

Straightforward Approach for Electrochemical Deposition and Modification of Conductive Polythiophene Thin Films for Bioreceptor Immobilization [†]

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Abstract: Biosensors offer exciting opportunities for various clinical applications and constitute a rapidly growing research branch due to new generations of bioreceptors, transducers, and biomaterials with versatile characteristics, such as conductive polymers. Polythiophene is a prominent example of conducting polymers and convinces with excellent conductivity and stability—and is yet barely used for the construction of biosensors. We want to address the fundamental lack of straightforward fabrication procedures for bioreceptor immobilization platforms based on polythiophene. We investigate the literature-known monomer 3-thiopheneacetic acid and present our newly developed method for electrochemical coupling of the linker p-aminobenzoic acid to deposited polythiophene films. Aminated bioreceptors can subsequently be immobilized via EDC/NHS click chemistry. Films were electropolymerized and modified by chronopotentiometry, characterized by electrochemical impedance spectroscopy, surface-enhanced Raman spectroscopy, as well as energy-dispersive X-ray spectroscopy. Both of the presented methods allow for the fabrication of functionalized polythiophene thin films of high conductivity and good reproducibility while convincing with their ease in synthesis.

Keywords: electrochemical biosensor; conductive polymers; polythiophene; electropolymerization; electrochemical impedance spectroscopy; chronopotentiometry; EDC/NHS click chemistry



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1. Introduction

Conductive polymers possess unique and interesting properties as they combine the advantages of both organic polymers and inorganic conductors. They can easily be electropolymerized and deposited on electrodes to form thin films, which serve as key components in trendsetting applications, such as chemical sensors and biosensors. The thin films demonstrate versatile and beneficial properties as they enhance electron transfer [1], reduce electrode poisoning [2] and fouling [3], and demonstrate great chemical and structural diversity, while being able to be co-deposited with other compounds of interest.

Polythiophene, consisting of interlinked sulfur heterocycles, is a prominent example of conductive polymers, which owes its electrical conductivity to the delocalization of electrons along the polymer backbone when being oxidized or reduced. Although polythiophene outperforms other conductive polymers, such as polypyrrole and polyaniline, regarding conductivity and stability [4], its application for the construction of biosensors can barely be seen.

We think that this owes to the fact that there is a fundamental lack of fast and easy-to-perform fabrication procedures for polythiophene-based bioreceptor immobilization platforms. Almost all of the published protocols require complex synthesis and downstream processing of new (macro)monomers with adequate know-how, well-equipped laboratories, potentially costly and hazardous chemicals, as well as a significant amount of time. This

way, biotinylated thiophene monomers [5,6], a 3-(oxyalkyl)-thiophene bearing arylsulfonamide group [7], a terthiophene monomer functionalized with acrylic acid and a methylhydroxyl group [8], a thiophene-functionalized polyphenylalanine macromonomer [9], and a thiophene-EDPT monomer with a central thiodiazole unit [10] were introduced. Therefore, there is a strong need for simple and cost-effective methods, which allow for covalent binding of the bioreceptor to the polythiophene immobilization platform.

The utilization of a ready-to-buy, carboxylic acid functionalized monomer, namely 3-thiophene acetic acid, is already reported [11–14]. After electropolymerization, the bioreceptor can be bound to the carboxylic group by simple EDC/NHS click chemistry. However, the nucleophilicity of the carboxylic group has to be considered, as it is reported to attack the radical cations of monomeric intermediates and thereby inhibits electropolymerization [15–17]. By co-polymerization with unfunctionalized thiophene monomers, however, this issue can be avoided [15,16].

We decided to rise to the challenge of working with polythiophene and could identify factors to significantly improve film properties. It is of utmost importance to carefully dry the working solution since even minor amounts of water negatively affect electropolymerization to the point where film formation can be inhibited completely. We have found that drying the solution of hygroscopic counter ion and solvent over a molecular sieve for at least a week constitutes a simple yet effective method to remove water and allow for successful electropolymerization [18]. This way, we could obtain polythiophene films with exceptionally low charge transfer resistance R_{CT} , which characterizes film conductivity in perpendicular direction and can be obtained from electrochemical impedance spectroscopy. By using the same conditions as described in the method section, the R_{CT} of obtained films was around 12 Ω and therewith significantly lower than that of the uncoated electrodes. The parameters deposition current and time, as well as monomer and supporting electrolyte concentration, were optimized to obtain films of remarkably smooth surface topography to inhibit sterical hindrance of subsequently bound bioreceptors. Scanning electron microscope (SEM) pictures of the optimized film in comparison to a film with rough surface topography can be seen in Figure 1. Lately, we also found and published that electropolymerization can be catalyzed, which also grants the films a smoother surface morphology and increases the selectivity of polymerization [19,20].



