Final report

Artificial receptors based on surface imprinted electrosynthesized polymer nanostructures for protein sensing

International Hungarian-German ERA Chemistry (OTKA-DFG) project
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1. Summary

The project aimed at exploring the potential of surface molecular imprinting to generate high affinity artificial polymeric receptors ("plastic antibodies") for biomacromolecules by implementing a broad range of original methodologies, materials and representative macromolecular targets. The research followed the submitted research plan, only minor changes were made with respect of the proteins used as templates. It should be noted that the proposal was submitted with a considerable amount of preliminary data to support the feasibility of the proposed approaches, which facilitated the progress especially during the first year. Overall the initial aims broadened to address the encountered difficulties and to better exploit the new findings. Therefore, we have requested a one-year extension of the originally 3-year project to accomplish the most difficult tasks and tackle the discoveries made during the previous years. The request was approved both by DFG and NKFI. As a summary we can report that the project resulted in 14 peer reviewed articles, with a cumulated impact factor of 78.88. Four additional papers are under preparation and close to submission. Three of the papers published in the project were selected as cover page articles in *Advanced Functional Materials*[1], *Analytica Chimica Acta*[2], and *Nanoscale*[3]. The research work generated also several invited keynote and plenary presentations at major analytical chemistry conferences (Pittcon, IMCS'18, International Conference on Chemical Sensors).

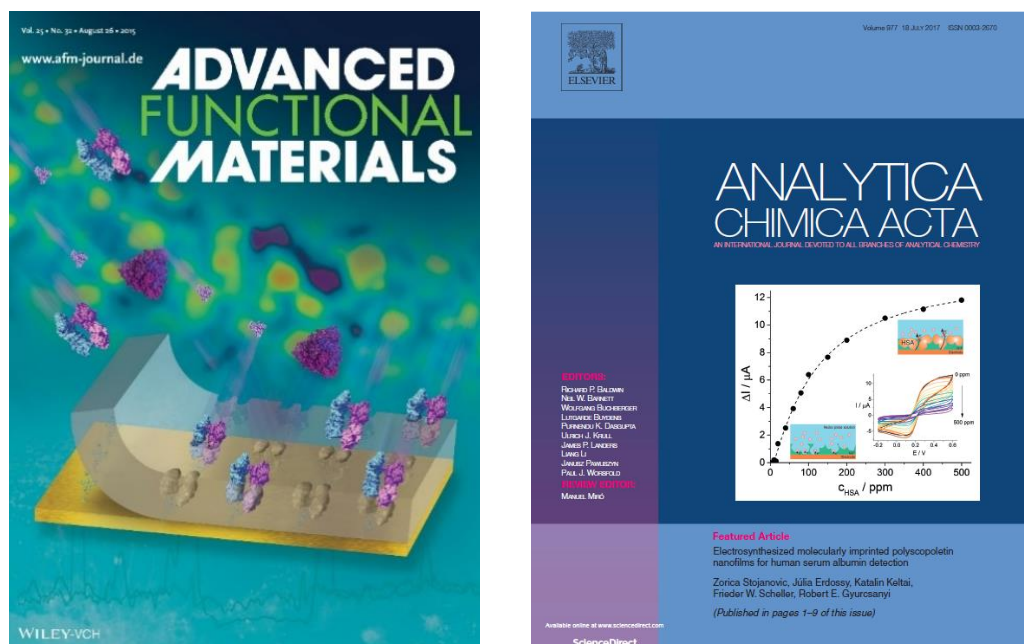

Figure 1. Cover page articles **(A)** Vectorially Imprinted Hybrid Nanofilm for Acetylcholinesterase Recognition. *Advanced Functional Materials* 2015, 25, 5178-5183 (IF_{2018}=15.621); **(B)**; Electrosynthesized molecularly imprinted polyscopoletin nanofilms for human serum albumin detection. *Analytica Chimica Acta* 2017, 977, 1-9.(IF_{2018}=5.256).

2. Introduction

Molecular imprinting is a universal concept to generate materials with "molecular memory" by performing a polymerization of suitable functional monomers in the presence of a target molecule acting as a template. The subsequent removal of the template is expected to create recognition sites in the molecularly imprinted polymer (MIP) that can, further on, selectively rebind the target. Imprinting with large molecular weight targets, such as proteins, which was the main aim of the project is extremely challenging as compared with small molecular weight molecules. This relates mainly with the fragility of the proteins, as well as the difficulty to avoid permanent entrapment of proteins in the imprinted polymeric matrix and generation of cross-reactive binding sites during the MIP synthesis. In the project we addressed these fundamental difficulties of protein imprinting.

