Molecularly imprinted polymer nanostructures for selective protein recognition

Ph.D. thesis

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Abbreviations

µSL microstereolithography
AChE acetylcholinesterase
AEDP 3-[(2-aminoethyl) dithio]propionic acid hydrochloride
AFM atomic force microscopy
APBA aminophenyl boronic acid
APS ammonium persulfate
APTES (3-amino-propyl)triethoxysilane
Av avidin
BSA bovine serum albumin
ConA concanavalin A
CPPA 4-cyano-4(phenylcarbonothioylthio)pentanoic acid
Cyt c cytochrome c
DMSO dimethyl sulfoxide
DTT DL-dithiothreitol
EA ExtrAvidin
Ebis N,N'-ethylenebis(acrylamide)
EDC 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
EDOT 3,4-ethylenedioxy-thiophene
ELISA enzyme-linked immunosorbent assay
eQCM electrochemical quartz crystal microbalance
FITC fluorescein isothiocyanate
FTIR Fourier-transform infrared (spectroscopy)
HPLC high performance liquid chromatography
HPLC-MS/MS high performance liquid chromatography hyphenated with a tandem mass spectrometer
HRP horseradish peroxidase
HS-TEG (1-mercaptoundec-11-yl) tetra(ethylene glycol)
IAM iodoacetamide
IF imprinting factor (ratio of the signals of the MIP and NIP after rebinding of the target)
Lys lysozyme
MIP molecularly imprinted polymer
Myo myoglobin
NA NeutrAvidin
NHS-SS-biotin succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate
NIP non-imprinted polymer
NSL nanosphere lithography
PBS phosphate buffered saline
PC polycarbonate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PDMS</td>
<td>polydimethylsiloxane</td>
</tr>
<tr>
<td>PEDOT/PSS</td>
<td>poly(3,4-ethylenedioxy-thiophene) doped with poly(styrene sulphonate)</td>
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<tr>
<td>PEW</td>
<td>polymerization by evanescent wave</td>
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<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PPy</td>
<td>polypyrrole</td>
</tr>
<tr>
<td>ProDOT</td>
<td>3,4-propylenedioxythiophene</td>
</tr>
<tr>
<td>PVA</td>
<td>poly(vinyl acetate)</td>
</tr>
<tr>
<td>QCM</td>
<td>quartz crystal microbalance</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SAM</td>
<td>self-assembled monolayer</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS- polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylene-diamine</td>
</tr>
<tr>
<td>TEOS</td>
<td>tetraethyl orthosilicate</td>
</tr>
<tr>
<td>TET</td>
<td>Tween containing Tris-EDTA buffer</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine Liquid Substrate System for ELISA</td>
</tr>
<tr>
<td>TPS</td>
<td>two-photon stereolithography</td>
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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 (Figure 1). The subsequent removal of the template creates recognition sites in the molecularly imprinted polymer (MIP) that can, further on, selectively rebind the target.

This concept has been developed for the recognition of small molecular weight compounds, but in the early 2000s a few papers appeared on protein imprinting as well and this field has grown ever since, reaching today ten percent of the total MIP publications each year. This growing interest is due to the need for selective recognition and quantitation of proteins as biomarkers and, recently, as therapeutics as well. The current gold standard of protein measurement is based on antibodies which are costly and time-consuming to develop and have a short shelf-life. Molecularly imprinted polymers, on the other hand, are much cheaper and easy to prepare and are more resistant to environmental conditions than antibodies. However, the structural variability of proteins, their large size and incompatibility with established MIP recipes made the development of protein imprinting to take a slow start. While MIPs for the solid-phase extraction of various small molecular weight compounds are already on the market, protein MIPs are still in the experimental phase; with most papers focusing on new fabrication methods and incorporating various materials to enhance the selectivity or sensitivity of protein recognition with MIPs, using model template proteins with distinct properties that facilitate the process or which are cheap to obtain.

Implementation of “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. In my thesis work I have set out to develop a generic microfabrication method which ensures accessibility of the formed binding sites for the target molecules. To this end, I have combined protein imprinting with nanosphere lithography, which not only enabled the straightforward confinement of imprint formation to the polymer surface but also made it possible to keep the template molecules in homogeneous orientation during polymerization, which has led to a higher selectivity than that obtained with the same polymer prepared with randomly oriented template molecules [Paper I].
In spite of a few reports claiming close to routine applicability 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.

In my thesis work I aimed at developing such an enabling technology for electrosynthesized MIPs. We have combined a standard microspotting technique with electrochemical polymerization and were able to deposit polymer spots of ca. 500 µm diameter and thicknesses in the order of proteins’ dimensions. Combining this fabrication process, termed microelectrospotting, with surface plasmon resonance imaging to monitor the template removal and rebinding processes, has led to a high-throughput screening technique which allowed the fast development and optimization of a ferritin-selective MIP [Paper II]. Microelectrospotting can also be applied to create an array of MIPs selective to different compounds, each, in order to simultaneously detect various analytes with the same chip.

A crucial step, often overlooked in the process of molecular imprinting, is the removal of the template. For this purpose I have developed and optimized a procedure of proteolytic digestion [Paper V]. The need for such a method was suggested by the low share of MIP papers using digestion for template removal and the lack of consensus between them with respect to a suitable procedure. Performing the digestion under inadequate conditions can lead to the false impression of this technique’s incapacity which in turn pulls back research of promising approaches which require effective digestion for template removal, such as imprinting with immobilized protein molecules. I have shown that proteins in their native state are often not even cleaved by proteinase K, an enzyme known for its ability to digest keratin as well. I have therefore developed a method for pre-treatment of the target protein, which allows its subsequent digestion by proteinase K. I have demonstrated the suitability of the described procedure in two different imprinting approaches, both using covalent immobilization of the template.

In Chapter 2 - Background I overview the challenges associated with protein imprinting and present the strategies to address them. Due to its relevance to my work, special emphasis is placed on electrochemical polymerization methods. This chapter is largely based on Papers III and IV in which I have compiled the literature of electrochemically prepared protein-MIPs.

In Chapter 3 - Materials and methods I describe the materials and procedures employed in my experimental work. The sectioning of this chapter follows the procedure of MIP preparation: polymer fabrication, template removal and characterization methods.

Chapter 4 presents the concept of nanosphere lithography-based surface imprinting of proteins (Paper I).
Chapter 5 focuses on the fabrication and pre-testing of the microelectrospotting device with a concise discussion of its application to optimize a MIP for ferritin recognition (Paper II).

An optimized method for template removal by proteolysis is presented in Chapter 6 (Paper V).

In Chapter 7 I give a final overview of my doctoral work and summarize my results in thesis points.
2. Background

2.1. Molecularly imprinted polymers

The first example of molecular imprinting was described as early as 1931 by Polyakov who has found that silica particles preferentially adsorbed the organic solvent in which they had been prepared. Imprinting in organic polymers, however, only appeared 40 years later when Wulff published a method known today as covalent imprinting, while the most widespread non-covalent approach was proposed by Mosbach’s group another decade later. These materials were first referred to as “molecularly imprinted adsorbent” or MIA, but the term was soon changed to “molecularly imprinted polymer” or MIP due to the wartime associations of the first abbreviation (“missing in action”).

In the classical bulk imprinting approach the template is mixed with monounsaturated compounds bearing appropriate functional groups (functional monomer) to form a so-called pre-polymerization complex by covalent (covalent approach) or secondary bonds (non-covalent approach). The structure of this complex is then fixed by adding bi- or triunsaturated (crosslinking) monomers and initiating the polymerization typically by UV irradiation or thermally. The obtained polymer monolith is rigid but porous because (in contrast to what polymer chemists call “bulk polymerization”) a pore-forming solvent (porogen) is also added to the polymerization mixture. For practical applications this monolith is ground and sieved to the desired size fraction, yielding particles of irregular shape. Generally, the covalent approach is believed to produce more selective and homogeneous binding sites than the non-covalent approach since the interactions between the template and the monomer are far more stable and better defined. Nevertheless, the non-covalent approach is much more abundant in the MIP literature due to its ease of synthesis and flexibility in terms of interactions that can be exploited and, therefore, the wider the range of templates it can be applied to.

In order to avoid the tedious processing and other disadvantages associated with the bulk imprinting technique, the main focus of MIP research in the 1990s was to develop synthesis methods that directly produce monodisperse, spherical MIP particles which are better suited for applications in separation. For this purpose, synthesis methods well-known in polymer chemistry, such as dispersion, precipitation and suspension polymerization were adapted, or pre-formed beads were used, around or in the pores of which the imprinted polymer was formed.

Another direction of research around the millennium was to speed up the process of preparing and testing dozens of MIPs with different recipes in order to find the optimal formulation for a given target molecule. A high-throughput version of the bulk imprinting method was developed where small volumes of pre-polymerization mixtures were placed into HPLC autosampler vials or microtiter plates to form polymer disks of only a few millimetres in thickness, named miniMIPs. This allowed fast template removal and rebinding kinetics simply by exchanging the supernatant in the vials, without the need for tedious crushing and sieving of the MIPs into smaller pieces.
Based on all these successful approaches, MIPs were demonstrated to be applicable in various fields. Probably their most notable utilization is in sample preparation: MIP-based adsorbents for solid-phase extraction are marketed for a wide range of analytes (e.g. SupelMIP by Sigma Aldrich, AFFINIMIP by Polyintell, AFFINILUTE by Biotage). Although many synthesis routes have been developed to ameliorate the chromatographic properties of MIP particles, their real potential in chromatography lies in chiral separations where, in addition to great enantioselectivity, MIPs have the advantage over generic chiral stationary phases of the elution order being predictable. MIPs have the prospects of replacing antibodies in every possible application, e.g. catalysis, ligand binding assays and as recognition elements in sensors, but such examples have mostly been demonstrated in laboratory context. Despite a few emerging companies that offer custom-made MIPs for specific demands (e.g. MIP Technologies, Semorex) the wide-spread practical application of molecularly imprinted polymers has yet to come.

2.2. Imprinting of proteins

The first example of imprinting a macromolecular target already appeared in 1985 but it was only followed by a few other papers in the late 1990s. Protein imprinting started to attract considerable attention in 2005 and since then, the number of publications on protein MIPs is exponentially increasing, making up 8-10% of all the MIP research each year.

Molecularly imprinted polymers 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 needs to be addressed by the implementation of new concepts in the molecular imprinting of proteins.

In the following sections I will describe the main challenges encountered in protein imprinting and some of the strategies that have been developed to address them.

2.2.1. Challenges in protein imprinting

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:

*Due to their fragility irreversible conformational changes may occur during polymerization and the rebinding of the native conformation to such imprinted sites is not favoured.*

Classical small-molecule imprinting is performed in organic solvents where proteins are prone to denaturation. In order to preserve the protein’s native conformation, we must work in aqueous medium. Even so, the protein might not remain intact, as shown by Kryscio et al.: albumin suffers conformational changes when exposed to more than 350-fold molar excess of typical monomers (e.g. acrylamide, methacrylic acid), which is still lower than the ratios generally used in MIP recipes. At the typical composition of pre-polymerization mixtures...
Background

(monomer:template > 1000:1) the protein lost all of its helicity. Similar observations were made with lysozyme and bovine hemoglobin indicating that this detrimental effect should be expected with other proteins as well\textsuperscript{19}. The fact that monomer-template interaction already occurs at lower monomer loadings where conformational changes are not yet observed on the mixtures’ circular dichroism spectra suggests that this problem could be avoided by careful choice of M:T ratio.

The importance of preserving the template in the same conformation as it is expected to be rebound in was demonstrated by Turner et al. who have imprinted poly(aminophenyl-boronic acid) thin films with different isoforms of β-lactoglobulin\textsuperscript{20}. Each MIP had a preference to bind its own template. Moreover, the larger the structural deviation of the template isoform was from the native conformation, the lower the MIP’s affinity was found to be toward the native form.

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.

MIPs were originally designed to be highly cross-linked in order to retain the structure of the binding sites. This is problematic when the template is much larger, since diffusion into and out of such dense networks is greatly hindered; the template might not be able to leave the binding pocket or reach it again upon rebinding. The amount of template recovered from the MIP is not always given in the literature but published values for protein MIPs range from 50% to 95%, depending on the template, matrix and removal method applied\textsuperscript{21}. The problematic diffusion of macromolecular templates is underlined by the fact that even a solution as harsh as 5-15% SDS+acetic acid is not always successful in quantitatively removing the protein from the MIP.

Entrapment of the template can also be caused by covalent bonding of the amino acid side chains (especially Trp, Tyr, Cys and His) to the growing polymer chains upon free radical polymerization, irrespective of the crosslinking density\textsuperscript{22} although this possibility has been ruled out in specific cases\textsuperscript{23}. Removal of covalently bonded template molecules (whether the bonding was intentional or not) is only possible by breaking up the bond(s) tethering the template, or by chopping the protein into small enough pieces so that any fragments remaining in the polymer do not interfere with subsequent rebinding. The latter can be achieved by proteolysis using enzymes of high cleaving specificity, as will be demonstrated in Chapter 6.

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 the polymeric material.

The successful imprinting of small molecules is generally based on a few but strong interactions between the template and the functional monomer(s) whereas proteinaceous
templates offer multiple weak interaction sites. In analogy with the antibody-antigen recognition, such multitude of secondary bonds can yield an altogether strong and selective binding between the protein and the polymer. However, the self-assembly equilibrium in the pre-polymerization mixture has to be shifted towards the formation of such weak, unstable bonds by applying a large excess of functional monomers over the template. These excess functionalities, in turn, will likely provide non-specific interaction sites in the obtained polymer.

By analysing the protein-imprinting literature of 2006-2015, Raim et al. have confirmed the general impression that greater selectivity can be obtained against competitor proteins of widely different isoelectric point from the template\textsuperscript{24}. The role of electrostatic interactions was also shown in Paper II, where the selectivity of a ferritin-imprinted polymer could be tuned by changing the ionic strength of the rebinding buffer. Furthermore, lower cross-reactivity was generally obtained with (i) larger competitors, which can be explained by their smaller mobility and thus reduced penetration in the MIP matrix, (ii) competitors with smaller surface roughness, as protrusions on a protein are more likely to reach the polymer and form nonspecific interactions, and (iii) if the aspect ratios of the competitor and the template were very different\textsuperscript{24}.

