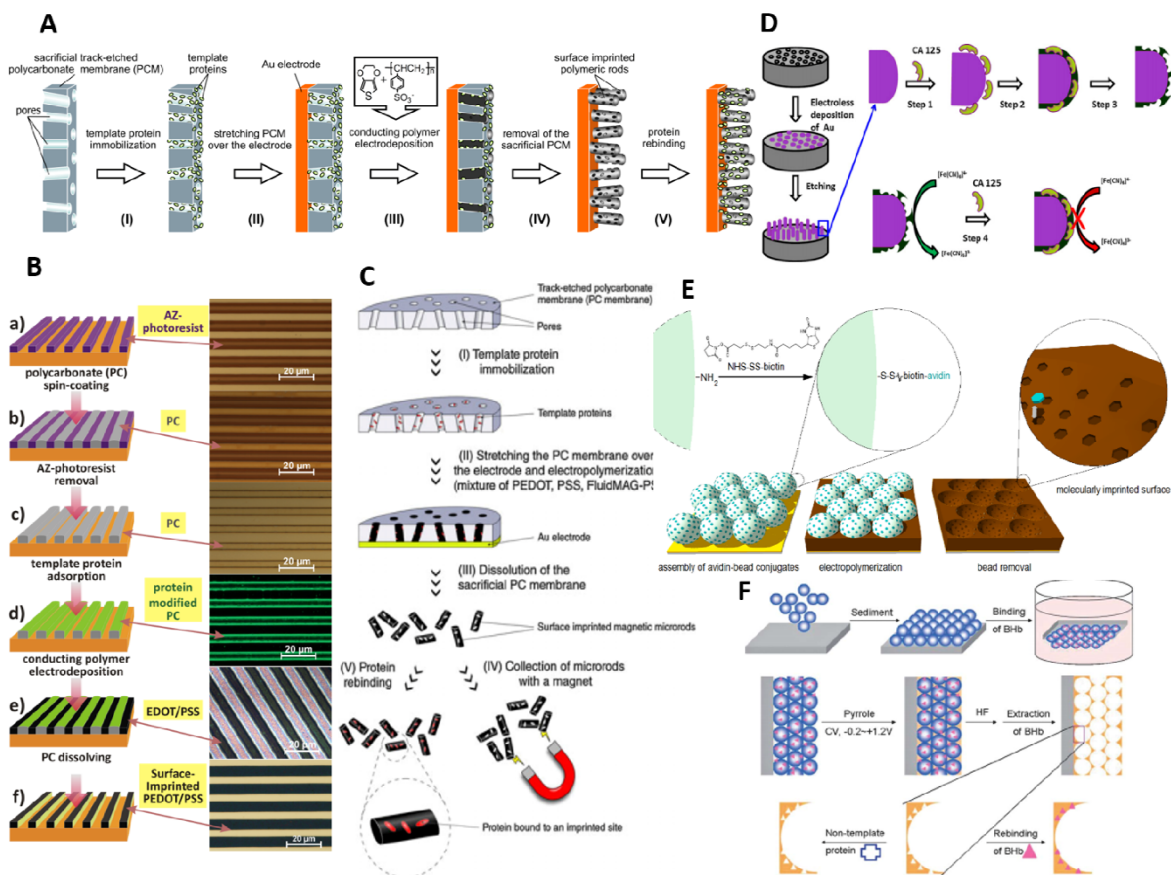
The immobilization of the protein through a self-assembled anchor layer offers advantages over random immobilization or electropolymerization from a protein-monomer mixture in terms of generating uniformly accessible binding sites. Kan et al. has demonstrated [81] that covalent immobilization of the template prior to polymer deposition can significantly enhance the MIP's analytical performance: their imprinting factor reached 20 due to this modification. Scheller's group has obtained a ca. 3-fold improvement in the imprinting factor when they used immobilized template [60] instead of free protein [58] to create selective recognition sites for cytochrome c in a polystyrene film. This approach was shown to be compatible with epitope imprinting, i.e. the terminal peptide of the native protein was immobilized onto the electrode and used as template in the subsequent electropolymerization step. The concept was demonstrated by Dechtrirat et al. [66] who immobilized the surface-exposed C-terminus peptide of cytochrome c (comprising of 9 amino acids) and showed that the epitope imprinted

polyscopoletin nanofilm could effectively rebind the native protein. An “inverse epitope-imprinting” method was proposed by Evtugyn et al. who imprinted poly(methylene green) with double-stranded DNA [85]. After template removal a short fragment of the DNA, an aptamer was rebound. Here, however, the MIP only served as a means of immobilizing the aptamer, which was in turn responsible for the recognition of the thrombin analyte.

