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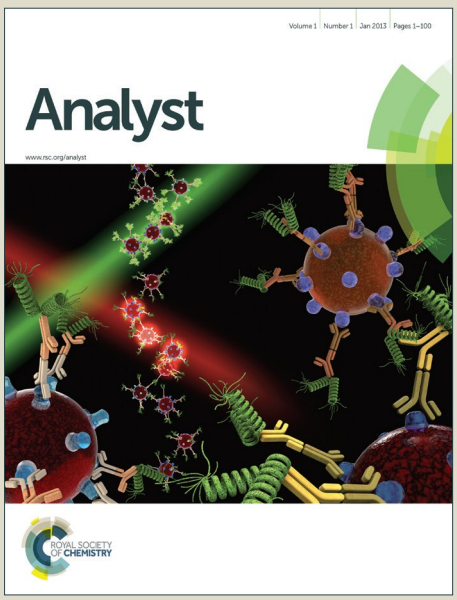

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Design of molecularly imprinted conducting polymer protein-sensing films via substrate-dopant binding

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Abstract

Addressing the challenge of protein biosensing using molecularly imprinted polymers (MIP), we have developed and tested a novel approach to creating sensing conducive polymer films imprinted with a protein substrate, Ricin Toxin Chain A (RTA). Our approach for creating MIP protein sensing films is based on a concept of substrate-guided dopant immobilization with subsequent conducting polymer film formation. In this proof-of-concept work we have tested three macromolecular dopants with strong protein affinity, Ponceau S, Coomassie BB R250 and ι-Carrageenan. The films were formed using sequential interactions of the substrate, dopant and pyrrole, followed by electrochemical polymerization. The films were formed on gold array electrodes allowing for extensive data acquisition. The thickness of the films was optimized to allow for efficient substrate extraction, which was removed by a combination of protease and detergent treatment. The MIP films were tested for substrate rebinding using Electrochemical Impedance Spectroscopy (EIS). The presence of macromolecular dopants was essential for MIP film specificity. Out of three dopants tested, RTA-imprinted polypyrrole films doped with Coomassie BB performed with highest specificity towards detection of RTA with a level of detection (LOD) of 0.1 ng/ml.

Keywords

MIP, conducting polymer, biosensor, Ricin, polypyrrole, molecularly imprinted polymer

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1. Introduction

Molecularly imprinted polymers (MIPs) are seen by many as an inexpensive synthetic alternative to antibodies and natural receptors. Performing polymerization in the presence of the substrate, one can produce a polymer with its molecular imprint once the substrate is removed. Molecular imprinting involves binding of the functional groups of a monomer with the substrate through a combination of hydrophobic and ionic interactions. The functional groups are locked in position during polymerization, forming a polymer network around the embedded substrate. Following the substrate removal, the polymer is left with molecular cavities, complementary to the substrate. The created receptor sites can be used for rebinding of the substrate. The ability of the imprinted polymers to selectively re-bind the substrate molecules has been researched and used for over seventy years for affinity separation and catalysis, and for over a decade for drug delivery and sensing applications, according to the vast database of MIP literature [**http://mipdatabase.com**], containing thousands of publications.

The current state of science and applications of MIPs, including chromatography, electrophoresis, catalysis, chemical sensing and biosensing, drug delivery, crystallization and cell culturing has been recently reviewed [**1-3**]. While molecular imprinting of small molecules with molecular weight below 1kD has been very successful and commercially viable, imprinting of biological macromolecules, such as proteins, polypeptides and/or DNA has been more challenging due to solubility, size and fragility of biological molecules [**4**]. A number of 2D and 3D imprinting approaches for macromolecules have been tested, defining the choice of solvents and variety of polymer precursors to allow desirable polar and hydrophobic interactions of the substrate and the polymer [**5**]. Performing successful imprinting of macromolecules, especially proteins is receiving a lot of attention lately as it opens many possibilities for label-free biosensing of a variety of biomarkers in ever-expanding medical diagnostics or bioenvironmental monitoring and can combine improved stability, price efficiency and versatility compared to conventional immunodetection methods [**6**].

Among different types of polymers used to produce MIP are conducting polymers, such as polypyrrole, polyaniline, polythiophene and others. The attractive quality of conducting polymers is that in addition to chemical polymerization, they can be polymerized electrochemically, incorporating the substrate into the polymer matrix. This can be a convenient way to confine a desired specificity to an electrode, which can be used for electrochemical sensing. Indeed, a wide range of low molecular weight substrates has been successfully imprinted into conducting polymers for sensing applications during the past decade, which included caffeine, dopamine, catechol, theophylline, sulfadimethoxine, atrazine, uric acid, doxycycline and others. Bulkier biological macromolecules, which have been imprinted for sensing purposes with conducting polymers include “model” proteins, bovine serum albumin and avidin, along with bovine hemoglobin, glycoprotein gp51 from bovine leukemia virus and human cardiac troponin. Recent publications on molecularly imprinted conducting polymer (MICP) sensors are summarized in **Table 1**, with the majority of them using electrochemical methods, such as amperometry, cyclic voltammetry (CV), differential pulse voltammetry (DPV)

and electrochemical impedance spectroscopy (EIS) to detect rebinding of the substrate to the imprinted sensing electrode.

MIP sensors using conducting polymers				
Substrate	***Conducting Polymer***	***Sensing method***	***Ref.***	***Year***
glycoprotein gp51 from Bovine Leukemia Virus	pPy	pulsed amperometry	**[7]**	2004
caffeine	pPy	pulsed amperometry	**[8]**	2006
caffeine	poly(o-phenelynediamine), pPy	piezoelectric quartz crystal	**[9]**	
uric acid	Amine-imide type conducting polymer	amperometry	**[10]**	2007
Amadori compound N-(1-deoxy-β-d-fructopyranose-1-yl)-l-valine (Fru-Val)	poly-aminophenylboronic acid	open circuit potential (Δ Eoc)	**[11]**	2009
atrazine	polythiophene derivative	CV	**[12]**	
catechol and dopamine	polyaniline derivative	CV	**[13]**	
avidin	PEDOT/PSS	fluorescence	**[14]**	
caffeine	pPy		**[15]**	2010
tyrosine	copper oxide-pPy	voltammetry	**[16]**	2011
D- and L-glutamic acid, 1- and 2- naphthalene-sulfonates;	o-pPy	EQCM	**[17]**	2012
para-nitrophenol	polyaniline-polyvinyl sulfonic acid	DPV	**[18]**	2013
doxycycline	pPy	CV	**[19]**	
bovine hemoglobin	pPy	DPV. EIS	**[20]**	
BSA	tetraethylene glycol 3-morpholin propionate acrylate/carbon nanotubes	DPV	**[21]**	
BSA	o-phenylenediamine and 3-aminophenylboronic acid monohydrate	Ec oxidation of grafted 6-ferrocenyl-hexanethiol	**[22]**	
human cardiac troponin	o-phenylenediamine	CV, EIS	**[23]**	
norepinephrine	o-aminophenol	SWV	**[24]**	2014
sulfadimethoxine	o-pPy	SDM, amperometric	**[25]**	
sulfadimethoxine	pPy	CV	**[26]**	
theophylline	pPy	gravimetry	**[27]**	
bacteria	o-pPy	dielectrophoresis.	**[28]**	

Table 1. MIP sensors using conducting polymers. Abbreviations: pPy: polypyrrole; o-pPy: oxidized polypyrrole; PEDOT: Poly(3,4-ethylenedioxythiophene); PSS: polystyrene sulfonate; BSA: bovine serum albumin; CV: Cyclic Voltammetry; DPV: Differential Pulse Voltammetry; EIS: Electrochemical Impedance Spectroscopy; SWV: square wave voltammetry; SDM: Stepwise Dissolution Measurement; EQCM: Electrochemical Quartz Crystal Microbalance.

Polypyrrole (pPy) is a highly biocompatible conducting polymer used widely for electrochemical biosensing, as reviewed by [**29**]. Solubility of pyrrole monomer in water allows to perform electrochemical polymerization in the presence of biological molecules while maintaining their native state, which can be used for their entrapment to either functionalize pPy or imprint it. Imprinting with pPy can involve physical entrapment for uncharged substrate molecules, while negatively charged substrates can be incorporated into pPy matrix as dopants, participating in the polymer's conductivity. In such case, removal of the substrate to create an imprint will result in

re-doping by an anion in the solution, thus occupying functionality and affecting the accuracy of the imprint. Over-oxidizing polypyrrole efficiently expels the anionic substrate, but results in diminishing of electrochemical activity of pPy. To maintain high electrochemical activity of a polymer, one should find a way to maintain polymer-dopant interactions while removing the substrate after the imprinting stage.

