

# Electrosynthesized molecularly imprinted polyscopoletin nanofilms for human serum albumin detection

Zorica Stojanovic<sup>a</sup>, Júlia Erdőssy<sup>b</sup>, Katalin Keltai<sup>c</sup>, Frieder W. Scheller<sup>d</sup>, Róbert E.  
Gyurcsányi<sup>b\*</sup>

<sup>a</sup> University of Novi Sad, Faculty of Technology, Department of Applied and Engineering  
Chemistry, Bul. cara Lazara 1, 21000, Novi Sad, Serbia

<sup>b</sup> MTA-BME “Lendület” Chemical Nanosensors Research Group, Department of Inorganic  
and Analytical Chemistry, Budapest University of Technology and Economics, Szent Gellért  
tér 4, H-1111 Budapest, Hungary

<sup>c</sup> Department of Internal Medicine, Faculty of Medicine, Semmelweis University, Kútvölgyi  
út 4, H-1125 Budapest, Hungary

<sup>d</sup> Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Strasse 25-  
26, Potsdam D-14476, Germany

\*Corresponding author: robertgy@mail.bme.hu

## Abstract

Molecularly imprinted polymers (MIPs) rendered selective solely by the imprinting with protein templates lacking of distinctive properties to facilitate strong target-MIP interaction are likely to exhibit medium to low template binding affinities. While this prohibits the use of such MIPs for applications requiring the assessment of very low template concentrations, their implementation for the quantification of high-abundance proteins seems to have a clear niche in the analytical practice. We investigated this opportunity by developing a polyscopoletin-based MIP nanofilm for the electrochemical determination of elevated human serum albumin (HSA) in urine. As reference for low abundance protein ferritin-MIPs were

1 also prepared by the same procedure. Under optimal conditions, the imprinted sensors gave a  
2 linear response to HSA in the concentration range of 20-100 mg/dm<sup>3</sup>, and to ferritin in the  
3 range of 120-360 mg/dm<sup>3</sup>. While as expected the obtained limit of detection was not  
4 sufficient to determine endogenous ferritin in plasma, the HSA-sensor was successfully  
5 employed to analyse urine samples of patients with albuminuria. The results suggest that  
6 MIP-based sensors may be applicable for quantifying high abundance proteins in a clinical  
7 setting.  
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12 **Keywords:** Human serum albumin; ferritin; molecularly imprinted polymer; scopoletin;  
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## 1. INTRODUCTION

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2 Since the fragility of antibodies is limiting in many biosensing applications their replacement  
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4 with more robust selective receptors received considerable attention.[1] In this respect  
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6 general synthetic processes with broad applicability in terms of targets to be recognized such  
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8 as molecular imprinting of polymers are especially appealing.[2, 3] Molecular imprinting  
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10 uses the target as a template during polymerization of functional monomers. Subsequent  
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12 removal of the template leads to recognition sites in the molecularly imprinted polymer  
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14 (MIP) that can selectively rebind the target. Extending this principle to macromolecules such  
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16 as proteins necessitated the implementation of a variety of enabling technologies (e.g.,  
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18 epitope imprinting,[4, 5] surface imprinting[6-10]) to cope both with the fragility of the  
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20 target[11] as well as with its limited diffusivity in the cross-linked polymeric matrices. The  
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22 electrochemical polymerization in this respect offers clear advantages such as performing the  
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24 polymerization in aqueous conditions that is compatible with the proteinaceous target.  
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26 Moreover, in terms of chemical sensor fabrication it enables the controlled deposition of MIP  
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28 nanofilms directly onto an electrode surface.[12-14] While the molecular imprinting concept  
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30 is fairly universal the functional monomers to provide selective recognition are not, i.e., the  
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32 library of monomers is rather limited and monomers adequate for a certain protein target may  
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34 not be necessarily optimal for another.[15] While high affinity MIPs were reported for a  
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36 number of targets[16, 17], the selectivity of protein-MIPs is often enhanced by incorporating  
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38 in the MIPs compounds known to interact with the target, e.g. substrates or inhibitors of an  
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40 enzyme target[18, 19], aptamers[20] and various nanomaterials[16, 21]. Alternatively,  
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42 rational design of monomers tuned for the specific target and semi-covalent imprinting was  
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44 also shown to give MIPs with high affinity towards protein templates [22].  
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57 However, target specific tuning of the molecular imprinting process departs from the basic  
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59 universal concept, i.e., to obtain a polymer rendered selective towards different proteins  
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1 solely by the molecular imprinting process. The difficulty to achieve this goal is largely due  
2 to the lack of universally applicable monomer library, i.e., in case of electropolymerized  
3 MIPs generally only a single monomer is used for the synthesis of MIPs.[15] Given the  
4 diversity of proteins in terms of physical chemical properties it is unlikely to have the full  
5 range of interactions for high affinity binding covered by these simple MIPs. Therefore, the  
6 success rate of this probabilistic approach was increased in many cases by focusing on  
7 proteins with distinctive properties[8, 23] that facilitates the likelihood of obtaining binding  
8 sites with both strong and selective interaction with the target. However, for “random”  
9 protein targets lacking such properties the resulting MIPs rendered selective solely by  
10 imprinting are likely to exhibit low to medium affinities, which prohibits their use for  
11 practical application where low or even trace amounts of proteins need to be detected. Still  
12 their implementation for the recognition of high-abundance proteins seems to have clear  
13 niche in the analytical practice. Here we investigated this opportunity through the  
14 electrochemical determination of elevated human serum albumin (HSA) in urine by HSA-  
15 imprinted MIP sensor. As a reference for a low abundance protein we used ferritin (normal  
16 levels are 12 to 300 ng/mL in the blood). In comparison up to 25 mg/L HSA in urine is  
17 considered normal and this value may increase orders of magnitude in case of kidney  
18 damage, e.g. elevated urinary excretion of albumin as in microalbuminuria[24] is an early  
19 indicator of kidney damage. Studies suggest that microalbuminuria defines a group at high  
20 risk of increased cardiovascular morbidity and mortality among patients with diabetes[25-27]  
21 or essential hypertension.[28] Moreover, microalbuminuria is associated with increased  
22 cardiovascular morbidity even in the non-diabetic, non-hypertensive population,[29] pointing  
23 out the necessity for routine screening of urinary albumin to enable prediction and prevention  
24 of future renal and cardiovascular diseases. There are many dye-binding procedures for rapid  
25 screening of elevated HSA[30] that are based on the interaction of the albumin and an anionic