The oriented immobilization of proteins via self-assembled anchor molecules which can reversibly bind to the protein through non-covalent bonds has been used also for the preparation of *hybrid molecularly imprinted polymers*. In such MIPs the binding site is generated both by the molecular imprinting process and the specific moieties of the anchoring molecule. The concept was demonstrated by using thiolated oligoethyleneglycol (OEG)/mannose monolayer self-assembled on the transducer surface to immobilize the carbohydrate binding protein, concanavalin A (ConA) [67]. Following the electropolymerization of a polyscopoletin film of comparable thickness with the protein, binding sites with enhanced binding properties were formed that comprised both the mannose moiety and the imprinted cavity. Similar methodology was implemented by Bossert et al. to induce oriented adsorption of cytochrome c by coating the electrode with an anionic self-assembled monolayer (SAM) which attracted the positively charged lysine residues of the protein neighboring its heme group [6]. Recently the enzyme acetylcholinesterase (AChE) has been “vectorially” bound via its peripheral anionic site to a propidium terminated SAM prior the deposition of an ultrathin polymer film of a ProDOT derivative [52] (Figure 3C). Propidium which is a weak inhibitor of AChE was thereby made part of the binding site in the MIP nanofilm. It was shown that in fact the binding is dominated by the propidium-protein interaction. The imprinted ProDOT film acts as a shape selective filter enabling the selective access of AChE to the propidium moiety and by that effectively suppressing interference from coexisting proteins. The selectivity was further enhanced by *in-situ* electrochemical detection of the enzymatic activity of AChE bound to the hybrid MIP.

The oriented protein imprinting can be achieved also by using an inert nanoparticle support for their immobilization [54, 86] instead of the surface of an electrode. The approach introduced by the group of Gyurcsányi used such particles to create nanostructured polymer films by assembling polystyrene nanospheres into a monolayer on the surface of an electrode, electropolymerizing PEDOT-PSS film in the voids between the spheres and then dissolving the nanospheres (Fig. 4E). This concept works best with conducting polymers alleviating the demand for ultrathin films while providing a higher binding capacity owing to the increased specific surface of the polymer. Most importantly, a significantly better discrimination could be achieved between avidin (used as target) and its analogues when the template

was immobilized through its biotin-binding site [54], than with the same type of polymer imprinted with randomly immobilized avidin [56].



**Figure 4.** Micro- and nanostructuring of protein-imprinted polymers based on sacrificial materials. Template synthesis using polycarbonate-based sacrificial materials of Av-imprinted PEDOT-PSS microstructures in the form of surface confined microrods (A) (adapted with permission of ref. [5]), microbands (B) (adapted with permission of ref. [56]) and solution dispersed magnetic core particles (C) (adapted with permission of ref. [63]) as well as for protein-imprinted polyphenol coated nanoelectrode arrays (D) (adapted with permission of ref. [87]). Nanosphere lithography based on latex (E) (adapted with permission of ref. [54]) and silica beads (F) (adapted with permission of ref. [86]) as sacrificial materials for the synthesis of surface imprinted PEDOT-PSS and polypyrrole films, respectively.

The sphere lithography concept could be extended to generate surface imprinted microporous MIPs by self-assembling several sacrificial bead layers [81] (Fig. 4D) as opposed to just one [49].

The efforts towards the electrosynthesis of micro- and nanostructured MIPs with high binding capacity were supported by the use of sacrificial scaffolds, which led to the formation of nanorods [88],[87] (Fig 4D), microbands [56] (Fig. 4), and microparticles [63] (Fig. 4C). In one of the earliest attempts Menaker

et al. [5] tightened an avidin coated track-etched polycarbonate membrane on top of a gold disk-electrode and deposited PEDOT-PSS within its pores. Afterwards, the membrane was detached from the electrode and dissolved in chloroform, liberating surface-imprinted polymer microrods that matched the size and shape of the original pores. The same concept and materials were implemented in a follow up study to enable the standard photolithographic fabrication of micropatterned surface-imprinted polymers [56] (Fig. 4B). By this methodology - compatible with large-scale fabrication - avidin-imprinted PEDOT-PSS conducting polymer microbands were prepared directly on surface plasmon resonance (SPR) chips. With the intent to enable high-throughput electrosynthesis and characterization of protein MIPs the so called microelectrospotting technique was developed by Bossert et al. [53]. The electrically connected spotting pin, which acted as a counter electrode, enclosed the mixture of monomer and template protein that upon contacting the surface of an electrode was *in-situ* electropolymerized. By using various templates and monomer mixtures surface confined polymers spots of ca. 500  $\mu\text{m}$  diameter were prepared on surface plasmon resonance imaging (SPRi) gold chips. It was shown that the thickness of the spots is in the low nanometer range that enables real time monitoring of template-MIP interactions by SPR. This novel format may open the route for high-throughput screening of protein-imprinted electrosynthesized MIPs given that a proper library of monomers and protein templates compatible with the microelectrospotting technology is established.

