A novel electrochemical impedance spectroscopy (EIS) sensor design, based on a standard interdigitated electrode arrangement in which the working electrode consists of gold and the combined counter and reference electrodes of polypyrrole doped with polystyrene sulfonate (PPy:PSS), is evaluated for biosensing applications. The performance is successfully proved by immobilization of a thiolated biotin as a self-assembled monolayer (SAM), followed by streptavidin and a biotinylated horseradish peroxidase. It is shown that specific binding of biomolecules takes place only at the gold electrode. The binding activities are not influenced by the addition of small amounts of the nonionic surfactant Pluronic F-68. The immobilization process is monitored online with EIS showing an excellent repeatability of the EIS signals in comparison with Au–Au electrode configuration even after electrode regeneration.

1. Introduction

Electrochemical impedance spectroscopy (EIS) is widely used to characterize electrode/electrolyte interfaces and provides the ability to sense chemical binding effects on and near the electrode. Especially in biosensor applications, EIS is utilized for studies of, e.g., DNA hybridization interactions, immunosensing protocols, virus detection, microbial cell layers, specific protein adsorption on self-assembled monolayers (SAMs), and evaluation of lectin binding kinetics on highly affine and multivalent glycopolymer brushes as well as for the real-time monitoring of protein insertion into block copolymer vesicle membranes.\(^1\)

EIS measurements are typically conducted in a two-electrode or three-electrode configuration. Compared with the three-electrode arrangement, counter electrode (CE) and reference electrode (RE) are combined with one large electrode (CE/RE) in the two-electrode setup, whereas the other smaller one serves as working electrode (WE). While using microstructured electrodes in the two-electrode configuration, a design of two interdigitated electrodes is preferred because of increased signal-to-noise ratio compared with unstructured electrodes.\(^2\) Typically, gold is used as a common electrode material for WE as well as for CE/RE. Gold is highly suitable as a sensing material for biosensing applications, because gold surfaces can easily be covered with an alkanethiolate-based SAM, which acts as an essential intermediate layer for successive biomolecule immobilization.\(^3\)

Recently, we have shown that EIS signals can be stabilized and highly repeatable without significant drift effects when the CE/RE made of gold is covered with a conductive conjugated polymer layer based on polypyrrole (PPy) doped with poly(4-styrenesulfonic acid) (PSS) by means of electropolymerization, assigned as PPy:PSS.\(^4\) During the electrodeposition, PPy is doped by poly(4-styrenesulfonate) anions.\(^5\) The sulfonate groups are incorporated into the polymer matrix and the film behaves like a redox cation exchanger, so PSS acts as a dopant which adds ionic conductivity to the system.\(^6\) It was shown that the coated CE/RE exhibited an increasing surface area. The increased surface area lowered the interfacial impedance in such a way that almost only the gold WE contributed to the overall impedance of the system.\(^3\) In addition, stable open-circuit potential (OCP) values at the PPy:PSS layer ensured that the drift behavior depended only on the interface conditions at the gold WE.

Based on these promising results, we here evaluate the novel electrode design as a biosensor by immobilizing a thiolated biotin as an SAM, followed by streptavidin and a biotinylated horseradish peroxidase (HRP) onto the gold WE. This protocol will be characterized with respect to the addition of small amounts of the nonionic surfactant Pluronic F-68, which is often used to prevent biofouling at hydrophobic surfaces.\(^7\) It will be shown that the surfactant does not harm the binding activity of the used biomolecules on the gold surface. Furthermore, specific binding of the biomolecules will only take place at the gold electrode but not on the PPy:PSS-coated electrode.
The article is structured as follows. First, the biotin-streptavidin-based protein stack is introduced, which enables photometric characterizations of binding events via a biotinylated HRP. Here, the influence of adding the surfactant is proofed. Then, the EIS sensor chip fabrication process will be briefly summarized.\(^\text{[3]}\) Afterward, EIS measurements will be conducted to monitor the adsorption of the biomolecules at the gold WE.