The formation of homogeneous binding sites is hindered by the conformational flexibility of proteins; small, rigid globular proteins were found to yield better defined imprints\textsuperscript{25}. However, in the more promising surface imprinting approaches (see section 2.2.3) only a fraction of the protein is embedded in the polymer. This can result in different imprints even of well-defined proteins unless we control which fraction of each template molecule is embedded in the MIP. Orientation of the template during polymerization can be achieved by specific anchors (e.g. a natural ligand\textsuperscript{26}) but such options are not available for every protein. Consequently, protein-MIPs are more often imprinted randomly, resulting in “polyclonal” recognition sites\textsuperscript{27}.

All these difficulties caused a considerable lag in the development of protein MIPs compared to small molecule imprinting. So far, the range of implemented protein targets is very narrow and rather restricted to templates having properties that facilitate 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 facilitate the formation of strong and/ or selective interactions such as electrostatic interaction between the positively charged proteins (e.g., lysozyme\textsuperscript{28}, avidin\textsuperscript{29}) and negatively charged polymers as well as between glycan moieties and aminophenyl boronic acid (APBA) monomers\textsuperscript{30}. 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.


2.2.2. Protein imprinting strategies

During the past decade different strategies have been introduced to overcome the barriers of protein imprinting and these were reviewed in several monographies\textsuperscript{21, 31-38}. 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 (Figure 2). The first approach, using \textit{lightly cross-linked hydrogels} similar to the ones used in gel electrophoresis, was initiated by Hjertén and his coworkers\textsuperscript{23, 39}. Though not many direct evidences, the formation of highly permeable polymer structures or gels seem to not fulfill expectations with respect to the quality and stability of the imprints owing to excessive chain flexibility\textsuperscript{38}. Another important approach is the \textit{“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\textsuperscript{40-41}. However, the rebinding of the targeted, much larger native protein may be still hindered in the MIP monolith. Therefore \textit{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 a solid surface\textsuperscript{42-43}. 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 \textit{microcontact imprinting} techniques\textsuperscript{28, 44} and \textit{sacrificial template support} methods\textsuperscript{29, 45}, or creating very \textit{thin polymer films} with thickness comparable to the template’s dimensions. Being relevant to my thesis work, the surface imprinting techniques will be discussed in detail in the following sections, with emphasis on sacrificial support methods and electrochemical polymerization as a tool for creating thin films.
2.2.3. Surface imprinting techniques

In the classical bulk imprinting method the template is dissolved in the pre-polymerization mixture and becomes randomly incorporated in the forming polymer. By chance, some of the generated binding sites will be found on the polymer’s surface but the majority will be buried in the polymer matrix. By limiting the polymer’s thickness to the size of the template, all the imprints will be inherently located on the polymer surface (Figure 2f-j). This can be achieved by various physical or chemical confinement techniques but the most precise control over the polymer growth is obtained with electrochemical deposition. These methods will be discussed in section 2.2.4 and 2.2.5.

Another approach to confine the binding site formation to the polymer surface is micro-contact printing whereby a stamp carrying the template protein is pressed against the forming polymer material and is removed when the polymerization is finished, much like in the process of stamping a sealing wax (Figure 2d). Generally, a stamp substrate (glass or quartz slide) is modified with the protein template by physical adsorption from its solution followed by drying, or by chemical tethering. Another substrate is then wetted with the monomer solution and the stamp is pressed against it while e.g. UV irradiation turns the
monomers into a polymeric network. The stamp is finally lifted off to reveal the imprinted sites on the polymer’s surface. Chou’s group used this approach to screen functional monomers for the most selective imprinting of various proteins\textsuperscript{28, 46-48}. By applying only 4 µl of monomer solutions and small glass slides as stamps, they could efficiently prepare dozens of samples simultaneously. Dickert’s group extended the range of templates imprinted with the micro-contact approach to viruses and cells\textsuperscript{44, 49-50}. Takeuchi’s group created a protein array as stamp and used it to fabricate an array of MIP spots, each imprinted with the corresponding protein on the stamp (Figure 3)\textsuperscript{51}. A 35-hole PDMS mold was used to immobilize different proteins on the stamp surface and the same mold was aligned later on the prepared MIP spots to create wells with MIP layers on their bottom. The binding properties of the polymers were evaluated in batch mode, using fluorescent labelled proteins. In a follow-up paper they prepared the MIP array on SPR chips and interrogated them using SPR imaging\textsuperscript{52}. Although the stamp could be theoretically re-used, it was not recommended due to the fragility of proteins.

A third approach is to immobilize the template to a \textit{sacrificial support} which is removed after the polymer formation around the template is finished (Figure 2e). The work of Shi et al. can be regarded as the first example of this method: they immobilized various blood proteins on atomically flat mica sheets which ensured that any topographical features created in the polymer layer deposited on top of it reflected the shape of the protein and not the substrate. Denaturation of the proteins upon immobilization was precluded by the hydrophilicity and negative charge of the mica, as well as a protective disaccharide layer coated on the proteins prior to radio frequency assisted polymer deposition. A second support slide was subsequently glued onto the back of the formed polymer layer, and finally the mica support was peeled off (Figure 4a) to expose the binding sites.

A precedent for chromatographically applicable surface-imprinted particles was described by Yilmaz and co-workers who covalently immobilized theophylline on the pore walls of porous silica gel particles which were subsequently wetted with just enough
monomer mixture to fill the pores. After polymerization, the silica was etched off with HF solution to yield macroporous polymer particles with theophylline imprints solely on their surface\(^9\) (Figure 4b). The same idea was pursued (and termed hierarchical imprinting) for the imprinting of peptides and proteins by the group of Sellergren\(^{53-54}\). This approach is especially suitable for creating MIP particles with good chromatographic properties since the diameter and pore size of the obtained particles are tuneable and determined by those of the sacrificial silica particles.

![Figure 4. Sacrificial support-based surface imprinting methods 1: (a) mica support-based thin film deposition\(^{55}\), (b) hierarchical imprinting in porous silica beads](image)

A different format, consisting of polymeric nanowires was developed by Li et al\(^45\). First, aldehyde groups were introduced onto the pore walls of a nanoporous alumina membrane in a multi-step reaction, to which the template protein BSA was covalently immobilized. Chemical polymerization of acrylamide occurred after rinsing away the unbound protein with a fresh monomer solution. Finally, the alumina membrane was dissolved in 1 M NaOH and the template was eluted with an acidified surfactant solution to obtain the surface-imprinted nanowires. Linares et al. have combined this approach with photolithography to create bundles of surface-imprinted nanowires confined to small spots on a glass slide\(^56\).

The sacrificial membrane approach was simplified and adapted to sensor application by Menaker et al. in cooperation with our research group\(^{29}\). The tedious modification of the alumina membrane was entirely omitted by using track-etched polycarbonate (PC) membrane instead, which readily adsorbed the template protein due to its hydrophobic nature by simple incubation with the protein solution. Further improvement compared to Li’s method consisted of replacing the chemical polymerization, which is very difficult to control and spatially confine inside the membrane’s pores with electrochemical polymerization. Menaker
et al. tightened the protein-modified membrane on top of a gold disk electrode and deposited poly(3,4-ethylenedioxy-thiophene) doped with poly(styrene sulphonate) (PEDOT/PSS) in the pores using a potentiostatic square-wave pulse sequence to control the thickness of the growing polymer. When the polymer had filled the pores, the electrode was immersed in chloroform to dissolve the PC membrane and leave behind surface-imprinted PEDOT/PSS microrods attached to the electrode (Figure 5A). By adding magnetic nanoparticles with surface PSS groups to the monomer solution, these were also incorporated in the growing polymeric rods which were then detached from the electrode and collected with a magnet (Figure 5B).

This concept was pursued by Wang’s group too, who sequentially deposited PEDOT/PSS, Pt, Ni and Pt layers on the avidin-modified pore walls of a polycarbonate membrane. After dissolving the membrane, they obtained surface-imprinted multi-layered microtubes which functioned as self-propulsion micromotors in the presence of hydrogen peroxide as fuel. These micromotors were demonstrated to be able to capture avidin while cruising in its solution by oxygen bubble-propulsion and navigation with an external magnet (Figure 5D).

Figure 5. Sacrificial support-based surface imprinting methods - the use of porous membranes. (A) preparation of polymeric microrods using a porous membrane, (B) a variation of concept A by addition of PSS-modified magnetic nanoparticles to the monomer solution in order to obtain magnetic microrods [adapted from ]; (C) fabrication of gold nanoelectrodes in the pores of a polycarbonate membrane for subsequent coating with a protein-imprinted polymer layer; (D) molecularly imprinted micromotors for protein capturing.
The same materials (polycarbonate as sacrificial support, PEDOT/PSS polymer and avidin as model template) were implemented by Lautner et al. to demonstrate the feasibility of large-scale production of surface-imprinted polymer microbands based on standard photolithographic technology (Figure 6). These studies served as basis for developing another sacrificial support-based surface imprinting method, using nanosphere lithography, which allows fine tuning of the binding site density (see Chapter 4).

In another approach, the sacrificial membrane was applied in the nanostructuring of a gold electrode: the pores of a polycarbonate membrane were filled with gold by electroless deposition. After partially etching away the membrane, gold nanoelectrodes were revealed, which were subsequently coated with a protein-imprinted polymer layer (Figure 5C).

2.2.4. Preparation of thin polymer films

As previously mentioned, the binding sites will be inherently located at the surface if the polymer’s thickness does not exceed the template’s dimensions. Very thin polymeric films can be created by physical or chemical confinement of the polymer formation. Physical constraints can be imposed on the system by applying only a very small amount or very thin layer of monomer solution on the substrate, while chemical constraint can appear in the form of limited availability of the initiating radicals or by localized triggering of the polymerization in an otherwise abundant monomer solution.
Physical confinement

Various techniques are available for dispensing very thin layers or minute amounts of precursor solutions including spray-, dip- and spin-coating or microspotting techniques such as dip pen or fountain pen nanolithography, but these techniques have scarcely appeared so far in the MIP literature.

The adaptation of *spin- and dip-coating* to molecular imprinting was retarded by the high volatility and very low viscosity of the solvents classically used in the MIP recipes. To overcome these problems, Schmidt et al. proposed to use a high viscosity solvent for spin-coating the monomer mixture prior to curing with UV light. They were able to tune the obtained films’ porosity and thickness in the 0.1-10 µm range by varying the poly(vinyl acetate) content of the PVA-diglyme porogen mixture.\(^{61-62}\) The aqueous pre-polymerization mixtures generally used in protein imprinting, on the other hand, are readily compatible with spin-coating. Rick and Chou simply mixed the solutions of aminophenylboronic acid functional monomer, cytochrome c or lysozyme template and ammonium persulfate initiator and spun an appropriate volume onto a quartz crystal. The film was then cured and washed with acidic surfactant to remove the template and any unreacted compounds.\(^{63}\) The thickness of the obtained polymer film was not measured in this case but it can be adjusted by varying the monomer concentration and the spin-coating regime.

*Microspotting* methods are used for dispensing nano- or even picoliters of solutions on a substrate. This is generally achieved with a pin (solid, or with an integrated fluidic channel) mounted on an automated stage capable of precise movement in xyz directions. The pin is first dipped in the solution then contacted with the substrate for a fraction of a second upon which a small droplet of the solution is left on the substrate. By repeating this process (generally without the need to re-immersing the pin in the solution after every spot) at different locations on the substrate, an array of droplets can be deposited. Microspotting is widely used in the analysis of biomolecular interactions where capture probes (e.g. various DNA sequences, aptamers or antibodies) are immobilized on the sensor surface by microspotting, followed by challenging the surface with the sample solution. Analyte in the sample will bind to the spots where its complementary probe is immobilized.

The principle of microspotting was adapted to creating molecularly imprinted polymer spots by Haupt’s group who used fountain pen nanolithography to deposit minute amounts of pre-polymerization mixtures on a substrate which was then illuminated with UV light to polymerize the monomer spots.\(^{64}\) An array of 10 silicon-based microcantilevers with integrated fluidic channel served as the “fountain pen”. By immersing the cantilevers in the monomer solution, the fluidic channels were filled by capillary forces. The cantilevers were then moved over and contacted with an activated glass slide using an xyz controller. Typically a few picoliters of solution were dispensed onto the substrate at each contact, and several spots could be deposited without the need to refill the cantilever. They obtained 30-60 µm diameter spots with ca. 0.6 µm thickness. The spot dimensions can be varied.
depending on the dimensions of the pin, the monomer content in the spotted solution and UV irradiation time.

MIP preparation by microspotting and UV curing involves several challenges, namely (i) the very small volumes of pre-polymerization mixture are prone to evaporation before the polymerization is finished, (ii) sufficient adhesion of the MIP layer to the substrate is necessary to ensure the film’s stability during washing steps, (iii) the monomer-content in the pre-polymerization mixture needs to be optimized to obtain the desired film thicknesses.

Chemical confinement

In a different approach the amount (volume) of monomer solution is copious but the polymerization is only triggered at well-defined locations instead of in the bulk. Such methods have mainly been introduced into the molecular imprinting process in order to achieve lateral patterning of the MIP but localization can also be achieved in the z direction.

The majority of the MIP literature is based on thermal or photo-initiation. Localized heating is very challenging to achieve but the precise confinement of electromagnetic fields is well established. However, only a few of the various photolithographic methods have been adapted so far to the field of molecular imprinting and the proof of concept is more often demonstrated with small molecular weight templates. The thicknesses obtained are not always given or exceed tens of nanometres, nevertheless the following methods carry the potential to be used for preparing protein-imprinted thin films.

UV mask projection and interference photolithography (or holography) are mainly suited for controlling the lateral expansion of the polymer, since these methods rely on the illumination of the entire thickness of a sample, through a mask or at locations of constructive interference, respectively. Microstereolithography (µSL), on the other hand, uses a sharply focused laser beam for illumination, and allows the polymerization to be confined in all three dimensions with 1-2 µm resolution. The substrate, covered with monomer solution, is translated in xyz directions with respect to the focal point of the laser beam, according to a pre-defined pattern and photopolymerization only occurs in the illuminated areas. Conrad et al. have demonstrated the feasibility of combining µSL with molecular imprinting by fabricating a waffle-like structure with features as small as 16-65 µm, from an adenine-imprinted polymer, but the principle could be applied to create distinct polymer spots of various compositions as well by exchanging the monomer solution over the substrate after polymerizing each spot. A similar approach was described by Henry et al. who combined microfluidics for liquid exchange with the localized polymerization effect of a laser beam, although in this case, the polymerization was triggered by the heat induced upon irradiation. Typically, 300-400 µm diameter spots were obtained but with the proper choice of parameters they managed to reduce this size to 70 µm. The thickness of the polymer features was not investigated in these works.