1 dye such as bromocresol purple.[31] Since the albumin bound dye has a different absorption  
2 maximum than the free dye the HSA can be detected by simple means. However, for very  
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4 specific HSA determinations clinical laboratories generally use immunoassays, e.g.  
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6 immunoturbidimetric assays, or separation based methods.[32] While most separation-based  
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8 methods do not cope with the requirements for high-throughput routine HSA measurements,  
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10 conventional immunoassays may underestimate the urinary albumin concentration as intact  
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12 albumin in urine may exist in both immunoreactive and unreactive forms.[33] Therefore, we  
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14 were interested to explore the use of MIPs, that are expected to give a broader specificity than  
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16 antibodies, for the quantitation of HSA in urine samples of patients showing  
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18 microalbuminuria. While several electropolymerizable monomers emerged for protein  
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20 imprinting applications [12] for preparing the HSA-imprinted MIP we used scopoletin as  
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22 monomer, which has been introduced by Gajovich-Eichelmann[34] for the electrosynthesis of  
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24 protein-MIPs and proved to enable the recognition of several protein targets.[34-38] By  
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26 electropolymerization scopoletin forms an insulating polymer film, the thickness of which  
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28 can be tuned to match the characteristic dimensions of the protein. The protein binding to the  
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30 MIP was detected by measuring the oxidative current of a redox probe on the underlying  
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32 electrode, i.e., the protein binding hinders the permeability of the redox probe through the  
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34 MIP nanofilm.  
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## 48 **2. EXPERIMENTAL**

### 49 **2.1. Chemicals and reagents**

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51 Scopoletin, human serum albumin (HSA) (isoelectric point (pI) 4.7), ferritin (pI 4,5), avidin  
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53 (pI 10), lysozyme (pI 11.35) and sodium dodecyl sulphate (SDS) were obtained from Sigma  
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55 (Steinheim, Germany). Potassium hexacyanoferrate(II) trihydrate ( $K_4Fe(CN)_6 \cdot 3H_2O$ ), sodium  
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1 hydroxide and Tween 20 were purchased from Fluka (Buchs, Switzerland). All other  
2 chemicals used were analytical reagent grade and were used without further purification.  
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4 Deionized water (DI) of 18.2 MΩ×cm resistivity prepared by a Millipore Milli-Q system was  
5 used in all experiments. Phosphate buffered saline (10 mM, pH 7.4 at 25°C, 137 mM NaCl,  
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7 2.7 mM KCl) was prepared according to the manufacturer's instructions by dissolution of one  
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9 phosphate buffered saline tablet (Sigma, Steinheim, Germany) in 200 ml DI water.  
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## 15 **2.2. Instrumentation**

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17 Electrochemical measurements were carried out with an Autolab potentiostat/galvanostat  
18 (model PGSTAT 12, Metrohm Autolab B.V, Utrecht, The Netherlands) controlled by a GPES  
19 (model PGSTAT 12, Metrohm Autolab B.V, Utrecht, The Netherlands) controlled by a GPES  
20 4.9 software package (Metrohm Autolab B.V, Utrecht, The Netherlands). A standard three  
21 electrode system was used to conduct all electrochemical experiments: a gold disk electrode  
22 (2 mm diameter) as working electrode, a Ag/AgCl/KCl (3.5 mol/dm<sup>3</sup>) reference electrode,  
23 and a platinum wire as counter electrode. Non-specific adsorption and rebinding capacity of  
24 MIPs and NIPs were evaluated by quartz crystal microbalance (eQCM 10M™ with  
25 Reference 600 potentiostat, Gamry Instruments Inc., Warminster, USA). 10 MHz Au-coated  
26 quartz crystals (Gamry Instruments Inc., Warminster, USA) were used to prepare MIPs and  
27 NIPs for AFM and QCM measurements.  
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## 43 **2.3. Electrosynthesis of polymer films**

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45 Molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs) were prepared  
46 by electropolymerization of scopoletin monomer on 2 mm diameter gold disk electrodes or  
47 on 10 MHz gold-coated quartz crystals. The crystals were used as received, while the disk  
48 electrodes were cleaned before modification by polishing successively with 1.0 and 0.05  
49 micrometer particle size alumina slurry (Buehler, Lake Bluff, USA) followed by thoroughly  
50 washing in an ultrasonic bath with DI water for 1 min. The electrode was electrochemically  
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1 cleaned afterwards by cycling the potential between -0.2 and 1.5 V in 0.1 M H<sub>2</sub>SO<sub>4</sub> at 50  
2 mV/s, until a steady cyclic voltammogram (CV) was obtained (generally 10 cycles). The  
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5 electrosynthesis of imprinted polyscopoletin film was performed after optimization by CV (3  
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7 cycles) in the potential range of 0.0 to 1.0 V at a scan rate of 150 mV/s. The polymerization  
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9 mixture contained 1 mM scopoletin and 7.5 μM HSA or 1 μM ferritin in PBS at pH 7.4. To  
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11 remove the template from the freshly prepared MIP films, the electrode was consecutively  
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13 immersed in 5 mL of gently agitated solutions of 5 mM NaOH (10 min), 5% SDS (5 min), 5  
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15 mM NaOH (10 min) for HSA, and additionally in 0.05% Tween20 (5 min), 5 mM NaOH (10  
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17 min) for ferritin, and finally in DI water for 5 minutes in both cases. The non-imprinted  
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19 polymer (NIP) electrodes were prepared with the same procedure but without protein  
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21 template during the electropolymerization of scopoletin.  
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#### 27 **2.4. Electrochemical detection of the MIP-target binding**

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29 The protein binding and removal process at MIP and NIP electrodes were characterized by  
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31 cyclic voltammetry in 10 mM K<sub>4</sub>Fe(CN)<sub>6</sub> in PBS at pH 7.4 in the range of -0.2 to 0.6 V at a  
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33 scan rate of 50 mV/s. For protein binding the MIPs and NIPs were equilibrated in the  
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35 ferrocyanide solution where a reference CV was recorded, followed by addition of HSA or  
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37 ferritin. After 10 min equilibration the CV was remeasured. The response for a certain target  
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39 protein concentration was determined as the difference of the oxidation current ( $\Delta I = I_0 - I_t$ ) of  
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41 the ferrocyanide before ( $I_0$ ) and after the protein addition ( $I_t$ ). For measuring in real samples  
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43 the sensor was immersed in the urine or plasma for 10 min, followed by 10 min in PBS and  
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45 rinsing. Finally, the electrode was transferred into the ferrocyanide solution to measure the  
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47 CV. All experiments were performed at room temperature.  
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#### 54 **2.5. Analysis of urine and plasma samples**