Figure 1. SEM pictures of (a) blank electrode, (b) polythiophene thin film that was electropolymerized under the optimized conditions described in the method section, (c) rough polythiophene film electropolymerized by a $6\times$ higher current density. Scale bare is applicable to all pictures.

In this communication, we investigate electropolymerization of the monomer 3-thiopheneacetic acid and present our newly developed method for covalent binding of a linker to the beforehand deposited polymer thin films. The linker p-aminobenzoic acid similarly possesses a carboxylic group for subsequent bioreceptor immobilization on one end and a primary amine for binding to polythiophene on the other end. The crucial step was to develop a suitable method for electrochemical coupling of the amine to the aromatic structure of the polymer film while not severely altering its conductivity. Both of the presented straightforward approaches offer the opportunity to construct a polythiophene immobilization platform for bioreceptor binding with EDC/NHS click chemistry—all it

takes is a potentiostat/galvanostat, affordable, low-hazard chemicals that can be used as received, as well as only a few minutes of time.

2. Materials and Methods

2.1. Electrodes and Equipment

Screen-printed thick-film electrode chips (SPE) with an integrated gold working electrode, silver reference electrode, and platinum counter electrode (model DRP-250AT) were obtained from Metrohm DropSens (Llanera, Spain). The integrated silver electrode was used for electropolymerization and modification, while an external Ag/AgCl reference electrode (Sensortechnik Meinsberg, Waldheim, Germany) was used for characterization.

Electrochemical procedures were performed using the SP-300 potentiostat/galvanostat with an impedance analyzer (Bio-Logic Science Instruments SAS, Claix, France). Films were further characterized by surface-enhanced Raman spectroscopy (SERS) with the QE65000 spectrometer (Ocean Insight, Orlando, FL, USA) and a 785 nm laser source (Fat Boy Laser Module, Innovative Photonic Solutions, Plainsboro Township, NJ, USA), and by energy-dispersive X-ray spectroscopy (EDS, Bruker, Microanalysis GmbH, Berlin, Germany), consisting of the detector XFlash[®] 5030 T 127 eV and the signal processing unit XFlash[®] SVE III.

2.2. Electrochemical Procedures

Polythiophene films were synthesized from 200 mM thiophene and 500 mM KPF₆ in acetonitrile by the application of a constant current of 0.5 mA, which equals 3.98 mA/cm² for our working electrodes of a diameter of 4 mm. A mix of thiophene and 3-thiopheneacetic acid was analogously electropolymerized, while the total monomer concentration was fixed at 200 mM. Successful binding of the linker was similarly performed: The films were incubated in different concentrations of p-aminobenzoic acid and 500 mM KPF₆ in acetonitrile, and a current of 0.5 mA was applied for 1 min. Electrochemical impedance spectroscopy (EIS) was carried out with a sinusoidal 7.07 mV rms excitation voltage around the DC potential of 0 V starting from a frequency of 100 kHz. The measurement buffer consisted of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM KCl, 1 mM MgCl₂, with an addition of 2 mM K₃[Fe(CN)₆] and 2 mM K₄[Fe(CN)₆]. Cyclic voltammetry (CV) was performed in acetonitrile with 500 mM KPF₆ as supporting electrolyte and a scan rate of 20 mV/s.

2.3. Electrode Cleaning

Polythiophene thin films were removed for electrode regeneration and reuse according to our recently developed procedure [21]. Briefly, the electrodes were incubated in 2 M sodium perchlorate in acetonitrile and a constant potential of 2.4 V was applied for one minute. The electrodes were then rinsed with distilled water. The remaining film fragments were wiped off with a Q-tip. Impedance measurement was deducted for quality assurance. Subsequently, the silver reference electrodes were regenerated by treatment with a Q-tip that was immersed in 30 mg/mL aqueous thiourea until the shiny silver surface was restored. The cleaned electrodes can be stored in 5 mL Eppendorf cups until the next usage.

3. Results

3.1. Covalent Binding of Carboxylated Linker

We decided to investigate a linker that contains a carboxylic group for subsequent bioreceptor binding and a primary amine for covalent linkage to the deposited polythiophene film. Primary amines oxidize at potentials greater than 1 V and form radicals, which can covalently bind to aromatic structures. This strategy was already reported for the modification of graphene-based materials, to which different amine-carrying compounds were bound via cyclic voltammetry [22–25].