We have previously explored preparation of thin pPy films from water solutions using negatively charged biological molecules as sole dopants [**30**]. In our experience, using bulky anionic macromolecular dopants resulted in solution-stable, electrochemically active and reusable functionalized pPy films. We have also found that when an anionic macromolecule is used as a dopant, any changes occurring to the dopant can be registered electrochemically. Combining this finding with the MIP concept, we aimed at creating a molecularly imprinted conducting polymer (MICP) sensing film by using the dopants which would not only contribute to stable conductivity of the pPy film but will also have affinity to the protein template. As compounds with sulfonate ($-SO_3^-$) groups are known to be among the best dopants for pPy, we have chosen three sulfonate-rich compounds with known affinity to proteins, Ponceau S, Coomassie BB and Carrageenan to be tested in MICP preparation. The dopants were used to create a mold around the protein substrate, which was followed by polymerization of pyrrole. Using this innovative approach, the macromolecular dopants contributed to the specificity of the imprint at the molecular level.

All steps of MICP film preparation and substrate rebinding were monitored by Electrochemical Impedance Spectroscopy (EIS), also known as AC Impedance. EIS is a popular method in electrochemical biosensing due to its versatility and sensitivity [**31**]. We have previously used EIS for electrochemical detection of proteins by means of electrode-immobilized specific aptamers. We have found that EIS is extremely sensitive to surface binding phenomena at the molecular level [**32, 33**]. Below we describe a new biosensing application with the specificity of the protein substrate binding provided by MICP film, while the binding event is registered through changes in EIS. To develop and test this method we have used Ricin Toxin Chain A (RTA) as a model substrate.

2. Material and Methods

2.1. Materials.

Phosphate-buffered saline (PBS) and DNAse-free, RNAse-free deionized water were purchased from Invitrogen. Coomassie Brilliant Blue R250, Ponceau S, Ricin Toxin Chain A (RTA), pyrrole, Bovine Serum Albumin (BSA), ι-Carrageenan (Type II) $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ and other chemicals were purchased from Sigma-Aldrich. Custom array electrodes were developed by Fractal Systems Inc. [**33**] and produced by Applied Biophysics.

2.2. Electrochemical measurements.

All electrochemical measurements were performed using CHI-760B Potentiostat (CH Instruments). The 9-electrode array (one side of the double-sided format, **Figure S1,**

Supplementary Information) was connected to the potentiostat using a switch-box, allowing to connect individual electrodes separately or simultaneously. The array chamber was fitted with Ag/AgCl mini reference electrode (Cypress Systems) and a counter wire electrode, positioned above the array electrodes. The array electrodes were cleaned electrochemically via cyclic voltammetry (CV: -0.9 V - +0.9 V, 100 mV/s, 10 segments) in 0.05 M H_2SO_4, rinsed with 0.05 M H_2SO_4, rinsed with copious amounts of deionized water, air dried and used immediately for substrate immobilization.

AC Impedance measurements of the MICP-modified array electrodes were performed in PBS buffer (pH 7.4) containing 2.5 mM $K_4Fe(CN)_6$, 2.5 mM mM $K_3Fe(CN)_6$. AC impedance spectra for each array electrode were recorded sequentially in a frequency range of 0.1 Hz to 100 kHz and AC amplitude of 0.005 V. AC impedance spectra were presented as a Nyquist plot (Z', real impedance, versus -Z", imaginary impedance). The -Z" value, which corresponds to the highest point of the semicircle on the Nyquist plot was used along with conventional R_{ct} (charge transfer resistance) value, obtained from the spectra fitted to Randles equivalent circuit by CH Instruments software. The relative values, i.e. % of Impedance -Z" change and % of R_{ct} change were found to be proportional, and % of -Z" value was routinely used for MICP evaluation.

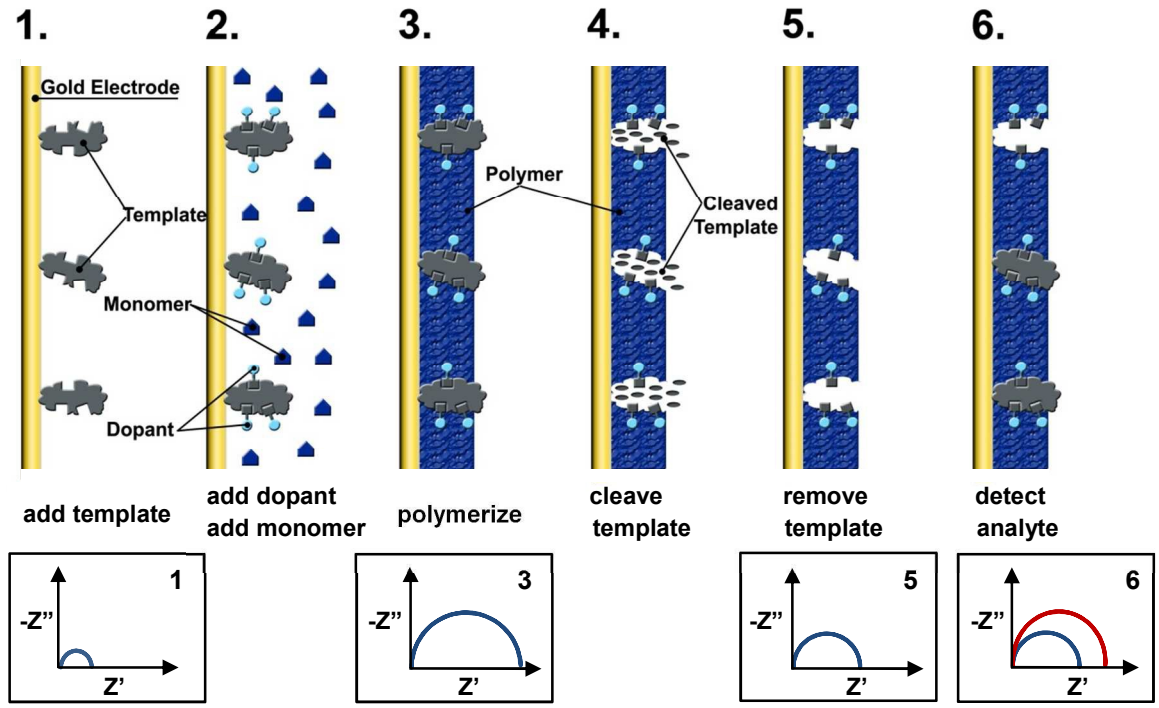

Figure 1. Preparation and testing of MICP sensing film: **(1)** Protein template is adsorbed on the surface of the gold electrode through amino groups (Impedance is low); **(2)** The dopant solution is applied to the absorbed protein and binds to it; **(3)** Electrochemical polymerization results in conducting polymer film with locked template (Impedance increase); **(4)** Protein template is cleaved by a protease; **(5)** Cleaved protein is extracted from the conductive polymer film (Impedance decrease); **(6)** Specific substrate is recognized by the imprinted film (Impedance increase). The inserts show schematic of AC Impedance spectra (Nyquist plot) changes in the process of film preparation and testing, with the numbers corresponding to the steps above. Insert 1: starting Impedance; Insert 3: impedance increases after conducting film polymerization; Insert 5: impedance decreases after substrate extraction; Insert 6: impedance increases after analyte rebinding.

2.3. MICP film preparation from polypyrrole.

Sensing film preparation consisted of five steps (steps 1-5, **Figure 1**). Each step was monitored by EIS. The procedure was optimized in a course of multiple experiments.

- ***Step 1, substrate immobilization.*** 5 µl drop of solution containing 0.9 mg/ml (30 µM concentration) of RTA, 40% glycerol, 10 mM phosphate, pH 6.0, 150 mM NaCl, 10 mM galactose, 0.5 mM dithioerythritol (original solution, Sigma-Aldrich L9514) was placed drop-wise on each pre-cleaned array electrode, incubated in a humidifying chamber for 30 min. at room temperature (R.T.) and rinsed with PBS.
- ***Step 2, targeted dopant application.*** One of the following dopants (see **Figure S2, *Supplementary Information,*** for chemical structures) was applied directly to each of the array electrodes in a 5 µl drop and incubated for 30 min. at R.T. (typically each dopant was applied to three of the nine electrodes): Ponceau S (3-Hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]phenylazo)-2,7-naphthalenedisulfonic acid sodium salt, 10 mM in PBS), Coomassie Brilliant Blue R250 (10 mM in PBS), ι-Carrageenan (5 mg/ml in PBS). After incubation the array was rinsed in PBS.
- ***Step 3, electrochemical polymerization***. Pyrrole (0.05M in PBS) was polymerized simultaneously on all array electrodes (CV, 0 - 0.7 V, 50 mV/s, 3 cycles, or 6 segments). To remove weakly-bound RTA, the array electrodes were washed with PBS/0.5% TWEEN 20 (Polyethylene glycol sorbitan monolaurate) for 30 min. on a rotary shaker. Alternatively, SDS could be used instead of TWEEN in this and subsequent washing steps.
- ***Step 4, substrate cleavage.*** Following the wash, RTA captured in polypyrrole was digested with 3 mg/ml of Proteinase K in PBS for 2 hours at 37° C.
- ***Step 5, substrate removal.*** To remove digested RTA and Proteinase K, the array electrodes were washed with PBS/ 0.5% TWEEN 20 for 1h with shaking.