- To overcome the fragility of the macromolecular templates we introduced novel electrosynthetic methods for the preparation of MIPs that enable imprinting in mild reaction conditions and aqueous solutions[4-6].
- To avoid the permanent entrapment of the macromolecular templates in the MIPs the electrosynthetic procedures were refined to generate surface imprinted polymers with utmost control, i.e. MIPs nanofilms with thicknesses comparable with the diameter of the protein targets.[2,6,7] Such MIP nanofilms enabled free exchange of the target between the recognition sites generated in the MIP nanofilms and the sample solution, which is the prerequisite of their use for affinity assays. It also offered means for effective optical and electrochemical transduction of the binding events.
- To avoid cross-reactivity of the protein MIPs, custom designed functional monomers[8] and monomer libraries were synthesized and used in combination with vectorial imprinting and hybrid MIPs.[1,9]

The development of selective MIPs is most often based on the empirical optimization of a complex set of experimental conditions, which is a rather slow process. Therefore, we attempted to address this problem as well by developing technologies for high-throughput preparation and screening of protein-imprinted MIPs.[6] We also identified a number of artifacts that may lead to report unrealistic values for the affinity constant of protein MIPs[7,9] and performed preliminary studies on the combination of synthetic receptors (aptamers) and MIPs within hybrid nanofilms[10] as well as the development of MIPs for the recognition of chemically modified nanoparticles (and viruses)[3,11]. Given the complexity of the interactions a large number of protein targets were tested, with some of the target systems already reaching practical applicability in the frame of the project.[2]

This final report will summarize only the most important results and some of the ongoing research close to publication, but yet unpublished.

3. Synthesis of surface imprinted polymers by microelectrospotting

A. Proof of concept of the microelectrospotting for protein MIP fabrication on surface plasmon resonance imaging chips

One of the main achievements of the project was the development of microelectrospotting - introduced by the Hungarian party - as a new approach for the preparation of protein-selective molecularly imprinted polymer microarrays on bare gold surface plasmon resonance imaging (SPRi) chips. The combination of microelectrospotting and SPRi was expected to be extremely advantageous as SPRi allows the label-free and real time detection of protein template removal and rebinding on each MIP spot simultaneously. For proof of principle, first we prepared electrospotting tips from 10 μL plastic micropipette tips (Fig. 1 A), comprising an inner cavity to uptake the monomer-template solution and an embedded electrode in contact with the uptaken solution.[6] During electrospotting both the gold SPRi chip and the spotting tip were electrically connected to a potentiostat as working and reference/counter electrodes, respectively. The spotting pin was placed on the chip surface followed by the application of a potential pulse program to locally electropolymerize the monomer-template protein cocktail onto the surface of the chip (ca. 500 μm spot diameter). The spotting tip was then removed, cleaned and refilled. By repeating this procedure at preprogrammed locations for various composition monomer-template mixtures we could generate MIP microspot arrays of nanometer-thin surface-imprinted films in a controlled manner. In the proof of concept study we used scopoletin (7-hydroxy-6-methoxycoumarin) monomer that forms an electrically insulating film upon polymerization, as well as the following protein templates/controls: Bovine Serum Albumin (BSA), Ferritin (from equine spleen), Urease (from Canavalia ensiformis), Myoglobin and cytochrome c (Cyt c) from equine heart.

A major challenge of the microelectrospotting methodology is that the thickness of the MIP spots deposited should be in the lower nanometer range, i.e. in the range of the hydrodynamic radius of the protein templates. This is required to enable free-exchange of the protein with the solution (thicker polymeric films may irreversibly entrap the protein template during synthesis), but also to enable the subsequent detection of the protein binding by SPR (thicker polymer films, with high optical density, can dramatically reduce the sensitivity). Very importantly, after optimization of the microelectrospotting both the non-imprinted polymer (NIP, prepared without template) and MIP spots proved to be compatible with the SPR detection. Thus the removal and rebinding kinetics of the template and of various potential interferents to the MIP microarrays could be monitored in real-time and multiplexed manner with SPR imaging. Atomic force microscopy (AFM) measurements revealed that for the same electrochemical settings the NIPs film were considerably thinner (~6 nm) than the MIP films (~18 nm) that again confirmed the effect of the protein template on the electrosynthesized nanofilms. The proof of concept paper was published in *Biosensors and Bioelectronics* (IF_{2018}=9.518).[6] During the collaborative work members of the German group (Maria Bosserdt, Xiaorong Zhang, Katharina Jetschmann, Aysu Yarman) spent time in the lab of the Hungarian PI where the microelectrospotting device was developed and set up.