Two-photon stereolithography (TPS) allows one to achieve even higher resolution, typically less than ~1 µm features since it relies on the strongly nonlinear process of two-
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Photon absorption. Nanofibers with less than 30 nm diameter as well as various intricate 3D structures were obtained with this method\textsuperscript{71}. Gomez et al. have adapted TPS to the localized polymerization of methacrylic acid and 4-vinylpyridine, two typical monomers used in MIP preparations\textsuperscript{72}. They have demonstrated the potential of TPS by creating various nanostructures, including (i) an intermixed array of MIP and NIP nanodots from which the MIP dots stood out upon incubation of the substrate with a fluorescent-labelled template (Figure 7); (ii) woodpile-type structures and (iii) MIP cantilevers. Typical dimensions of these structures were a few hundred nanometers.

\textbf{Figure 7.} An intermixed array of imprinted and non-imprinted polymer spots. Fluorescence microscopy image was taken after equilibrating the array with the fluorescently labelled template.\textsuperscript{72}

\textit{Polymerization by evanescent wave} (PEW) inherently yields very thin films. An evanescent wave is created in the vicinity of the phase boundary where total internal reflection of a light beam occurs, and this evanescent field can only penetrate a few hundreds of nanometres into the adjacent phases, one of which can be the polymerization medium. The polymer formation cannot exceed this range and the film thickness can be further controlled by adjusting the intensity of the light and the exposure time. Fuchs et al. have demonstrated the feasibility of creating MIP thin films by PEW but the smallest thickness they have prepared was 45-60 nm which still exceeds the dimensions of proteins.\textsuperscript{73} Chegel et al. have used the evanescent wave field generated by a surface plasmon resonance (SPR) sensor to deposit thin MIP film spots \textit{in situ} at the locations of interrogation of the SPR chip surface.\textsuperscript{74} They were able to deposit polymer films with 20-200 nm thickness, depending primarily on the concentration of a light absorbing dye (a precursor of the initiator) but further control could be exercised through the monomer concentration and irradiation time. This method is therefore very promising in terms of creating surface-imprinted thin polymer films although in this paper such application was not demonstrated.
Confinement of the polymerization can also be achieved by limiting the availability of initiator molecules. A widely applied example to this is the immobilization of the initiator to a solid surface, especially of spherical particles which are mixed into the solution of monomers and template. By UV or thermal initiation, the initiator decomposes and chain propagation starts at the particle surface. By varying the irradiation time, 2-10 nm thin layer can be grafted from the surface. However, the most commonly used initiators are azo compounds which undergo homolytic scission yielding two identical radicals, only one of which remains tethered to the surface. The other radical can freely diffuse into the bulk of the solution, initiating polymerization there as well. This not only consumes reagents but can result in the conglutination of the particles. More controlled versions of radical polymerization are therefore preferred, using e.g. dithioesters. When these compounds capture a radical generated e.g. from an azo initiator, they decompose by heterolytic scission into an active radical and a more stable, nonactive radical which does not propagate. By immobilizing the dithioester to the core particles through its active moiety, solution polymerization can be avoided.

While the great majority of MIP preparation methods are variants of chemical polymerization, the electropolymerization of functional monomers emerged as a particularly attractive approach to prepare MIPs for chemical sensing applications. 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 utilize MIPs to confer selectivity. 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, electropolymerization most often requires no external initiator. The lack of initiators is also a major advantage with respect to chemically initiated polymerization, as for instance, the commonly used persulfate initiators are strong oxidizing agents, which may alter the structure of proteins that are susceptible to oxidation.

The precise control over electrosynthesis enables the fine tuning of the polymer layer thickness, which is particularly important for surface imprinting with protein templates. Electrosynthesis can be used straightforwardly to create several nanometer thick polymer films and can be combined with the use of sacrificial materials to prepare micro- and nanostructures with surface confined binding sites to enhance selectivity or binding capacity.

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.
compared to small molecules, not many macromolecular MIPs have been synthesized to date by electrochemical polymerization. Nevertheless, the intrinsic compatibility of the method with protein imprinting and the previously mentioned advantages make it a promising technique. Therefore in the following, separate section (2.2.5) the functional monomers and strategies applied for the generation of electropolymerized MIPs, as well as typical detection techniques will be presented.

2.2.5. Electrochemical polymerization

Functional monomers

Most of the functional monomers traditionally applied in molecular imprinting cannot be polymerized electrochemically unless suitably derivatized. It must be noted, however, that beside direct electrooxidation the versatility of electrochemical synthesis enables polymer deposition also by electrochemically generating the “active” initiator or by electrochemically changing the local pH in the close vicinity of the electrode.

Electrochemical polymerization of monomers (Figure 8) 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) and/or a sufficiently compact film is formed to halt the permeation of the monomer to the electrode.

The first examples of electrosynthesized MIPs were published in a narrow timeframe (1998-1999) by three different groups, introducing phenol, pyrrole, o-phenylenediamine and a porphyrin-compound to the arsenal of monomers used in imprinting. Since then, various other electropolymerizable materials have been explored too, which were reviewed by Sharma et al.

Electrically conducting protein-imprinted polymers are mainly based on the electrochemical oxidation of pyrrole and ethylenedioxythiophene (EDOT) monomers (Figure 8f,g) to form polypyrrole (PPy) and poly(3,4-ethylenedioxythiophene) (PEDOT) 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. Polypyrrole can be additionally overoxidized by applying high potentials (e.g., 1.0 V vs. Ag/AgCl) in buffer or in alkaline solutions in the presence of oxygen. Such overoxidation induces the formation of additional functionalities, e.g., carboxyl groups, along with the loss of conductivity of the respective polymers.

The use of PEDOT-PSS (PEDOT doped with polystyrene sulfonate) for creating different protein-imprinted nanostructures was pioneered in our lab by Gyurcsányi’s group.
The functional groups of this polymer offer various interactions, including hydrogen-bond, electrostatic and π-π interactions and yet it has been found to show very low nonspecific adsorption of proteins. These properties make PEDOT-PSS an excellent candidate for protein imprinting. However, electrosynthesized PEDOT MIPs have only been prepared to date for a single protein template, avidin, and a few small molecular targets such as morphine and atrazine. PEDOT films have also been applied as immobilization matrix of methacrylic acid-based MIP particles in order to provide electrochemical transduction of the binding event.

**Figure 8.** Representative monomers applied for the electrosynthesis of protein-imprinted polymers: (a) phenol, (b) o-aminophenol, (c) o-phenylenediamine, (d) aniline, (e) scopoletin, (f) pyrrole, (g) 3,4-ethylenedioxythiophene, (h) proDOT-COOH, (i) aminophenylboronic acid, (j) dopamine and (k) 2,2′-bithiophene-5-carboxylic acid. Monomers within the dashed line can polymerize into conductive films.

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. 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. Imprinting of polyscopoletin with cytochrome c and concanavalin A has been demonstrated by Scheller’s group and as a results of a collaboration, our group has also started to exploit this polymer [see Papers II and VI].
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\(^{106}\).

Aniline\(^{107}\) and related compounds such as \(o\)-phenylenediamine\(^{108-109}\) and \(o\)-aminophenol\(^{110}\) were also favoured 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 3-aminophenylboronic acid. This is suited for the recognition of glycoproteins through the ability of the \(-\text{B(OH)}_2\) moiety to form under alkaline conditions reversible covalent bonds with vicinal diols that are commonly found in carbohydrates\(^{111}\) and glycoproteins\(^{112}\). This opportunity, although well exploited in case of chemically synthesized pAPBA MIPs\(^{113-114}\), has not been employed with electropolymerized films, which have only been prepared to date for the recognition of BSA\(^{115}\), lysozyme and cytochrome c\(^{116}\); 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\(^{117}\). Future works with electrosynthesized pAPBA MIPs for glycoprotein recognition should consider oriented immobilization of the template glycoprotein, which was found essential in obtaining satisfactory selectivity against other glycoproteins\(^{113}\). Beside biocompatibility, a wide variety of functional groups and extensive knowledge about the formed polymers motivated the use of dopamine\(^{118-119}\) and acrylamide\(^{89,120}\) 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 also enables 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\(^{57}\), which in turn enables their application for separation and pre-concentration.

**Electrosynthesis strategies**

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. Generally, excessively 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\(^{98,121}\). Potentiostatic deposition lacks control over film compactness, but the film thickness can be
precisely adjusted by the charge consumed during electropolymerization\textsuperscript{57, 86}. Furthermore, the applied potential can be pulsed in order to create films with enhanced adhesion to the electrode surface\textsuperscript{29}. The application of pulsed potential aids also the replenishment of the depleted solution layer adjacent to the electrode by the low diffusivity macromolecular template\textsuperscript{122}, and thus enhances the incorporation of the protein template into the growing polymer.

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 Paper III 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 a clear imprinting effect (Figure 9A). 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\textsuperscript{108, 123}; while Kan\textsuperscript{98} and Li\textsuperscript{89} 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\textsuperscript{117} (Figure 9B). 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.\textsuperscript{106}. 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\textsuperscript{122}.

The immobilization of the protein through a self-assembled anchor layer offers advantages over random immobilization or electropolymerization from a protein-monomer solution in terms of generating uniformly accessible binding sites. Li et al. have demonstrated\textsuperscript{124} that covalent immobilization of the template prior to polymer deposition can significantly enhance the MIP’s analytical performance: the 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\textsuperscript{105} instead of free protein\textsuperscript{30} to create selective recognition sites for cytochrome c in a polyscopoletin film. This approach was shown to be compatible with epitope imprinting by Dechtrirat et al.\textsuperscript{105} They 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\textsuperscript{125}. After template removal a short fragment of the DNA, an aptamer was rebound. Here, however, the MIP only served as a
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means of immobilizing the aptamer, which was in turn responsible for the recognition of the thrombin analyte.

Figure 9. Surface imprinting methodologies for the electrosynthesis of MIP films for protein recognition: 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) [Paper III]

The oriented immobilization of proteins via self-assembled anchor molecules which can reversibly bind to the protein through noncovalent 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 interaction with the anchoring molecule. The concept was demonstrated by using thiolated oligoethylene glycol (OEG)/mannose monolayer self-assembled on the transducer surface to immobilize the carbohydrate binding protein, concanavalin A (ConA)\textsuperscript{26}. 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 Bosserdt 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\textsuperscript{30}. Recently the enzyme acetylcholinesterase (AChE) was “vectorially” bound via its peripheral anionic site to a propidium terminated SAM prior to the deposition of an ultrathin polymer film of a ProDOT derivative\textsuperscript{84} (Figure 9C). Propidium which is a weak inhibitor of AChE was thereby made part of the binding site in the MIP nanofilm and was shown to be mainly responsible for the selective binding of AChE. The imprinted ProDOT film acts as a shape selective filter enabling the selective access of AChE to the propidium moiety and 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.

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 in this respect and the first report on solution dispersed electrosynthesised MIP particles used the pores of an avidin-coated track-etched membrane as a microreactor to generate surface imprinted microrods\(^{57}\) (Figure 5B). The combination of electrosynthesis with the use of sacrificial supports, has led to the creation of various micro- and nanostructures with high binding capacity, including nanorods\(^{58, 126}\) and microbands\(^{60}\) (Figure 5). These methods were presented in section 2.2.3.

### 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 method is a trade-off between complete removal of the imprinted protein\(^{60, 109}\) and preservation of the integrity of the binding sites\(^{58, 117}\). Accordingly, different methods were applied that range from the application of chaotropic agents to the denaturation, dissociation or lysis of the protein templates.

Globular proteins have been extracted by 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\(^{30, 122, 125}\) or basic\(^{57}\) solutions and/or surfactants (sodium dodecylsulfate (SDS)\(^{106, 127}\) or Tween 20\(^{116-117}\)), sometimes at elevated temperatures\(^{98, 108}\). 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 with mercaptoethanol\(^{119}\), while the imine bond is easily hydrolyzed using oxalic acid\(^{124}\). Sometimes the protein is removed together with the sacrificial support to which it was immobilized, as in the works of Menaker et al.\(^{29}\), Lautner et al.\(^{60}\) and Orozco et al.\(^{59}\), where the polycarbonate-based support was dissolved in chloroform or dichloromethane without any further steps.

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\(^{105}\), while Kong et al. changed the charge of the polypyrrole film by overoxidation, which thereby rejected the now identically charged template\(^{128}\).
An alternative to harsh solvent extraction is the degradation of the protein target by proteolytic digestion using a protease enzyme. This approach has been scarcely used even though digestion should be much more efficient than simple solvent extraction methods, especially in the case of covalently immobilized templates. The reported examples of template removal by enzymatic digestion were performed under largely different conditions and their efficiency was rarely determined. These facts have motivated us to investigate the digestion procedure with proteinase K and to develop a generally applicable procedure for effective template removal using proteolysis. For further details, see Chapter 6.

2.4. Detection of the target binding

The most important parameters characterizing the performance of MIPs are the affinity and selectivity of target rebinding which determine the dynamic concentration range including the lower limit of detection and the cross reactivity. In the early papers MIPs were characterized as chromatographic stationary phases or in batch rebinding assays where the analyte solution of known concentration was equilibrated with the polymer followed by quantitating the unbound fraction in the supernatant. The development of various novel MIP formats and integration of MIP layers with sensor surfaces opened the route for many other detection methods. In general, the rebinding of the target is quantified either by methods which indicate directly the presence of the target in the MIP layer or by “indirect” methods which evaluate the change in the signal of a marker molecule. In the following section I will focus on protein detection methods but many of them are not exclusive to macromolecular targets.

Direct detection of the template protein by Raman and FTIR spectroscopy offers a rather specific mean to confirm 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 and the assessment of local enzymatic activity, 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 a competitive manner. This is not the case for SPR and QCM 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 the presence of such 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 of the polymer with a protein template, in fact the generated surface will be a mixture of imprinted and non-imprinted polymer (NIP) so the lack of selectivity may easily originate from binding to the NIP fraction 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 lysozyme when testing the selectivity of cytochrome c-imprinted polymers by SPR. However, when
injecting lysozyme first and then the template the signal increased roughly with the same amount as when the template was injected on a lysozyme-free MIP. This suggests that the lysozyme does not bind to surface imprinted sites, but elsewhere on the MIP, while still contributing to the SPR signal. Despite this deficiency both SPR and QCM offer very sensitive means to evaluate the MIP-target interactions. 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)\(^{84}\). While this method is by no means suitable for sensing application it offered clear evidence for the formation of protein imprints (cavities) in highly conformal surface imprinted polymer layers and for the subsequent binding of proteins.