One of the main drawbacks of electrosynthetic methods is that electrochemical polymerization requires contact with an electrode and as such the fabrication of free-standing polymeric structures/particles is difficult. However, the use of sacrificial scaffolds proved to be an enabling technology also in this respect and the first report on solution dispersed electrosynthesized MIP particles used the pores of an avidin-coated track etch membrane as a microreactor to generate surface imprinted microrods [55]. To enable the recovery and handling of such nanoparticles after the removal of the sacrificial membrane, PSS modified magnetic nanoparticles were incorporated during the electrosynthesis into the PEDOT microrods. The same concept was pursued by Wang's group to prepare surface imprinted tubular microengines [64] that can actively move by bubble propulsion to capture and carry the template protein (avidin) in the sample solution.

### 2.3. Template removal

Template removal is essential to liberate accessible binding sites for latter rebinding of the protein. The essential prerequisite for any procedure is not to alter the polymer structure in an extent that would affect the rebinding of the template. This is not as straightforward as many studies suggest and in fact the choice of the regeneration solvent is a trade-off between complete removal of the imprinted protein [56],[71] and preservation of the integrity of the binding sites [79],[87]. Accordingly, different methods were applied that range from the application of chaotropic agents to the denaturation, dissociation or lysis of the protein.

Globular proteins have been extracted by chaotropic reagents; alcohols or surfactants which induce a collapse of the tertiary structure. This method is applicable to imprints created with free or physically immobilized proteins. A wide range of treatments were reported including the use of highly acidic [6],[84],[85] or basic [55] solutions and/or surfactants (sodium dodecylsulfate (SDS) [68],[89] or Tween 20 [78],[79]), sometimes at elevated temperatures [62],[6],[70]. For oligomeric proteins the dissociation into monomeric units by changing the pH and/or employing detergents may also be used to generate free binding sites in the MIP. Covalently immobilized proteins are removed according to the nature of the bond: a disulfide-containing crosslinker can be cleaved by reduction with mercaptoethanol [54],[81], while the imine bond is easily hydrolyzed using oxalic acid [86]. Sometimes the protein is removed together with the sacrificial support to which it was immobilized, as in the works of Menaker et al. [5], Lautner et al. [56] and Orozco et al. [64], where the polycarbonate-based support was dissolved in chloroform or dichloromethane without any further steps.

An alternative to harsh solvent extraction is the degradation of the protein target by proteolytic digestion using a protease enzyme. Proteinase K – a protease enzyme with broad cleaving specificity – offers a particularly attractive alternative for protein removal under mild conditions. This approach was used for the removal of concanavalin A [67] and myoglobin [72] templates. The obtained peptides and amino acid residues, as well as the remaining protease were then washed out of the polymer with a buffer solution.