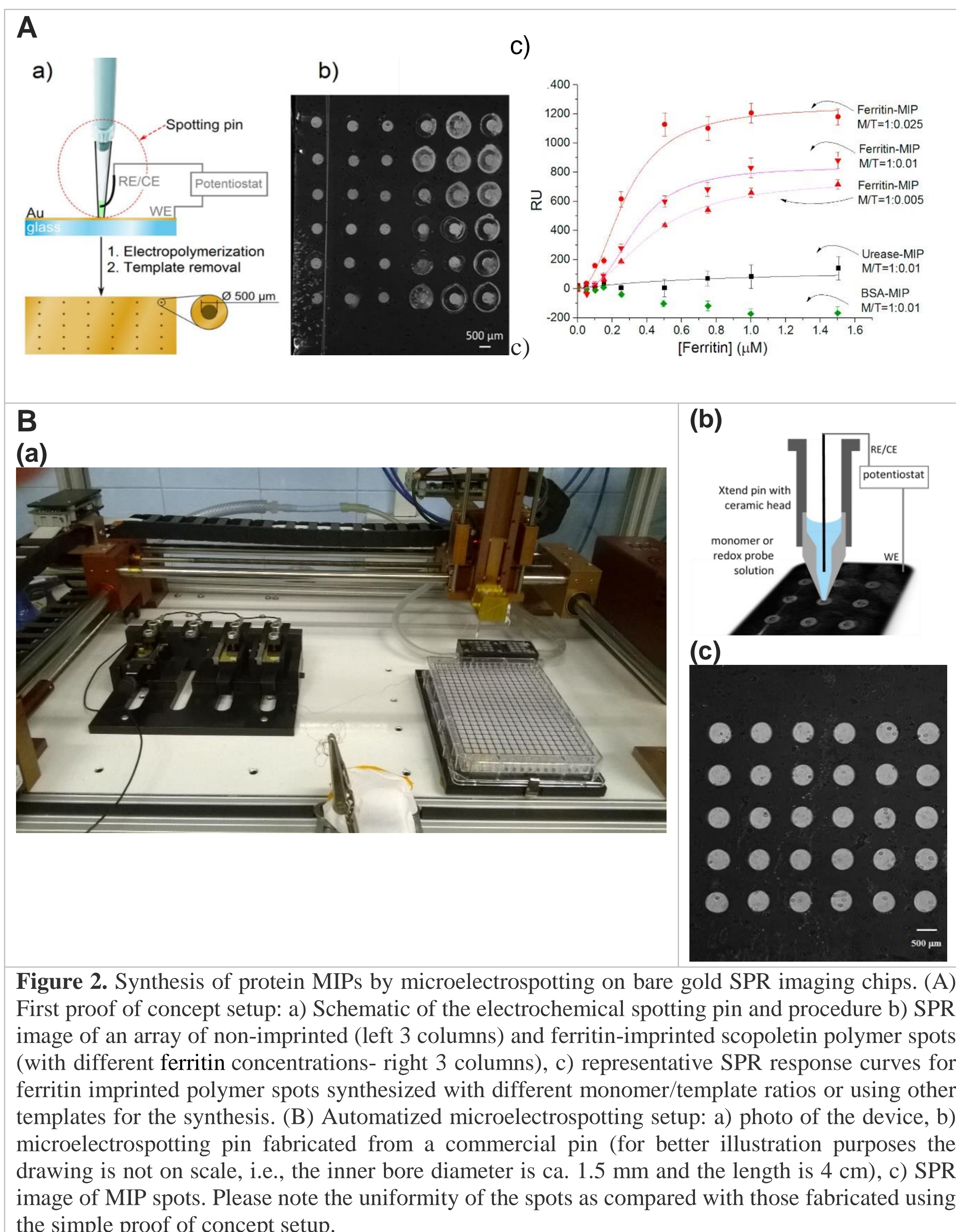

Figure 2. Synthesis of protein MIPs by microelectrospotting on bare gold SPR imaging chips. (A) First proof of concept setup: a) Schematic of the electrochemical spotting pin and procedure b) SPR image of an array of non-imprinted (left 3 columns) and ferritin-imprinted scopoletin polymer spots (with different ferritin concentrations- right 3 columns), c) representative SPR response curves for ferritin imprinted polymer spots synthesized with different monomer/template ratios or using other templates for the synthesis. (B) Automatized microelectrospotting setup: a) photo of the device, b) microelectrospotting pin fabricated from a commercial pin (for better illustration purposes the drawing is not on scale, i.e., the inner bore diameter is ca. 1.5 mm and the length is 4 cm), c) SPR image of MIP spots. Please note the uniformity of the spots as compared with those fabricated using the simple proof of concept setup.

B. Automatized system for the microelectrospotting of protein MIPs

We have further developed the electrochemical microspotting technology for high throughput electrosynthesis of MIP nanofilms. The system set up for proof of concept was replaced with a robotic system for fully automatic and micrometer precision positioning of the electrospotting tip (Fig. 2B (a)), which also includes automatic tip washing steps to avoid cross-contamination between the electrospotted monomer-protein mixtures. In the

automatized system we implemented a microchannel-based stainless steel spotting pin with an electrically insulating ceramic tip (Fig. 2B (b)) in which a reference/counter electrode was introduced. This electrochemical microspotting system was found to enable the generation of MIP arrays with 5-15 nm thickness on the surface of bare gold SPRi chips so that the protein rebinding could be followed in real time and label-free for all MIP spots on its surface[7].