2.4. MICP film testing

AC Impedance was measured before and after incubation with the analytes as described in section 2.2. The measurements were followed by PBS/ 0.5% Tween rinse to remove ferrocyanides prior to further incubations. For binding, the electrodes were incubated for 30 min. at R.T. in a drop-like fashion with one of the analytes: 1 µg/ml of RTA solution (~33 nM, specific), or 10 µg/ml of BSA solution (10 times weight excess, nonspecific) in PBS buffer containing 0.04% glycerol, 11 µM galactose, 0.55 µM dithioerythritol. Thus, one of three electrodes carrying MICP film with a particular dopant was exposed either to a specific analyte, or nonspecific protein or buffer. The difference between the Impedance values (expressed as % of Impedance (-Z") change) was used to characterize the binding to MICP film. The array electrodes were reusable after washing in PBS/ 0.5% TWEEN overnight at R.T. with shaking, AC Impedance measurements and incubations were repeated up to 4 times.

3. Results and Discussion

3.1. MICP preparation

Our approach for creating MICP protein sensing films is based on a novel concept of substrate-guided dopant immobilization subsequent conducting polymer film formation. Step-wise MICP preparation (**Figure 1**) was chosen to allow for efficient molecular interaction of substrate and dopant in kinetically unrestricted conditions.

First, the protein substrate, Ricin Toxin Chain A (RTA) was immobilized on the electrode surface by absorption, as proteins are known to be immobilized on gold through a combination of electrostatic and hydrophobic interactions [**34**]. Immobilization was confirmed by EIS after 10, 20, 30, 40 and 60 min., resulting in Impedance increase compared to the pure gold electrode. 30 minutes was sufficient as the immobilization rate did not increase beyond this time.

Next, the immobilized substrate was allowed to interact with a dopant. Three compounds, Ponceau S, Coomassie BB R250 and ι-Carrageenan were selected as dopants for their known affinity to proteins, which they bind mostly electrostatically due to the presence of sulfonate ($-SO_3^-$) groups **[35-37].** Compounds with sulfonate groups are also excellent dopants for pPy, contributing to its conductivity [**38**]. Thus, after binding the protein substrate and creating a mold around it, the dopants were incorporated into ultra-thin pPy films, which were polymerized on the electrodes around the immobilized substrate coated by the dopant. The concentration (w/v) of the dopants used for binding the substrate was in 5 - 10 times excess relative to the original concentration of RTA used in immobilization (the molar concentration of Ponceau and Coomassie exceeded molar concentration of RTA ~300 times). The excess of dopant was needed to ensure complete coverage of the immobilized substrate with the dopant molecules, while sulfonate groups would contribute both to protein binding and doping pPy. The unbound dopant was removed prior to film polymerization.

PPy sensing films were polymerized simultaneously on array electrodes by cyclic voltammetry (CV) from a solution of pyrrole in PBS. The presence of the buffer was needed to preserve the conformation of immobilized RTA. We hypothesized that while the anions present in PBS buffer would also dope the growing pPy film, the immobilized dopants would have an advantage due to their immobilized state, bulkiness and the presence of multiple sulfonate groups. As pPy films were repeatedly washed and incubated for prolonged times in solutions, this proved to be the case, i.e. the films were stable in solution and maintained their binding properties.

The thickness of the pPy films depended on the number of cycles in CV polymerization. The thickness of the pPy film defined whether the protein substrate molecules were completely or only partially embedded. We have compared films prepared with 5, 4 and 3 cycles of CV. The first two resulted in thicker films embedding the protein molecules, which made it impossible to extract them later on. Films prepared with 3 cycles of CV performed well in the substrate extraction step.

The efficiency of substrate extraction_defines the sensitivity of MIP. For bulky biological substrates of complex shapes, this step of MIP preparation poses the most challenge. After testing, comparing and combining different approaches, such as washing MICP with detergent, over-oxidation of conducting polymer and digestion of the substrate with a protease, we have found that a combination of protease treatment, preceded and followed by a detergent wash resulted in most efficient protein substrate removal.

In an optimized extraction procedure, the substrate was digested by Proteinase K and extracted by intensive one-hour washing in the presence of 0.5% TWEEN 20 (which was preferential to 0.5% SDS with respect to more efficient substrate re-binding during testing). Using detergent solution or a protease treatment separately also resulted in protein substrate extraction, but to a lesser extent. The combination of the two allowed efficient substrate removal, which was confirmed by EIS, registered as impedance decrease. Different times of proteinase K treatments were tested and after 2 hours the digestion and removal were mostly complete when compared to overnight digestion (**Figure 2A**). Control experiments, performed on the film polymerized in the absence of RTA (no substrate), did not show any extraction-related Impedance decrease (**Figure 2B**).

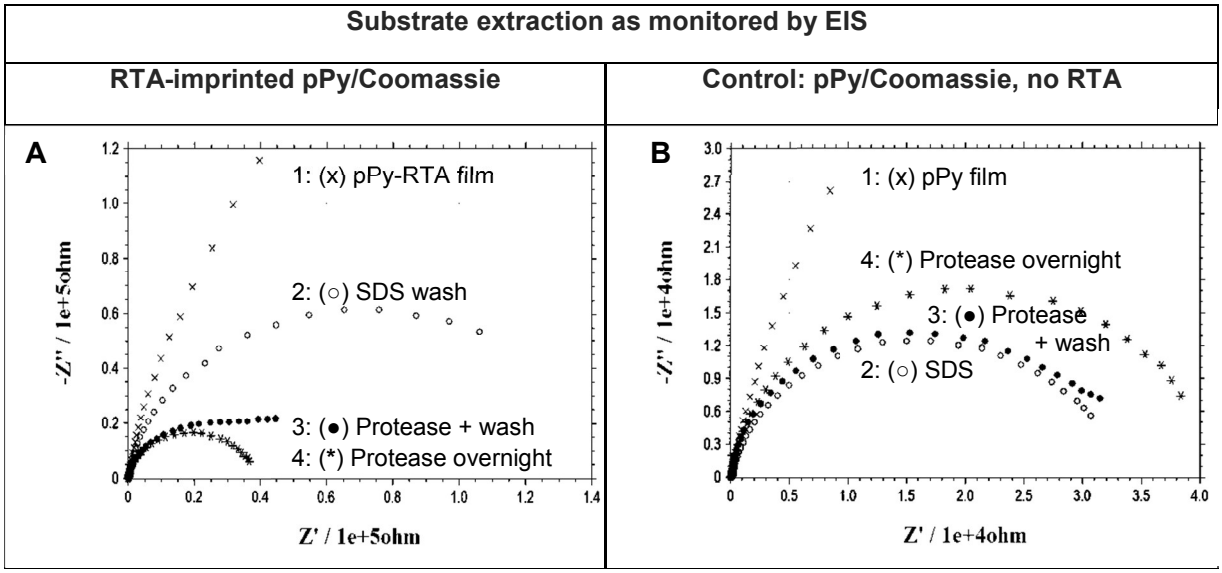

Figure 2. Substrate removal from MICP film. (A): MICP pPy/Coomassie film imprinted with RTA; **(B)** same without RTA. (A&B): 1: after pPy polymerization by CV; 2: after 1h wash PBS/ 0.5% SDS; 3: after protease treatment 2h and repeated PBS/SDS wash; 4: after protease treatment overnight.

Polymerized pPy film consists of positively charged pPy molecules stabilized with negatively charged dopant. Upon over-oxidation, polypyrrole loses its conductivity and charge. Over-oxidation of pPy MICP has been previously used to facilitate dopant and substrate release [**17, 25, 28**]. We have found that although over-oxidizing pPy MICP by applying 1 V (vs. Ag/AgCl) for 1, 2 and 5 min. indeed resulted in substrate release (confirmed by impedance decrease), it was less pronounced compared to Proteinase K/detergent treatment, plus more substrate could be released if Proteinase K/detergent treatment was applied to previously over-oxidized films

(**Figure S3, *Supplementary Information***). We hypothesize that the complex shape of the substrate requires splitting the substrate into pieces for efficient removal. After substrate removal pPy imprinted films were ready for testing.

3.3. Testing of MICP films.

MICP films on array electrodes were tested individually by either specific analyte (RTA), nonspecific analyte (BSA) or blank incubation in a matching buffer, to account for experimental noise. Following the initial AC Impedance spectra recording, the analyte or buffer was placed directly on the electrode in a small drop and allowed to interact with the MICP film for 30 min., which was followed by a second Impedance measurement. Specific analyte would bind to the imprinted molecular cavity and cause Impedance increase (which signifies molecular absorption of the analyte by the MIP electrode and a corresponding decrease in redox activity of ferrocyanides present in the solution.