2. Experimental Section

2.1. Materials

All protein solutions were prepared using a phosphate buffer (PB) containing sodium chloride. The buffer consists of $50 \times 10^{-3}$ m Na$_2$HPO$_4$ and $150 \times 10^{-3}$ m NaCl, yielding a pH value of 7.4. PB was filtered using a polyvinylidene difluoride (PVDF) membrane filter (0.2 μm pore size). Bovine serum albumin (BSA, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), streptavidin (STR, Biornol GmbH, Hamburg, Germany), and a biotinylated HRP (Thermo Fisher Scientific, Waltham, MA, USA) were dissolved in the buffer solution to achieve concentrations of 2 mg mL$^{-1}$, 0.01 mg mL$^{-1}$, and 62.5 ng mL$^{-1}$, respectively. For blocking purposes on the sensor chips, the BSA concentration was set to 0.2 mg mL$^{-1}$ due to the need of blocking different areas. A biotinylated SAM reagent (product number 746622, Sigma-Aldrich Chemie GmbH, Munich, Germany) served to generate a self-assembled biotin-terminated monolayer on gold to later immobilize streptavidin, as described by Simoncelli et al.\(^\text{[7]}\) In some cases, a nonionic surfactant Pluronic F-68 (10% solution, Thermo Fisher Scientific, Waltham, MA, USA) was added to the SAM, buffer, and protein solutions in a concentration of 0.05% w/v. These solutions are hallmarked with a small “p.” For analysis by photometric measurements, a ready-to-use substrate including 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide (Kem-En-Tec Diagnostics A/S, Taastrup, Denmark) was used for HRP.

For photolithography processes, all photoresists were purchased from MicroChemicals GmbH (Ulm, Germany).

For the electrochemical deposition of PPy:PSS, pyrrole (reagent grade, 98%, Sigma-Aldrich Chemie GmbH, Munich, Germany) and poly(4-styrenesulfonic acid) solution (Sigma-Aldrich Chemie GmbH, Munich, Germany) were diluted with deionized water to a concentration of $200 \times 10^{-3}$ m each. Electropolymerization was conducted by mixing both solutions in a ratio of 1:1.

Electrode cleaning was conducted using a solution of deionized water, ammonium hydroxide (NH$_4$OH, 25%, Th. Geyer GmbH & Co. KG, Renningen, Germany), and hydrogen peroxide (H$_2$O$_2$, 30%, MicroChemicals GmbH, Ulm, Germany) in the volume ratio of 7:2:1 before every experiment and before PPy:PSS deposition, respectively.

2.2. Protein Stack

The experiments for creating the protein stack were conducted in a standard 96-well microtiter plate (MTP). MTPs have the advantage that experiments can be performed in parallel. For the experiments, oxidized silicon wafers were coated with a 30 nm Ti layer and a 100 nm Au layer by sputter deposition and diced into 3.5 mm $\times$ 3.5 mm chips. The chips were used to bind first the SAM to the gold surface, then block the uncovered surface area with BSA, and subsequently immobilize STR to the biotinylated surface. Finally, a biotinylated HRP was used as a reporter molecule. A sketch of the protein stack is shown in Figure 1. In detail, a chip was inserted in an empty MTP well. The well was filled with 50 μL of 0.5 $\times$ 10$^{-3}$ m biotinylated SAM and the chip was incubated for 30 min. During SAM incubation, the well was sealed with an adhesive foil to avoid evaporation of ethanol. Afterward, the chip was transferred again to an empty well and 200 μL of BSA solution was added. Using the same procedure, the chip was consecutively immersed in STR and HRP solution for 30 min incubation time each. After each incubation step, the chip was grabbed with tweezers and rinsed three times with 500 μL PB containing 0.05% w/v Pluronic F-68 below denoted as PBp. Then, the chip was immersed in 70 μL ready-to-use substrate including 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide for 30 s in a fresh well. The reaction observed by a color change was stopped by transferring 50 μL of the reaction volume into a separate well pre-filled with 50 μL of 3 m hydrochloric acid. Note that the aforementioned description does not distinguish between solutions with or without the surfactant Pluronic F-68. When Pluronic F-68 was added to solutions, it was indicated by “p” (Section 3.2).

Negative control experiments were conducted, omitting one selected protein in the corresponding solution, which prevents binding of the subsequently applied proteins. In addition, negative control experiments on PPy:PSS were conducted. Here, the chips were coated with PPy:PSS, as described later, and the full protein stack was built-up, whereas Pluronic F-68 was added to each solution. All experiments were conducted in a threefold determination, averaged, and blank corrected.