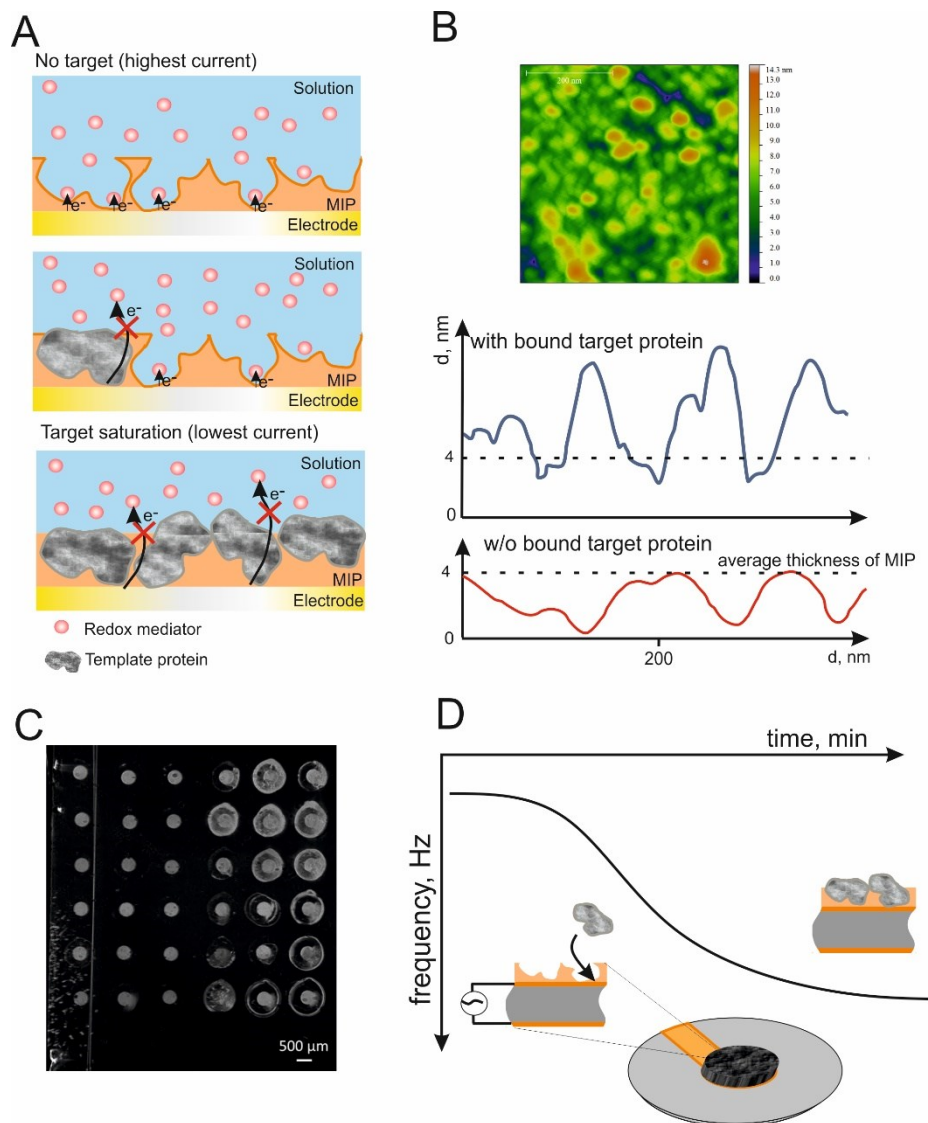
Electrochemical means have only been applied so far for the removal of small molecular weight templates, but in principle can be used also to remove protein targets. Dechtrirat et al. oxidized the thiol group tethering the template peptide chain to the electrode at a high potential [66], while Kong et al. changed the charge of the polypyrrole film by overoxidation, which thereby rejected the now identically charged template [90].



#### *2.4. Detection of the template binding*

The most important parameters characterizing the performance of MIPs are the affinity and specificity of target rebinding which determine the dynamic concentration range including the lower limit of detection and the cross reactivity. The rebinding of the target is quantified by methods which indicate directly the presence of the protein in the MIP layer and by “indirect” methods which evaluate the change in the signal of a marker molecule. Direct detection of the template protein by Raman and FTIR spectroscopy [72] offers a rather specific mean to detect the presence of the protein template in the MIP and by that to evaluate the efficiency of the template removal. In case of redox active proteins and certain enzymes the direct electron transfer[6] and the assessment of local enzymatic activity [52], respectively, offer sensitive means for the direct detection of the template protein binding to the MIP. As the signal originates in principle solely from the template bound to the MIP, nonspecific protein adsorption may interfere only in competitive manner. This is not the case for SPR [53, 56, 66, 77, 79] and QCM [54, 67, 81](Fig. 5D) where the signal reflects overall mass (refractive index change) of the MIP layer, which can be induced not only by the target but also by nonspecific adsorption and other type of changes of the chemical environment. It is very important to realize that such interfering effects in case of nonspecific signals does not necessarily mean that the imprinted sites lack sufficient selectivity. The reason is that it is hardly possible to imprint the whole sample exposed surface with a protein template, meaning that in fact the generated surface will be a mixture of imprinted and non-imprinted polymer (NIP). Accordingly, the NIP is expected to exhibit inherent adsorption properties of the bulk polymeric material and the lack of selectivity may easily originate from binding to the NIP fraction of the surface and not from the insufficient selectivity of the imprinted sites. A conclusive demonstration of this eventuality was provided by Dechtrirat et al. who observed a very significant interference from lysine when testing the selectivity of cytochrome c-imprinted polymers by SPR [66]. However, when injecting lysine first and then the template the signal increased roughly with the same amount as when the template was injected on a lysine-free MIP. This suggests that the lysine does not bind to surface imprinted sites, but elsewhere on the MIP, while still contributed to the SPR signal. Despite of this deficiency both SPR and QCM offer very sensitive means to evaluate the MIP-target interactions. In particular SPR imaging [56] coupled with local deposition of MIPs (Fig. 5C) having different compositions or imprinted by different templates [53] is particularly promising for high throughput optimization of MIP compositions and protein analysis. Following on the line of label-free methods the presence of the proteins in surface imprinted polymer films was successfully detected also by atomic force microscopy

(AFM) [52, 53]. While this method is by no means suitable for sensing application it offered clear evidence for the formation of protein imprints (cavities) (Fig. 5B) in highly conformal surface imprinted polymer layers and for the subsequent binding of proteins [52].