C. Synthesis of hybrid MIPs with layered molecular architectures by microelectrospotting

We have investigated the synergistic use of aptamers and molecularly imprinted polymers to create a MIP for selective determination of IgE (an indicator of allergic reactions). A self-assembled monolayer (SAM) of terminal thiol-labeled IgE aptamers was first formed on gold SPRi chips to anchor the IgE protein that was followed by microelectrospotting of copolymer nanofilms from dopamine and scopoletin monomer mixtures. The copolymer nanofilm was formed around the IgE targets and after the IgE template was removed binding cavities complementary in shape were released that integrated at their bottom the IgE aptamers. By such “molecular architecture” we expected that the selectivity of the aptamer is further enhanced by the shape selectivity of the MIP, i.e., the access of proteins to the aptamer is restricted in a shape selective manner by the MIP nanofilm. While the proof of concept was made, further work is needed to ensure the appropriate stability and reproducibility of such hybrid nanofilms for analytical applications. To enable better control over the synthesis of DNA aptamer-MIP hybrid nanofilms we have introduced a new method based on using a small molecular weight marker (ruthenium hexamine) to visualize and quantitatively assess the DNA probe density in a multiplexed manner on DNA microchips by SPRi (published in *Analytica Chimica Acta*).[12] This approach allows the exact adjustment and quantitation of the aptamer layers for the hybrid MIPs. Original aptamers were also successfully developed and applied for virus detection (human respiratory syncytial virus, RSV)[3] that can be further used in hybrid MIP development for selective RSV recognition.

D. Multiplexed electrochemical readout of protein MIPs with the microelectrospotting system

A new method was introduced to electrochemically monitor in a multiplexed manner the target protein (IgE) binding to MIP spots prepared by microelectrospotting on gold chips. The oxidation current of a small molecular weight redox probe (e.g., ferrocenecarboxylic acid, ferrocyanide) is measured to characterize the permeability of the MIP nanofilms which in turn is influenced by the occupancy of the binding cavities by the target (Fig. 4). After full template removal all the binding cavities are empty allowing the redox probe to access the underlying electrode and undergo oxidation. As template macromolecules bind into the cavities, the access of the redox probe to the electrode is hindered resulting in a target concentration-dependent decrease of the oxidation current. We demonstrated that such electrochemical readout can be performed on microelectrospotted thin polymer films using an electrospotting pin filled with redox probe solution. The methodology makes use of the same microelectrospotting device but using a smaller tip diameter pin (d = 350 μm) than the one used for spotting, which is filled with the solution of a redox probe (2 mM ferrocenecarboxylic acid in phosphate buffer). After template removal the chip was aligned and the microelectrospotting pin was moved over the MIP spots one by one following the microelectrospotting layout used for the generation of MIP spots. The smaller tip size than the diameter of MIP spot ensures that the readout is confined solely to the MIP spots. On each spot, cyclic voltammograms were recorded and the oxidation peak current was determined. To

assess IgE rebinding, the chip was then incubated for 20 min in various concentration IgE solutions and the same electrochemical measurement was performed. This method by avoiding the use of high end imaging instruments as SPRi provides a cost-effective alternative for multiplexed MIP-target binding measurements (a paper on the multiplexed electrochemical readout methodology of MIP-protein binding is in preparation for submission in *Electrochemistry Communications*).

4. Development of protein-imprinted hybrid nanofilms for selective protein sensing

Our studies confirmed that it is possible to obtain highly selective MIPs for given proteins. However, random synthetic approaches are generally not equally successful in generating selective imprints for all protein targets, characterized by an inherently large diversity of physico-chemical properties. Therefore, for protein targets with high practical importance it is more desirable to use a rational approach and custom tune the MIP synthesis for improved selectivity. For this purpose we developed a series of vectorially imprinted hybrid nanofilms in which the selective recognition is assisted by an additional selective recognition unit that is embedded in the MIP film. The major discovery at the core of this approach was that even if the MIPs generated for a protein is not sufficiently selective for practical use, the very conformal, compact MIP nanofilms made by electrosynthesis can act as a shape recognition nanofilters[7], i.e., it is rather straightforward to generate shape complementarity with target proteins even if the chemical complementarity does not result in sufficient affinity for fully selective recognition.