2.3. Sensor Fabrication

The fabrication process was already described in detail and is briefly summarized here.\(^\text{[3]}\) Interdigitated electrodes made of gold were fabricated on 4 in. glass wafers by standard lithography, sputter deposition of a 30 nm-thick titanium adhesion layer, a 220 nm-thick gold layer, a lift-off process followed by deposition and structuring of Parylene-C as the protective layer. Surface modification of the larger CE/RE was conducted by electropolymerization, as described by El Hasni et al.\(^\text{[8]}\) A 200 μL droplet
containing $200 \times 10^{-3}$ m PPy and $200 \times 10^{-3}$ m PSS was pipetted onto the electrodes and a potential of 1 V was applied to the CE/RE for 30 min, whereas the WE was connected to ground. Atomic force microscopy (AFM) pictures were made using a Veeco Dimension 3100 atomic force microscope (Veeco, Plainview, NY, USA).

2.4. Electrode Cleaning

The chips were cleaned in ammonium hydroxide–hydrogen peroxide solution for 60 min. This procedure removed residual proteins from the gold surface as well as the PPy:PSS film. We recently showed by EIS measurements that a similar treatment reveals a clean gold surface.

2.5. Photometric Measurements

In experiments conducted in MTPs, light absorption was measured in a Spectra Max Plus photometer (Molecular Devices, Biberach, Germany) at a wavelength of 450 nm. The readout has been conducted using the software XFlour4 (version 4.51) in absorbance mode.

2.6. EIS Measurements

The bare gold and the PPy:PSS-coated electrode were first characterized separately using a standard three-electrode setup. A platinum wire served as a CE, an Ag/AgCl electrode pellet (World Precision Instruments, Inc., Sarasota, FL, USA) as pseudo-RE, and one of the aforementioned electrodes as WE. A Novocontrol Technologies Alpha-A High Performance Frequency Analyzer (Novocontrol Technologies GmbH & Co. KG, Montabaur, Germany) was used as impedance spectrometer. EIS spectra were obtained from 10 Hz to 1 MHz, applying a voltage amplitude of 10 mV.

Transient EIS measurements during protein immobilization were carried out in a two-electrode arrangement using a Hewlett Packard 4294A Precision Impedance Analyzer (Palo Alto, CA, USA), as described previously. A sensor chip was clamped in a custom-made fixture and electrically contacted by pogo pins (Mill-Max Manufacturing Corp., Oyster Bay, NY, USA) and coaxial cables to the analyzer to enable four-point measurements. Impedance values were obtained by applying a voltage of 10 mV with 50 frequencies ranging from 100 Hz to 3 MHz, uniformly distributed at the logarithmic axis. A full spectrum was taken within approximately 15 s.

2.7. OCP Measurements

OCP was measured over 1 h for Au and PPy:PSS-coated electrodes in PB using a Potentiostat/Galvanostat Model 283 (EG&G Instruments, Princeton Applied Research). A two-electrode setup with an Ag/AgCl double-junction reference electrode (World Precision Instruments, Inc., Sarasota, FL, USA) as combined CE and RE was used. PPy:PSS and Au were connected to the WE terminal of the potentiostat, respectively.

3. Results

3.1. Sensor Fabrication

The microscope and AFM images of the fabricated sensor are shown in Figure 2. In Figure 2a, a circularly patterned interdigitated Au electrode design is shown with lines and spaces of 20 μm each. In comparison, Figure 2b shows the design after PPy:PSS deposition on the larger CE/RE. The PPy:PSS layer exhibits a brownish color. AFM images taken from randomly picked spots of 5 μm × 5 μm of the two surfaces reveal that the bare gold electrode in Figure 2c shows a grainy structure as usual for sputter-deposited thin films, whereas the deposited PPy:PSS layer exhibits a rough surface morphology (Figure 2d).

From the AFM images, the root-mean-square (RMS) roughness $S_q$ was calculated by the software Gwyddion and was determined to be 3.7 nm for gold and 20.5 nm for the PPy:PSS layer, respectively. The profile scan along the arrow shown in Figure 2b, which was conducted by a Tencor P-10 surface profiler (KLA-Tencor, Milpitas, CA, USA), is shown in Figure 3, indicating that the combined titanium/gold layer is 0.263 μm thick, whereas the PPy:PSS layer on top of the metal electrodes has an average thickness of around 1.058 μm. The spikes at the edges of the polymer layer were not considered for calculation of the layer thickness.

The electrical impedance of the CE/RE before and after electropolymerization of PPy:PSS was measured using the three-electrode setup. Figure 4 shows the magnitude of the electrical impedance of the bare gold and PPy:PSS-coated electrode in PB, respectively. Compared with the bare gold electrode, the PPy:PSS-coated electrode exhibited a lower magnitude of impedance in the low-frequency regime.