**Figure 5.** Representative general detection methods of the protein binding to the MIP films: (A) Electrochemical detection based on the modulation of the flux of redox mediators to the supporting electrode upon target protein binding, (B) atomic force microscopy (adapted with permission of ref. [52]), (C) surface plasmon resonance imaging (adapted with permission of ref. [53]), (D) quartz crystal microbalance.

Labeled targets were also used for the direct assessment of template removal and rebinding. Measurement of MIP film fluorescence after interaction with a fluorescently labeled target protein

[61],[27, 62] or of the enzymatic activity (HRP)[79] of enzyme labeled targets allows highly sensitive and selective quantification of rebinding. A frequently applied indirect method for the characterization of template rebinding to thin films of MIPs is based on measuring by CV [71],[86],[83] or electrochemical impedance spectroscopy [91],[89] the permeability of the polymer film for a redox marker such as ferrocyanide or ferrocenecarboxylic acid [68]. In the absence of the target protein the templated voids (binding sites) in the MIP film permit the access of the redox mediator to the underlying electrode surface while the target binding will gradually decrease the permeability of the MIP film in a concentration dependent manner, i.e., the current signal of the redox marker is diminished upon target binding (Fig. 5A). The detection scheme resembles to that of ion-channel sensors with logarithmic concentration dependence and dynamic range over several orders of magnitude[92]. This approach provides indeed a convenient procedure to follow the work flow of MIP preparation by comparing the signals of the MIP after electropolymerization, template removal and rebinding. However, it may not be fully suited for the accurate determination of binding parameters, such as binding isotherm and equilibrium constant. A major disadvantage of the method is that at low target concentrations minute decreases in the current are to be detected in a large base current. As such the fluctuation of the background current makes the uncertainty of the determinations very high in this range, especially, considering contingent swelling-shrinking of the polymer film upon change in the ionic strength and/or pH of the sample solution. Therefore, it is surprising that several papers describing MIPs for both low and high molecular weight targets (using the redox marker ferrocyanide) claim measuring ranges over several orders of magnitude with sub-nanomolar lower limit of detection. Many trials were made to enhance the sensitivity of the electrochemical readout of protein MIPs by using different coatings on the electrode surface prior to polymer deposition. Among these the application of graphene and carbon nanotubes stand out. Graphene, beside its excellent mechanical properties and electrical conductivity, provides a large surface that leads to an increased current response and thus better sensitivity [71],[91]. Carbon nanotubes [69],[89] are usually drop-casted onto the electrode surface prior to polymer deposition in random orientation, but Choong et al. prepared a vertically aligned array by directly growing the carbon nanotubes onto the electrode surface and electrochemically deposited an imprinted polypyrrole layer on their lateral wall [88]. Although developed with caffeine as template, this procedure is promising for the imprinting of large molecules as well because the thickness of the imprinted polymer layer can be readily tuned to match the dimensions of the template. Moreover, the capacity and the binding site density of the polymer can be controlled by varying the length and packing density of the carbon nanotubes. Indeed, later on spectacular results were reported for arrays of carbon-nanotube tips

with an imprinted polyphenol coating that could achieve pg/L limit of detection for ferritin and a human papillomavirus derived protein using ferrocene carboxylic acid as marker and electrochemical impedance spectroscopy for detection [68]. Microdendrites can also improve electronic transfer rates and the effective electrode area [57].

The imprinting factor IF is defined as the ratio of the signals of the MIP and NIP after rebinding of the target. It should reflect the ratio of “specific” binding of the MIP to the nonspecific binding at the NIP surface. At saturation concentrations it returns the ratio of binding capacities to the imprinted and nonimprinted polymer. As an alternative, the ratio of the slopes in the linear region of the binding curve is determined. However, for negligible nonspecific binding this method will provide nonrealistic high IF values. It is obvious that the determination of the imprinting factor is influenced by the method which was used to measure the rebinding to the MIP and NIP. For SPR and QCM the signal can be influenced by structural changes of the polymer. In this respect the evaluation of the enzymatic activity or of direct electron transfer is considerably more specific and gives a “functional” imprinting factor. The frequently used measurement of the current of a redox marker is influenced not only by the occupancy of the cavities by the target but also by the formation of “nonspecific” pores during the extraction of the template. Therefore the conditions of template removal are crucial.