A. Vectorially imprinted hybrid nanofilms for acetylcholinesterase recognition

By developing hybrid materials, we attempted in collaboration with the group of Prof. Scheller (who initiated it) to implement a sophisticated rational approach towards the synthesis of highly selective protein MIPs. The concept - demonstrated through the detection of acetylcholinesterase (AChE), a potential biomarker of Alzheimer disease - is based on a stepwise synthesis procedure and the combined use of molecular imprinting and a selective protein-binding compound within the same hybrid nanofilm.[1] The synthesis of the hybrid nanofilm involved first the formation of a self-assembled monolayer (SAM) on the surface of an electrode to enable the oriented and reversible immobilization of the template protein. This was followed by the non-covalent anchoring of the template protein and finally by the controlled electrodeposition of the polymer film. As anchoring SAM we implemented a propidium derivative (Prop), which is a reversible week inhibitor of AChE that specifically binds to the peripheral anionic site of the enzyme so that the enzyme activity is preserved. By this procedure the enzyme is immobilized on the surface of a gold electrode in a proper orientation and acts as a molecular template for the polymer film electrodeposited in the subsequent step. For this purpose an original functional monomer, a carboxylate-modified derivative of 3,4-propylenedioxythiophene (ProDOTCOOH), was synthesized. The polymer film was grown with utmost control to a thickness of ca. 5 nm, which enables the removal of the template protein after the synthesis and subsequent rebinding of AChE to the liberated binding sites (Fig. 3A).

The presence of the binding sites and their "filling" with AChE upon binding was confirmed by AFM measurements. For AChE sensing the binding of the enzyme to the hybrid nanofilm was detected via its enzymatic activity using acetylthiocholine as substrate (Fig. 3B). The AChE catalyzed hydrolysis of the substrate generated thiocholine, which was oxidized directly at the underlying gold electrode. The main role of the AChE-imprinted negatively charged poly(ProDOTCOOH) film proved to be the shape specific access of AChE to the Prop-SAM binding sites, while the non-specific binding of other proteins was effectively suppressed. Thus the new hybrid nanofilm was shown to provide a threefold selectivity gain by the synergistic effect of: (i) selective binding of AChE to the propidium-SAM, (ii) "shape-specific" filtering by the MIP film, and (iii) signal generation only by the AChE bound to the nanofilm. The results were published in *Advanced Functional Materials* and the relevant paper was chosen for the cover page (Fig. 1)[1].