In addition, the OCP of bare gold as well as of PPy:PSS-coated electrode was recorded over time in PB versus Ag/AgCl RE for 1 h (Figure 5). After 30 min exposure to the medium, the electrode under investigation was taken out of the solution, stream dried under nitrogen, and immersed in the buffer solution again.

The OCP progression for the bare gold electrode exhibits a decreasing trend, starting at 151 mV, and approaches around 91 to 97 mV after approximately 5 min exposure time. After the second immersion, the OCP increases significantly and decreases within approximately 15 min, approaching a slightly higher steady-state value ($\approx 110$ mV). It is worthwhile to denote that the course of the OCP for the bare gold electrode exhibits a lot of fluctuations. The same electrode was then coated with PPy:PSS, and OCP was recorded using the same procedure described previously. Here, the OCP value is lower, starting at 37 mV after the first immersion. A slight decrease can be observed down to stable values of 10 mV. After the second immersion, the OCP remains almost stable starting at 8 mV and slightly drifts within the next 30 min to 13 mV. In comparison, fluctuations have not been observed for the PPy:PSS-coated electrode.

3.2. Protein Stack

The adsorption of different biomolecules was first developed in MTPs and verified by photometric measurements. The results are shown in Figure 6. The blue bar in the bottom row corresponds to the described SAM–BSA–STR–HRP stack (called full
model) without the addition of Pluronic F-68 to the solutions. Light absorption of around 0.26 was observed, indicating the successful formation of the protein stack on the gold surface. The three additional bars (yellow, gray, and orange) assigned in the bottom row show comparable light absorption values when Pluronic F-68 was added to certain protein solutions, as indicated by a small “p” in the legend. In general, the blue bars represent experiments without Pluronic F-68, whereas for experiments represented by the yellow bars, Pluronic F-68 was added to the SAM

Figure 2. a) The unmodified sensor consists of interdigitated gold electrodes with lines and spaces of 20 μm, respectively. A layer of Parylene C insulates the leads electrically. b) The sensor area with the electrodeposited PPy:PSS film on CE/RE in brownish color. c) AFM image of the bare gold CE/RE. d) AFM image after electrodeposition of PPy:PSS onto CE/RE.

Figure 3. The thickness profile of the electrodes was measured after electrodeposition. The profile scan was conducted in the direction indicated by the arrow in Figure 2b.

Figure 4. Magnitude of the electrical impedance of CE/RE before coating (Au) and after electrodeposition of PPy:PSS. Spectra were obtained in a three-electrode setup in PB.
and the protein solutions. For experiments, which are assigned by the orange and gray bars, Pluronic F-68 was added to only the protein solutions (BSAp, STRp, and HRPp) or SAM, respectively. Negative controls, indicated by the three following upper rows, were conducted by omitting one of the proteins during the stack preparations, either without Pluronic F-68 or with Pluronic F-68, as denoted on the vertical axis. These three negative controls reveal negligible adsorptions at the gold surface. For comparison, the full model with the addition of Pluronic F-68 to all solutions was applied to PPy:PSS-coated chips (top row). In this case, a negligible light absorption signal was observed.

3.3. EIS Measurements of Protein Stack Formation

It has to be mentioned that the preparation of the protein stack on the interdigitated electrodes followed a slightly different procedure. Here, a 20 μL droplet of SAMp was pipetted onto the interdigitated electrodes and incubated for 1 h. For subsequent incubation of BSAp, STRp, and HRPp, a 10 μL droplet of each solution was pipetted on the sensor spot for 30 min. Washing in between was conducted by rinsing the electrode area three times with 500 μL PBp and drying the sensor area in a stream of nitrogen. During all incubation steps, the droplets were covered with a lid of a falcon sealed with a gasket made of Parafilm (Parafilm M, Sigma-Aldrich Chemie GmbH, Munich, Germany) to reduce evaporation.

The protein stack formation was monitored online by EIS. As an example for a typical EIS spectrum, a Bode plot of the last obtained spectrum during BSAp incubation at t = 1800 s on chip B is shown in Figure 7.

The plots in Figure 7 show a typically capacitive behavior in the low-frequency range and a typical resistive behavior at higher frequencies. This spectrum was analyzed by equivalent electric circuits. For the frequency range between 100 Hz and 100 kHz, a modified Randles circuit including a resistor R\text{S} representing the solution resistance, the constant phase element CPE\text{DL} as double-layer capacity, and the charge-transfer resistance R\text{CT}.