Labelled targets were also used for the direct assessment of template removal and rebinding. Measurement of MIP film fluorescence after interaction with a fluorescently labelled target protein\(^{26, 29, 105}\) 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 cyclic voltammetry\(^{109, 123-124}\) or electrochemical impedance spectroscopy\(^{121, 127}\) the permeability of the polymer film for a redox marker such as ferrocyanide or ferrocenecarboxylic acid\(^{106}\). 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 (Figure 10b). 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 stands 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\textsuperscript{109, 121}. Carbon nanotubes\textsuperscript{107, 127} 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.\textsuperscript{126} 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.\textsuperscript{106} Microdendrites can also improve electronic transfer rates and the effective electrode area.\textsuperscript{89}

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 of 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 unrealistic high IF values. Moreover, the determination of the imprinting factor is influenced by the method which was used to measure the target 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.
# Materials and methods

## 3.1. Chemicals and reagents

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<tr>
<th>name</th>
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<tr>
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<tr>
<td>toluene</td>
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### Materials and methods

#### Coupling and other reagents

<table>
<thead>
<tr>
<th>name</th>
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<th>purity, properties</th>
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<td>succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin)</td>
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<td>tris(2-carboxyethyl)phosphine hydrochloride (TCEP)</td>
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<td>&gt;98%</td>
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<td>&gt;99%</td>
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<td>3,3’,5,5’-Tetramethylbenzidine Liquid Substrate System for ELISA (TMB)</td>
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<td>BCA assay kit</td>
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<td>Polysciences</td>
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<td>Polybead Amino</td>
<td>Polysciences</td>
<td>polystyrene beads with primary amine surface groups, nominal Ø750 nm</td>
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</table>

*SA – Sigma Aldrich, TFS – Thermo Fischer Scientific

### 3.2. Patterning methods

#### 3.2.1. Nanosphere lithography

**Synthesis of avidin-polymer bead conjugates:**

To be used for preparing **Av-MIP1**: The Polybead Amino stock solution was diluted 10 times with PBS followed by the addition of NHS-SS-Biotin (100 µL, 10 mM) in DMSO. The suspension was incubated under continuous shaking for 60 min and then centrifuged at 16 660g for 10 min. The supernatant was discarded, and the particles were resuspended in PBS (900 µL). This procedure was repeated five times to separate the biotinylated nanoparticle from the free reagent. Further modification with Av or FITC-labelled Av was made by mixing the protein in PBS (100 µL, 10 mg mL⁻¹) with biotinylated bead suspension (900 µL) for 60 min. The beads were collected by centrifugation (at 16 660g for 10 min) and washed five times with DI water, followed by resuspension in DI water (1000 µL) to give a final concentration of ca. 10¹⁰ particles mL⁻¹.
To be used for preparing Av-MIP2 and Av-MIP3: 6.25 mg of 0.792 µm diameter Polybead Carboxylate beads were suspended in 85 µl phosphate buffer (PB) pH 5 and activated with 10 µl EDC (200 mg/mL) for 15 minutes. Next, AEDP (450 µl, 5 mg/mL) was added to the activated beads and allowed to react for 60 min. The beads were then collected by centrifugation (10 min, 12000g) and were washed 5 times with PB, and finally resuspended in PB to give a concentration of 29 mg/mL. The carboxyl group of the AEDP moiety was activated with 10 µl 200 mg/mL EDC for 15 min with end-over-end mixing, followed by diluting the suspension ten times with PBS pH 7.4 and adding 2.5 mg avidin to the beads. After 60 min mixing the beads were collected by centrifugation (10 min, 12000g) and were washed twice with PBS and thrice with ultrapure water, and finally resuspended in ultrapure water to give a concentration of 2.5 mg/mL (ca. $10^{10}$ particles mL$^{-1}$).

**Self-assembly of the beads:**

The surface of the gold electrode (0.205 cm$^2$) on a 10 MHz AT-cut gold-coated quartz crystal resonator (Gamry Instruments, Warminster, PA, USA) was pretreated in a UV ozone cleaner (Novascan PSD Pro UV Ozone System) for 15 min. An aqueous suspension of Av-modified beads (0.13 w/v%) was drop cast onto the surface to provide a calculated surface coverage of $8.98 \times 10^8$ beads cm$^{-2}$ and slowly dried at a controlled relative humidity of 75% (T = 23°C). The relative humidity in the sealed drying chamber was kept constant by the presence of wet NaCl crystals. The subsequently deposited polymer film was patterned with the obtained bead array.

**3.2.2. Microelectrospotting**

For microelectrospotting a 10 µL polypropylene micropipette tip (epTIP 0.5–20 µL; Eppendorf, Hamburg, Germany) integrating a Ag/AgCl wire that served as reference and counter electrode was used. To prepare this electrospotting tip the side wall of the micropipette tip was pierced and a 500 µm diameter Ag/AgCl wire was inserted inside until its end was at ca. 2 mm from the tip opening. Then the hole around the wire was sealed with melted Parafilm M®. The tip was filled with freshly prepared monomer cocktail and moved to a precise location on the gold SPR chip that acted as the working electrode. After the polymer spot was formed, the spotting tip was withdrawn and rinsed with water, and the gold SPR chip was repositioned for the next spot using a motorized stage (Newport). Repeating the microelectrospotting at different locations resulted in a protein-MIP-microarray with ca. 500 µm diameter spots.

**3.3. Polymerization methods**

**3.3.1. Electrochemical polymerization**

**Potentiostatic polymer deposition**

PEDOT/PSS was deposited potentiostatically within the voids of the self-assembled particle array (3.2) by polarizing the surface of the gold-coated QCM crystal at 0.9 V (reference electrode: Ag/AgCl/3M NaCl, counter electrode: Pt, potentiostat: µAutolab Type
II) in an aqueous solution of 10 mM EDOT and 25 mM NaPSS. The amount of film deposited (the thickness of the layer) was controlled by the electrical charge passed during the electropolymerization. (Av-MIP1-3)

**Potentiodynamic deposition with potential pulses**

The microelectrosploting tip (with integrated Ag/AgCl wire as reference/counter electrode) was filled with monomer solution (aqueous solution of 1 mM scopoletin, 10 mM NaCl and 5, 10 or 25 µM ferritin) and positioned on the SPR chip. A potential pulse program was applied involving a number of cycles in which the working electrode (the SPR chip) was polarized at 0 V for 1 s and at 0.9 V for 0.1 s (potentiostat: Autolab PGSTAT12). The thickness of the polymer film was controlled by the electrical charge passed during electropolymerization. (fer-spotMIPs)

**3.3.2. Free radical polymerization**

An HRP-imprinted polyacrylamide shell was created by free radical polymerization on the surface of functionalized silica core particles. (HRP-MIP)

First, silica core particles were synthesized by the Stöber method. Briefly, 10 mL TEOS, 133 mL ethanol, 5.667 mL 25% ammonia solution and 1.575 mL water were mixed and stirred for 24h. The obtained particles were collected by centrifugation at 2,934 relative centrifugal force for 60 min, washed four times with ethanol and dried under vacuum at room temperature. Second, surface amino groups were introduced by reacting 0.67 g silica particles, dispersed in 12 mL anhydrous toluene, with 0.24 mL APTES for 15h under stirring. The particles were washed 3 times with acetone and dried under vacuum at room temperature. In a third step CPPA was immobilized to the aminated silica particles by EDC coupling. To this end, 25 mg NH$_2$-functionalized silica particle was mixed with 64 µmol CPPA and 128 µmol EDC in 1.25 mL 80:20 v/v% acetonitrile:water and was agitated for 2 hours at room temperature. The particles were washed with acetonitrile three times and resuspended in 0.5 mL acetonitrile. In the fourth step, 158 nmol (6.912 mg) periodate activated HRP and 369 µmol sodium cyanoborohydride in 7 mL PBS pH 7.4 were added to 48 mg CPPA-functionalized silica particle and gently agitated for 1 hour at room temperature. The particles were then washed with PBS 5 times. The fifth step consisted of polymerizing a molecularly imprinted shell on the particles by mixing 47 mg HRP-conjugated silica particle with 42.9 mg N-isopropylacrylamide, 3.4 mg EBis, 3.17 mg APS and 2.05 µl TEMED in 9.2 mL water. After 72h at 45°C the particles were washed with water 5 times.
3.4. Template removal methods

3.4.1. Solvent extraction

The template proteins were removed from the polycaprolactin film prepared with free template by rinses or immersion in various wash solutions. Namely, the SPR chip containing microelectrospotted ferritin-imprinted films (fer-spotMIPs) was first rinsed with water to remove the unreacted monomers. In order to reduce non-specific protein adsorption to the unspotted gold surface, these areas were blocked by incubating the chip for 30 min in 1 mM HS-TEG solution made with PBS buffer followed by rinsing with water and drying with a stream of nitrogen. The template was removed in the SPR flow-cell by flowing 5 mM NaOH solution over the films for 20 min followed by ultrapure water for another 20 min.

3.4.2. Chemical cleaving and solvent extraction

Covalently immobilized templates cannot be removed by simple solvent extraction. Therefore, in the case of Av-MIP1 and Av-MIP2 the disulphide bridge of the linker attaching avidin to the latex beads was first cleaved with 50 mM TCEP for 30 min to release the protein which was then rinsed out of the polymer with ethanol or 0.05% Tween20, respectively, and water. After drying with nitrogen, the crystals were immersed in toluene to dissolve the latex beads. The bare gold surfaces exposed upon this step were finally blocked with 0.1 mM HS-TEG in water for 60 min.

3.4.3. Enzymatic cleaving and solvent extraction

In the case of Av-MIP3 and HRP-MIP, the covalently attached template was hydrolysed into smaller fragments with a protease. Briefly, 10 µl 0.2% RapiGestTM SF surfactant was added either to the quartz crystal coated with avidin-imprinted MIP (pre-wetted with 25 µl ammonium acetate buffer, 10 mM, pH 8) or to 3 mg of HRP-imprinted core-shell beads suspended in 25 µl buffer. After addition of 3 µl 100 mM DTT the sample was shaken at 250rpm for 60 min. Next, 27.5 µl CaCl2 (final concentration: 4.3 mM) and 4.7 µl 200 mM IAM were added and the sample was kept in the dark for 45 min. Finally, 25 µl 1 mg/mL proteinase K was added and the digestion was allowed to proceed at 37°C with gentle shaking for at least 8 hours. The supernatant was removed and acidified for subsequent HPLC-MS/MS analysis while the polymer samples were treated as follows. The Av-MIP3 coated crystal was rinsed with 0.05% Tween20 and water, in order to remove the protein fragments from the polymer. After drying with nitrogen, the crystal was immersed in toluene to dissolve the latex beads. The bare gold surfaces exposed upon this step were finally blocked with 0.1 mM HS-TEG in water for 60 min. The HRP-MIP core-shell beads were washed with methanol:water 1:1 mixture containing 1% acetic acid to remove the fragments.

In all cases, control polymers (non-imprinted polymers, NIPs) were also prepared in the exact same manner as the MIPs but with omission of the protein template from the process.
3.5. Characterization methods

3.5.1. Particle size

The size distribution of the Stöber silica particles was determined by dynamic light scattering on a ZetaSizer Nano ZS instrument (Malvern Instruments, Malvern, UK). The silica particles, dispersed in the reaction medium or in fresh ethanol, were transferred to a 1 cm disposable cuvette. Measurement position and attenuation were automatically selected by the Malvern Zetasizer Software v7.01.

3.5.2. Morphology

The morphology of the self-assembled bead array in the nanosphere lithography experiments was examined by optical and scanning electron microscopy (SEM). Optical micrographs were recorded with a MicroQ W130 digital eyepiece (1.3 MP resolution, Budapesti Távcső Centrum, Budapest, H) mounted in the ocular tube of an Olympus BH-2 optical microscope, using ToupView software (ToupTek, Hangzhou, CN). Electron micrographs were recorded with a JEOL JSM-5500LV scanning electron microscope.

The morphology of the NSL-patterned polymer films was investigated with an easyScan2 atomic force microscope (Nanosurf AF, Liestal, CH) in dynamic force mode. The thickness of these polymer films was estimated from the topography images.

3.5.3. Polymer thickness

The thickness of polyacrylamide shell grown on silica core particles was estimated from thermogravimetric measurements performed on an SDT 2960 simultaneous thermal analyser (TA Instruments, New Castle, DE, USA). The sample was heated to 750°C with 10°C/min ramp using a 15min isotherm period at 105°C, in 130 mL/min air flow. The thickness of the polymer shell was calculated from the difference in the total weight loss between a polymer-coated and uncoated silica sample.

3.5.4. Particle coatings

Latex and silica particles were coated with proteins or other molecules throughout the work. The following methods were used to examine the success of these coatings.

Eriochrome Black T dye-binding assay

The amount of surface amine groups introduced onto silica particles by reaction with APTES (for the preparation of HRP-MIP) was determined by equilibrating the particles with a concentrated solution of Eriochrome Black T dye in 1 mM HCl followed by washing the particles with 1 mM HCl to remove the excess, unbound dye. By transferring the particles into a basic medium (1 mM NaOH) the bound dye molecules were released from the particles’ surface and their amount was calculated from the supernatant’s absorbance at 620 nm. The NH₂-coverage of the particles was calculated from this value assuming 1:1 stoichiometry between the dye and surface NH₂ groups.
Further modification of the silica particles with CPPA was quantified with the same assay from the difference between the NH\textsubscript{2}-coverage measured before and after CPPA-coupling.

**TMB enzyme activity assay**

The amount of HRP immobilized on the silica particles (for the preparation of HRP-MIP) was quantified by measuring the peroxidatic activity with TMB liquid substrate system following the manufacturer’s manual. Briefly, HRP-modified particles were incubated for 30 min with TMB followed by addition of 1 M H\textsubscript{2}SO\textsubscript{4} to stop the reaction. The absorbance of the supernatant was measured at 450 nm from which the amount of HRP in the sample was determined based on a calibration curve constructed with HRP solutions. This method is expected to underestimate the HRP-coverage of the particles because the enzyme activity of immobilized HRP might be lower than that of the free enzyme.