Two potential linkers were investigated, namely p-aminobenzoic acid and 6-aminohexanoic acid, of which the latter unfortunately was found to be insoluble in acetonitrile, while

p-aminobenzoic acid (PABA) showed solubility of at least 100 mM. To investigate its applicability as a linker, cyclic voltammetry (CV) experiments were conducted. As can be seen from Figure 2, p-aminobenzoic acid owes its electrochemical activity solely to the oxidation of its primary amine since benzoic acid is electrochemically stable. Therefore, p-aminobenzoic acid can be used as a linker to equip polythiophene films with carboxylic groups.

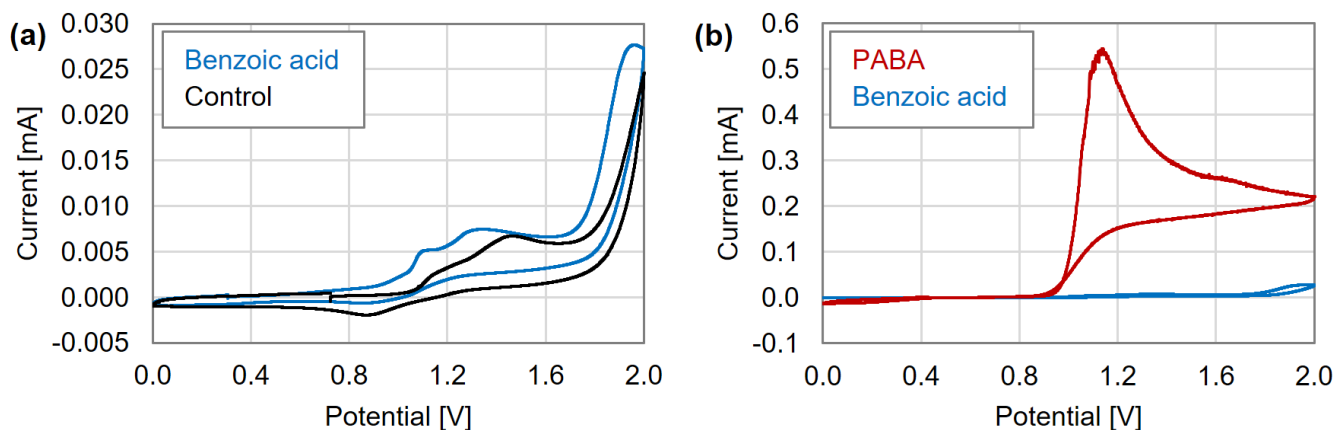


Figure 2. Cyclic voltammetry of (a) 100 mM benzoic acid and the control of just solvent and supporting electrolyte without any compound under investigation, (b) 100 mM p-aminobenzoic acid (PABA) in comparison to benzoic acid.

Analogous to literature reports [22–25], cyclic voltammetry was used to link PABA to previously deposited polythiophene. As can be seen from Figure 3a, in the presence of 25 mM PABA, a significantly higher current can be measured above 1 V compared to the control of polythiophene cycling in the absence of PABA. Therefore, it can be concluded that the amine of PABA was oxidized and presumably bound to the polymer film. As the vertex potential in CV, 1.13 V was chosen since it constituted the potential corresponding to the maximum current measured in Figure 2b. The CV scan ended at the vertex potential to ensure that polythiophene was in its oxidized state and, therefore, conductive.