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1 The practical applicability of the electrodes was tested for the quantification of HSA and  
2 ferritin in human urine and plasma samples, respectively. Spot urine samples were obtained  
3 from healthy volunteers and from diabetic patients. Depending on the content of HSA, the  
4 samples were used either undiluted or diluted 2 or 10 times with PBS pH 7.4 for analysis.  
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6 Plasma from healthy donors was obtained from the Hungarian National Blood Transfusion  
7 Service. The quantitative determination of microalbumin in human urine was made by  
8 standard immunoturbidimetric method on a Beckman Coulter AU analyser.  
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12 The total protein content of urine samples was determined after desalting the urine samples  
13 (Zeba Microspin desalting columns, 7K MWCO, Thermo Fisher) following the standard or  
14 enhanced protocol (incubation at 37 or 60°C) of the Pierce BCA Protein Assay kit (Thermo  
15 Fisher). To correct for contingent losses during sample clean-up HSA standards were  
16 subjected to the same desalting protocol and the resulting HSA was measured..  
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## 19 **2.6. Determination of the MIP and NIP thickness**

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21 The thickness of polymer films deposited on quartz crystals was measured using an atomic  
22 force microscope (AFM, FlexAFM, Nanosurf, Liestal, Switzerland). The polymer was  
23 mechanically removed over a 1x1  $\mu\text{m}$  area by scanning it in contact mode using a  
24 TAP190GD-G tip (Budget Sensors, force constant: 48 N/m, length: 225  $\mu\text{m}$ ) with 500 nN  
25 force in five consecutive cycles. A 3x3  $\mu\text{m}$  area, including the scratched area was then  
26 scanned in tapping mode and depth profiles were taken across the image.  
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## 29 **3. RESULTS AND DISCUSSION**

### 30 **3.1. Electrosynthesis of the molecularly imprinted film**

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32 The first cycle during the electropolymerization of scopoletin by CV revealed a current peak  
33 at 0.5 V corresponding to the oxidation of the monomer: The oxidative current decreased  
34 with each additional cycle due to the gradual enclosing of the insulating polyscopoletin film  
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1 that hinders the access of monomers to the electrode (Figure 1 f). The complete suppression  
2 of the oxidation current at the end of the CV program suggests that the film is non-porous  
3 given that not even the small molecular weight monomer can permeate through, which is  
4 beneficial in terms of providing a highly conformal and dense polymeric matrix for protein  
5 imprinting. During electropolymerization of the MIPs, the protein molecules in the  
6 electropolymerization mixture are entrapped in the polymer matrix due to the molecular  
7 imprinting effect, i.e., given the high ionic strength of the polymerization solution most likely  
8 through formation of hydrogen bonds and hydrophobic interactions with scopoletin. After  
9 washing the MIPs to remove the template the current of the redox probe increases (Figure  
10 1c,e) but the obtained peak currents were smaller than that on the bare gold electrode. This  
11 suggested that the polymer film remains on the electrode and only some pores were liberated  
12 by template extraction. This was confirmed by the lack of change in the current response of  
13 the NIP film subjected to the same washing procedure. Similarity between CVs for the MIPs  
14 and NIP electropolymerization indicated no electrochemical activity of HSA or ferritin within  
15 -0.2 to 0.4 V.  
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### 41 **3.2. Optimization of the surface protein imprinting of polyscopoletin**

42 The analytical MIP performance is expected to be influenced by the conditions of the  
43 electrodeposition and the measurement procedure. Therefore, the electrosynthesis of the  
44 MIPs in terms of thickness and physical stability of the polymer film was investigated as a  
45 function of the number of polymerization cycles and scan rate, template concentration and  
46 extraction method as well as rebinding time. The performance of the prepared polymer films  
47 was evaluated through their template rebinding properties using the template concentration  
48 dependent changes of the oxidation peak current of ferrocyanide ( $\Delta I$ ).  
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### 3.2.1. Effect of the electropolymerization conditions

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2 Since polyscopoletin forms an insulating film, the polymer growth stops after a compact film  
3 is obtained, but these films can reach a few tens of nanometers in thickness.[39] Such  
4 thicknesses may already irreversibly entrap the template proteins, therefore the film thickness  
5 needs to be controlled. The main parameters of the electropolymerization affecting the  
6 thickness and the compactness of the deposited polymer films are the number of  
7 polymerization cycles and the scan rate, respectively,[12] while the concentration of the  
8 template in the polymerization cocktail is expected to influence the binding site density. In  
9 our experiments, five or more cycles led to the formation of thicker polymer films from  
10 which the template could not be extracted quantitatively (data not shown). On the other hand,  
11 films formed with only one or two cycles were too thin and were not stable during subsequent  
12 washing steps. Thus, the optimal film thickness was obtained with three polymerization  
13 cycles. The effect of scan rate on template recognition was examined in the range of 50 mV/s  
14 to 200 mV/s in the case of ferritin imprinting (Figure 2a). The lowest scan rate resulted in the  
15 formation of compact polyscopoletin film from which the template could not be removed  
16 efficiently as suggested by the low rebinding ability of this film (data not shown). The film  
17 prepared with 100 mV/s scan rate had more than twice as high sensitivity which further  
18 improved slightly with the increase of scan rate. Ultimately, 150 mV/s scan rate was chosen  
19 for further experiments.  
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46 The effect of the template concentration in the polymerization mixture was investigated by  
47 preparing MIPs with 1 to 15  $\mu\text{M}$  HSA as template and measuring the response to 100  $\text{mg}/\text{dm}^3$   
48 HSA. The results indicated that the response of the MIP films to HSA increased with  
49 template concentration up to 7.5  $\mu\text{M}$  suggesting that an increasing number of imprints were  
50 formed in the polymer (Figure 2b). This value was chosen for subsequent experiments.  
51 Higher template concentrations led to a slight decrease in the analytical signal, which is most  
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1 likely due to surface aggregation of HSA at high concentrations, which may in fact reduce the  
2 binding site density. [40]  
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### 9 **3.2.2. Template removal**