**Fluorescence microscopy**

The modification of the latex beads with Av and their removal from the PEDOT/PSS surface (Av-MIP\textsubscript{1}) was confirmed by using fluorescent labelled Av (Av-FITC) and a hyperspectral optical imaging system consisting of an Olympus IX71 inverted epifluorescence microscope and a Pariss hyperspectral imaging system (LightForm Inc., Asheville, NC, USA). The fluorescence intensities were recorded in at least 240 different spots on the surface using an LCPLFL20XPH/0.40 long working distance objective and a U-MNB2 fluorescence filter cube (narrow band excitation (470–490 nm), 500 nm cut-off dichroic mirror and a long pass 520 nm emission filter).

**Zeta potential**

The modification of the latex beads with Av (Av-MIP\textsubscript{1}) was also confirmed by measuring the shift in their zeta potential. Commercial polystyrene latex beads, unmodified or bearing surface carboxyl or amine groups, as well as the Av-coated beads were suspended in 10 mM KCl in ~10\textsuperscript{8} mL\textsuperscript{-1} concentration. The zeta potential was measured with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) dynamic light scattering instrument.

**BCA total protein assay**

In the course of preparing Av-MIP\textsubscript{2} and Av-MIP\textsubscript{3} the modification of the latex beads with Av was quantified with a modified biuret test, the bicinchoninic acid (BCA) total protein assay following the manufacturer’s instructions. Briefly, the bead suspension was mixed with an equal volume of the Micro BCA working reagent (Thermo Fischer) and shaken for 60 min at 60°C. After cooling to room temperature the suspension was centrifuged and the supernatant was transferred to a microplate where its absorbance at 562 nm was measured from which the amount of Av on the beads was determined based on a calibration curve constructed with Av solutions.
3.5.5. Rebinding

**QCM**

Protein binding to the Av-MIP films was monitored by quartz crystal microbalance. The crystal was mounted into a combined cell (ALS Co. Ltd, Tokyo, JP) with the polymer-modified side facing either the flow or the static cell piece and was connected to a Gamry eQCM 10M electrochemical quartz crystal microbalance. In experiments conducted in flow mode (volume of flow cell: 120 µL) the protein solutions (250 µL) were injected into TET carrier solution flown at a rate of 60 µL min^{-1}. Calibrations were carried out by consecutive injections of increasing concentrations of Av solutions, without regenerating the surface between the injections. During the selectivity measurements, the polymer surface was regenerated by injecting 1 M NaCl into the flow cell after each protein adsorption step.

In other experiments the static cell was filled with 500 µl PBT. After obtaining a stable baseline, the binding of avidin was measured by replacing 50 µl portions of the solution with avidin stock solutions of increasing concentration and recording the stabilized frequency after each step.

In both cases the surface concentration of the adsorbed protein was calculated from changes in the resonant frequency using the Sauerbrey equation.

**SPR**

Protein binding to the fer-spotMIPs was monitored by surface plasmon resonance imaging in either a SPRi-Plex II (Horiba Jobin Yvon, Palaiseau, FR) or a FELXChip instrument (Biacore, Uppsala, SE). The SPR signal was simultaneously measured on typically 36 polymer spots and 4 reference spots (on the unspotted gold surface blocked with HS-TEG) for background correction. The baseline was obtained in 0.5 mL/min 10 mM HEPES buffer pH 7.4 followed by injections of 1.6 mL of samples. *In situ* template removal experiments were performed by changing the running buffer to various washing solutions.

**Equilibrium batch rebinding assay**

The amount of HRP bound by the HRP-MIP and NIP was determined by equilibrating the core-shell particles for 1h with HRP solutions of different concentration in a 2500:1 phase ratio (e.g. V=500 µl protein solution added to m=0.2 mg particles) after which the HRP concentration in the supernatant was determined with TMB liquid substrate system following the manufacturer’s manual, as described above. The amount of bound HRP was calculated by subtracting the HRP concentration measured in the supernatant (c_e, mg/mL) from the initial concentration (c_0, mg/mL).

\[
Q \left[ \frac{mg}{g} \right] = (c_0 - c_e) \cdot \frac{V}{m} \cdot 1000 \frac{mg}{mg}
\]
3.5.6. Other methods

HPLC-MS/MS

A Perkin Elmer Series 200 HPLC system was used for analysing the digest samples. 10 µl of the digest was injected on a SeQuant ZIC-pHILIC column (Merck, 150x2.1 mm, 5 µm, 200 Å polymeric beads) with a 20x2.1 mm guard column. The autosampler was set at 10°C. Gradient elution at a flow rate of 0.1 mL/min was performed with acetonitrile (eluent A) and 20 mM ammonium acetate buffer pH 4 (eluent B): equilibration with initial conditions at 33% B, increase to 80% B from 0 to 12 min, reversion to the starting conditions (33% B) from 12 to 12.5 min, re-equilibration with the initial composition from 12.5 to 30 min (total run time: 30 min).

The HPLC system was interfaced with an AB Sciex 4000 QTRAP mass spectrometer (Applied Biosystems, Framingham, MA, USA). The positive electrospray ionization parameters were as follows: curtain gas: 35, collision gas: medium, ionspray voltage: 5500, temperature: 350, nebulizer gas 50, drying gas: 40, entrance potential: 11. The selected amino acids were quantified in the multiple reaction monitoring (MRM) mode, the settings for each monitored transition are listed in Table 1. Analysis and data acquisition were performed using Analyst software, version 1.4.2 (AB SCIEX, Framingham, MA, USA).

Table 1. MS settings for amino acid monitoring

<table>
<thead>
<tr>
<th>amino acid</th>
<th>MRM transition (m/z)</th>
<th>DP</th>
<th>CE</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>valine</td>
<td>118 → 72</td>
<td>40</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>threonine</td>
<td>120 → 103</td>
<td>70</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>(iso)leucine</td>
<td>132 → 86</td>
<td>45</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>166 → 120</td>
<td>35</td>
<td>25</td>
<td>10</td>
</tr>
</tbody>
</table>

DP: declustering potential, CE: collision energy, CXP: collision cell exit potential
4. Nanosphere lithography based protein imprinting

4.1. Introduction

The essential prerequisite of generating macromolecular imprints is to create accessible binding sites amenable for free target exchange between the MIP and the sample phase, i.e. to have binding sites confined to the surface of the MIPs. As described in section 2.2.3, surface imprints can be created by ensuring that the polymer’s thickness is less than the protein’s diameter or by various techniques involving immobilization of the template. In fact, template immobilization can be combined with thin film deposition as well, since it provides several advantages over the template being freely dissolved in the pre-polymerization mixture:

- by restraining the protein’s movement it yields better defined binding sites than those obtained with template molecules freely changing their conformation
- if available, specific anchors can be used to immobilize the template in an oriented manner which further improves homogeneity of the formed imprints
- the protein doesn’t need to be soluble in the monomer solution (although some level of compatibility with the applied solvent is still required to avoid denaturation of the template).

For high binding capacities the surface localization of the binding sites should be combined with fabrication methods leading to high surface area/volume ratios of the respective MIPs. Therefore, generation of polymeric micro- and nanostructures comes as a natural necessity to take full advantage of the surface-imprinted sites. For this purpose, we have adapted nanosphere lithography, a method well-known in the field of plasmonics, where the self-assembly of nanospheres creates a colloidal crystal which serves as mask in the subsequent deposition of the desired material. This method itself can be considered a form of imprinting as well, since after removing the nanospheres the deposited layer retains the mask’s pattern. The introduction of nanosphere lithography to the MIP field was further motivated by the fact that in contrast to imprinting with free template it offers the possibility of oriented imprinting. NSL puts less stringent constraints on the polymer thickness compared to methods where the template is immobilized directly on the electrode hence limiting the appropriate thickness of the embedding polymer layer to the protein’s dimensions. By immobilizing the protein on support beads, the polymer thickness can reach the bead radius which can be orders of magnitude larger than the protein itself without hindering access to the formed binding sites.
The proof of concept (Figure 11) was demonstrated using avidin as template based on previous works\textsuperscript{29, 60} by the group. Avidin is a tetrameric protein found mainly in egg white. Its strong affinity towards biotin is exploited for immobilization in various biochemical assays where the reagent to be immobilized and the recipient surface are biotinylated and conjugated with avidin, respectively. Similarly, we have biotinylated the surface of 0.75 micron aminated latex beads which could then capture avidin in an oriented manner through one of its biotin-binding sites. The protein-modified beads were then drop-casted onto a gold-coated quartz crystal, followed by electrochemical deposition of PEDOT/PSS in the voids of the beads. The choice of PEDOT was motivated by its inherently high biocompatibility and its excellent electrical conductivity. The duration of the electropolymerization was adjusted to grow PEDOT/PSS layers with thicknesses on the order of the radius of the deposited beads. Dissolving the beads resulted in complementary cavities decorated with the protein’s imprint, as demonstrated by the polymer’s ability to selectively rebinding avidin.
Figure 12. Reaction steps of nanosphere lithography based molecular imprinting of avidin with the characterization methods of the respective stages indicated in dashed boxes.
4.2. Results and discussion

The reaction steps of preparing an avidin-imprinted polymer based on nanosphere lithography are summarized in Figure 12, along with the characterization methods applied at various stages of the procedure. In the following sections I will present the details of the polymer synthesis.

4.2.1. Immobilization of avidin

We have immobilized avidin on Polybead Amine latex spheres as described in 3.2.1. Initial experiments have shown that the polystyrene spheres can only be dissolved in toluene if the protein coating is first removed, otherwise a layer of precipitate forms. We have therefore chosen a cleavable crosslinker, succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate to immobilize avidin on the spheres. After coating the spheres with the crosslinker, avidin was added to bind to the biotin end of the crosslinker. Since one avidin molecule contains four biotin-binding sites, this step had to be optimized in terms of avidin-excess and bead concentration in order to avoid aggregation of the beads by avidin-crosslinks.

The protein coating was verified by measuring the zeta potential of the beads before and after coating. The modification with Av having a pI of ca. 10.5 resulted in a significant shift of the zeta potential of the aliphatic amine modified beads to more positive, i.e. from −29.6 mV to +6.7 mV (Figure 13).

4.2.2. Deposition of Av-coated beads onto gold-coated quartz crystals

The latex spheres were simply drop-casted on the surface of a gold-coated quartz crystal and the suspension was allowed to evaporate to deposit the beads. We have found that the beads’ arrangement was closer to the ideal hexagonal packing if (i) the gold surface was pretreated in a UV/ozone chamber for 15-30 min immediately prior to drop-casting the beads, (ii) the suspension was allowed to dry slowly by setting the relative humidity to 75%, and (iii) the amount of beads deposited was ca. four times the amount required for a closely-packed monolayer to account for the fraction of particles inevitably expended to multilayer
formation especially near the cell walls. Even so the Av-coated spheres did not arrange as closely as the unmodified (or aminated) spheres (Figure 14). Hypothesizing that the protein quickly adsorbed to the gold substrate thereby anchoring the beads and preventing further migration we have tried to block the gold surface against protein adsorption by coating with a self-assembled monolayer of a thiolated tetra(ethyleneglycol) (HS-TEG). This in fact improved the sphere array’s uniformity but adhesion of PEDOT/PSS polymer film to this HS-TEG layer was poor.

**Figure 14.** Scanning electron micrographs of a layer of (a) unmodified and (b) avidin-coated polystyrene beads. The bars represent 5 µm.

### 4.2.3. Synthesis of the surface-imprinted PEDOT/PSS film

The voids of the sphere array were filled by electrodeposition of PEDOT/PSS from an aqueous solution of 10 mM EDOT and 25 mM NaPSS at 0.9 V (vs. Ag/AgCl). The thickness of the deposited polymer film was controlled by monitoring the polymerization current. Polymers were deposited with different amounts of charge passed and their thickness was determined with atomic force microscopy by Júlia Szűcs. By simple geometrical calculations, the maximum ratio of imprinted to non-imprinted surface could be obtained by embedding the spheres approximately up to their radius, corresponding to 17 mC/cm² charge density.

To liberate the formed binding sites, avidin was first detached from the latex spheres by reducing the disulphide bond of the crosslinker and washing the polymer with water and ethanol. After drying, the film was immersed in toluene where the latex spheres instantly dissolved. The protein removal was necessary to avoid its precipitation in toluene which prevented complete dissolution of the beads. A MIP film prepared using Av-FITC coated spheres was examined with fluorescent microscopy at different stages of its preparation. Removal of the labelled protein from the MIP film was demonstrated by the decrease in fluorescence of the film (Figure 15a,b). The remaining fluorescence at this stage is about the same intensity as the fluorescence of a layer of uncoated latex spheres (Figure 15b'). Dissolving the spheres from the MIP film resulted in further decrease of its fluorescence. The residual fluorescence of the MIP after complete template removal can be attributed to the autofluorescence of the PEDOT/PSS material (Figure 15c').
Nanosphere lithography based protein imprinting

Figure 15. Averaged fluorescence intensity at 528 nm from 240 different spots on the QCM chip surface (excitation: 470-490 nm): (a) Av-FITC modified latex spheres after deposition and embedding in PEDOT/PSS polymer; (b) after removal of Av-FITC by reduction of the disulphide linker; and (c) after dissolution of the latex spheres. Average fluorescence intensities recorded in similar conditions of: (b’) a layer of unmodified latex spheres and (c’) a plain PEDOT/PSS film are shown for comparison. The inset shows the relevant averaged fluorescence emission spectra of the chip’s surface under the conditions described above. The large standard deviation is due to the fact that the fluorescence is measured both from spots where fluorescent spheres are deposited as well as from inter bead voids.

Please note that both the unmodified spheres and the PEDOT/PSS film show “autofluorescence”.

4.2.4. Nanogravimetric measurements of protein binding

The surface-imprinted polymer film-modified 10 MHz quartz crystal chips were mounted into a flow cell and after stabilization of the frequency, increasing concentrations of Av were injected in the carrier buffer and the frequency change was monitored in real-time. To determine the effect of the surface imprinting on the binding properties of the polymer the Av binding to non-imprinted polymer film (NIP) was recorded in similar conditions, but in a separate experiment. The NIP films were synthesized in exactly the same way as the MIPs but nanospheres not modified with Av were used for patterning the PEDOT/PSS film.