![Figure 5. OCP of bare gold (Au, before electrodeposition) and PPy:PSS-coated CE/RE versus Ag/AgCl RE in PB. At t = 0 s, the electrode was immersed in the buffer solution, whereas at t = 1800 s, the electrode was removed out of the solution, dried in a stream of nitrogen, and immediately immersed in the solution again.](image1)

![Figure 6. Photometric measurements to characterize protein stack formation. The absorption was measured at a wavelength of 450 nm with a photometer. The four upper rows show negative controls with a negligible color reaction, whereas the positive controls in the bottom row show strong absorption signals, even when Pluronic F-68 is added to certain protein solutions.](image2)

![Figure 7. Bode plot of the last obtained spectrum obtained by EIS during BSAp incubation on chip B in Au–PPy:PSS electrode configuration.](image3)
The applied equivalent circuit is shown in Figure 8.

The CPE$_{DL}$ for modeling the double-layer capacity represents the roughness of the nonideal capacitive electrode. The impedance of CPE$_{DL}$ is given by $Z = 1/(Q_2 \omega + j)$ for fitting, the Powell algorithm (1000 iterations) and the open-source software “EIS Analyser” were used. Table 1 shows the fitted parameters and the relative errors. In addition, the impedance of each element is calculated at 664 Hz.

For the following transient EIS measurements, the imaginary part of the impedance at 664 Hz was selected, reflecting information on the double-layer capacitance behavior and therefore information on the adsorption of proteins at the electrode/electrolyte interface. Three different sensor chips in the Au–PPy:PSS electrode configuration labeled with letters were used. Figure 9 shows the transient of the imaginary part of the impedance signal at 664 Hz during protein incubation. The values are normalized to the last value measured during BSAp incubation (compare Bode plot in Figure 7).

This value is used as reference because the BSAp incubation serves only as a blocking step and does not represent specific binding of the analytes to the SAM$_p$ primed surface. In addition to blocking purposes, BSAp incubation is added to further stabilize the CE/RE potential. The three sections in Figure 9 correspond to 30 min-long consecutive incubations of BSAp, STR$_p$, and HRP$_p$ on SAM$_p$ pretreated chips. During each incubation, a fast increase in the EIS signal is observed followed by saturation and a slight decrease in the sensor signal. Each incubation is clearly resolved by a significant increase in the impedance signal.

For comparison, the same immobilization protocol and corresponding EIS measurements were conducted using gold for both electrodes, for WE as well as for CE/RE, respectively. The values of the imaginary part of the impedance for the Au–Au electrode configuration are shown in Figure 10. Here, the imaginary part has been normalized to the last value of each BSAp incubation ($t = 1800$ s), for better comparison with the courses obtained with the Au–PPy:PSS electrode configuration (compare Figure 9). In these experiments, the normalized imaginary part of the impedance signals at 664 Hz drops irreproducibly for the first minutes and then increases and saturates after each media change. The impedance values in the saturation phase of STR$_p$ and HRP$_p$ incubation are nearly the same and settle around the value obtained in the BSAp saturation phase ($\approx 1$).

Exemplarily, the measurement on chip G has been evaluated at different frequencies to investigate the origin of the irreproducible drops after media exchange on the Au–Au electrode configuration. Figure S1, Supporting Information, shows the

![Figure 8](image)

**Figure 8.** Equivalent circuit of the electrode/electrolyte interface for the frequency range between 100 Hz and 100 kHz. CPE$_{DL}$ represents the electric double-layer capacity and $R_s$ the resistance of the buffer solution. The charge-transfer resistance is modeled by $R_{CT}$.

| Table 1. Circuit elements of the equivalent circuit obtained by Powell algorithm using the software “EIS Analyser.” The relative error is shown additionally. The impedance of each circuit element has been evaluated at 664 Hz, which is the frequency of interest during the transient measurements. |
|---|---|---|---|---|
| $R_s$ | $R_{CT}$ | $Q$ (CPE$_{DL}$) | $n$ (CPE$_{DL}$) |
| Value | 214.94 $\Omega$ | 22.1 M$\Omega$ | 2.89 $nF \times s^{-1}$ | 0.96 |
| Rel. error | 6.91% | 24.53% | 3.99% | 0.04% |
| Impedance at 664 Hz | 214.94 $\Omega$ | 22.1 M$\Omega$ | 115.763 k$\Omega$ |

![Figure 9](image)

**Figure 9.** EIS measurements during protein stack formation using the Au–PPy:PSS electrode configuration. The imaginary part of the impedance is measured at 664 Hz normalized to the last value obtained during BSAp incubation. Media exchanges are designated with the vertical solid lines every $1800$ s (30 min). The protein solution used in each incubation step is indicated in the graph. Different chips have been used corresponding to the letter in the inset.