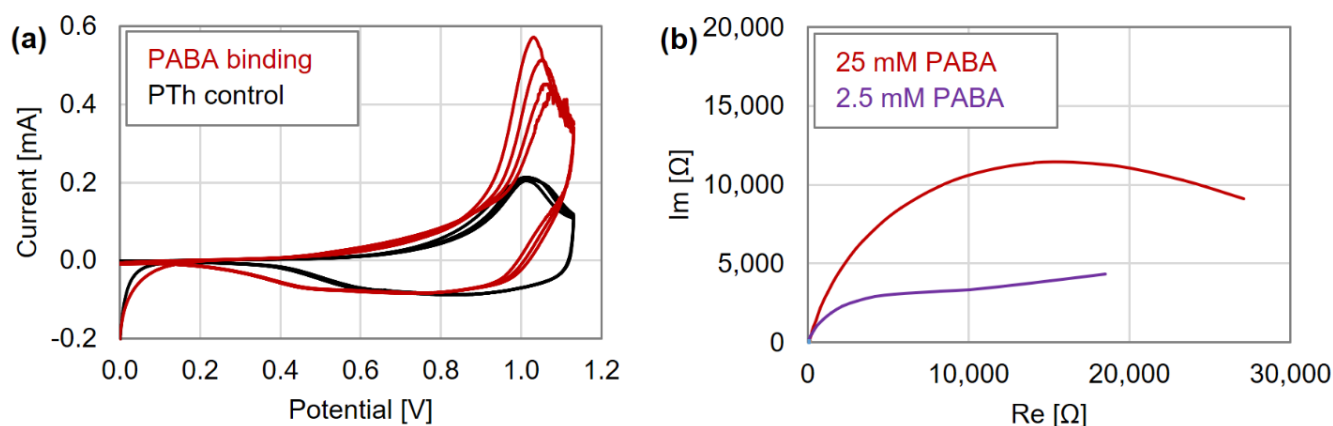


Figure 3. (a) Cyclic voltammetry of polythiophene (PTh) in the absence and presence of 25 mM p-aminobenzoic acid (PABA). Three cycles were each performed with a vortex/end potential of 1.13 V. (b) Nyquist plots of PTh modified with 25 mM and 2.5 mM PABA by cyclic voltammetry.

When the modified film was characterized by electrochemical impedance spectroscopy (EIS), however, the charge transfer resistance R_{CT} had increased from 12 Ω of the unmodified film to several 10,000 Ω after modification with PABA. The experiment was repeated with a tenfold lower concentration of 2.5 mM PABA, but a similarly high R_{CT} was obtained (see Figure 3b). Since the potential measured during electropolymerization of thiophene equals 1.65 V, the vertex/end potential was set to this value to improve film conductiv-

ity. Here, equally high values for R_{CT} were obtained, so it must be concluded that linker binding by cyclic voltammetry severely blocks electron transfer of the modified film.

While graphene is stable and conductive in a wide potential window, this does not account for polymer films, whose conductivity depends on its oxidative state and, therefore, the applied potential. A gentle method is, hence, needed that does not severely alter the conductivity of the polymer film. This, logically, should be achievable under the conditions that are used for thiophene electropolymerization, which were optimized to obtain films of good reproducibility and low charge transfer resistance. Therefore, binding of 25 mM PABA was performed by the application of 0.5 mA ($=3.98 \text{ mA/cm}^2$) for 1 min, similar to the conditions used for electropolymerization (see Figure 4a). The measured current during PABA binding by chronopotentiometry lies between the currents resulting from the control experiments of PTh oxidation in the absence of PABA and PABA oxidation in the absence of PTh, which was overloaded and stopped after 10 s. The modified film was characterized by EIS and revealed its great conductivity, as can be seen in Figure 4b. It can, therefore, be concluded that linker binding under the same conditions used for monomer electropolymerization can apparently be performed successfully and is additionally highly beneficial for the conductivity of the modified film.

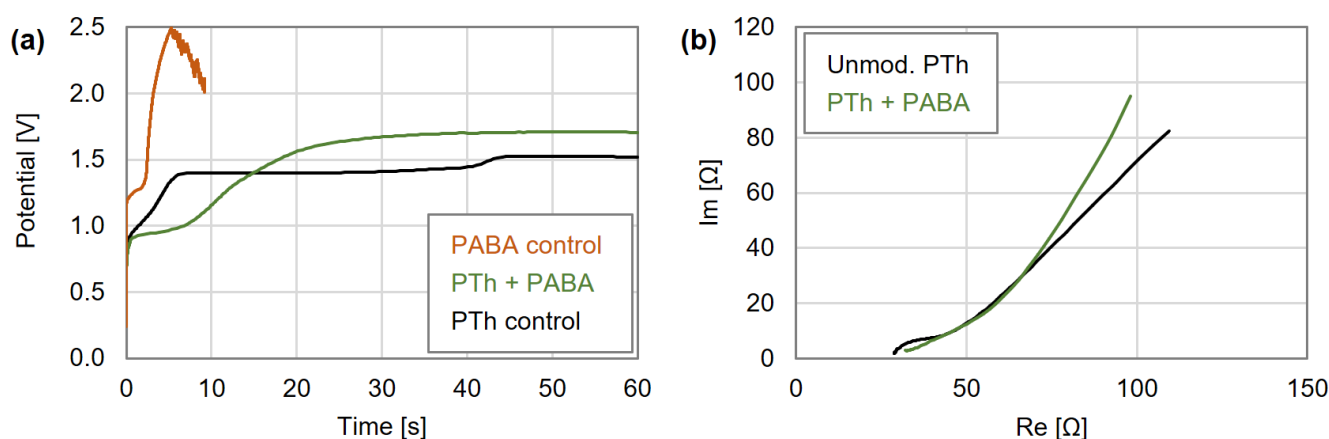


Figure 4. (a) Chronopotentiometry for binding of 25 mM p-aminobenzoic acid (PABA) to polythiophene (PTh). Control experiments of PABA oxidation in the absence of PTh, and of PTh oxidation in the absence of PABA are included. (b) Nyquist plots of PTh before and after binding of 25 mM PABA by chronopotentiometry.