Preliminary measurements by Júlia Szűcs suggested high levels of nonspecific protein adsorption which was significantly reduced by (i) blocking with HS-TEG the small gold spots on the MIP-coated quartz crystal exposed upon bead removal and (ii) by adding 0.01% Tween20 to the Tris-EDTA carrier buffer (Figure 16a and b).

Figure 16. Reduction of nonspecific adsorption by (a) blocking with HS-TEG after template removal (filled circles); the same MIP surface without HS-TEG-blocking (open circles) and (b) adding 0.01% Tween20 to the carrier buffer: Av binding to MIP (filled shapes) and NIP (open shapes) in Tris-EDTA buffer (circles) or in Tris-EDTA+0.01% Tween20 buffer (triangles).
Avidin rebinding was repeatedly measured under such conditions on several polymers and the maximum surface coverage observed at the highest injected concentration was 1.384 µg/cm² on the MIP and 0.208 µg/cm² on the NIP (Figure 17) which gives an imprinting factor (IF) of 6.65. This value is higher than the vast majority of imprinting factors reported for acrylic polymers (generally between 1.2 and 4.5) and was among the highest IFs reported for protein MIPs at the time of the publication. However, we could not reassert our previous result of IF≈10 obtained with PEDOT/PSS polymer microbands, most likely due to the relatively large nonimprinted fraction of the surface as a result of the non-compact Av-modified bead layer.

![Figure 17](image)

Figure 17. (a) Typical frequency response of Av binding to MIP and NIP. Injections of 250 µl Av solutions of the following concentrations into 60 µl/min carrier buffer are marked by arrows: 10⁻³, 10⁻², 2×10⁻², 5×10⁻², 10⁻¹, 2×10⁻¹, and 3×10⁻¹ mg mL⁻¹. (b) Binding isotherms of Av to the imprinted (filled squares) and nonimprinted (open squares) PEDOT/PSS patterns. The data points are the average of measurements performed on three different MIPS and NIPS modified quartz crystals.

The selectivity of the Av-imprinted polymer was determined for three avidin species: ExtrAvidin (EA), NeutrAvidin (NA), and Streptavidin (SA); all of them having a near-neutral isoelectric point (EA: 6.5, NA: 6.3, SA: 5.5) and various degrees of structural similarity with avidin. ExtrAvidin and NeutrAvidin are deglycosylated forms of avidin, while Streptavidin is a biotin-binding protein found in the bacterium *Streptomyces avidinii* with only 33% of the amino acid residues identical to those found in Av. The selectivity study further included Lysozyme (Lys), a representative for high isoelectric point (pI) proteins and bovine serum albumin (BSA) with largely different pI, but similar molecular weight. The respective proteins were injected at a concentration of 0.1 mg mL⁻¹ and the amount of protein bound to MIP and NIP surfaces was determined by QCM. The surface was regenerated between measurements with 1 M NaCl. The Av-imprinted PEDOT/PSS film showed clearly the greatest affinity for Av, followed in order by Lys, BSA and the avidin-analogues (Figure 18). The significant Lys adsorption is not surprising as it is one of the most surface active proteins that can form multilayer aggregates upon adsorption to charged surfaces. The relatively high amount of adsorbed Lys having a pI of 11.4 suggests that electrostatic interactions play an important role in the recognition process. However, the Lys adsorption is practically the same on both MIP and NIP surfaces and also similar to the Av adsorption on the NIP, which
suggest inherent adsorption to the non-imprinted PEDOT/PSS material. Given the fivefold increase in the Av adsorption on the MIP as compared with the NIP and Lys adsorption on both MIP and NIP surfaces clearly demonstrates that the Av recognition on the MIP cannot be solely attributed to electrostatic interactions. A major benefit of the proposed method is the remarkably suppressed adsorption of the avidin analogues on the MIP, most spectacularly of EA, which is even lower than that of BSA and significantly better than in earlier studies using randomly immobilized Av for imprinting.

![Figure 18](image.png)

**Figure 18.** Adsorbed amounts of different proteins to Av-imprinted polymer (blue) and NIP (grey). The relative size of the proteins is represented by circles the colour of which denotes the protein’s isoelectric point (pI).

**4.3. Conclusions and outlook**

We have demonstrated that nanosphere lithography can be used to generate surface-imprinted polymers for selective protein recognition. This work was featured on the cover page of *Advanced Functional Materials*. A remarkable selectivity and an imprinting factor close to state of the art were obtained despite of the non-compact nanosphere assembly, which exposes a significant fraction of the non-imprinted surface for interaction. The method proposed has the following potential advantages: (i) the oriented immobilization of the macromolecular template on the nanosphere surface makes feasible site-directed imprinting of the respective macromolecules, (ii) the binding site density on the surface-imprinted polymer can be controlled by adjusting the surface density of the protein coating of the nanospheres, (iii) the nanosphere carrier imposes less strain on the control of the polymer film thickness as compared with surface-confined direct macromolecular imprinting requiring ultrathin smooth films.
Reproducibility of the method was confirmed when new polymers were prepared later with a slight alteration of the Av-coating of the latex spheres (Av-MIP2 and Av-MIP3, 3.2.1) and we obtained almost identical surface coverages when measuring rebinding of Av by QCM in static mode (see chapter 6.2.3).

In the past few years there has been a growing interest in the application of nanosphere lithography for creating various protein sensing elements. Soon after the publication of our work a similar approach was published\(^{124}\) where several layers of silica microspheres were deposited on a gold electrode followed by modification of the array surface with BSA and electrodeposition of polypyrrole in the voids of the array. Finally, the silica was etched to obtain a macroporous MIP film. Yoshizawa et al. also coated the particles with protein after depositing the array\(^{52}\). Although this approach could result in a compact, orderly array but this was not achieved in these works according to microscopy images. Having an ordered bead array opens the way to the precise control over the number of layers incorporated in the polymer. This idea was pursued by Dabrowski et al.\(^{134}\) who used Langmuir-Blodgett technique to deposit exactly four compact layers of silica nanospheres which were subsequently coated with HSA-monomer conjugate. During potentiostatic deposition of a crosslinking monomer they observed slight perturbation in the current corresponding to the embedding of each half layer of the beads. This phenomenon allowed direct control over the thickness of the deposited polymer layer.

The oriented immobilization was achieved in our case by taking advantage of the affinity of avidin towards biotin. Various interactions can be exploited to immobilize other proteins in an oriented manner, e.g. natural ligands, substrate or inhibitor of an enzyme or strong electrostatic interactions (see Electrosynthesis strategies in 2.2.5). However, such specific anchors are not available or not known for every protein which makes the generalization of this method somewhat complicated. If no simpler solution is available, genetic engineering can always be applied to introduce a linker peptide in the target protein, as demonstrated by Liu et al.\(^{135}\)
5. Microelectrospotting

5.1. Introduction

The development of MIPs for new targets usually follows a highly empirical strategy whereby the composition of the pre-polymerization cocktail and the polymerization conditions are optimized by preparing and testing a large number of polymers. This is costly and time-consuming therefore a progress in the field 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.

A method for the rapid screening of reduced-scale bulk polymers have been developed in parallel by Sellergren’s and Takeuchi’s group\textsuperscript{10-11}. They prepared small volumes of monomer cocktails in HPLC autosampler vials which, after polymerization, formed thin polymer disks at the bottom of the vials, called mini-MIPs. The small thickness eliminated the need for grinding and sieving and allowed rapid template elution and rebinding simply by exchanging the supernatant in the vial which then could be analysed by HPLC. The method was further improved, partly in our research group, by automation of the liquid handling\textsuperscript{136-137} and by preparing the polymers on the surface of microfiltration membranes in multiwell filterplates\textsuperscript{138}. These examples were demonstrated with low molecular weight templates while a similar format, resulting in ca. 100 nm thin films of poly(aminophenylboronic acid) on the bottom of microplate wells, was used by Bossi et al. to prepare MIPs for a range of proteins\textsuperscript{139}. An ELISA-mimic assay was developed by Shea’s group to rapidly screen a library of nanoparticles made from various monomers to identify the highest or lowest affinity composition towards a chosen protein target\textsuperscript{140}. Lin et al. combined molecular imprinting with a micro-contact approach which allowed the parallel preparation of dozens of samples\textsuperscript{28}. Real high-throughput optimization, however, can only be performed if both the polymer synthesis and the detection are multiplexed. For this, surface plasmon resonance (SPR) imaging seems to be the most promising technique, coupled with the formation of a MIP microarray.

Lee et al. deposited MIP spots on an SPR slide by UV polymerization through a photomask and developed a microfluidic device which even allowed different analytes to be flown simultaneously over the spots\textsuperscript{141}. In another microfluidic approach, a focused laser beam was applied to thermally polymerize the monomer cocktail at a precise location followed by rinsing the chamber, filling with the next monomer cocktail and repeating the polymerization at a different location\textsuperscript{66}. This method allowed a library of different monomers to be tested quickly but the polymer thickness was difficult to control due to the different reactivities of the monomers. This might not be crucial in the case of small molecular weight templates (although SPR detection does require the sensing layer thickness to be lower than 100 nm) but good protein MIPs can only be prepared by surface imprinting, i.e. by limiting the polymer’s thickness to the size of the template. The polymer’s thickness was inherently
limited in Haupt’s approach where only picoliter volumes of monomer solutions were deposited to each spot using a cantilever with an integrated fluidic channel (fountain pen microlithography) but the concept was only shown for small molecular weight targets. As described in section 2.2.4, Chegel et al. have used the evanescent wave field to deposit thin MIP film spots in situ at the locations of interrogation of the SPR chip surface and have obtained very promising results in terms of precise control of the polymer thickness.

The above examples have all used free radical polymerization and high-throughput techniques for the electrosynthesis of MIPs has not been demonstrated. This hiatus and the inherent suitability of electrosynthesis for protein imprinting and its aptitude for precise control over the polymer deposition process, have motivated us to develop such a technique for the rapid synthesis and screening of electrosynthesized protein MIPs. To achieve this, we have adapted the microspotting technique and fabricated a custom hollow microspotting tip which accommodated a counter. The tip was filled with monomer cocktail then brought into contact with a gold-coated SPR slide. By applying an appropriate potential program between the gold surface, acting as working electrode, and the counter/reference electrode in the tip we successfully deposited polymer spots of ca. 500 µm diameter. By repeating the deposition process, termed microelectrospotting, at different locations on the substrate and by filling the tip with different monomer cocktails we created an array of protein-imprinted polymer films on which the template removal and rebinding process could be tested in real-time and in a multiplexed manner by SPR imaging.

5.2. Results

5.2.1. Fabrication of the microelectrospotting device

SPR chips accommodating an array of biomolecular probes (“biochips”) are routinely fabricated with dedicated microspotter instruments which accommodate a metallic pin attached to a robotic arm. This pin is dipped into the well of a microplate where it becomes coated with the biomolecule solution. The pin is then brought into contact with the SPR slide for the fraction of a second whereby a few nanoliters of the solution are left on the substrate. In order for electropolymerization to be feasible simultaneously with spotting of the solution (to avoid short-circuiting), the metallic pin had to be extended with an insulating capillary in which the pre-polymerization solution is accommodated. I have fabricated such an extension from a piece of a 10 µl Eppendorf pipette tip (Figure 19) on a 375 µm diameter solid stainless steel pin (BioRad Calligrapher). A diagonal cut and hydrophilization in oxygen plasma or coating with polyvinylpyrrolidone of the pipette tip was necessary to obtain satisfactory elevation of the liquid in the capillary extension. With this device I managed to deposit 600 µm diameter PEDOT/PSS spots onto a gold surface but with poor reproducibility. The irregular shape of the polymer spots (Figure 19b) was partly due to the fact that the diagonal edge of the pipette tip could not be evenly pressed against the substrate, and partly to air bubbles being sometimes trapped in the capillary. To eliminate these problems, associated
with the passive filling mechanism of the capillary, we have decided against its use in the microspotting robot and I have fabricated another electrospotting tip.

Figure 19. (a) First prototype of electrospotting tip, based on a BioRad Calligrapher pin, and (b) PEDOT/PSS spots deposited with this tip at 2.0 V for the specified amounts of time, in the absence or presence (where indicated) of 1 mg/mL BSA in the monomer solution of 10 mM EDOT+25 mM NaPSS.

In the second prototype active filling of the tip was at aim. To this end, I used a 10 µl Eppendorf pipette tip and drilled a hole in its side where a Ag/AgCl wire was introduced. After sealing the hole around the wire with parafilm, the tip could be attached to a pipette and the monomer solution could be aspirated and dispensed as usual (Figure 20). The pipette was fixed in a vertical position above a motorized stage (Newport) holding the substrate. The pipette could be lifted or lowered manually with a micrometer screw while the stage could be moved in the x,y directions, controlled by a computer.

Figure 20. (a) Schematic and (b) photo of the second prototype of electrospotting pin, based on a 10 µl pipette tip.

5.2.2. Preparation and testing of polymer arrays

The high-throughput testing potential of the microelectrospotting technique was demonstrated by optimizing the polymerization and rebinding conditions of a ferritin-imprinted MIP. Scopoletin was used as monomer which polymerizes into an insulating film. This is advantageous in terms of inherently limiting the attainable polymer thickness to a few tens of nanometers thereby remaining in the sensitive range of the SPR.
The spotting tip, loaded with 10 µL of monomer solution (with or without the protein template) was placed on a gold SPR chip surface which was polarized as working electrode (Figure 20a) against the Ag/AgCl reference electrode. The scopoletin monomer was intermittently oxidized at 0.9 V to form a thin insulating polymer film over an area delimited by the micropipette tip. The application of pulsed potential was expected to enhance the incorporation of the protein template in the growing film by allowing time for the solution layer adjacent to the electrode to be replenished in the low diffusivity macromolecule. This electrospotting procedure was then repeated at different positions while the composition of the monomer–protein mixture and the electropolymerization conditions were changed to obtain microarrays of surface-imprinted nanometer-thin polymer spots (fer-spotMIPs and NIPs) (Figure 21). The chip was then inserted in the SPR imaging instrument to monitor the rebinding experiments in a label-free manner for each spot. As visible in Figure 21, the area of the MIP spots was larger than those of the NIPs, i.e. the growth of the NIP films was confined to the inner diameter of the pipette tip while the MIP extended to the outer diameter. Since the only difference between the spotted cocktail was the protein content, it is likely that the tip/surface contact area is wetted by the proteins adsorbed on the respective surfaces. For evaluation, in all instances we used the centre of the polymer spots corresponding to the inner area of the spotting tip.