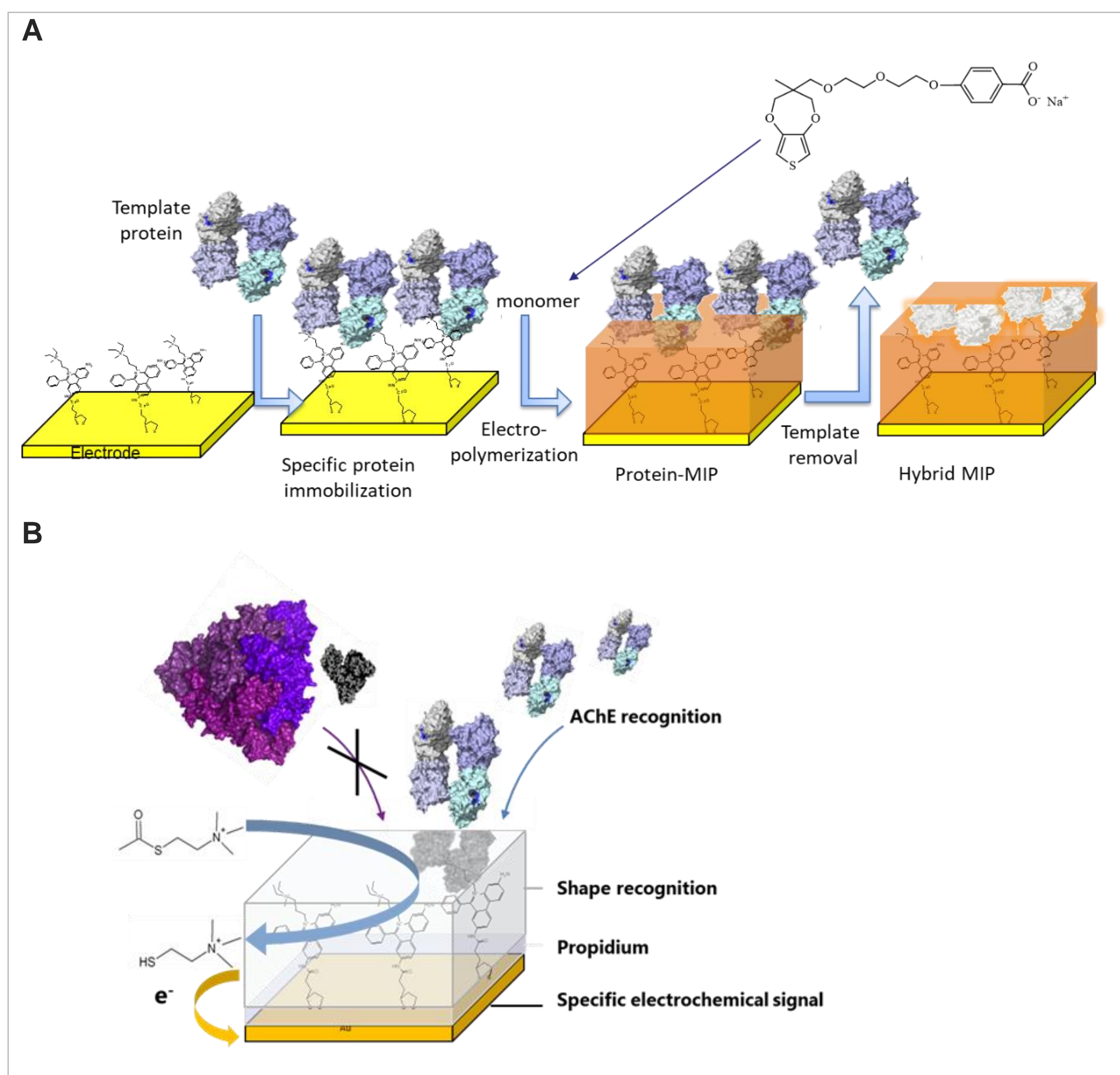

Figure 3. (A) Schematic representation of the preparation of the vectorially imprinted polymer for AChE by using a propidium-SAM on gold in hybrid architecture with an electrosynthesized polymer film from ProDOT-COOH monomer. For the synthesis of the propidium-self assembled monolayer a lipoic-acid-SAM was first formed on a gold electrode to which the propidium was coupled using *N*,*N* '-dicyclohexylcarbodiimide (DCC) activation. The AChE was reversibly attached to the Prop-SAM and then a ≈5 nm thick polymer film is deposited, which after removal of the AChE template

possesses binding sites for AChE. (B) The rebinding of the template is detected via the generation of thiocholine from acetylthiocholine, which is oxidized at the underlying gold electrode.

We consider that the surface imprinted hybrid nanofilm design we introduced by tuning the size of the specific protein binding sites show potential in discriminating among different forms of a template protein and can be generalized for other biomolecular targets, where simultaneously assessing the state of association, post-translational modifications, and, if relevant, enzymatic activity is of interest.

B. Electrosynthesis of vectorially imprinted polymers (VIPs) for cytochrome P450cam

We developed a new "vectorial" imprinting strategy for the P450cam enzyme by using a surface exposed binding motif of Putidaredoxin (Pdx), a natural redox partner of P450cam, as non-covalent anchor.[9] The binding motif comprises a 15 aminoacid peptide chain from the largest continuous sequence of the Pdx's interaction site, that was slightly modified to facilitate its application as an anchor unit for P450cam in the VIP synthesis: (i) internal cysteines were replaced by serines to prevent disulfide bond formation; (ii) 2 polyethylene glycol units were attached to the N-terminus as a spacer region (iii) an N-terminal cysteine was added to allow the immobilization on the electrode surface. After immobilization of P450cam via the peptide anchor to the electrode surface it served as template for the subsequent electrochemical synthesis of an electrically insulating hydrophilic polyscopoletin MIP film. This VIP nanofilm enabled reversible oriented docking of P450cam and catalytic oxygen reduction via direct electron transfer between the enzyme and the underlying electrode. Catalysis of oxygen reduction by P450cam bound to the VIP-modified glassy carbon electrode was used to measure rebinding to the VIP. Interaction of the enzyme with the VIP layer on top of the Pdx-peptide-SAM was additionally characterized by AFM and quartz crystal microbalance. The "mild" coupling of an oxidoreductase with the electrode may be appropriate for realizing electrode-driven substrate conversion by instable P450 enzymes without the need of NADPH co-factor. The paper was published in *ChemElectroChem*. [9]

5. Synthesis of monomer library for the electrosynthesis of protein MIPs

Most MIPs use a very limited number of monomers, many times just one, that seems insufficient to cover the whole range of non-covalent interactions with a protein template.[5] Preliminary experiments with various electropolymerizable monomers indicated that the difference in their oxidation potential will influence the extent of their integration in copolymer films, which is difficult to control. Therefore, we changed our initial approach and set to the synthesis of novel monomers with different substituents attached to the same electropolymerizable unit in such a way to do not influence the oxidation potential of the latter. We designed such a library based on 3,4-ethylenedioxythiophene (EDOT) as electropolymerizable monomer using the 1,4-dioxane ring to attach various functional molecules as side chains. During the project the synthesis of conjugated EDOT molecules was realized by click chemistry with lysine, aspartate and succinate, along with EDOTs bearing linear hydrophobic side chains. This library by providing positively, negatively charged, zwitterionic and hydrophobic units in principle can cover the full range of non-covalent interactions. The electropolymerization and co-electropolymerization of these monomers was systematically studied by using fluorescent labeled IgG to enable high throughput fluorescence

imaging of the template amounts retained in the electrosynthesized MIP nanofilms as well as their removal and rebinding. These measurements led to very interesting results as indeed such films were found to bind strongly the template and in fact one of the main challenges turned out to be the removal of the template after the imprinting process to free the binding sites for subsequent affinity interactions. A very fine tuning of the composition of the different monomers seems to be the solution and it currently is under investigation. We expect this new concept based on much closer synthetic mimicry of antibodies than ever attempted will be of very high importance and therefore we refrained to publish partial results. However, some of the inherently hydrophobic monomers and hydrophobic side chain EDOT momomers that we investigated (synthesized) proved to be extremely useful as solid contacts for ISEs and was published in a different context in *Analytical Chemistry*[8,13] and *Analyst*[14].