**Table 1.** Electrosynthesized protein MIPs

TEMPLATE	POLYMERIZATION METHOD	TEMPLATE REMOVAL [method of verification]	DETECTION	PERFORMANCE	SELECTIVITY		COMMENTS	REF
				PARAMETERS <sup>a</sup> [binding model]	WELL DISCRIMINATED	INTERFERING (>25%)		
<b>acrylamide (AAM), methylenebis(acrylamide) (MBA)</b>								
BHb	CV: (-1.2)-(-0.4) V, 20 mV/s	10% AcOH + 10% SDS [CV]	CV, LSV	IF = 7.6	cyt c, OvA, BSA, Lys	n.a.	polymer film on electrode coated with Au microdendrites and PrB	[57]
BHb	CV: (-0.2)-(-1.4) V, 20 mV/s	10% AcOH + 10% SDS, 0.5 M H <sub>2</sub> SO <sub>4</sub> , PB pH 6.8	CV, DPV	no response on NIP	BSA, OvA, GOx, cyt c	n.a.		[82]
<b>aminophenol</b>								
Myo	CV: (-0.2)-0.8 V, 50 mV/s	protK digestion [EIS, CV, FTIR]	EIS, SWV	IF ~ 4, K <sub>d</sub> = 5.88·10 <sup>-7</sup> M [La]	troponinT	n.a.		[72]
<b>aminophenylboronic acid (APBA)</b>								
BSA	CV: (-0.1)-1.4 V, 20 mV/s	3% AcOH + 0.1% Tween20	SPR	LR: 2·10 <sup>-2</sup> -10 <sup>-1</sup> g/l, IF ~ 2	n.a.	Hb, Lys	co-monomer: oPD	[77]
cyt c, Lys	CV: (-0.2)-0.7 V, 100 mV/s	3 v/v% AcOH, 0.1 v/v% Tween20	CV	IF = 6.7 (Lys), ~ 2.3 (cyt c)	n.a.	n.a.	PPy and non-imprinted pAPBA supporting layers	[78]
PHb	CV: (-0.1)-1.1 V, 20 mV/s	3% AcOH + 0.1% Tween20	electrochemical SPR	LR: 5·10 <sup>-2</sup> -50 g/l, IF ~ 1.6	BSA	OvA, Lys		[79]
<b>aniline</b>								
BSA	CV: (-0.4)-1.2 V, 50 mV/s	5% AcOH + 10% SDS [DPV]	DPV, EIS	LR: 10 <sup>-7</sup> -10 <sup>-1</sup> g/l	HSA, BHb	n.a.	polymer film on GR and MWCNT-modified electrode	[69]
<b>dopamine</b>								
IgG	CV: (-0.45)-0.55 V, 50 mV/s	0.1 M ME (EtOH), 3 M NaCl+0.1% SDS (100°C)	QCM	IF = 1.66, K <sub>d</sub> = 2.96·10 <sup>-7</sup> M [Fr-La]	n.a.	n.a.		[81]
tumor marker Ags	CV: (-0.5)-0.5 V, 20 mV/s	1 wt% SDS	ECLIA	IF = 2 (CEA), 1.23 (CA199)	BSA, IgG, AFP, Lys	n.a.		[80]
<b>3,4-ethylenedioxythiophene (EDOT)</b>								
Av	potentiostatic: 0.9 V	disrupt Av-bead linkage, dissolve beads in toluene	QCM	IF = 6.5	SAv, NAV, (EAv), BSA	Lys	nanostructured film, vectorially immobilized template	[54]
Av	potentiostatic: 0.75 V	1 M NaOH, DCM, 50% MeOH	fluorescence	IF ~ 5	n.a.	n.a.	microrods with embedded SPM NPs	[55]
Av	potentiostatic: 0.75 V	DCM; regeneration: 1 M NaCl.	SPR	IF ~ 10; K <sub>d</sub> = 1.25·10 <sup>-9</sup> M [sp]	BSA, SAv, NAV	Lys, EAv	polymer microbands formed with lithography	[56]
Av	pulses: (-0.2) / 1.1 V, 5 Hz	chloroform	fluorescence	IF = 6, K <sub>d</sub> = 3.49·10 <sup>-9</sup> M [Sc-Ro]	BSA	n.a.	microrods prepared using a sacrificial membrane	[5]
Av-FITC	potentiostatic: 0.8 V	DCM	fluorescent microscopy	no binding observed on NIP	anti-IgG	n.a.	microrods prepared using a sacrificial membrane	[64]