![Figure 10](image)

**Figure 10.** EIS measurements during protein stack formation using Au–Au electrode configuration. The imaginary part of the impedance is measured at 664 Hz normalized to the last value obtained during BSAp incubation. Media exchanges are designated with the vertical solid lines every $1800$ s (30 min). The protein solution used in each incubation step is indicated in the graph. Different chips have been used corresponding to the letter in the bottom right inset. The number indicates multiple uses of the indicated chip after electrode cleaning. The inset in the bottom left shows a close-up of the red frame in the graph ranging from $t = 1800$ s to $t = 2000$ s on the time axis and from 0.97 to 1.01 on the normalized impedance axis.
transient course of the EIS measurements at 232, 1902, 5445, and 15591 Hz, respectively. For comparison, the curve at 664 Hz already shown in Figure 10 is added. The irreproducible signal drops are observed at low frequencies. At elevated frequencies, the magnitude of the drops decreases. Signal drops after media exchange completely disappear at 15591 Hz.

4. Discussion

An interdigitated EIS sensor design, which consists of a gold WE and PPy:PSS as combined CE and RE, was evaluated as a biosensor. The two-electrode sensor design is shown in Figure 2, consisting of a smaller WE of gold and a larger CE/RE made of an electropolymerized PPy:PSS layer. The electropolymerization process was conducted potentiostatically in deionized water, which is a common process. The increased electroactive surface area of the PPy:PSS layer decreases the interface impedance, as discussed in detail in a previous study. In a previous publication, we already have shown that this sensor type yields stable and highly repeatable EIS spectra.

In this work, we prove the sensor design as a biosensor. The protein layer stack shown in Figure 1 was stepwise formed on the sensor surface. Although it is of interest to know details of the molecular structure of the applied SAM, the supplier gave no information concerning this matter. It has to be noted that this type of SAM is assigned especially for biotin–streptavidin assays and was already used.

The deposition of the SAM terminated with biotin, followed by streptavidin, and biotinylated horse serum peroxidase onto the sensor surface shows impressively that the biomolecule stack only binds to the gold electrode and not to the PPy:PSS electrode (Figure 6). In general, BSA is always used as a blocking agent in such assays to avoid the unspecified binding of consequently applied biomolecules. Although BSA with \(\approx 66 \text{kDa}\) is a large molecule compared with biotin (\(\approx 244 \text{Da}\)) and streptavidin (\(\approx 52 \text{kDa}\)), control experiments revealed that subsequently applied proteins are not hindered when binding to the biotin of the SAM. Kazuma et al. pointed out that BSA does not bind to the SAM itself by means of localized surface plasmon resonance. Omitting the SAM in the MTP experiments led to absorption values in the photometric measurements of 0.19 without BSA blocking and 0.02 with blocking, respectively. This shows that the use of BSA avoids unspecified binding of consequently applied proteins. Due to missing of the SAM in the false-positive negative control (absorption 0.19), the binding mechanism is only unspecified on gold. For the following STR offer, it is known that streptavidin tends to unspecific binding of consequently applied biomolecules.

Although BSA with \(\approx 66 \text{kDa}\) is a large molecule compared with biotin (\(\approx 244 \text{Da}\)) and streptavidin (\(\approx 52 \text{kDa}\)), control experiments revealed that subsequently applied proteins are not hindered when binding to the biotin of the SAM. Kazuma et al. pointed out that BSA does not bind to the SAM itself by means of localized surface plasmon resonance. Omitting the SAM in the MTP experiments led to absorption values in the photometric measurements of 0.19 without BSA blocking and 0.02 with blocking, respectively. This shows that the use of BSA avoids unspecified binding of consequently applied proteins. Due to missing of the SAM in the false-positive negative control (absorption 0.19), the binding mechanism is only unspecified on gold. For the following STR offer, it is known that streptavidin tends to unspecific binding of consequently applied biomolecules.