To verify PABA binding, surface-enhanced Raman spectroscopy (SERS) and energy-dispersive X-ray spectroscopy (EDS) were employed. The bands of polythiophene in SERS can be assigned to the vibrational modes pictured in Figure 5 [26,27]. Various benzoic acid derivatives show a band at around $1370\text{--}1380 \text{ cm}^{-1}$, which is identified as the symmetrical stretching vibrations of COO^- groups and can also be found in the SERS spectra of PABA in this work. In addition, a broad band of similar intensity can be found with a maximum at 230 cm^{-1} , which is associated with oxygen but could not yet be clearly identified [20]. The spectrum of the PABA-modified PTh film contains both the band of $\nu_s(\text{COO}^-)$ as well as the oxygen-associated band, proving the successful binding of PABA to PTh.

The $\nu_s(\text{C}=\text{C})$ band of PTh cannot be seen at the expected wavenumbers since PABA binding shifted or suppressed the ring $\text{C}=\text{C}$ stretch in SERS. It is reasonable to assume that PABA binds to the C_β position of polythiophene, as pictured in the proposed binding mechanism in Figure 6. The introduced radicals can easily move along the polymer backbone and recombine because the conjugated system, accounting for the film's conductivity, is not disrupted. We assume that amine binding thereby shifts the quinoid structure of the doped polythiophene to benzenoid structure or vice versa, which could be observed in SERS by a shift of the $\nu_s(\text{C}=\text{C})$ band that now might overlap with the $\nu_s(\text{COO}^-)$ of PABA.

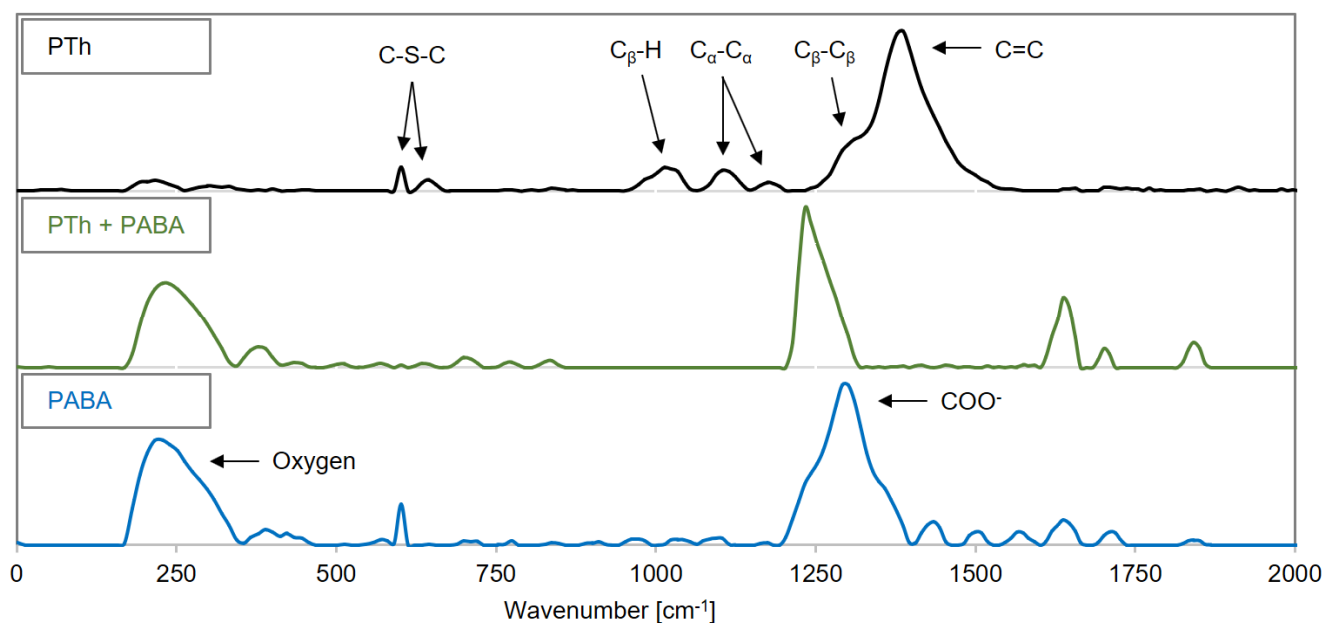


Figure 5. SERS of polythiophene (PTh), p-aminobenzoic acid (PABA), and PABA modified PTh.