TEMPLATE	POLYMERIZATION METHOD	TEMPLATE REMOVAL [method of verification]	DETECTION	PERFORMANCE	SELECTIVITY		COMMENTS	REF
				PARAMETERS <sup>a</sup> [binding model]	WELL DISCRIMINATED	INTERFERING (>25%)		
				<b>methylene green</b>				
thrombin (target) DNA aptamer (template)	CV: (-0.3)-1.3 V, 100 mV/s	5 M HCl	EIS, potentiometry	IF = 1.83	HSA	n.a.	"inverse epitope imprinting"	[85]
				<b>phenol</b>				
HFer, HPV E7 protein, Cal ovarian cancer antigen	CV: (0.3 V * 30 s), 0-0.9 V, 50 mV/s	DI water or 5% AcOH or 10% SDS	DPV, EIS	9.8·10 <sup>-15</sup> M [Hill]	horse (apo)Fer, Ca <sup>2+</sup> -free conformation of Cal	n.a.	arrays of carbon-nanotube tips	[68]
	CV: 0-0.9 V, 50 mV/s	DI water or 1% AcOH + 3% SDS [EIS]	DPV, EIS		HSA, human serum proteins	n.a.	polymer coating on nanoelectrodes	[87]
				<b>o-phenylenediamine (oPD)</b>				
troponinT	CV: 0-1.1 V, 50 mV/s	0.25 m NaOH (EtOH:H <sub>2</sub> O 2:1) [CV]	CV, EIS, DPV	LR: 9·10 <sup>-9</sup> -8·10 <sup>-7</sup> g/l, K <sub>d</sub> = 2.4·10 <sup>-12</sup> M [sp.+nsp], no response on NIP	serum proteins	n.a.		[70]
troponinT	CV: 0-1.1 V, 50 mV/s	0.25 m NaOH (EtOH:H <sub>2</sub> O 2:1) [CV]	CV, EIS, DPV	no response on NIP, K <sub>d</sub> = 2.3·10 <sup>-13</sup> M [sp.+nsp]	n.a.	n.a.		[83]
				<b>proDOT-COOH</b>				
AChE	potentiostatic: 0.75 V * 5 s, 0 V * 5 s	glycine-HCl [AFM, enzyme activity]	enzyme activity, AFM	IF = 5.3, K <sub>d</sub> = 4.2·10 <sup>-7</sup> M [La]	BSA	urease		[52]
				<b>pyrrole</b>				
BHb	CV: (-0.2)-1.2 V, 100 mV/s	1 M H <sub>2</sub> SO <sub>4</sub> [EIS]	DPV	LR: 10 <sup>-5</sup> - 1 g/l	BSA, HSA, Lys, Bln, ATP	n.a.	film on electrode coated with GR and IL	[91]
BHb	CV: (-0.2)-1.2 V, 100 mV/s	1 M H <sub>2</sub> SO <sub>4</sub> , CVs in PBS (-0.6)-1 V	DPV, EIS	IF = 7.72	BSA	EAv, Lys		[62]
BHb	CV: (-0.2)-1.2 V, 100 mV/s	4% HF, 1 M oxalic acid [DPV]	EIS, DPV	IF ~ 20	BSA, EAv	Lys	nanostructured film	[86]
BLV protein	pulses: 0.95 V * 1 s, 0.35 V * 10 s	1 M H <sub>2</sub> SO <sub>4</sub> [PA]	PA, ELISA assay	IF = 22	BSA	n.a.		[84]
BSA	CV: (-0.35)-0.85 V, 50 mV/s	5% AcOH + 10% SDS [EIS]	DPV, EIS	LR: 10 <sup>-7</sup> - 10 <sup>-1</sup> g/l	HSA, BHb	n.a.	polymer film on MWCNT and magnetic NPs-modified electrode	[89]