5. Practical applications of surface imprinted polymers

A. *Quantitation of human serum albumin (HSA) in urine by MIP-based sensors*

HSA can appear in urine in up to 25 mg/dm^3 concentration for healthy individuals. In case of microalbuminuria the urinary albumin excretion elevates further to ca. 200 mg/dm^3, which can be an early indicator of kidney damage. For preparing the HSA-imprinted MIP we used scopoletin as monomer in the presence of HSA template.[2] Scopoletin by electropolymerization formed an insulating polymer film the thickness of which was tuned to match the characteristic dimensions of the target protein (Fig.4).

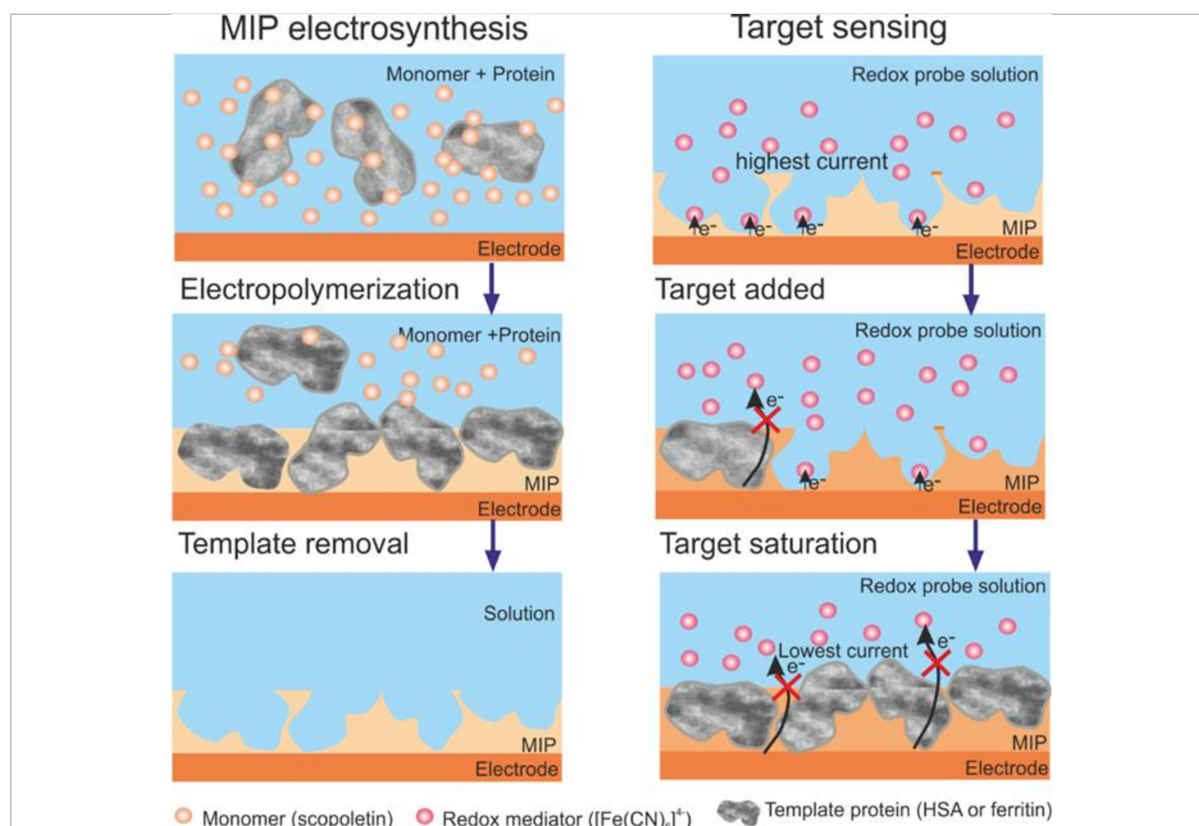

Figure 4. Schematics of the electrosynthesis of surface imprinted polyscopoletin nanofilms on a gold electrode surface and the electrochemical sensing scheme of the target protein binding. The synthetic approach results after the removal of the template protein in a few nanometer thick conformal and

electrically insulating MIP nanofilm on the surface with free binding cavities. Through these cavities the electrode surface can be accessed by redox probes that undergo oxidation resulting in a large current. The template binding by hindering the access of the redox probes through the MIP nanofilm will decrease the measured current in a concentration dependent manner.