10 Extraction of the template molecules liberates the binding sites formed during the MIP  
11 synthesis and is therefore a prerequisite for all subsequent binding experiments and sensing  
12 applications. For optimal template removal, several procedures were examined with regard to  
13 their ability to remove the ferritin molecules from the imprinted polymer matrix.  
14 Electrochemical template removal[41] was performed by immersing the polymer-modified  
15 electrode in 0.1 M H<sub>2</sub>SO<sub>4</sub> and cycling the potential between -0.2 and 1.5 V at 200 mV/s and  
16 the CV of 10 mM K<sub>4</sub>Fe(CN)<sub>6</sub> was obtained intermittently to monitor the progression of the  
17 template removal. A sudden increase in the peak current was observed between 30 and 40  
18 removal cycles indicating the necessity to perform at least 40 cycles to quantitatively remove  
19 the template (Figure 3a). On the other hand, the NIP film remained impermeable to the redox  
20 probe even after 70 cycles confirming that the polymer itself is intact. Unfortunately, the  
21 procedure was not reproducible for all conditions and gradual degradations of the polymer  
22 film turned us to seek milder conditions for template removal.  
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44 Therefore, template removal was attempted by enzymatic digestion of the template using  
45 500 mg/dm<sup>3</sup> proteinase K (in 10 mM NH<sub>4</sub>OAc pH 8 + 5 mM CaCl<sub>2</sub>) during gently shaking.  
46 Proteinase K has a broad cleavage specificity that results in very small peptide fragments or  
47 even single amino acids[42] which are then expected to be easily removed from the polymer.  
48 However, we found that this mild treatment, i.e., 1h incubation with the enzyme, is  
49 insufficient for the complete template removal while longer times were impractical.  
50 Nevertheless, the enzyme digestion may still be considered when the template removal is  
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1 performed simultaneously as the last step of MIP fabrication on a large batch of sensors (e.g.  
2 mass produced microfabricated electrodes).  
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5 The enzyme digestion was complemented with further intensive washing sequence with 5  
6 mM NaOH, 5% SDS and 5 mM NaOH (Figure 3b), which was found to be efficient.  
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8 However, since this post-treatment setbacks the benefits of the mild enzymatic digestion we  
9 explored the possibility to remove the template solely by washing the electrodes in the  
10 mentioned solutions. We found that 10 min washing in 5 mM NaOH followed by 5 min in  
11 5% SDS and 10 min in 5 mM NaOH was equally or even more effective (Figure 3b, red  
12 curve) as the same sequence applied after digestion. Further testing of various washing  
13 solutions including 5 mM H<sub>2</sub>SO<sub>4</sub>, 0.05% Tween20, ethanol and PBS confirmed that for HSA-  
14 MIPs, the above mentioned sequence was the most effective, while ferritin was best removed  
15 by this sequence followed by an additional 5 min in 0.05% Tween 20 and 5 min in 5 mM  
16 NaOH. In both cases, the washing procedure was completed by rinsing the electrodes in  
17 water.  
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### 36 **3.2.3. Template rebinding**

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38 The time required to reach a steady sensor response in a given analyte solution was  
39 determined by equilibrating the electrodes in 10 mM K<sub>4</sub>Fe(CN)<sub>6</sub> solution in PBS (pH 7.4)  
40 followed by addition of 1 g/dm<sup>3</sup> ferritin or 150 mg/dm<sup>3</sup> HSA. The sensor response (CV  
41 curve) was then periodically recorded over a time range of at least 35 minutes. The peak  
42 current of ferrocyanide oxidation decreased rapidly with the increase of incubation time ( $\Delta I$   
43 increased, Figure 4.) as the template molecules rebound to the MIP blocked the electron  
44 transfer between the electrode and the redox probe. A steady cyclic voltammogram indicating  
45 that the rebinding equilibrium is reached was obtained after 35 min for the ferritin-imprinted  
46 polymer. However, the signal did not change significantly after 20 min which therefore was  
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1 chosen for further experiments to minimize the analysis time. In the case of HSA an  
2 incubation time of 10 minutes was sufficient. Furthermore, we found that adding the analyte  
3 directly to the ferrocyanide solution or incubating the electrode in a separate solution with the  
4 analyte for the same duration and then transferring it into ferrocyanide solution did not affect  
5 the response (data not shown). Therefore, the simpler, additive method was applied to  
6 construct calibration curves, while the selectivity and biological sample measurements were  
7 performed in separate solutions to remove by rinsing unbound interferents.  
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### 21 **3.3. Surface characterization**

22 The thickness of the polymer films was determined by mechanically removing them from the  
23 gold over a rectangular area by using AFM in contact mode then remapping the surface in  
24 tapping mode[19]. Representative depth profiles taken across the indented area reveal a  
25 polymer film thickness close to 3 nm for the non-imprinted and 12-14 nm for the ferritin-  
26 imprinted film (Figure 5). Protein sensing with the surface imprinted polymer-modified  
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#### 39 **3.3.1. Concentration dependence and limit of detection**

40 To investigate the concentration dependence of the response of HSA and ferritin-imprinted  
41 polymers, the background solution containing 10 mM  $K_4Fe(CN)_6$  in PBS was spiked with  
42 various amounts of the target proteins and the CV of the redox probe was recorded after each  
43 protein addition. The analytical response of the sensors, i.e. the change in oxidation current of  
44 ferrocyanide ( $\Delta I$ ) in relation with the concentration of the target proteins is shown in Figure  
45 6. A close to linear range was observed between 20 and 100  $mg/dm^3$  for HSA while the  
46 dynamic range of the ferritin sensor was between 40 and 360  $mg/dm^3$ . The HSA and ferritin  
47 imprinted MIPs showed saturation around 400 (ca. 6  $\mu M$ ) and 600  $mg/dm^3$  (ca. 1.3  $\mu M$ )  
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1 respectively. The limit of detection, calculated as  $3 \cdot \sigma_{\text{intercept}}/\text{slope}$  from the parameters of a  
2 linear fitting to the lowest concentration ranges, was found to be  $3.7 \text{ mg/dm}^3$  (56 nM) for  
3 HSA and  $10.7 \text{ mg/dm}^3$  (10 nM) for ferritin. Interestingly the 5-6 times lower saturation  
4 concentration and LOD in case of ferritin seems to match roughly the ratio in the footprints of  
5 the two molecules, i.e., the spherical shape ferritin has a radius of 9.1 nm[43] while HSA 3.51  
6 nm[44] which means that the ratio of their footprints is 3.9. The HSA response could be well  
7 fitted (adjusted R-square of 0.986) with the Hill equation having the Hill coefficient (n)  
8 indicative of binding cooperativity set to 1, which resulted in a  $K_d$  of  $2.8 (\pm 0.4) \mu\text{M}$ .  
9

10 The NIP electrodes were practically impenetrable for the redox probe and consequently  
11 showed only a very low response even to high concentrations of HSA or ferritin (data not  
12 shown). However, QCM measurements confirmed that increasing concentrations of HSA did  
13 not provoke any frequency changes suggesting that non-specific adsorption was negligible  
14 (data not shown).  
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### 17 **3.3.2. Repeatability and stability**

18 The repeatability of the HSA-MIP and ferritin-MIP modified electrodes was determined at  
19  $150 \text{ mg/dm}^3$  HSA and  $100 \text{ mg/dm}^3$  ferritin concentration levels in five subsequent template  
20 binding-removal cycles, respectively. The relative standard deviation of the HSA and ferritin  
21 sensors was 6.8% and 6.3%, respectively, demonstrating the possibility to regenerate and  
22 repeatedly use the MIP-based sensors. The reproducibility of the sensor fabrication was  
23 tested by preparing four MIP-coated electrodes under the same conditions and measuring  
24 their additive response to  $50\text{-}250 \text{ mg/dm}^3$  HSA. The relative standard deviation of the  
25 sensitivities was 5.8%, which matched within the experimental error the repeatability of the  
26 MIP-based sensors. The short term stability was evaluated by keeping the MIP-based  
27 electrode in DI water for seven days and measuring intermittently its response to  $100 \text{ mg/dm}^3$   
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1 HSA or 200 mg/dm<sup>3</sup> ferritin. The largest deviation from the original response was 5.7% and  
2 8%, respectively, over the studied time range.  
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### 5 **3.3.3. Selectivity**