![Figure 21. SPR image of an array of imprinted (D-F) and non-imprinted (A-C) scopoletin polymer spots and the details of the layout.](image)

Preliminary experiments with different polymer thicknesses were performed in Budapest while systematic optimization was conducted at the Fraunhofer Institute by the German co-authors. First the optimal polymer thickness had to be determined because spots with very different thickness were not possible to measure simultaneously with the SPR instrument available at the time due to their largely different plasmon resonance curves. The thickness of the polymer film was optimized in order to incorporate as much of the template as possible without irreversibly entrapping it. As a compromise between the instability of very thin
layers and the restricted target accessibility of increasing film thickness, 200 pulses have been chosen for the electrospotting of ferritin-imprinted MIPs.

**Table 2. Washing solutions tested for template removal**

<table>
<thead>
<tr>
<th>solution</th>
<th>targeted interaction</th>
<th>result</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM glycine-HCl pH 2.0</td>
<td>H-bonds</td>
<td>no effect</td>
</tr>
<tr>
<td>0.05% Tween20</td>
<td>hydrophobic interactions</td>
<td>signal decreased both on MIP and NIP spots</td>
</tr>
<tr>
<td>5 mM NaOH</td>
<td>H-bonds</td>
<td>signal decreased on MIP spots, NIP spots unaffected</td>
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Next, various regeneration solutions able to disrupt different non-covalent bonds were tested (Table 2) and 5 mM NaOH was found to remove the template most efficiently. Rebinding of ferritin was performed in HEPES buffer (10 mM, pH 7.4). Since the polycoproletin surface was found to be negatively charged, NaCl was added to the buffer to shield electrostatic repulsion of ferritin (pI 4.5) which is also negative at the applied pH. Addition of at least 150 mM NaCl to the buffer was found to be effective but even higher ionic strengths further promoted the rebinding of ferritin to the MIP spots. The signal on the NIP was negligible up to 600 mM salt concentration, where further measurements were made. However, the highest imprinting factor, reaching a remarkable value of 13, was observed at 300 mM NaCl concentration.

The amount of template used during polymerization was found to affect the polymer’s binding capacity: more ferritin was rebound by the spots prepared with more template (Figure 22a). The selectivity of the ferritin-MIP was tested by injecting other proteins on the spots. Myoglobin and cytochrome c were barely retained by the ferritin-MIP spots, while some binding was observed with urease and BSA. However, the SPR signal change for these interferents was only 30% of that of ferritin (Figure 22b).

**Figure 22.** (a) Binding isotherms of ferritin on polycoproletin spots imprinted with different templates, measured by SPR in 10 mM HEPES pH 7.4 + 600 mM NaCl at 25°C. Solid lines are fitted Hill functions. (b) Typical SPR sensorgrams of ferritin-imprinted spots (M/T = 1:0.025) obtained upon injections of different proteins at 0.5 µM in 10 mM HEPES pH 7.4 + 600 mM NaCl.
5.3. Conclusions and outlook

With the intent to fill the gap and establish a high throughput synthesis and testing method for the optimization of protein MIPs, we have developed the so called microelectrospotting technique in combination with SPR imaging. The electrically connected spotting tip, which acted as a counter electrode, enclosed the mixture of monomer and template protein, which 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 µm diameter were prepared on SPR imaging gold chips. It was shown that the thickness of the spots is in the low nanometre 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.

Based on these promising results a more sophisticated microelectrospotting device has been purchased and installed in our lab. The HORIBA SPRi-Arrayer is a microarrayer especially suited for spotting on SPR slides and biochips with an option for electrochemical experiments made possible by the application of a metallic pin with ceramic (insulating) tip. Coupling the arrayer with a potentiostat remains to be worked out but the automation of the liquid handling and the uniformity of the obtained spots (Figure 23a) are clear advantages compared to the tedious microelectrospotting using the modified micropipette. The real high-throughput potential of microelectrospotting can be further exploited by using an SPR instrument capable of measuring the reflectivity at multiple resonant angles simultaneously. Such an instrument (HORIBA XelPleX) has also been installed in our lab since the publication of the microelectrospotting method. The need for such a feature soon became apparent, since the resonant angle of each spot depends on its thickness, and spots with widely differing thicknesses are not possible to measure sensitively at one given resonant angle. The thickness of the polymer films was not only affected by the number of potential

![Image of polymer array created by microelectrospotting using the HORIBA SPRI Arrayer](image)

Figure 23. (a) A polymer array created by microelectrospotting using the HORIBA SPRI Arrayer. Electrodeposition by 50 pulses of (0V*5s, 0.9V*1s) from 0.5 mM scopoletin in 10 mM NaCl, additionally containing the indicated amounts of transferrin. (b) Corresponding plasmon curves: background (grey), spots made with 0-1-5-10 µM template (yellow - green - blue - black).
pulses applied for deposition but also on the concentration of the template in the monomer solution (Figure 23b).

Beside MIP optimization, the presented approach may be used to generate MIP arrays as a platform for recognition of various protein panels where the array format might be exploited also as a mean to compensate for contingent cross-reactivities or deficiencies in the selectivity of individual MIPs.
6. Enzymatic digestion as a tool for template removal

6.1. Introduction

Template removal is a key step in the preparation of a molecularly imprinted polymer since this process will liberate selective binding sites. Incomplete template removal leads to loss of binding capacity and the remaining template might later leach out, falsifying measurements. Macromolecular templates, such as proteins, can be challenging to remove from the polymer network due to their large size and hindered mobility. The protein might even become covalently bound to the polymer during free radical polymerization although this has been excluded experimentally in special cases. Intentional immobilization of the template, on the other hand, is often applied to increase the binding site density in thin polymer films or to perform site-directed imprinting for better selectivity. Weak, reversible bonds might break during polymerization, therefore the formation of stable, covalent bonds is preferred for this purpose. This approach usually entails proteolytic digestion as a means of template removal unless a cleavable linker or a sacrificial support is applied for protein immobilization, as for example in Paper I.

Enzymatic digestion has emerged as a tool for template removal in imprinting strategies using free, dissolved template, too, since it can be performed under milder conditions and the obtained peptide fragments are expected to be more easily washed out of the MIP than the intact protein. Therefore proteases with broad cleaving specificity are preferred which are able to break down the protein to very short peptides or even single amino acids, e.g. subtilisin, proteinase K, pronases or pepsin. Trypsin, which breaks down proteins to relatively large peptide fragments was also applied in some cases but satisfactory rebinding was only obtained if the digestion was followed by extensive washes with a surfactant solution. When tryptic digestion was compared with surfactant + acetic acid washes as template removal method it was found that despite the higher template recovery the digested MIP’s binding capacity was 4.5 times poorer than that of the washed MIP. It was hypothesized that the protein fragments remaining in the digested polymer hindered rebinding. Others, in contrast, suppose that such fragments might even be partly responsible for target recognition. Taguchi et al. experimentally proved this concept by creating MIPs in which the template protein was covalently bonded to the polymer at three or five positions, leaving behind three or five peptide fragments after peptic digestion. It is noteworthy that peptic fragments are generally smaller than tryptic fragments so it is possible that larger fragments sterically hinder, while small fragments promote the rebinding by offering additional interaction points. This would further underline the importance of choosing a protease with broad cleaving specificity and ensuring the completion of all possible cleavings.

Current protein digestion protocols used in MIP template removal are rather simple. In most cases the MIP is incubated with a buffered solution of the proteolytic enzyme. The efficiency of template removal is scarcely given in the literature and the experimental
conditions (enzyme concentration, buffer composition and pH, temperature, length) are generally not optimized; only the protease:protein ratio was varied in one paper, and the necessary incubation length was determined in a few others. Pre-treatment of the protein template was never performed, except for three cases, where the template was denatured with surfactant, despite the fact that the folded or denatured state can influence a protein’s susceptibility to proteolytic degradation. Moreover, the presence of disulphide bonds or glycosylation also contributes to the proteolytic resistance of proteins.

We therefore aimed at investigating the efficiency of digestion and setup a generally applicable protocol for the removal of protein templates from molecularly imprinted polymers. For this purpose we have selected one of the proteases with broad cleaving specificity: proteinase K, as it is a single, well-defined enzyme (unlike e.g. pronase, which is a mixture of proteases) and works very efficiently, it can digest even keratin. Proteinase K has already been introduced in the field of molecular imprinting by the group of Sales. They applied the enzyme in 0.4-0.5 mg/mL concentration and used 2.5h or overnight incubation followed by washes with buffer and water. Electrochemical impedance measurements on the MIP indicated the liberation of some binding sites. FTIR, however, suggested that some protein remained in the film. Komarova et al. applied proteinase K in a higher concentration (3 mg/mL) and found that a 2h incubation was enough, the polymer’s resistance did not change further after prolonged incubation. However, the amount of template recovered from the MIP was not estimated. Cutivet et al. compared various methods for template removal, one of which was denaturation of the polymer-entrapped protein at elevated temperature in surfactant solution prior to digesting it with proteinase K. They added Ca$^{2+}$ ions to the protease solution which stabilizes the enzyme against self-digestion. SDS-PAGE analysis of the polymer particles confirmed complete template removal but due to the harsh conditions introduced in their digestion protocol, a different template removal method was chosen for further experiments.

The above examples demonstrate the need for a procedure which ensures complete digestion of the protein but avoids the use of extreme conditions. We first optimized the digestion conditions on HRP, a model protein dissolved in buffer, by monitoring the amount of single amino acids in the digest by HPLC-MS/MS. The optimized procedure was then tested on another protein, avidin in solution, and finally on molecularly imprinted polymers fabricated with covalently immobilized HRP or avidin.
6.2. Results

6.2.1. Optimizing proteinase K digestion on dissolved HRP

We first investigated the digestion of a native protein in solution using proteinase K. We chose HRP as our model protein due to its high stability; its four disulphide bridges and eight glycosylated sites make it a more challenging candidate for digestion. We hypothesized that a procedure able to digest HRP will also be successful on other, less stable proteins. To evaluate the completion of the process we performed an in silico digestion of HRP with proteinase K. PeptideCutter predicts the cleavage sites and resulting peptides of a given amino acid sequence using a given protease or chemical. Proteinase K was predicted to perform 148 cleavages on horseradish peroxidase (sequence according to Welinder et al.) resulting in 1-7 amino acid long peptides. The majority of the single amino acids in the digest are predicted to be leucines (7 mol isoleucine and 15 mol leucine per 1 mol HRP), valine (11 mol), alanine (10 mol), phenylalanine (10 mol) and threonine (9 mol). We have therefore developed an HPLC-MS/MS method to measure the amount of these amino acids in experimental digests and compare them with the predicted values. Alanine was finally not included in our investigations due to difficulties in its quantitation.

Native HRP was digested by mixing it with proteinase K in 1:1 ratio in 10 mM NH₄OAc pH 8 buffer and reacting for 5h at 37°C. In this digest we did not find significantly more amino acids than in a blank digest (containing only buffer and proteinase K) suggesting that most of the amino acids detected are the result of the enzyme’s self-digestion (Figure 24a). We assumed that the possible cleavage sites are not accessible in the folded, native state of the protein and therefore we tried two approaches to facilitate cleaving: (i) denaturation of the protein with a surfactant, and (ii) reduction and alkylation of the disulphide bridges in the protein. For the first purpose, RapiGest™ SF was applied due to its compatibility with MS analysis: this surfactant decomposes into two products in low pH environment, one of which can be removed from the digest as a precipitate, and the other is an ionic compound that does not interfere with MS analysis. The application of RapiGest™ alone, however, was not effective; the amount of amino acids in the digest did not exceed self-digestion levels (Figure 24a). Reduction of the disulphide bridges was performed with DL-dithiothreitol (DTT) and the obtained cysteines were stabilized by alkylation with iodoacetamide (IAM). We have found that disruption of the disulphide bridges made it possible for the proteinase K to access some of the cleavage sites in HRP but the digestion was more complete when the protein was first denatured with the surfactant (Figure 24b). In the previous examples proteinase K was added to HRP in equimass quantities. Proteases are usually applied in much smaller quantities than the protein to be digested (10-1000:1) to minimize self-digestion we therefore performed a digestion on HRP pre-treated with RapiGest™, DTT and IAM using only 10% proteinase K compared to HRP’s mass. As expected, the amount of amino acids ascribable to self-digestion was significantly smaller than with 10 times more proteinase K but the amount of amino acids in the HRP’s digest also decreased. The net digestion was less complete than with a larger amount of protease (Figure 24b). In conclusion, the optimal
performance is obtained when the protein is unfolded, its disulphide bridges are reduced and alkylated prior to digestion which is performed with an equimass quantity of proteinase K.

In order to optimize the duration of the proteolysis we performed digestions for different lengths of time. After 24h the amount of amino acids reached already 78-90% of that obtained after a 70h digestion which could be considered complete (Figure 25). Therefore, we have decided to use a 24 h digestion in the final protocol. The amount of single amino acids obtained with this protocol is 10-87% of those predicted by simulation suggesting that not all of the predicted cleavages were performed. It has to be noted, however, that the accuracy of the simulation is limited since it works with quite simple rules and does not take into account any post-translational modifications in the protein, e.g. glycosylation, which could block a cleavage site. On the other hand we believe it is unpractical to further expand the digestion procedure because the obtained amounts of amino acids suggest that the protein is cut into small enough pieces to be easily washed out of a molecularly imprinted polymer. The final protocol is described in Figure 26.

Figure 24. Amino acid quantities found in the digests of HRP. (a) Digest of native (empty bars) and RapiGest™-denatured HRP (chequered bars) and blank digest (self-digestion of proteinase K, striped), (b) digest of HRP pre-treated with DTT and IAM (empty), pre-treated with RapiGest™, DTT and IAM (striped), pre-treated with RapiGest™, DTT and IAM using only 10% proteinase K (chequered). Values in (b) are corrected with the corresponding blank values. Horizontal lines indicate the amount of each amino acid predicted to be found in the digest by the PeptideCutter software.

Figure 25. Evolution of the amount of selected amino acids found in the digest of HRP compared to their maximum amounts found after 70h digestion; threonine (diamond), phenylalanine (circle), leucines (square), valine (triangle).
6.2.2. Testing the optimized digestion on avidin

We further tested the applicability of the optimized digestion procedure on avidin. This protein, found in egg white, consists of four identical polypeptide chains, each of which contain a disulphide bridge and a glycosylated residue. Here again we compared the amount of amino acids found in the digest with those predicted by \textit{in silico} digestion of avidin. This protein also had to be pre-treated prior to digestion and the combination of denaturation with reduction and alkylation was found to be the most effective. Recoveries were between 7 and 61% for the selected amino acids (Figure 27). Threonine was not monitored in this case because it was not predicted by PeptideCutter to appear in the digest of avidin.