The protein binding to the MIP was detected by measuring the oxidative current of a redox probe on the underlying electrode, i.e., the protein binding hinders the permeability of the redox probe through the MIP nanofilm in a concentration dependent manner. This label-free sensing method was used successfully to quantify HSA in urine samples from diabetic patients. The results were in agreement with immunoturbidimetric determination, suggesting that this approach can be used for detection of HSA in urine sample for diagnostic purposes as alternative to more expensive antibody based assays. It is also an alternative to the simple colorimetric tests as the HSA recognition proved to be selective also in the presence of other proteins in urine samples. The results were published in *Analytica Chimica Acta*[2] and the paper was selected for the cover page of the journal by the Editorial Board (Fig. 1).

B. MIPs for the recognition of the N-terminal peptides of human adult hemoglobin (HbA) and glycated Hb (HbA1c)

The N-terminal peptides of HbA and HbA1c are the analytes in the IFCC (International Federation of Clinical Chemistry) Reference Method for the determination of the long-term biomarker for diabetes because the increased glucose levels result in higher fraction of the glycated hemoglobin. The challenge for a synthetic MIP receptor is to differentiate between the glycated and non-glycated forms that differ in a single fructosyl residue. MIPs for the recognition of the respective N-terminal pentapeptides were synthesized by using them either in a mixture with scopoletin or immobilized on the surface of gold electrodes in the form of a self assembled monolayer using a cystein-extended N-terminal pentapeptide of the ß-chain. In both cases a MIP nanofilm was prepared by electropolymerizing scopoletin. All steps of MIP-synthesis and rebinding were analyzed by square wave voltammetry (SWV) of the redox marker ferricyanide and by surface-enhanced infrared absorption (SEIRA) spectroscopy of both the bound target, i.e., the peptides or the parent protein, and the polymer polyscopoletin. For the MIP prepared on the SAM-covered electrode the relative decrease of the voltammetric peak (obstruction of the redox marker permeation through the MIP nanofilm to the electrode by the bound target) was linearly dependent on the peptide concentration up to 100 nM and reached saturation above 200 nM. Substitution of the histidine residue by alanine or introduction of a fructosyl residue at the N-terminal valine decreased the saturation value by a factor of almost eight. On the other hand, the measuring range for the MIP produced by polymerizing the mixture of scopoletin with the N-terminal peptide of HbA was in the µM range reaching saturation above 10 µM. This MIP showed almost the same concentration dependence of HbA-binding as the MIP formed on top of the SAM of the cystein-extended N-terminus. Because the affinity of the N-terminal petide differs by a factor of almost ten this relation is also expected for the binding of HbA to the epitope –shaped cavities of the different MIPs. The most important finding was that the electropolymerization of scopoletin in the presence of fructosyl valine -the N-terminus of glycated HbA (HbA1c)- brought about a MIP which recognized its target in the lower µM range whilst the N-terminal peptide of (non-glycated) HbA was not bound. Combination of the respective MIPs should allow the quantification of both the glycated and the non-glycated N-terminal peptides of HbA1c and

HbA after splitting the red blood cells and enzymatic digestion as it is proposed in the IFCC Reference Method for the HbA1c. The paper is in preparation for submission.

Published Papers:

1. Jetzschmann KJ, Jágerszki G, Dechtrirat D, Yarman A, Gajovic-Eichelmann N, Gilsing HD, Schulz B, Gyurcsányi RE, Scheller FW. Vectorially Imprinted Hybrid Nanofilm for Acetylcholinesterase Recognition. **Advanced Functional Materials** 2015;25:5178-5183.
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Manuscripts under preparation:

Supala E, Tamás L, Erdőssy J, Gyurcsányi R.E, (2019) Multiplexed electrochemical readout of electrochemically microspotted molecularly imprinted polymer chips, in preparation

Zhang X., Caserta G., Yarman A., Supala E., Caserta G., Zebger I., Wollenberger U., Gyurcsányi R.E., Scheller F.W. (2019) Electrochemical and Surface-Enhanced Infrared Absorption Analysis of electrosynthesized MIPs for the N-terminal peptides of glycated and non-glycated human hemoglobin, to be submitted

Yarman A., Caserta G., Supala E., Frielingsdorf S., Borrero P., Kurbanoglu S., Neumann B., Zebger I., Lenz O., Mroginski M.A., Wollenberger U., Gyurcsányi R.E., Scheller F.W. (2019) Fully Electrochemically Prepared MIPs for Strep-Tag II Peptide and Strep-Tagged Proteins, in preparation

Supala E., Bojtár M., Gyurcsányi R.E (2020) Functional amino acid-based monomer libraries for the electrosynthesis of molecularly imprinted polymers, in preparation