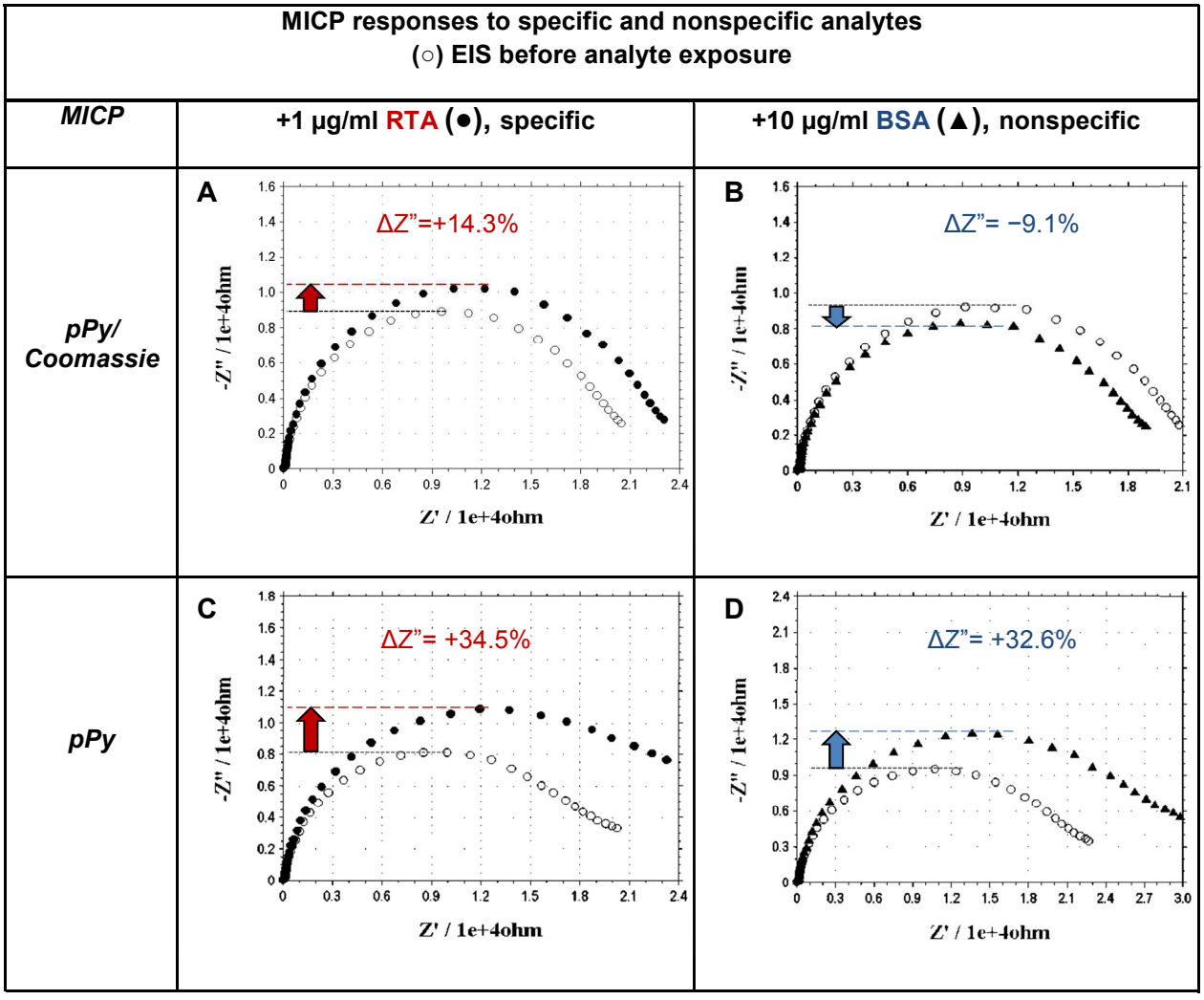

Figure 3. **Macromolecular dopant is needed for specificity of MICP.** Impedance changes in RTA-imprinted pPy/Coomassie MICP (**A&B**) and in pPy MICP (**C&D**) upon interaction with specific analyte (●): 1 µg/ml RTA, **A&C**; and nonspecific analyte (▲): 10 µg/ml BSA, **B&D**. (○): EIS spectra before incubation.

For example, a typical specific response, like the one shown in **Figure 3A** of pPy/Coomassie MICP for 1 μg/ml of RTA would be 10 - 20% increase in Impedance, calculated from the changes in the values of –Z", obtained directly from the Nyquist plot. Nonspecific analyte (BSA) used in 10 times excess relative to RTA, at 10 μg/ml, ideally should cause no change. However we have often noticed a decrease in Impedance in response to nonspecific analyte or buffer, as shown in **Figure 3B**, which is probably related to the diffusion phenomena in the film submerged in the buffer in the absence of the protein blocking the surface. The nonspecific response was clearly different from the one caused by substrate binding. The nature of this nonspecific response will be a subject of a future investigation.

The challenge we needed to address was nonspecific BSA binding to the surface of the film, which was significant prior to procedure optimization. EIS as a method cannot differentiate between specific and nonspecific surface binding, both resulting in increased surface coverage and decreased charge transport, registered as impedance increase. In our approach nonspecific binding was diminished when PBS/0.5% TWEEN (or 0.5% SDS) wash was used immediately before exposing the MICP to the analyte solution. As RTA used for imprinting was stabilized in a buffer containing glycerol, galactose and dithioerythritol, these components were carefully matched with appropriate dilution in the negative controls. Control experiments with MICP prepared without a macromolecular dopant showed no specificity and responded with Impedance increase to both RTA and BSA (**Figure 3C, D**).

Using an array approach allowed us to simultaneously test 9 electrodes in each experiment, containing three types of dopant for a given polymer, with either specific or nonspecific analyte or buffer for each type of dopant (**Figure S4**, ***Supplementary Information***). We have found that the arrays of MICP films were functional for more than one testing, and the binding and measurements could be repeated after a washing step containing 0.5% detergent. During the repetitions the testing sequence was changed in order to avoid repetitive measurement of a specific analyte on the same MICP film, which could lead to a possible loss of sensitivity due to saturation.

A set of data from a typical array with three repetitive incubations is presented in **Figure S5** ***Supplementary Information.*** The binding of RTA at 1 μg/ml to RTA-imprinted pPy MICP films prepared with either of the macromolecular dopants was stronger than binding of BSA at 10 μg/ml to the same films. The specificity of pPy/Coomassie MICP was the best as it showed no nonspecific binding and the highest specific response on the average. While the specificity of MICP films was clearly pronounced, the variability of the specific response remained high. The reproducibility and calibration of the MICP response will be a subject for further optimization.

We have tested RTA-imprinted pPy/Coomassie MICP films with various concentrations of RTA to detect the LOD. The films remained responsive down to RTA dilutions of 0.1 ng/ml, or 3.3 pM/L, **Figure S6,** ***Supplementary Information***. This is similar to the LOD for Ricin obtained by ELISA [**39**] and below the Ricin detection range obtained by portable colorimetric assays (1.1 - 100 ng/mL, [**40**]) and several amplification-free colorimetric, fluorescent and other methods [**41-44**].

Using macromolecular dopants with high protein affinity was important for MICP specificity. In control experiments, we have prepared MICP while omitting the substrate-dopant interaction step (step 2 in **Experimental section 2.3**). After the substrate was immobilized on gold electrode, pPy was polymerized by CV. Due to the fact that polymerization was performed in PBS, phosphate and chloride ions served as dopants during pPy film formation. We have noticed that films prepared without macromolecular dopant had low specificity to RTA and high nonspecific binding with BSA (**Figure 3C, D**) when tested in identical conditions to films containing macromolecular dopants (**Figure 3A, B**). We hypothesize that it is the affinity of the macromolecular dopants to protein substrate that contributes to the specificity of analyte recognition. In addition, the EIS of MICP pPy films simultaneously polymerized without macromolecular dopants on an electrode array were significantly less reproducible than pPy/Coomassie MICP films (**Figure S7, *Supplementary Information***). Using macromolecular dopants also enhanced film stability in solution. Overall, we consider macromolecular dopants possessing high affinity to proteins to be a key component of our MICP preparation. Macromolecular dopants create a "mold" around a protein substrate, while subsequent polymerization locks them in place. Upon substrate digestion and removal, the resulting imprint shows clear specificity for a specific analyte.

4. Conclusions

1. In this proof-of-concept work we have developed and tested a novel approach to creating MICP films imprinted with a protein substrate, Ricin Toxin Chain A (RTA). The novelty of our approach consists of using substrate-guided dopant immobilization with subsequent conducting polymer film formation. It results in creating a mold around a protein substrate by the dopant molecules, which are then locked in place by the polymer.

2. To create MICP, we have tested three macromolecular dopants with high affinity to proteins, Ponceau S, Coomassie BB R250 and ι-Carrageenan. The films were formed in a multistep procedure using sequential interactions of the protein substrate, dopant and pyrrole monomer, which was followed by electrochemical polymerization using Cyclic Voltammetry (CV). The films were formed on gold array electrodes allowing for performing parallel experiments. The thickness of the films was optimized to allow for efficient substrate extraction, which was removed by a combination of protease and detergent treatment. All MICP preparation steps were verified by Electrochemical Impedance Spectroscopy (EIS).