6 A quick assessment of the selectivity of the HSA-sensor towards proteins was made beside  
7 ferritin (much larger than HSA, MW 450 kDa) with avidin (similar size as HSA) and  
8 lysozyme (considerably smaller size than HSA) at 100 mg/dm<sup>3</sup> level. Since the  
9 polyscopoletin film is negatively charged and the latter two proteins with high pIs are  
10 notorious for their nonspecific adsorption they were expected to be indicative of the worst  
11 scenario, i.e., highest nonspecific adsorption.[35] After immersed in the examined protein  
12 solutions for 5 min, the sensor was washed with deionised water and then CV experiments  
13 were performed in 10 mM K<sub>4</sub>Fe(CN)<sub>6</sub> in PBS. As shown in Figure 7a the sensor response to  
14 avidin and lysozyme was less than 30% of the response given to the same concentration of  
15 HSA. As expected the much larger ferritin (450 kDa) with a pI of 5.5 produced the smallest  
16 interference.  
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35 To eliminate the eventuality that electroactive molecules such as ascorbic acid and uric acid  
36 are interfering with the current signal, i.e., adsorbed during incubation in the sample on the  
37 MIP which may interfere with the ferrocyanide signal, the MIP modified electrodes were  
38 incubated in 5.7 mM ascorbic acid and 4.5 mM uric acid and then subjected to the same short  
39 washing procedure as in case of protein detection. Cycling the potential of the electrodes in  
40 ferrocyanide solution the observed change in the current observed was less than 6%  
41 indicating that they were easily removed from the cavities of the MIP during the rinsing step  
42 (Figure 7b).  
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### 54 **3.4. Urine albumin and plasma ferritin measurements**

1 The ferritin levels in the plasma of healthy patients range from 12 to 300  $\mu\text{g}/\text{dm}^3$  while the  
2 LOD of MIP based ferritin sensor is 40  $\text{mg}/\text{dm}^3$ . Thus this MIP sensor is clearly not suitable  
3 for clinical application. However, to check the assumption that high abundance proteins may  
4 be determined even in complex samples we spiked the plasma with ferritin concentrations  
5 (40-150  $\text{mg}/\text{dm}^3$ ) assessable with the ferritin-MIP-based electrode. The recoveries were  
6 surprisingly good given the complexity of the matrix, i.e. within 5% except for the smallest  
7 tested concentration where ca. 20 % error was observed, most likely due to uncertainty of the  
8 measurements close to the LOD (Table 1).  
9

10 On the other hand, albumin is the most abundant protein in blood being present in 35-  
11 50  $\text{g}/\text{dm}^3$  concentration and it can also appear in the urine in up to 25  $\text{mg}/\text{dm}^3$  concentration  
12 for healthy individuals. In case of microalbuminuria the urinary albumin excretion elevates  
13 further to ca. 200  $\text{mg}/\text{dm}^3$ , which can be an early indicator of kidney damage. Given its  
14 operating range of 20 to 300  $\text{mg}/\text{dm}^3$  these level should be readily accessible with HSA-MIP  
15 based sensor. First a spot urine sample from a healthy volunteer was measured where the  
16 albumin concentration was found to be below the limit of detection. The sample was then  
17 spiked with 20-120  $\text{mg}/\text{dm}^3$  albumin and the added concentrations could be determined with  
18 less than 5% relative error (Table 2) indicating that the sensor is suitable for rapid testing of  
19 albumin in urine.  
20

21 In order to further evaluate the applicability and reliability of the developed MIP sensor  
22 device in clinical analysis, three urine samples from diabetic patients were analysed directly  
23 or after dilution with PBS pH 7.4. The electrodes were incubated for 5 min in the urine  
24 samples and after washing CV experiments were performed in 10 mM  $\text{K}_4\text{Fe}(\text{CN})_6$  dissolved  
25 in PBS to determine the oxidation current. Quantification of HSA was performed using the  
26 multiple standard addition method and the results were compared with albumin levels  
27 determined with a standard immunoturbidimetric method. The results show less than 10 %  
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1 deviation between the HSA contents determined by the two methods (Table 3), which given  
2 the general uncertainties of HSA measurements, i.e., HSA present in various forms and  
3 molecular associates seems satisfactory. To further show that the MIPs are selectively  
4 determining the HSA content of the samples the total protein content of the urine samples  
5 were estimated by the BCA assay. The total protein content was found to be significantly  
6 higher than the HSA content for each sample, suggesting that the HSA-MIP can be used to  
7 determine the HSA in urine in the presence of other proteins as well.  
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#### 17 **4. CONCLUSIONS**

18 In this study we have shown that polyscopoletin nanofilms can be made sensitive to two  
19 different proteins, HSA and ferritin, by surface imprinting. These MIPs were rendered  
20 selective only by the imprinting process as the employed monomers and proteins had no  
21 distinct features to predestine the formation of binding sites with strong interaction with the  
22 template. Indeed, the  $K_d$  of the HSA-imprinted film was in the lower micromolar range, while  
23 the saturation concentration and LODs of the two MIPs indicates fairly similar responses  
24 scaling mainly with the size of the template proteins. As expected the relatively high LODs  
25 obtained ( $10.7 \text{ mg/dm}^3$  for ferritin) did not allow the assessment of endogenous ferritin in  
26 blood. However, ferritin spiked plasma samples falling in the dynamic range of the ferritin-  
27 MIP have shown excellent recoveries, despite the complexity of the matrix. On the other  
28 hand, the more prevalent albumin was successfully quantified with the HSA-MIP-based  
29 sensor in urine samples from diabetic patients. The results were in satisfactory agreement  
30 with immunoturbidimetric determination, suggesting that this approach can be used for  
31 detection of HSA in urine sample for diagnostic purposes as alternative to more expensive  
32 antibody based assays. This application seems to not require extremely high affinity  
33 MIPs[22] as reported earlier and overall suggests as a perspective application for genuine  
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1 “randomly” imprinted protein MIPs the quantification of high abundance proteins of clinical  
2 relevance that, however, still require a selective recognition process.  
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## 8 **Acknowledgement**