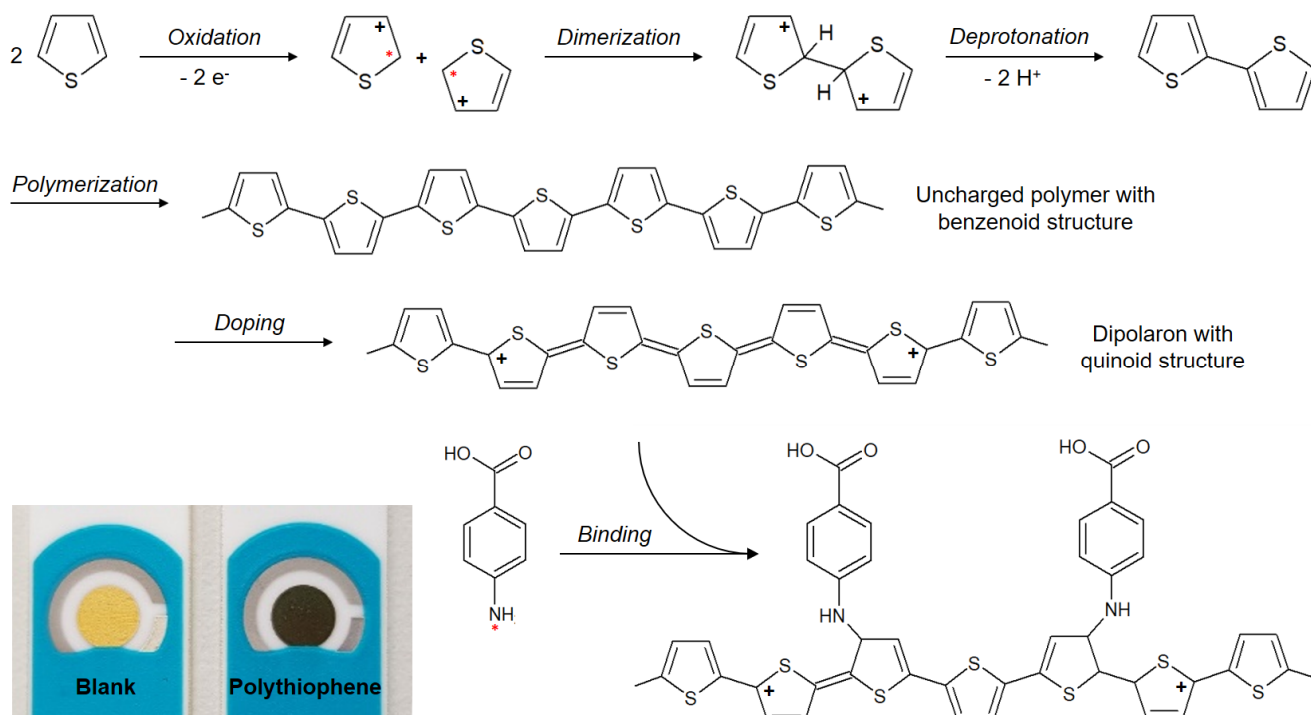


Figure 6. Schematic representation of electropolymerization mechanism [26] and proposed binding of p-aminobenzoic acid (PABA). Due to electrochemical oxidation, PABA forms radicals and binds to polythiophene in, as we propose, the C_β position.

In correlation with SERS results, energy-dispersive X-ray spectroscopy revealed increasing amounts of oxygen (from $7.3 \pm 1.8\%$ to $12.2 \pm 0.6\%$) and carbon (from $55.8 \pm 2.7\%$ to $56.7 \pm 0.6\%$), while nitrogen was now present on the modified films (0.26%). The sulfur amount decreased (from $36.9 \pm 4.3\%$ to $30.8 \pm 0.8\%$) since the relative abundance of polythiophene decreased due to the newly present PABA on the modified films. The combined data from chronopotentiometry, SERS, and EDS, therefore, proves the successful binding of p-aminobenzoic acid to polythiophene via the presented method.