3. The created MICP films were tested for substrate re-binding using EIS. Excess of nonspecific protein was used for control. While MICP films prepared with different dopants showed variable degree of specificity, the films prepared without macromolecular dopants showed no specificity towards rebinding the substrate, which proves the essential role of the dopant in the imprint formation. RTA-imprinted polypyrrole films doped with Coomassie BB exhibited the highest specificity towards detection of RTA with a LOD of 0.1 ng/ml. Optimization of response reproducibility, calibration of the MICP response as well as expanding the sensing approach to other protein substrates will be a subject of further investigation.

5. Acknowledgements
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Design of molecularly imprinted conducting polymer protein-sensing films via substrate-dopant binding

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Abstract

Addressing the challenge of protein biosensing using molecularly imprinted polymers (MIP), we have developed and tested a novel approach to creating sensing conducive polymer films imprinted with a protein substrate, Ricin Toxin Chain A (RTA). Our approach for creating MIP protein sensing films is based on a concept of substrate-guided dopant immobilization with subsequent conducting polymer film formation. In this proof-of-concept work we have tested three macromolecular dopants with strong protein affinity, Ponceau S, Coomassie BB R250 and ι-Carrageenan. The films were formed using sequential interactions of the substrate, dopant and pyrrole, followed by electrochemical polymerization. The films were formed on gold array electrodes allowing for extensive data acquisition. The thickness of the films was optimized to allow for efficient substrate extraction, which was removed by a combination of protease and detergent treatment. The MIP films were tested for substrate rebinding using Electrochemical Impedance Spectroscopy (EIS). The presence of macromolecular dopants was essential for MIP film specificity. Out of three dopants tested, RTA-imprinted polypyrrole films doped with Coomassie BB performed with highest specificity towards detection of RTA with a level of detection (LOD) of 0.1 ng/ml.

Keywords

MIP, conducting polymer, biosensor, Ricin, polypyrrole, molecularly imprinted polymer

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1. Introduction

Molecularly imprinted polymers (MIPs) are seen by many as an inexpensive synthetic alternative to antibodies and natural receptors. Performing polymerization in the presence of the substrate, one can produce a polymer with its molecular imprint once the substrate is removed. Molecular imprinting involves binding of the functional groups of a monomer with the substrate through a combination of hydrophobic and ionic interactions. The functional groups are locked in position during polymerization, forming a polymer network around the embedded substrate. Following the substrate removal, the polymer is left with molecular cavities, complementary to the substrate. The created receptor sites can be used for rebinding of the substrate. The ability of the imprinted polymers to selectively re-bind the substrate molecules has been researched and used for over seventy years for affinity separation and catalysis, and for over a decade for drug delivery and sensing applications, according to the vast database of MIP literature [**http://mipdatabase.com**], containing thousands of publications.

The current state of science and applications of MIPs, including chromatography, electrophoresis, catalysis, chemical sensing and biosensing, drug delivery, crystallization and cell culturing has been recently reviewed [**1-3**]. While molecular imprinting of small molecules with molecular weight below 1kD has been very successful and commercially viable, imprinting of biological macromolecules, such as proteins, polypeptides and/or DNA has been more challenging due to solubility, size and fragility of biological molecules [**4**]. A number of 2D and 3D imprinting approaches for macromolecules have been tested, defining the choice of solvents and variety of polymer precursors to allow desirable polar and hydrophobic interactions of the substrate and the polymer [**5**]. Performing successful imprinting of macromolecules, especially proteins is receiving a lot of attention lately as it opens many possibilities for label-free biosensing of a variety of biomarkers in ever-expanding medical diagnostics or bioenvironmental monitoring and can combine improved stability, price efficiency and versatility compared to conventional immunodetection methods [**6**].

Among different types of polymers used to produce MIP are conducting polymers, such as polypyrrole, polyaniline, polythiophene and others. The attractive quality of conducting polymers is that in addition to chemical polymerization, they can be polymerized electrochemically, incorporating the substrate into the polymer matrix. This can be a convenient way to confine a desired specificity to an electrode, which can be used for electrochemical sensing. Indeed, a wide range of low molecular weight substrates has been successfully imprinted into conducting polymers for sensing applications during the past decade, which included caffeine, dopamine, catechol, theophylline, sulfadimethoxine, atrazine, uric acid, doxycycline and others. Bulkier biological macromolecules, which have been imprinted for sensing purposes with conducting polymers include “model” proteins, bovine serum albumin and avidin, along with bovine hemoglobin, glycoprotein gp51 from bovine leukemia virus and human cardiac troponin. Recent publications on molecularly imprinted conducting polymer (MICP) sensors are summarized in **Table 1**, with the majority of them using electrochemical methods, such as amperometry, cyclic voltammetry (CV), differential pulse voltammetry (DPV)

and electrochemical impedance spectroscopy (EIS) to detect rebinding of the substrate to the imprinted sensing electrode.

MIP sensors using conducting polymers				
Substrate	***Conducting Polymer***	***Sensing method***	***Ref.***	***Year***
glycoprotein gp51 from Bovine Leukemia Virus	pPy	pulsed amperometry	**[7]**	2004
caffeine	pPy	pulsed amperometry	**[8]**	2006
caffeine	poly(o-phenelynediamine), pPy	piezoelectric quartz crystal	**[9]**	
uric acid	Amine-imide type conducting polymer	amperometry	**[10]**	2007
Amadori compound N-(1-deoxy-β-d-fructopyranose-1-yl)-l-valine (Fru-Val)	poly-aminophenylboronic acid	open circuit potential (Δ Eoc)	**[11]**	2009
atrazine	polythiophene derivative	CV	**[12]**	
catechol and dopamine	polyaniline derivative	CV	**[13]**	
avidin	PEDOT/PSS	fluorescence	**[14]**	
caffeine	pPy		**[15]**	2010
tyrosine	copper oxide-pPy	voltammetry	**[16]**	2011
D- and L-glutamic acid, 1- and 2- naphthalene-sulfonates;	o-pPy	EQCM	**[17]**	2012
para-nitrophenol	polyaniline-polyvinyl sulfonic acid	DPV	**[18]**	2013
doxycycline	pPy	CV	**[19]**	
bovine hemoglobin	pPy	DPV. EIS	**[20]**	
BSA	tetraethylene glycol 3-morpholin propionate acrylate/carbon nanotubes	DPV	**[21]**	
BSA	o-phenylenediamine and 3-aminophenylboronic acid monohydrate	Ec oxidation of grafted 6-ferrocenyl-hexanethiol	**[22]**	
human cardiac troponin	o-phenylenediamine	CV, EIS	**[23]**	
norepinephrine	o-aminophenol	SWV	**[24]**	2014
sulfadimethoxine	o-pPy	SDM, amperometric	**[25]**	
sulfadimethoxine	pPy	CV	**[26]**	
theophylline	pPy	gravimetry	**[27]**	
bacteria	o-pPy	dielectrophoresis.	**[28]**	

Table 1. MIP sensors using conducting polymers. Abbreviations: pPy: polypyrrole; o-pPy: oxidized polypyrrole; PEDOT: Poly(3,4-ethylenedioxythiophene); PSS: polystyrene sulfonate; BSA: bovine serum albumin; CV: Cyclic Voltammetry; DPV: Differential Pulse Voltammetry; EIS: Electrochemical Impedance Spectroscopy; SWV: square wave voltammetry; SDM: Stepwise Dissolution Measurement; EQCM: Electrochemical Quartz Crystal Microbalance.

Polypyrrole (pPy) is a highly biocompatible conducting polymer used widely for electrochemical biosensing, as reviewed by [**29**]. Solubility of pyrrole monomer in water allows to perform electrochemical polymerization in the presence of biological molecules while maintaining their native state, which can be used for their entrapment to either functionalize pPy or imprint it. Imprinting with pPy can involve physical entrapment for uncharged substrate molecules, while negatively charged substrates can be incorporated into pPy matrix as dopants, participating in the polymer's conductivity. In such case, removal of the substrate to create an imprint will result in

re-doping by an anion in the solution, thus occupying functionality and affecting the accuracy of the imprint. Over-oxidizing polypyrrole efficiently expels the anionic substrate, but results in diminishing of electrochemical activity of pPy. To maintain high electrochemical activity of a polymer, one should find a way to maintain polymer-dopant interactions while removing the substrate after the imprinting stage.

We have previously explored preparation of thin pPy films from water solutions using negatively charged biological molecules as sole dopants [**30**]. In our experience, using bulky anionic macromolecular dopants resulted in solution-stable, electrochemically active and reusable functionalized pPy films. We have also found that when an anionic macromolecule is used as a dopant, any changes occurring to the dopant can be registered electrochemically. Combining this finding with the MIP concept, we aimed at creating a molecularly imprinted conducting polymer (MICP) sensing film by using the dopants which would not only contribute to stable conductivity of the pPy film but will also have affinity to the protein template. As compounds with sulfonate ($-SO_3^-$) groups are known to be among the best dopants for pPy, we have chosen three sulfonate-rich compounds with known affinity to proteins, Ponceau S, Coomassie BB and Carrageenan to be tested in MICP preparation. The dopants were used to create a mold around the protein substrate, which was followed by polymerization of pyrrole. Using this innovative approach, the macromolecular dopants contributed to the specificity of the imprint at the molecular level.