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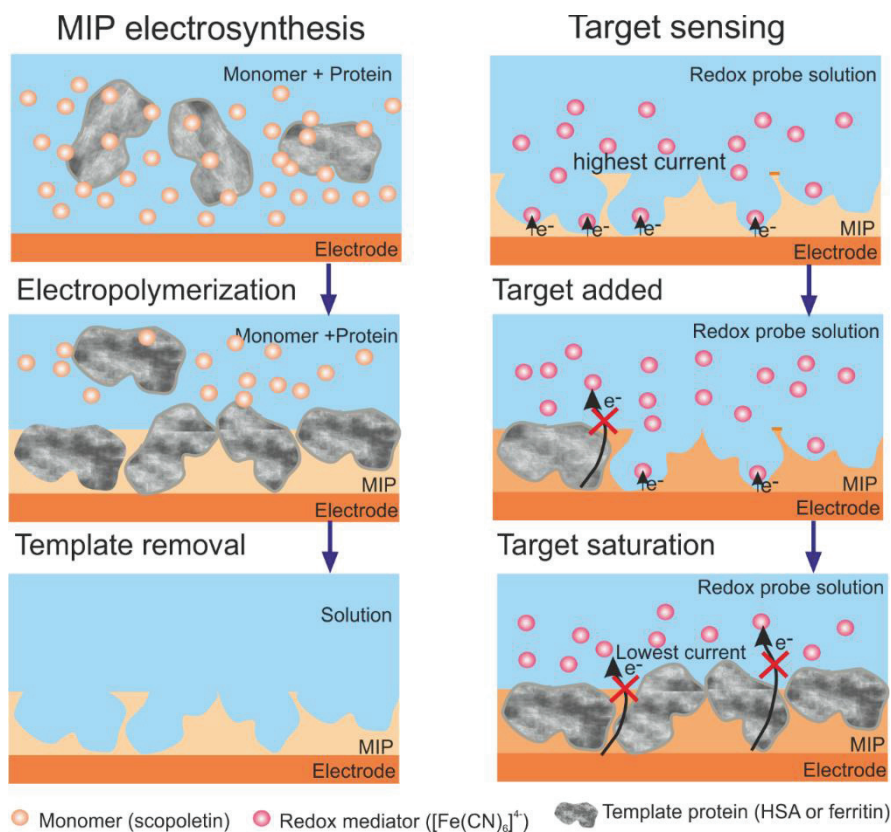
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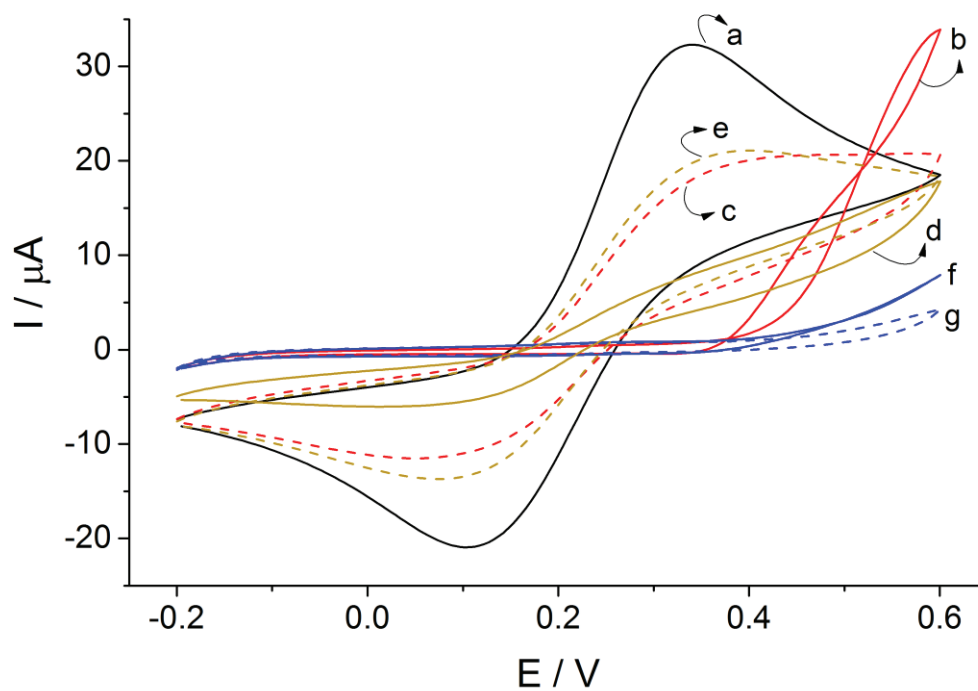
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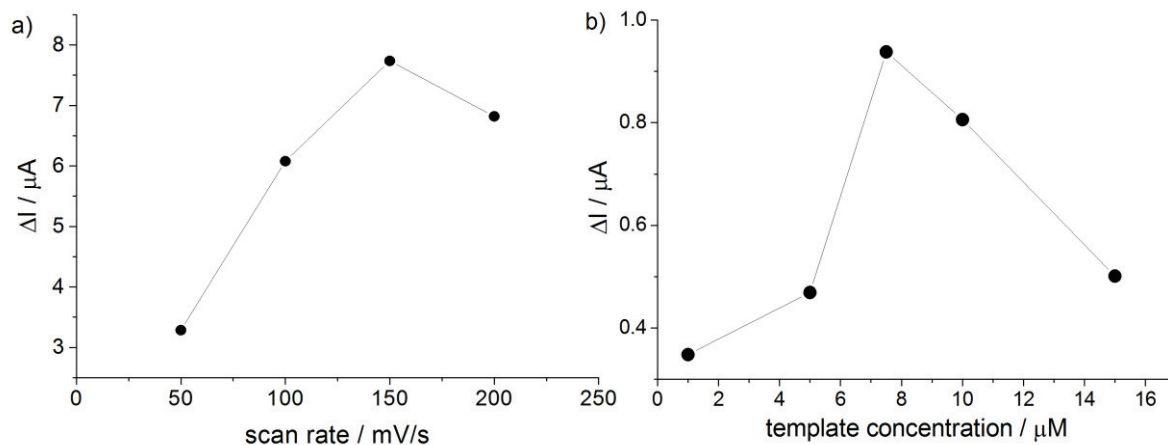
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**Scheme 1.** Schematics of the electrosynthesis of surface imprinted polyscopoletin nanofilm on a gold electrode surface and its use for the detection of the target. Please note that while not indicated in the scheme, in case of biological samples the MIP-modified electrodes were first incubated in these samples and after washing the electrode was transferred in the redox probe solution for the measurement.



**Figure 1.** Cyclic voltammograms of 10 mM  $K_4Fe(CN)_6$  redox probe in 10 mM PBS pH 7.4 recorded on bare Au disk electrode (a), on a MIP film coated electrode before (b,d) and after (c,e) removal of the HSA and ferritin template, respectively, and NIP film coated electrode before (f) and after washing (g).