With this optimized protocol, different concentrations of PABA were bound to polythiophene films, and the resulting potential was monitored. With an increasing PABA concentration, increasing potentials could be observed, as can be seen in Figure 7a. Film conductivity and reproducibility of modification were evaluated by electrochemical impedance spectroscopy. The Nyquist plots of polythiophene films can easily be fitted with the Randles–Ershler equivalent circuit [28,29], which is typically used for the interpretation of impedance spectra of biosensors. However, this is not the case for PABA-modified films since the straight line, which is associated with the diffusion-controlled regime, occurs with an angle greater than 45° . Therefore, a new equivalent circuit was developed for accurate fitting of the obtained measurement curves, as can be seen in Figure 7b.

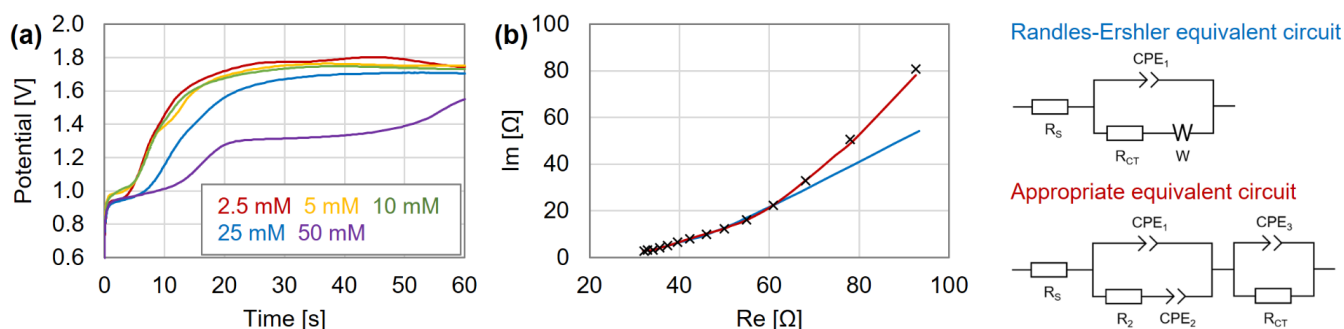


Figure 7. (a) Chronopotentiometry for binding of different concentrations of p-aminobenzoic acid (PABA) to polythiophene films. (b) Identification of a new equivalent circuit for appropriate fitting (red) in comparison to the commonly used Randles–Ershler equivalent circuit (blue).

The charge transfer resistance R_{CT} of the PABA modified films were calculated and are listed in Table 1. The modification with a PABA concentration of 2.5 mM did not lead to reproducible results, while the binding of 50 mM PABA led to an R_{CT} of several thousand Ohm. In EIS, the diffusion of the redox mediator—in this case, the ferro/ferricyanide redox couple—gets more and more impeded, the denser and bigger bound molecules on the electrode are. Seemingly, 50 mM PABA built such a dense layer on the conductive polymer film that electron transfer was severely hindered so that a high R_{CT} resulted. By using 5–25 mM PABA, modified films could be obtained that convince with great reproducibility and conductivity.

Table 1. Charge transfer resistance R_{CT} of polythiophene films modified with different concentrations of p-aminobenzoic acid. Experiments were performed in triplicates, and measurement curves were fitted with the new equivalent circuit pictured in Figure 7b.

2.5 mM	5 mM	10 mM	25 mM	50 mM
196.2 Ω	76.7 Ω	71.9 Ω	131.0 Ω	>1000 Ω
±90.7%	±13.9%	±8.7%	±7.3%	

3.2. Electropolymerization of Carboxylated Monomer 3-Thiopheneacetic Acid

The utilization of a carboxylated monomer constitutes another opportunity for the synthesis of a polythiophene-based immobilization platform. The nucleophilicity of its carboxylic group is already known to attack radical cations of monomeric intermediates, which inhibits electropolymerization but can be avoided by co-polymerization with the unfunctionalized thiophene monomer [15–17]. We can confirm that 3-thiopheneacetic acid cannot be polymerized alone, although a current can be measured during chronopotentiometry: The current should correspond to monomer oxidation, however, films that visibly fully covered the utilized electrodes could never be obtained.

While increasing the applied current did not solve this issue, mixing 3-thiopheneacetic acid (Th-COOH) with unfunctionalized thiophene (Th), however, did. By using at least three equivalents of thiophene, a co-polymer film that fully covers the electrodes could

be obtained. Therefore, ratios Th:Th-COOH of 3:1, 5:1, and 10:1 were investigated while keeping an absolute monomer concentration of 200 mM. Although a reciprocal tendency was expected, the R_{CT} of the synthesized films increased with decreasing concentration of Th-COOH. From the 10:1 mix, films with a mean R_{CT} of $824.5 \pm 8.5\%$ resulted, while films synthesized from monomer ratios of 3:1 and 5:1 showed R_{CT} values of $48.6 \Omega \pm 20.1\%$ and $53.3 \Omega \pm 6.8\%$, respectively. Experiments were performed in triplicates, and the equivalent circuit pictured in Figure 7b was used for fitting. While film qualities of all tested monomer ratios demonstrated acceptable reproducibility, a lower R_{CT} of the immobilization platform is more beneficial for the construction of biosensors since it usually leads to more sensitive sensing.