As a novelty, the protocol was characterized with respect to the addition of small amounts of the nonionic surfactant Pluronic F-68. Nonionic surfactants are typically added to solutions containing biomolecules to prevent biofouling on hydrophobic surfaces. In future, we intend to use the novel electrode design in a digital microfluidic platform where droplets containing proteins will be manipulated on hydrophobic surfaces by application of electric fields to underlying electrodes, called electrowetting. Our results reveal, on the one hand, that Pluronic F-68 does not affect the adsorption behavior of the biomolecules to the electrode surface and, on the other hand, that Pluronic F-68 does not harm the activity of the HRP. To our knowledge, the impact of Pluronic F-68 on the adsorption behavior of the presented molecule stack has not been investigated in detail.

Although the results obtained by photometer measurements only reflect the final preparation condition (end-point detection), online EIS measurements of the protein stack formation on the gold WE in the Au–PPy:PSS electrode configuration were conducted (Figure 9). It must be noted that the BSA concentration in the droplet-based experiments was significantly reduced compared with the experiments in the MTPs. The higher concentration of BSA in the MTP-based experiments was used because the wetted surface area in the MTP is much larger than the wetted surface on the sensor spot of the droplet-based approach compared with the volume. It was shown that the BSA concentration in MTP could not be reduced for sufficient blocking. In the droplet-based approach, we lowered the BSA concentration as much as possible, because of future application in a digital microfluidic system.

The values of the imaginary part of the impedance measured at 664 Hz, representing the double-layer capacity (Figure 7), were normalized to the last value measured during BSAp incubation, because after this blocking step the sensor surface was prepared for specific binding of streptavidin to the biotinylated SAM. Unspecific binding is prevented due to BSAp blocking. It has to be mentioned that the progressions of BSAp incubation showed a slightly different behavior in each measurement. During SAMP incubation, the CE/RE is wetted by ethanol and diH2O. Upon BSAp application, the electrochemical equilibrium changes and settles due to the replacement from an alcohol-containing solution to a water-based solution and the corresponding change in salt concentration. This leads to compensation processes at the electrode superimposed by the monitoring of unspecific binding of BSA. The media exchange to STRp and later to HPRp (same buffer solution) does not affect the electrode conditions, which has been shown by the measurement of the OCP (compare Figure 5).

Table 1 shows the values of the elements of the chosen equivalent electric circuit defined in Figure 8. The calculated impedance values for each element at 664 Hz show clearly that the double-layer capacity \(C_{PEI\Omega}\) dominates the impedance in that frequency regime. \(R_s\) is negligible compared with the circuit elements modeling the double layer. Because the \(R_{CT}\) branch connected in parallel to \(C_{PEI\Omega}\) owns a significantly higher electrical impedance at the chosen frequency, the resulting impedance of the parallel circuit equals almost only the impedance of the double-layer capacity. The high value of the charge transfer resistance \(R_{CT}\) of 22.1 M\(\Omega\) indicates that mainly non-Faradaic processes occur at the electrodes. This is due to the lack of redox couples in the solution.

As shown in Figure 9, the evolution of the normalized imaginary part of the impedance is repeatable and shows only a slight drift behavior. It seems that the drift depends on the type of the incubated protein. We assume that biotin/streptavidin interaction is quick and straightforward. Streptavidin/biotin-HRP is also quick but drifts more, maybe some biotin-HRPs do not find their binding partner streptavidin due to steric hindrance by the already-bound biotin-HRP.
Furthermore, the saturation of the gold electrode surface appears shortly after access of STRp and HRPp, indicating fast biomolecule binding, as shown in Figure 9. This result perfectly matches with the results obtained by photometric measurements where the incubations saturate within at least 30 min. Fast binding kinetics of biotin and streptavidin have been shown by Duan et al. on functionalized silicon nanowire transistors and reveal also quick binding.\[22\]

In contrast, EIS measurements conducted at the Au–Au electrode configuration reveal signal peaks directly after each media exchange (Figure 10). As shown in Figure 5, the OCP progression at the bare gold electrode also shows a similar sharp peak after media exchange. Both signal behaviors can be correlated with the settling of the OCPs at the Au electrode/electrolyte interface after each media exchange. Using the Au–Au electrode configuration, biomolecule adsorptions and the disturbance of the electrochemical equilibrium take place at both electrodes, which influence the behavior of double-layer capacity and the OCP settling at both electrodes. For comparison, EIS progressions at the Au/PPy:PSS electrode combination show no occurrence of peaks after media exchange (compare Figure 9).