**Figure 2.** Effect of (a) scan rate during electropolymerization and (b) template concentration on the response of MIP-based sensors. (a) The polymers were deposited from 1 mM scopoletin in PBS pH 7.4 containing 1  $\mu\text{M}$  ferritin by 3 cycles between 0 and 1 V with the specified scan rates. The sensor responses to 1 mM ferritin were measured after template removal. (b) The polymers were deposited from 1 mM scopoletin in PBS pH 7.4 containing 1-15  $\mu\text{M}$  HSA by 3 cycles between 0 and 1 V at 150 mV/s. The sensor responses to 100  $\text{mg}/\text{dm}^3$  HSA were measured after template removal.

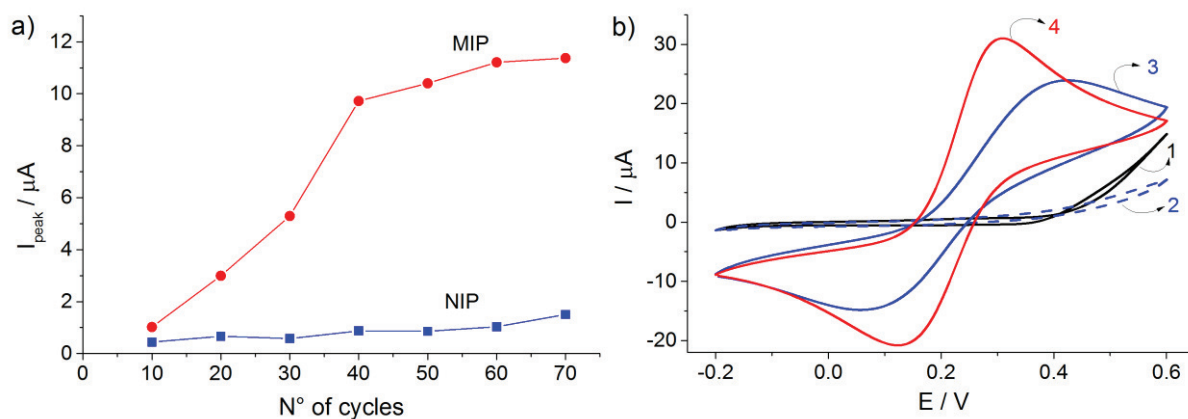
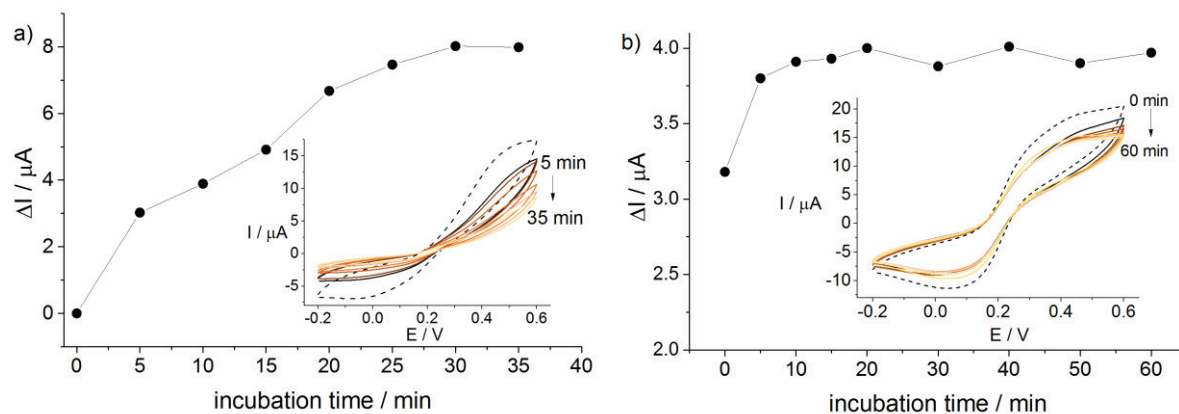
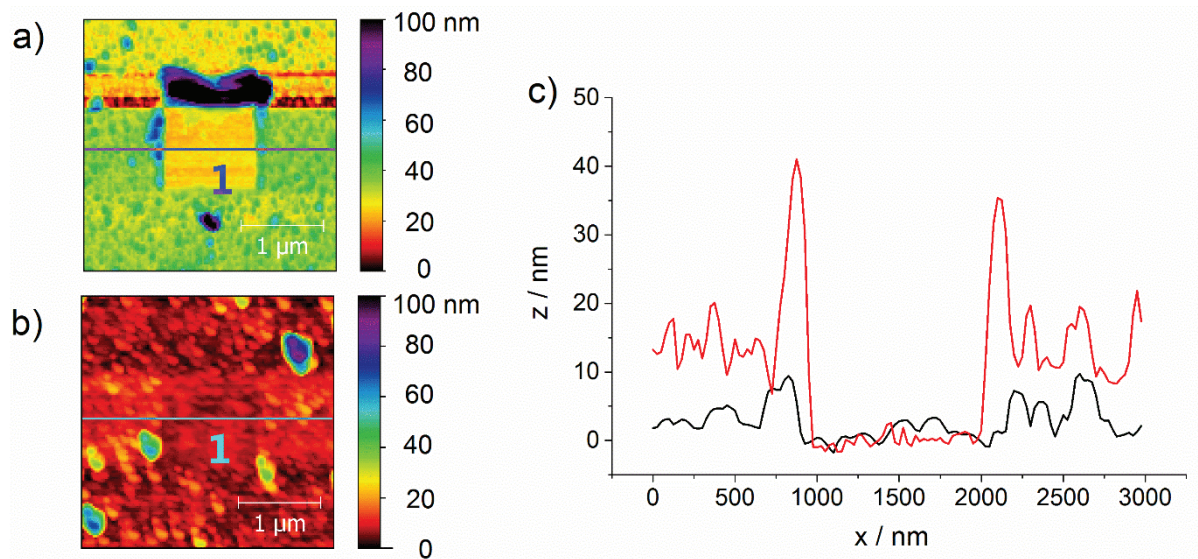


Figure 3. Removal of ferritin template by electrochemical cycling in 0.1 M  $H_2SO_4$  (a) and by digestion with 500  $mg/dm^3$  proteinase K (b). (a) Oxidation peak current of 10 mM  $K_4Fe(CN)_6$  with the MIP (circle) and NIP (square) covered electrodes after up to 70 cycles in  $H_2SO_4$ . (b) CVs of 10 mM  $K_4Fe(CN)_6$  on MIP after polymer deposition (1-black), after 1h in proteinase K (2-dashed blue), further washed with 5 mM NaOH and 5% SDS (3- solid blue), a MIP only washed with 5 mM NaOH and 5% SDS (4- red).