![Figure 27. Amino acid quantities found in the digest of avidin pre-treated with DTT and IAM (empty bars) or pre-treated with RapiGest\textsuperscript{TM}, DTT and IAM (striped bars). Values are corrected with the corresponding blanks. Horizontal lines indicate the amount of each amino acid predicted to be found in the digest by the PeptideCutter software.]

6.2.3. Application of digestion in template removal from MIPs

We tested the applicability of digestion as a tool for removing covalently immobilized template proteins from molecularly imprinted polymers in two different systems: in core-shell particles (HRP-MIP) and thin films (Av-MIP2-3). In the first case we immobilized HRP on the surface of aminated silica particles of 56±3 nm diameter (3.3.2). For this purpose
we oxidized the vicinal diols in the protein’s carbohydrate groups with sodium periodate to aldehydes, which reacted with the amine groups of the silica particles. The obtained Schiff-base was reduced to create a stable covalent bond. A portion of the surface amine groups was reacted with CPPA, a dithioester compound which helped to avoid polymer growth in the solution phase in the subsequent polymerization step. In fact, CPPA was coupled to the beads before HRP in order to avoid exposure of the protein to the organic medium required for CPPA coupling. We added varying amounts of CPPA to the aminated silica beads and determined the obtained surface coverage with Eriochrome Black T dye-binding assay (3.5.4). For further experiments we have chosen to add 1000 nmol CPPA/mg particle which resulted in ~0.4 CPPA/nm² coverage, corresponding to approximately a third of the amine groups, leaving enough anchor points for the protein. The modified particles exhibited the characteristic red colour of the CPPA (Figure 28a).

![Graph](image1)

**Figure 28.** Optimization of (a) CPPA and (b) HRP-coupling to silica beads, with representative photos of the obtained particles.

Similarly, the amount of HRP immobilized on the CPPA-modified beads was investigated. The amount of immobilized protein was determined with BCA total protein assay (3.5.4). In order to maximize the number of protein imprints we chose the highest HRP-amount for further experiments, which resulted in ~32% of a monolayer coverage (Figure 28b).

A poly(methacrylamide-co-bisacrylamide) shell was then allowed to grow around the particles. The thickness of the obtained polymer shell was estimated by thermogravimetry from the weight loss of the poly(NiPAAm) coated particles compared to the NH₂-modified silica particles to be cca. 2 nm, while the corresponding non-imprinted particles had a cca. 3 nm thick shell (Figure 29).
Finally, the particles were subjected to the optimized digestion procedure in order to remove the protein template. The necessary amount of reagents was determined based on the amount of HRP immobilized on the particles (~13 µg HRP/mg particle, roughly estimated by enzymatic activity assay). After digestion and washing, the particles retained a weak brown colour indicative of HRP (Figure 30) which led us to perform an additional digestion cycle. After the second cycle, the particles were almost colourless.

The amount of selected amino acids in the two digests were measured by HPLC-MS/MS and added up. Knowing the approximate amount of template immobilized on the MIP particles, PeptideCutter predicted the maximum amount of each amino acid that would form if the digestion was complete. Based on the recovery of the different amino acids, 9-133% template removal was calculated. The high variation of recovery is due to the fact that in silico digestion does not reflect experimentally obtained ratios of the monitored amino acids, because not all predicted cleavages are actually performed. Figure 31 shows that the Phe and Thr yields are quite well predicted: the amount of these amino acids found in the experimental digest of dissolved HRP (striped bars) is close to 100% of the values predicted by PeptideCutter, but the predicted cleavages next to valines and leucines are mostly not performed (only 10-15% of the predicted amounts were found from these amino acids). The same trends were observed in the digest of the MIP (Figure 31, empty bars): close to the predicted amounts were found from Phe and Thr while much lower recoveries were obtained.
from Val and Ile+Leu. By taking the amounts of amino acids obtained experimentally after solution digestion as reference instead of the in silico prediction, amino acid recovery from the HRP-MIP was between 84-147%.

![Recoveries comparison](image1)

**Figure 31.** Recoveries (compared to in silico prediction) of selected amino acids found in the digest of MIPS-embedded (empty bars) or dissolved (striped bars) HRP.

The binding properties of the HRP-MIP particles were tested in equilibrium batch rebinding assay (0) and compared with the NIP particles which were prepared identically but without HRP. The higher binding on the MIP indicated successful imprinting (Figure 32a).

![Adsorption isotherm and dose-response curves](image2)

**Figure 32.** (a) Adsorption isotherm of HRP on MIP (filled squares) and NIP (empty squares) core-shell particles. (b) Dose-response curves of avidin rebinding to MIPs prepared by template removal with digestion (circles) or chemical cleavage of the crosslinker (diamonds).

In another example the nanosphere lithography-based imprinting method (Chapter 4) was adapted. Briefly, the template protein was covalently immobilized to polystyrene beads with a cleavable crosslinker, resulting in ~24 µg avidin/mg bead coverage as estimated by BCA assay. 0.16 mg of the beads was drop-casted to a quartz crystal and embedded up to half height into a polymer layer which was deposited by electrochemical means. Template removal was then performed either by cleaving the crosslinker to release the protein from the beads (Av-MIP2) or by digestion (Av-MIP3). In both cases the polymer was washed in the end to remove the proteins, amino acids or peptide molecules, and finally the beads were dissolved in toluene to obtain the imprinted polymers.
The amount of avidin rebound by the chemically cleaved Av-MIP2 and the proteinase K digested Av-MIP3 was tested with quartz crystal microbalance. The digested Av-MIP3 could rebind even somewhat greater amounts of avidin than the chemically cleaved Av-MIP2 confirming the suitability of the proposed method for template removal (Figure 32b). It has to be noted that digestion allows direct immobilization of the template (without spacer) and the cleavable crosslinker was only used for better comparability of the two template removal methods.

6.3. Conclusions and outlook

Proteolysis is a prerequisite for washing out covalently immobilized protein templates and can also replace more harsh solvent extraction methods. Nevertheless enzymatic digestion has been used for template removal from protein imprinted polymers only in a few instances and optimal conditions or its efficiency have not been investigated. In most cases the protease enzyme was added to the polymer without pre-treating the template protein. This could have led to inefficient template removal which further on negatively influenced the binding properties of the MIP.

We have demonstrated that proteins in their native state are often not sufficiently cleaved even by proteinase K, an enzyme known for its ability to digest keratin as well. We have therefore developed and optimized a protocol that includes denaturation of the protein with a surfactant followed by reduction of disulphide bonds in order to make it ready for enzymatic digestion. The proposed procedure was tested on two different proteins that are difficult to digest and is expected to be generally suitable. Its applicability to template removal was demonstrated on two different imprinting systems and was found to be effective in removing the protein templates. Our procedure is especially useful for removing protein templates covalently immobilized to a support, but it can be advantageously applied in other imprinting techniques as well.
Summary and thesis points

In my thesis work I have developed new formats and enabling techniques for the efficient imprinting of protein templates. The need for such research was apparent from the narrow spectrum of protein templates imprinted so far, demonstrating the difficulties of such undertaking. While molecularly imprinted polymers for the selective recognition of small molecules are already on the market, macromolecular targets have been mainly restricted to those with specific properties facilitating their imprinting, and to proofs-of-concept of intricate fabrication methods. The development of novel approaches for protein imprinting seems necessary until one or more of the published methods find their way into widespread practical application. Given the inherent suitability of electrochemical polymer synthesis for protein imprinting, which nevertheless has not been pursued as extensively as free radical polymerization, I have focused on surface imprinting by electrodeposition.

As a new fabrication concept, I have demonstrated that nanosphere lithography (NSL) can be advantageously applied in the synthesis of protein MIPs. I have covalently coated commercially available polystyrene latex nanospheres with the template avidin and allowed the beads to self-assemble on a gold coated quartz crystal. The voids of the bead array were then filled by electrodeposition with PEDOT/PSS. After removal of the protein and the beads, the microstructured polymer film was demonstrated to bear the imprints of avidin on its surface. A remarkable selectivity and an imprinting factor close to the state of the art were achieved, in spite of the rather large portion of non-imprinted surface resulting from the non-compact assembly of the protein-coated nanospheres on the substrate. The proposed method combines several advantages: (i) oriented immobilization of the template protein on the nanospheres makes feasible its site-directed imprinting which can yield highly homogeneous binding sites; (ii) the binding site density can be tuned by adjusting the surface coverage of the beads with protein molecules; (iii) the bead carrier, being orders of magnitude larger than the protein itself, imposes less stringent requirements on the control of polymer film thickness, since accessibility of the binding sites and easy template removal is ensured until the polymer thickness reaches the bead radius and not that of the protein molecule.

I have also addressed another problem associated with preparing selective sorbents by molecular imprinting: that of polymer optimization. Imprinting new templates requires the testing of a great number of monomers and polymerization conditions in order to find the optimal regime. Currently, such optimization is largely based on trial-and-error strategies, i.e. on preparing and testing many MIPs. Therefore, high-throughput synthesis and detection methods would be of clear benefit as these could accelerate the determination of the optimal MIP compositions offering high affinity and selectivity. Such high-throughput methods have only been proposed for the free radical synthesis of polymers until now, and mainly with small molecule imprinting in mind. I have developed a method for the high-throughput fabrication and testing of electrochemically synthesized protein MIPs, electropolymerization being a particularly beneficial approach for protein imprinting. I have adapted the well-
established microspotting technique to electrosynthesis conditions and fabricated a modified micropipette tip to demonstrate the feasibility of MIP synthesis by what we named microelectrospotting. The proposed method consists of filling the micropipette tip accommodating a counter/reference electrode with the pre-polymerization mixture and contacting it to a substrate where electropolymerization occurs. We were able to deposit ca. 500 µm diameter polymer spots with this method, on the surface of a surface plasmon resonance (SPR) slide. The combination of microelectrospotting with imaging SPR detection to monitor the template removal and rebinding process, has led to a high-throughput screening technique which allowed the fast development and optimization of a ferritin-selective MIP.

A crucial step in the preparation of MIPs is template removal, i.e. the liberation of the formed binding sites. Generally, various solvents are applied to extract the template but proteolysis has also appeared as an alternative to these harsh and sometimes inefficient methods. In contrast to the wide literature on solvent extraction the optimal conditions or efficiency of enzymatic digestion have not been investigated. I have demonstrated that proteins in their native state are often not cleaved even by proteinase K, an enzyme known for its ability to digest keratin as well. Therefore I have developed and optimized a protocol that includes denaturation of the protein with a surfactant followed by reduction of disulphide bonds in order to make it ready for enzymatic digestion. The proposed procedure was tested on two different proteins that are difficult to digest, and is expected to be generally suitable for other proteins as well. Its applicability to remove covalently immobilized template was demonstrated on two different imprinting systems and was found to be effective. The proposed method is especially suited for the promising approach of imprinting with immobilized template but it can be advantageously used in other methods as well.

The main results of this thesis can be summarized in the following points:

1. I have demonstrated that nanosphere lithography can be applied to generate surface-imprinted polymers for the recognition of proteins. As a proof-of-concept I have electrosynthesized an avidin-imprinted poly(3,4-ethylenedioxythiophene)/poly(styrene sulfonate) film on a gold coated quartz crystal microbalance using self-assembled avidin-coated polystyrene nanospheres. The imprinted polymer was able to bind 6.5 times as much avidin as the non-imprinted control polymer, an imprinting factor close to the state of the art. [Paper I]

2. I have demonstrated the potential of site-directed imprinting in the creation of homogeneous binding sites. In the above system the avidin template was immobilized to the polystyrene beads in an oriented manner through one of its biotin-binding sites. This polymer showed higher selectivity against avidin-analogues than a previously
reported MIP prepared with the same polymer but with randomly immobilized avidin. [Paper I]

3. I have developed a method for the localized electrochemical deposition (termed microelectrospotting) of molecularly imprinted polymers. I have fabricated a prototype micropipette tip accommodating a counter/reference electrode and demonstrated the microelectrospotting of ferritin-imprinted polyscopoletin spots of ca. 500 µm diameter on SPR chips. [Paper II]

4. I have demonstrated that the combination of microelectrospotting and surface plasmon resonance (SPR) imaging leads to a high-throughput screening method which accelerates the optimization of the molecular imprinting process. This was demonstrated by the fast optimization of a ferritin-imprinted polymer, resulting in an outstanding imprinting factor of 13. [Paper II]

5. I have proposed a widely applicable, improved enzymatic digestion protocol for template removal from protein imprinted polymers based on observations that HRP and avidin were not efficiently digested by proteinase K in their native state. The novel approach differs from existing proteolytic template removal protocols in that it contains a pre-treatment step involving denaturation of the protein with a surfactant and reduction of its disulphide bonds. I have demonstrated the applicability of the improved digestion protocol to remove covalently immobilized HRP and avidin from a core-shell and a microstructured thin MIP film, respectively. [Paper V]
8. List of publications

IF: 10.44, Cited by: 35 (independent: 29)

IF: 7.47, Cited by: 9 (independent: 5)

IF: 7.49 (2015), Cited by: 10 (independent: 7)

Cited by: 1 (independent: 1)


Contribution of the author:
Paper I. The author did all experiments except for the polymer thickness optimization.
Paper II. The author fabricated and tested the prototype of the microspotting device.
Paper V. The author performed most of the digestions and HPLC-MS/MS measurements as well as fabrication and characterization of the core-shell particles and rebinding test on the digested avidin-MIP.

Papers not related to this thesis


IF: 2.55, Cited by: 12 (independent: 6)

Oral presentations:


**Posters:**


Declaration

I, the undersigned Júlia Erdőssy (née Júlia Bognár) hereby declare that I prepared the present Ph.D. thesis by myself and I used only the given sources. Every part that I literally adapted or rephrased with the same content, I cited unambiguously with the indication of the source.

Nyilatkozat

Alulírott Erdőssy Júlia (született Bognár Júlia) kijelentem, hogy ezt a doktori értekezést magam készítettem és abban csak a megadott forrásokat használtam fel. Minden olyan részt, amelyet szó szerint, vagy azonos tartalomban, de átfogalmazva más forrásból átvettem, egyértelműen, a forrás megadásával megjelöltem.
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