A film resulting from the monomer ratio 3:1 was exemplarily characterized by SERS. From the spectra pictured in Figure 8, it is evident that the deposited polythiophene backbone is of the same quality compared to polymers origin from electropolymerization of unfunctionalized monomers, while the broad peak around $200\text{--}400\text{ cm}^{-1}$ indicates the presence of carboxylic groups. It can therefore be concluded that the synthesis of a co-polymer film can be performed successfully so that films of reproducible and low charge transfer resistance can be obtained that contain the desired carboxylic groups for subsequent bioreceptor binding.

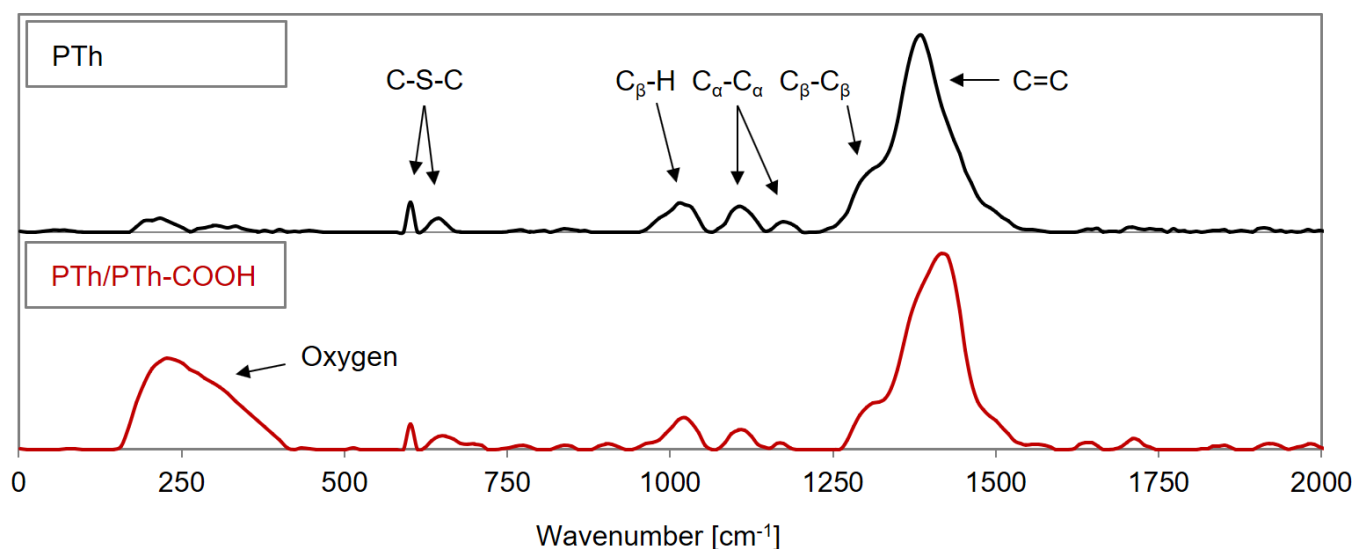


Figure 8. SERS of polythiophene (PTh) and poly(thiophene-co-3-thiopheneacetic acid) (PTh/PTh-COOH). The co-polymer film was polymerized from a mix of 150 mM Th and 50 mM Th-COOH.

4. Conclusions

In this communication, we investigated the electropolymerization of the literature-known functionalized monomer 3-thiopheneacetic acid and presented our newly developed protocol for electrochemical modification of polythiophene with the carboxylated linker p-aminobenzoic acid. Successful binding could be verified with SERS and EDS, while the films were characterized by EIS, where they demonstrated great reproducibility and conductivity. Both of the presented strategies allow for the straightforward synthesis of polythiophene immobilization platforms, to which bioreceptors can be subsequently bound by simple EDC/NHS click chemistry. The platforms can be implemented with affordable, low-hazard chemicals that can be used as received, a potentiostat/galvanostat, as well as only a few minutes of time. With this communication, we hope to support other research groups in the utilization of polythiophene thin films for the construction of electrochemical biosensors for exciting new clinical applications.

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