All steps of MICP film preparation and substrate rebinding were monitored by Electrochemical Impedance Spectroscopy (EIS), also known as AC Impedance. EIS is a popular method in electrochemical biosensing due to its versatility and sensitivity [**31**]. We have previously used EIS for electrochemical detection of proteins by means of electrode-immobilized specific aptamers. We have found that EIS is extremely sensitive to surface binding phenomena at the molecular level [**32, 33**]. Below we describe a new biosensing application with the specificity of the protein substrate binding provided by MICP film, while the binding event is registered through changes in EIS. To develop and test this method we have used Ricin Toxin Chain A (RTA) as a model substrate.

2. Material and Methods

2.1. Materials.

Phosphate-buffered saline (PBS) and DNAse-free, RNAse-free deionized water were purchased from Invitrogen. Coomassie Brilliant Blue R250, Ponceau S, Ricin Toxin Chain A (RTA), pyrrole, Bovine Serum Albumin (BSA), ι-Carrageenan (Type II) $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ and other chemicals were purchased from Sigma-Aldrich. Custom array electrodes were developed by Fractal Systems Inc. [**33**] and produced by Applied Biophysics.

2.2. Electrochemical measurements.

All electrochemical measurements were performed using CHI-760B Potentiostat (CH Instruments). The 9-electrode array (one side of the double-sided format, **Figure S1,**

Supplementary Information) was connected to the potentiostat using a switch-box, allowing to connect individual electrodes separately or simultaneously. The array chamber was fitted with Ag/AgCl mini reference electrode (Cypress Systems) and a counter wire electrode, positioned above the array electrodes. The array electrodes were cleaned electrochemically via cyclic voltammetry (CV: -0.9 V - +0.9 V, 100 mV/s, 10 segments) in 0.05 M H_2SO_4, rinsed with 0.05 M H_2SO_4, rinsed with copious amounts of deionized water, air dried and used immediately for substrate immobilization.

AC Impedance measurements of the MICP-modified array electrodes were performed in PBS buffer (pH 7.4) containing 2.5 mM $K_4Fe(CN)_6$, 2.5 mM mM $K_3Fe(CN)_6$. AC impedance spectra for each array electrode were recorded sequentially in a frequency range of 0.1 Hz to 100 kHz and AC amplitude of 0.005 V. AC impedance spectra were presented as a Nyquist plot (Z', real impedance, versus -Z", imaginary impedance). The -Z" value, which corresponds to the highest point of the semicircle on the Nyquist plot was used along with conventional R_{ct} (charge transfer resistance) value, obtained from the spectra fitted to Randles equivalent circuit by CH Instruments software. The relative values, i.e. % of Impedance -Z" change and % of R_{ct} change were found to be proportional, and % of -Z" value was routinely used for MICP evaluation.

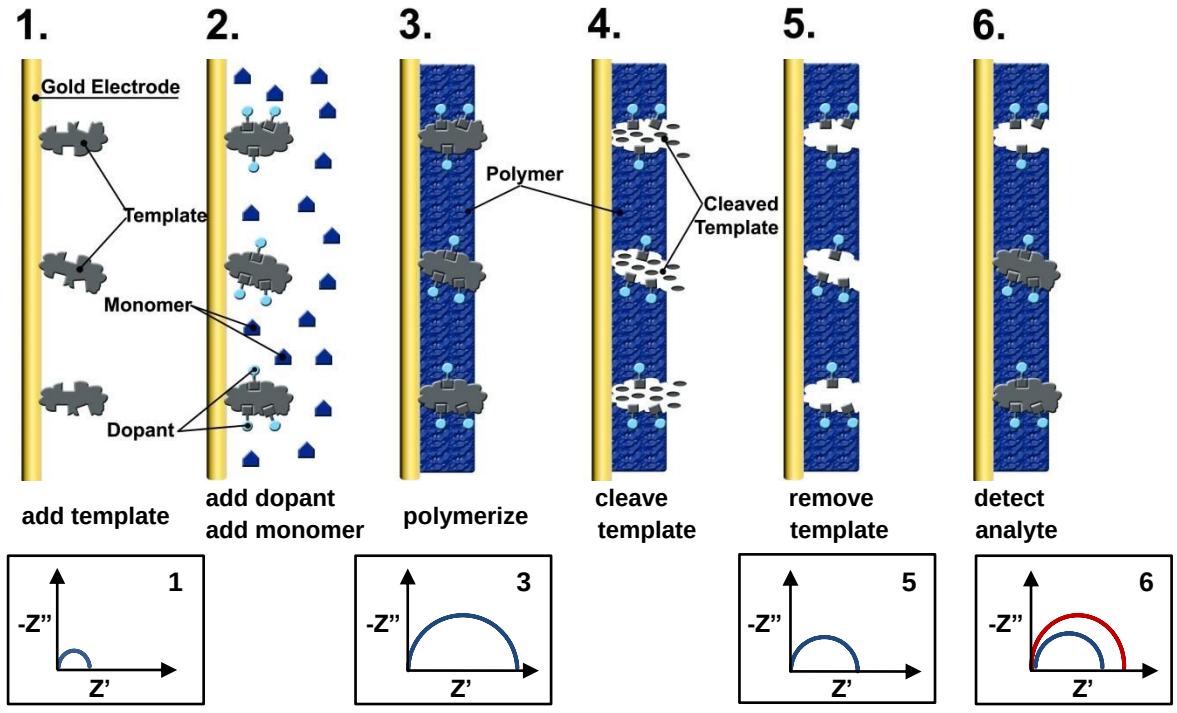

Figure 1. Preparation and testing of MICP sensing film: **(1)** Protein template is adsorbed on the surface of the gold electrode through amino groups (Impedance is low); **(2)** The dopant solution is applied to the absorbed protein and binds to it; **(3)** Electrochemical polymerization results in conducting polymer film with locked template (Impedance increase); **(4)** Protein template is cleaved by a protease; **(5)** Cleaved protein is extracted from the conductive polymer film (Impedance decrease); **(6)** Specific substrate is recognized by the imprinted film (Impedance increase). The inserts show schematic of AC Impedance spectra (Nyquist plot) changes in the process of film preparation and testing, with the numbers corresponding to the steps above. Insert 1: starting Impedance; Insert 3: impedance increases after conducting film polymerization; Insert 5: impedance decreases after substrate extraction; Insert 6: impedance increases after analyte rebinding.

2.3. MICP film preparation from polypyrrole.

Sensing film preparation consisted of five steps (steps 1-5, **Figure 1**). Each step was monitored by EIS. The procedure was optimized in a course of multiple experiments.

- ***Step 1, substrate immobilization.*** 5 µl drop of solution containing 0.9 mg/ml (30 µM concentration) of RTA, 40% glycerol, 10 mM phosphate, pH 6.0, 150 mM NaCl, 10 mM galactose, 0.5 mM dithioerythritol (original solution, Sigma-Aldrich L9514) was placed drop-wise on each pre-cleaned array electrode, incubated in a humidifying chamber for 30 min. at room temperature (R.T.) and rinsed with PBS.
- ***Step 2, targeted dopant application.*** One of the following dopants (see **Figure S2, *Supplementary Information,*** for chemical structures) was applied directly to each of the array electrodes in a 5 µl drop and incubated for 30 min. at R.T. (typically each dopant was applied to three of the nine electrodes): Ponceau S (3-Hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]phenylazo)-2,7-naphthalenedisulfonic acid sodium salt, 10 mM in PBS), Coomassie Brilliant Blue R250 (10 mM in PBS), ι-Carrageenan (5 mg/ml in PBS). After incubation the array was rinsed in PBS.
- ***Step 3, electrochemical polymerization***. Pyrrole (0.05M in PBS) was polymerized simultaneously on all array electrodes (CV, 0 - 0.7 V, 50 mV/s, 3 cycles, or 6 segments). To remove weakly-bound RTA, the array electrodes were washed with PBS/0.5% TWEEN 20 (Polyethylene glycol sorbitan monolaurate) for 30 min. on a rotary shaker. Alternatively, SDS could be used instead of TWEEN in this and subsequent washing steps.
- ***Step 4, substrate cleavage.*** Following the wash, RTA captured in polypyrrole was digested with 3 mg/ml of Proteinase K in PBS for 2 hours at 37° C.
- ***Step 5, substrate removal.*** To remove digested RTA and Proteinase K, the array electrodes were washed with PBS/ 0.5% TWEEN 20 for 1h with shaking.

2.4. MICP film testing

AC Impedance was measured before and after incubation with the analytes as described in section 2.2. The measurements were followed by PBS/ 0.5% Tween rinse to remove ferrocyanides prior to further incubations. For binding, the electrodes were incubated for 30 min. at R.T. in a drop-like fashion with one of the analytes: 1 µg/ml of RTA solution (~33 nM, specific), or 10 µg/ml of BSA solution (10 times weight excess, nonspecific) in PBS buffer containing 0.04% glycerol, 11 µM galactose, 0.55 µM dithioerythritol. Thus, one of three electrodes carrying MICP film with a particular dopant was exposed either to a specific analyte, or nonspecific protein or buffer. The difference between the Impedance values (expressed as % of Impedance (-Z") change) was used to characterize the binding to MICP film. The array electrodes were reusable after washing in PBS/ 0.5% TWEEN overnight at R.T. with shaking, AC Impedance measurements and incubations were repeated up to 4 times.