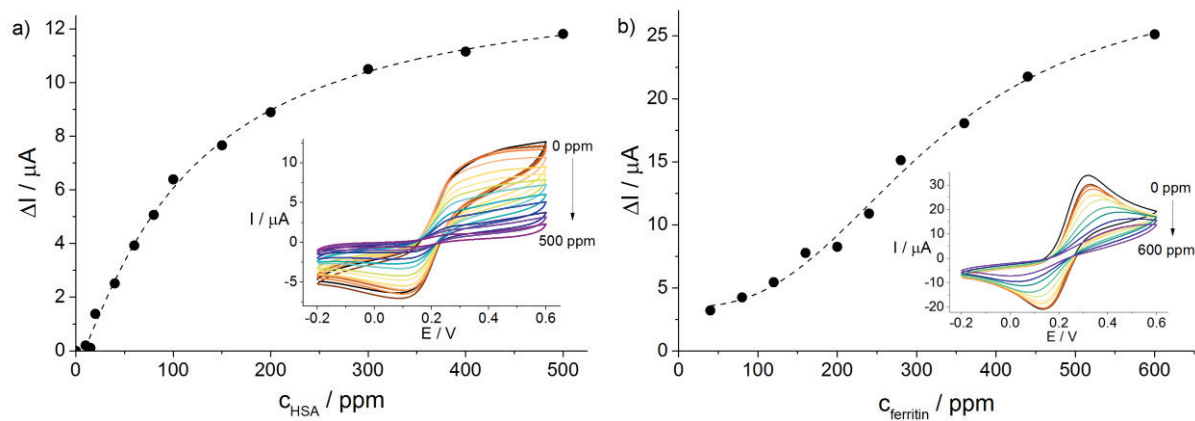




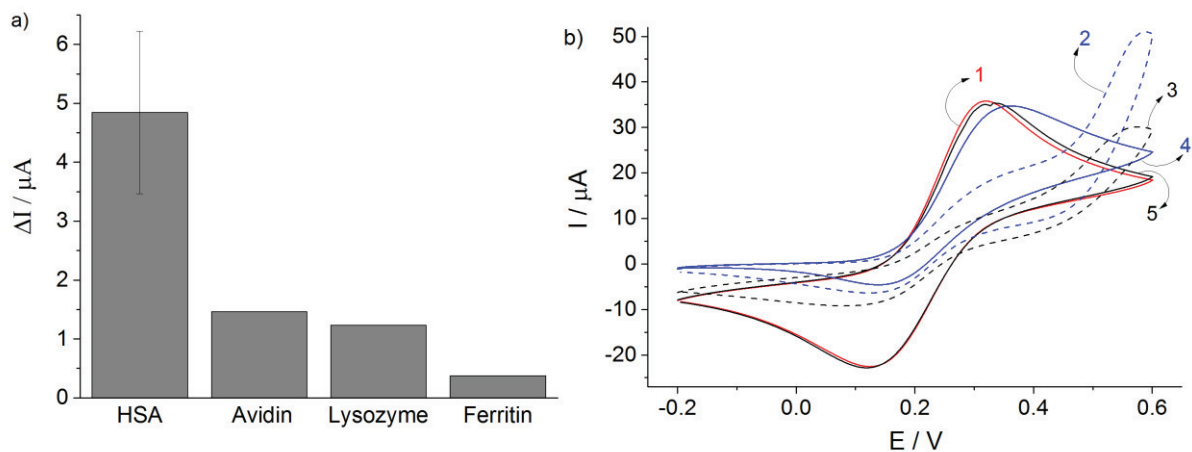
**Figure 4.** Effect of incubation time on the response of ferritin (a) and HSA (b) imprinted polymers to template rebinding. Insets: CVs of MIP-modified electrode before protein addition (dashed line) and after different incubation times in 1 g/dm<sup>3</sup> ferritin (a) or in 150 mg/dm<sup>3</sup> HSA (b). The CVs were recorded in 10 mM K<sub>4</sub>Fe(CN)<sub>6</sub> (PBS pH 7.4).



**Figure 5.** Representative morphology of the ferritin-imprinted (a) and non-imprinted (b) polymer layer after removing the film by contact mode AFM over a  $1 \times 1 \mu\text{m}$  area and the corresponding cross-sectional profiles (c, MIP: red, NIP: black). The polymers were deposited from 1 mM scopoletin in PBS pH 7.4, (containing additionally  $1 \mu\text{M}$  ferritin in the case of MIP) by 3 potential cycles between 0 and 1 V at 150 mV/s. A TAP190GD-G tip (force constant: 48 N/m, length:  $225 \mu\text{m}$ ) was used for five consecutive scans in contact mode (500 nN force) to remove the polymer layers, followed by scanning a larger ( $3 \times 3 \mu\text{m}$ ) area in tapping mode.



**Figure 6.** Concentration dependence of sensor response: 5-300  $\text{mg}/\text{dm}^3$  HSA rebinding to a HSA-imprinted sensor (a) and 40-600  $\text{mg}/\text{dm}^3$  ferritin rebinding to a ferritin-imprinted sensor (b). Insets: corresponding CV curves in 10 mM  $\text{K}_4\text{Fe}(\text{CN})_6$  in PBS.



**Figure 7.** The HSA-MIP based sensor's response to (a) various proteins at 100 ppm concentration level and (b) small molecules: CV curve of 10 mM  $K_4Fe(CN)_6$  in PBS pH 7.4 on HSA-imprinted MIP electrode before (1-red) and after incubation in 5.7 mM ascorbic acid (2-dashed blue) or 4.5 mM uric acid (3-dashed black) and after subsequent washing (after ascorbic acid: 4-solid blue, after uric acid: 5-solid black).

**Table 1.** Determination of ferritin in spiked plasma samples

added concentration, mg/dm <sup>3</sup>	determined concentration, mg/dm <sup>3</sup>	recovery, %
40	48.4	121.0
60	62.8	104.7
80	83.2	104.0
100	103.9	103.9
120	128.6	107.2
150	154.2	102.8

**Table 2.** Determination of albumin in spiked spot urine samples

added concentration, mg/dm <sup>3</sup>	determined concentration <sup>a</sup> , mg/dm <sup>3</sup>	recovery, %
20	20.93±1.48	104.7
60	62.98±2.36	105.0
80	83.57±3.45	104.5
100	125.78±5.02	104.8

<sup>a</sup> mean±2SD, n=3**Table 3.** Determination of HSA in urine samples obtained from diabetic patients

Sample	MIP (mg/L)	Immunoturbidimetry (mg/L)	Relative error (%)	Total protein (mg/mL)
1	86.0	89.2	3.7	165
2	7.7	8.3	8.8	107
3	546.2	506.5	7.83	728