TEMPLATE	POLYMERIZATION METHOD	TEMPLATE REMOVAL [method of verification]	DETECTION	PERFORMANCE PARAMETERS <sup>a</sup> [binding model]	SELECTIVITY		COMMENTS	REF
					WELL DISCRIMINATED	INTERFERING (>25%)		
<b>scopoletin</b>								
con A	potentiostatic: 0.7 V * 5 s, 0 V * 5 s	protK digestion [fluorescence]	QCM	IF = 8.6, K <sub>d</sub> = 7.2·10 <sup>-10</sup> M [La]	BSA, Myo, RNase, lectins	n.a.		[67]
cyt c	potentiostatic: 0 V * 15 s, 0.5 V * 35 s	1 M H <sub>2</sub> SO <sub>4</sub> [CV]	CV (DET), pseudo-peroxidase activity	IF = 2, K <sub>d</sub> = 10 <sup>-5</sup> M [Sc]	BSA, Myo	Lys	oriented template adsorption	
cyt c derived peptide	potentiostatic: 0.7 V * 5 s, 0 V * 15 s	electrochemical stripping, PBS+0,1% Tween [fluorescence]	fluorescence, SPR	IF = 10 (peptide), 6 (cyt c), K <sub>d</sub> = 2.51·10 <sup>-6</sup> M (peptide), 8.5·10 <sup>-6</sup> M (cyt c) [Sc]	single mismatch peptide, BSA, Myo	Lys		[66]
ferritin	pulses: 0 V * 1 s, 0.9 V * 0.1 s	5 mM NaOH injections in iSPR	iSPR	IF = 13	cyt c, Myo	BSA, urease	thin film confined to spots	[53]
<b>bithiophene-derivatives</b>								
HSA	CV: 0-1.2 V, 50 mV/s	30% NaOH 45°C	DPV, EIS	IF = 26.8, LR: 8·10 <sup>-4</sup> -2·10 <sup>-2</sup> (DPV) or 4·10 <sup>-3</sup> -8·10 <sup>-2</sup> (EIS) g/l	creatinine, uric acid, cyt c, Myo, artificial serum	urea, glucose, Lys		[93]

<sup>a</sup> LR: linear range, IF: imprinting factor (signal of MIP divided by signal of NIP), binding models: (n)sp: (non)specific binding, Sc: Scatchard, R: Rosenthal, L: Langmuir, Fr: Freundlich

ACHe: acetylcholinesterase, AFM: atomic force microscopy, Ags: antigens, Av: avidin, Av-FITC: avidin labelled with fluorescein isothiocyanate, BHb: bovine hemoglobin, BIn: bovine insulin, BLV: bovine leukemia virus, BSA: bovine serum albumin, Cal: calmodulin, cyt c: cytochrome c, con A: concanavalin A, CV: cyclic voltammetry, DCM: dichloromethane, DET: direct electron transfer, DPV: differential pulse voltammetry, EAv: ExtrAvidin®, ECLIA: electrochemiluminescent immunoassay, EIS: electrochemical impedance spectroscopy, FTIR: Fourier-transform infrared spectroscopy, GOx: glucose oxidase, GR: graphene, HFer: human ferritin, HPV: human papilloma virus, HSA: human serum albumin, IL: ionic liquid, LSV: linear sweep voltammetry, Lys: lysozyme, ME: mercaptoethanol, Myo: myoglobin, n.a.: not available, NAv: NeutrAvidin, OvA: ovalbumin PA: pulsed amperometry, PC: polycarbonate, PHb: porcine hemoglobin, PrB: prussian blue, protK: proteinase K, QCM: quartz crystal microbalance, SAv: Streptavidin, SPM: superparamagnetic, (i)SPR: surface plasmon resonance (imaging), SWV: squarewave voltammetry

### **3. Conclusions and outlook**

As compared with antibodies protein MIPs have been prepared only for a restricted spectrum of proteins starting with RNase by Mosbach's group [25]. Almost half of the papers still use hemoglobin, serum albumins and avidin as model templates. Point-of-care detection of marker proteins for cardiac [70],[72] or tumor disease [53, 68][72],[87], Alzheimer's disease (AChE)[52] or virus infections is the prospective aim in the generation of electrochemically addressable MIPs. In spite of a few reports claiming close to routine applicability [68, 72] MIPs need still substantial improvement to overcome disturbances caused by constituents of real samples. Presently the synthesis of MIPs follows a highly empirical strategy therefore a progress is expected either from a rational design or from the association of an empirical approach with high-throughput synthesis and detection methods. The latter could accelerate the determination of the optimal MIP compositions offering high affinity and selectivity. The proof of principle of such enabling technology was already made for electrosynthesized MIPs by using microelectrospotting [53]. As an additional benefit "microfabrication" methods can drastically reduce the required amount of template making available for imprinting presently unaffordable proteins, i.e., expensive proteins or proteins available in restricted quantities, e.g. proteins produced by cell-free protein synthesis. However, it is still a question whether such high-throughput approaches can be universally applied for a wide range of monomers and proteins with distinct physico-chemical properties. With respect to the rational design the complexity of the protein-imprinted systems is a major obstacle, which may be reduced by epitope imprinting and/or the implementation of hybrid materials involving bioinspired moieties with known binding properties. Irrespective of the approach adopted the implementation of nanomaterials and controlled oriented imprinting features as an optimal choice to increase the number and homogeneity of binding sites and by that improving the sensitivity and the selectivity of protein MIPs. The field would have also to benefit from establishing standardized testing procedure and relevant quality control criteria of the prepared protein MIPs.

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