3. Results and Discussion

3.1. MICP preparation

Our approach for creating MICP protein sensing films is based on a novel concept of substrate-guided dopant immobilization subsequent conducting polymer film formation. Step-wise MICP preparation (**Figure 1**) was chosen to allow for efficient molecular interaction of substrate and dopant in kinetically unrestricted conditions.

First, the protein substrate, Ricin Toxin Chain A (RTA) was immobilized on the electrode surface by absorption, as proteins are known to be immobilized on gold through a combination of electrostatic and hydrophobic interactions [**34**]. Immobilization was confirmed by EIS after 10, 20, 30, 40 and 60 min., resulting in Impedance increase compared to the pure gold electrode. 30 minutes was sufficient as the immobilization rate did not increase beyond this time.

Next, the immobilized substrate was allowed to interact with a dopant. Three compounds, Ponceau S, Coomassie BB R250 and ι-Carrageenan were selected as dopants for their known affinity to proteins, which they bind mostly electrostatically due to the presence of sulfonate ($-SO_3^-$) groups **[35-37].** Compounds with sulfonate groups are also excellent dopants for pPy, contributing to its conductivity [**38**]. Thus, after binding the protein substrate and creating a mold around it, the dopants were incorporated into ultra-thin pPy films, which were polymerized on the electrodes around the immobilized substrate coated by the dopant. The concentration (w/v) of the dopants used for binding the substrate was in 5 - 10 times excess relative to the original concentration of RTA used in immobilization (the molar concentration of Ponceau and Coomassie exceeded molar concentration of RTA ~300 times). The excess of dopant was needed to ensure complete coverage of the immobilized substrate with the dopant molecules, while sulfonate groups would contribute both to protein binding and doping pPy. The unbound dopant was removed prior to film polymerization.

PPy sensing films were polymerized simultaneously on array electrodes by cyclic voltammetry (CV) from a solution of pyrrole in PBS. The presence of the buffer was needed to preserve the conformation of immobilized RTA. We hypothesized that while the anions present in PBS buffer would also dope the growing pPy film, the immobilized dopants would have an advantage due to their immobilized state, bulkiness and the presence of multiple sulfonate groups. As pPy films were repeatedly washed and incubated for prolonged times in solutions, this proved to be the case, i.e. the films were stable in solution and maintained their binding properties.

The thickness of the pPy films depended on the number of cycles in CV polymerization. The thickness of the pPy film defined whether the protein substrate molecules were completely or only partially embedded. We have compared films prepared with 5, 4 and 3 cycles of CV. The first two resulted in thicker films embedding the protein molecules, which made it impossible to extract them later on. Films prepared with 3 cycles of CV performed well in the substrate extraction step.

The efficiency of substrate extraction_defines the sensitivity of MIP. For bulky biological substrates of complex shapes, this step of MIP preparation poses the most challenge. After testing, comparing and combining different approaches, such as washing MICP with detergent, over-oxidation of conducting polymer and digestion of the substrate with a protease, we have found that a combination of protease treatment, preceded and followed by a detergent wash resulted in most efficient protein substrate removal.

In an optimized extraction procedure, the substrate was digested by Proteinase K and extracted by intensive one-hour washing in the presence of 0.5% TWEEN 20 (which was preferential to 0.5% SDS with respect to more efficient substrate re-binding during testing). Using detergent solution or a protease treatment separately also resulted in protein substrate extraction, but to a lesser extent. The combination of the two allowed efficient substrate removal, which was confirmed by EIS, registered as impedance decrease. Different times of proteinase K treatments were tested and after 2 hours the digestion and removal were mostly complete when compared to overnight digestion (**Figure 2A**). Control experiments, performed on the film polymerized in the absence of RTA (no substrate), did not show any extraction-related Impedance decrease (**Figure 2B**).

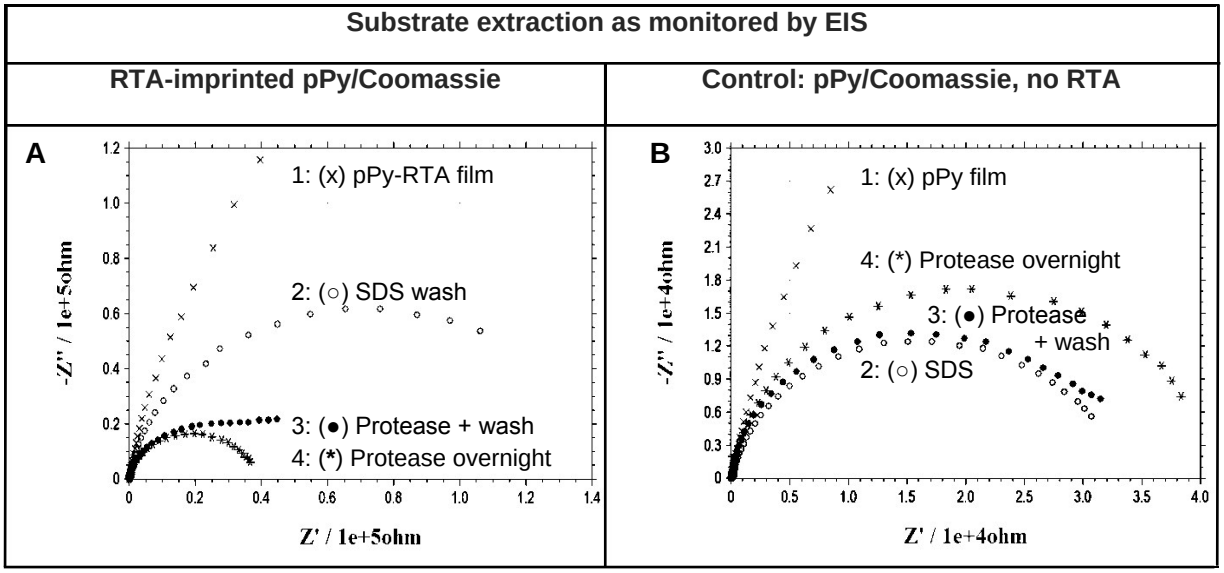

Figure 2. Substrate removal from MICP film. (A): MICP pPy/Coomassie film imprinted with RTA; **(B)** same without RTA. (A&B): 1: after pPy polymerization by CV; 2: after 1h wash PBS/ 0.5% SDS; 3: after protease treatment 2h and repeated PBS/SDS wash; 4: after protease treatment overnight.

Polymerized pPy film consists of positively charged pPy molecules stabilized with negatively charged dopant. Upon over-oxidation, polypyrrole loses its conductivity and charge. Over-oxidation of pPy MICP has been previously used to facilitate dopant and substrate release [**17, 25, 28**]. We have found that although over-oxidizing pPy MICP by applying 1 V (vs. Ag/AgCl) for 1, 2 and 5 min. indeed resulted in substrate release (confirmed by impedance decrease), it was less pronounced compared to Proteinase K/detergent treatment, plus more substrate could be released if Proteinase K/detergent treatment was applied to previously over-oxidized films (**Figure S3, *Supplementary Information***). We hypothesize that the complex shape of the

substrate requires splitting the substrate into pieces for efficient removal. After substrate removal pPy imprinted films were ready for testing.

3.3. Testing of MICP films.

MICP films on array electrodes were tested individually by either specific analyte (RTA), nonspecific analyte (BSA) or blank incubation in a matching buffer, to account for experimental noise. Following the initial AC Impedance spectra recording, the analyte or buffer was placed directly on the electrode in a small drop and allowed to interact with the MICP film for 30 min., which was followed by a second Impedance measurement. Specific analyte would bind to the imprinted molecular cavity and cause Impedance increase (which signifies molecular absorption of the analyte by the MIP electrode and a corresponding decrease in redox activity of ferrocyanides present in the solution).