While looking to the OCP performance in Figure 5, the OCP at PPy:PSS-coated electrodes remains nearly constant independently of media exchange, approaching the performance of a (pseudo-)RE. The PPy:PSS-coated electrode serves as CE/RE, where the feature of CE exhibiting a low interface impedance to deliver sufficient compensation current is combined with the feature of RE providing a stable OCP. It can be argued that the OCP-stable PPy:PSS-coated CE/RE, which exhibits a significantly larger effective surface area due to its porosity than the gold WE, reduces on the one hand the peak height of OCP settling, because OCP settling takes place only at the gold WE. On the other hand, a smaller peak might be covered by the previously discussed fast and efficient SAM and subsequent protein adsorption processes. This favorable behavior of PPy:PSS as CE/RE leads to EIS measurements of protein adsorptions without occurrence of a OCP settling peak when the electrochemical equilibrium is disrupted by media exchange and drying of the sensor spot. In addition, transient EIS progressions have been taken at different frequencies (compare Figure S1, Supporting Information). The signal drop due to media exchange is more severe at low frequencies, whereas at higher frequencies these instabilities were not observed. In addition, a pronounced overall signal increase is observed after each biomolecule incubation step only in measurements at high frequencies. The peaks in the impedance signal during media exchange can be attributed to the settling of the OCP after disturbance of the electrochemical equilibrium because OCP-related effects are more dominant in the low-frequency regime.

The sensor signal obtained with the Au–Au electrode configuration shows highly irreproducible transient responses by forming a peak and a negligible EIS signal increase in the saturation phase with respect to the saturation phase of the previous incubation step. In contrast, the sensor signals in the saturation phase increase about 2% when using the Au–PPy:PSS electrode configuration. The different behaviors are due to the severe change of the OCP at the gold CE/RE. The unsteady OCP behavior shown in Figure 5 observed for the Au electrode is significantly improved when PPy:PSS is used as electrode material. Here, the OCP stays almost constant after the media exchange, which provides a stable reference potential at the PPy:PSS-coated CE/RE. In addition, it has to be mentioned that OCP changes by adsorption of molecules at the electrode surface.\[23\] In this work, it is shown that no binding of STR and HRP to the PPy:PSS electrode occurs (Figure 6), so OCP stays stable at the PPy:PSS-coated CE/RE.

Setups for EIS applications with interdigitated electrodes are often used in continuous flow environments.\[11,24\] In continuous flow conditions, the electrode potentials can stabilize and are not disturbed by a turbulent media exchange and by drying the sensor spot as in our case. Therefore, studies show transient EIS spectra after initial signal stabilization or are not monitored online but show the final preparation step after, e.g., DNA hybridization.\[24e\]

It must be noted that the objective of the work is not the investigation of the binding reaction kinetics. As shown in Figure 9, the time resolution of the signals is not sufficient to calculate valuable kinetic constants. In these experiments, the impedances were measured over a large frequency range from which the values of the imaginary part at 664 Hz were extracted for Figure 9 and 10. In future experiments, the impedances will be monitored only at one frequency in the low-frequency regime, being able to characterize the change of the double-layer capacitance with a subsecond temporal resolution.

5. Conclusion

We proved an EIS sensor design for biosensor applications based on an interdigitated electrode arrangement in which the WE was made of gold, whereas the combined CE and RE consisted of a PPy layer doped with PSS. The performance of the new design was successfully tested by monitoring the immobilization of a stack of thiolated biotin as an intermediate SAM, followed by streptavidin and a biotinylated horseradish peroxidase on the bare gold finger. The immobilization process was monitored online by EIS, showing repeatable EIS signals, where the protein formation state can be clearly resolved, and increased sensor signal. Furthermore, it was shown that the addition of the nonionic surfactant Pluronic F-68 did not influence the protein stack formation and did not harm the HRP activity.

The results showed that the novel two-electrode design is very suitable for EIS-based biosensors, especially in long-term applications, in which a three-electrode configuration with separated WE, CE, and RE cannot be used, such as in microfluidic applications, providing highly stable and repeatable sensor signals.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors express their sincere thanks to Dorothee Breuer, Ewa-Janina Sekula, and Jochen Heiss for assistance in fabricating the sensor chips, Vivek Pachauri for making AFM images, and Benjamin Schick and Anabel Mernitz for assistance conducting some experiments.
Conflict of Interest

The authors declare no conflict of interest.

Keywords
biosensors, electrochemical impedance spectroscopy, polypyrrole doped with polystyrene sulphonate, proteins, surfactants

Received: September 30, 2019
Revised: February 11, 2020
Published online: 2019

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