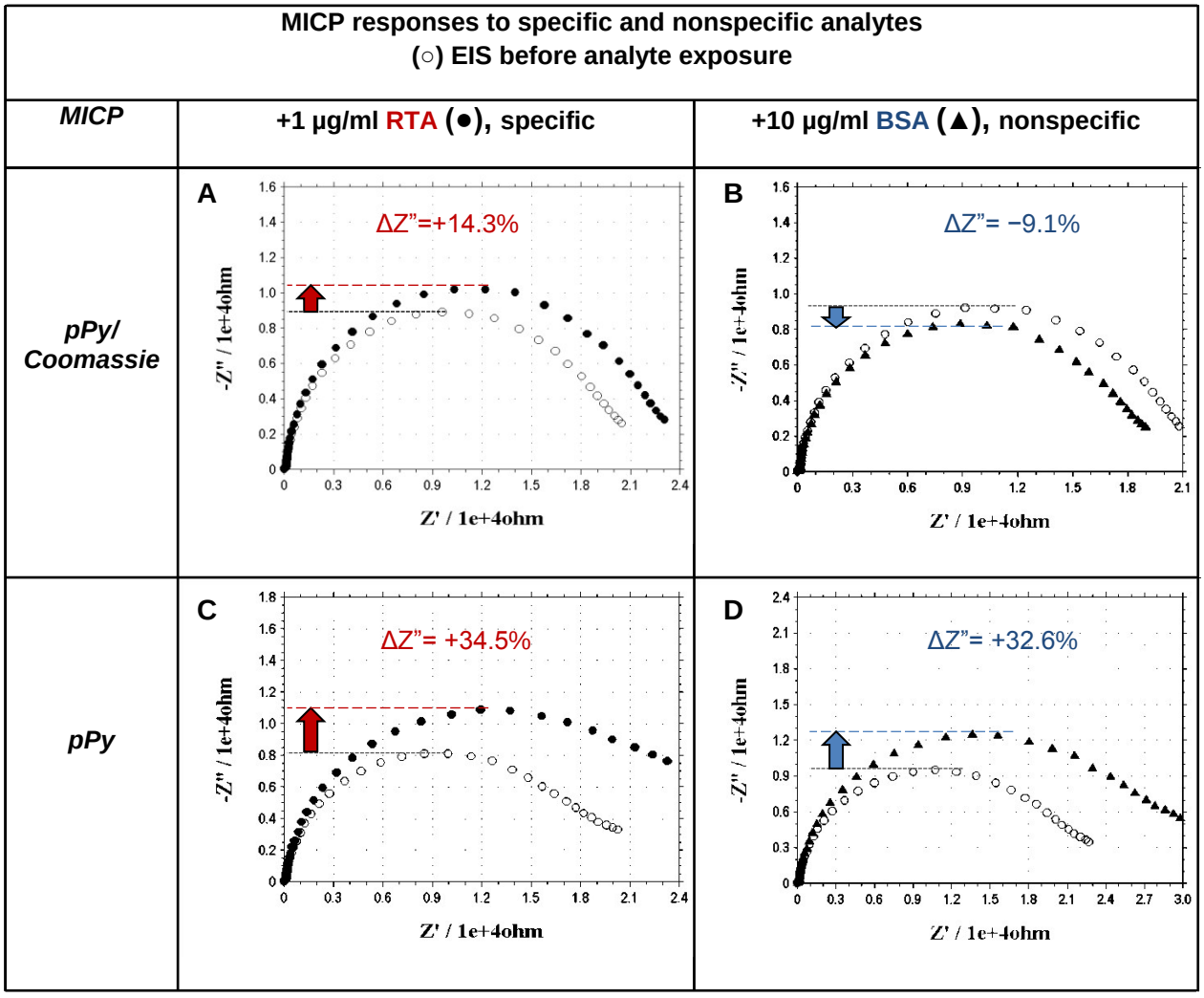

Figure 3. **Macromolecular dopant is needed for specificity of MICP.** Impedance changes in RTA-imprinted pPy/Coomassie MICP (**A&B**) and in pPy MICP (**C&D**) upon interaction with specific analyte (●): 1 µg/ml RTA, **A&C**; and nonspecific analyte (▲): 10 µg/ml BSA, **B&D**. (○): EIS spectra before incubation.

For example, a typical specific response, like the one shown in **Figure 3A** of pPy/Coomassie MICP for 1 µg/ml of RTA would be 10 - 20% increase in Impedance, calculated from the

changes in the values of –Z", obtained directly from the Nyquist plot. Nonspecific analyte (BSA) used in 10 times excess relative to RTA, at 10 µg/ml, ideally should cause no change. However we have often noticed a decrease in Impedance in response to nonspecific analyte or buffer, as shown in **Figure 3B**, which is probably related to the diffusion phenomena in the film submerged in the buffer in the absence of the protein blocking the surface. The nonspecific response was clearly different from the one caused by substrate binding. The nature of this nonspecific response will be a subject of a future investigation.

The challenge we needed to address was nonspecific BSA binding to the surface of the film, which was significant prior to procedure optimization. EIS as a method cannot differentiate between specific and nonspecific surface binding, both resulting in increased surface coverage and decreased charge transport, registered as impedance increase. In our approach nonspecific binding was diminished when PBS/0.5% TWEEN (or 0.5% SDS) wash was used immediately before exposing the MICP to the analyte solution. As RTA used for imprinting was stabilized in a buffer containing glycerol, galactose and dithioerythritol, these components were carefully matched with appropriate dilution in the negative controls. Control experiments with MICP prepared without a macromolecular dopant showed no specificity and responded with Impedance increase to both RTA and BSA (**Figure 3C, D**).

Using an array approach allowed us to simultaneously test 9 electrodes in each experiment, containing three types of dopant for a given polymer, with either specific or nonspecific analyte or buffer for each type of dopant (**Figure S4**, ***Supplementary Information***). We have found that the arrays of MICP films were functional for more than one testing, and the binding and measurements could be repeated after a washing step containing 0.5% detergent. During the repetitions the testing sequence was changed in order to avoid repetitive measurement of a specific analyte on the same MICP film, which could lead to a possible loss of sensitivity due to saturation.

A set of data from a typical array with three repetitive incubations is presented in **Figure S5** ***Supplementary Information.*** The binding of RTA at 1 µg/ml to RTA-imprinted pPy MICP films prepared with either of the macromolecular dopants was stronger than binding of BSA at 10 µg/ml to the same films. The specificity of pPy/Coomassie MICP was the best as it showed no nonspecific binding and the highest specific response on the average. While the specificity of MICP films was clearly pronounced, the variability of the specific response remained high. The reproducibility and calibration of the MICP response will be a subject for further optimization.

We have tested RTA-imprinted pPy/Coomassie MICP films with various concentrations of RTA to detect the LOD. The films remained responsive down to RTA dilutions of 0.1 ng/ml, or 3.3 pM/L, **Figure S6,** ***Supplementary Information***. This is similar to the LOD for Ricin obtained by ELISA [**39**] and below the Ricin detection range obtained by portable colorimetric assays (1.1 - 100 ng/mL, [**40**]) and several amplification-free colorimetric, fluorescent and other methods [**41-44**].

Using macromolecular dopants with high protein affinity was important for MICP specificity. In control experiments, we have prepared MICP while omitting the substrate-dopant interaction

step (step 2 in **Experimental section 2.3**). After the substrate was immobilized on gold electrode, pPy was polymerized by CV. Due to the fact that polymerization was performed in PBS, phosphate and chloride ions served as dopants during pPy film formation. We have noticed that films prepared without macromolecular dopant had low specificity to RTA and high nonspecific binding with BSA (**Figure 3C, D**) when tested in identical conditions to films containing macromolecular dopants (**Figure 3A, B**). We hypothesize that it is the affinity of the macromolecular dopants to protein substrate that contributes to the specificity of analyte recognition. In addition, the EIS of MICP pPy films simultaneously polymerized without macromolecular dopants on an electrode array were significantly less reproducible than pPy/Coomassie MICP films (**Figure S7, *Supplementary Information***). Using macromolecular dopants also enhanced film stability in solution. Overall, we consider macromolecular dopants possessing high affinity to proteins to be a key component of our MICP preparation. Macromolecular dopants create a "mold" around a protein substrate, while subsequent polymerization locks them in place. Upon substrate digestion and removal, the resulting imprint shows clear specificity for a specific analyte.

4. Conclusions

1. In this proof-of-concept work we have developed and tested a novel approach to creating MICP films imprinted with a protein substrate, Ricin Toxin Chain A (RTA). The novelty of our approach consists of using substrate-guided dopant immobilization with subsequent conducting polymer film formation. It results in creating a mold around a protein substrate by the dopant molecules, which are then locked in place by the polymer.

2. To create MICP, we have tested three macromolecular dopants with high affinity to proteins, Ponceau S, Coomassie BB R250 and ι-Carrageenan. The films were formed in a multistep procedure using sequential interactions of the protein substrate, dopant and pyrrole monomer, which was followed by electrochemical polymerization using Cyclic Voltammetry (CV). The films were formed on gold array electrodes allowing for performing parallel experiments. The thickness of the films was optimized to allow for efficient substrate extraction, which was removed by a combination of protease and detergent treatment. All MICP preparation steps were verified by Electrochemical Impedance Spectroscopy (EIS).

3. The created MICP films were tested for substrate re-binding using EIS. Excess of nonspecific protein was used for control. While MICP films prepared with different dopants showed variable degree of specificity, the films prepared without macromolecular dopants showed no specificity towards rebinding the substrate, which proves the essential role of the dopant in the imprint formation. RTA-imprinted polypyrrole films doped with Coomassie BB exhibited the highest specificity towards detection of RTA with a LOD of 0.1 ng/ml. Optimization of response reproducibility, calibration of the MICP response as well as expanding the sensing approach to other protein substrates will be a subject of further investigation.

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