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ELECTROCHEMICAL POLYMERIZATION OF FUNCTIONALIZED THIOPHENE DERIVATIVES FOR THE IMMOBILIZATION OF PROTEINS

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Abstract: The hydroxy group of 3-(2-hydroxyethyl)thiophene was protected as methyl ether **1** and as dimethyl tert-butyl silyl ether **5** before anodic polymerization. The poly[3-(2-methoxyethyl)thiophene] **2** was prepared by electrochemical homopolymerization of **1**. Ether cleavage was carried out in the polymer film **2** and the resulting poly[3-(2-hydroxyethyl)thiophene] (**3**) was activated with cyanogen bromide to immobilize alcohol dehydrogenase. Silylether **5** did not undergo homopolymerization but copolymerization of **5** with 3-methylthiophene (**4**) was successful. After cleavage of the protecting group the resulting copolymer 7 was activated by cyanuric chloride, and chymotrypsin was immobilized. Electrocopolymerization of thiophene-3-acetic acid (**8**) and 3-methylthiophene (**4**) under various conditions produces copolymer **9**. By activation of the carboxylic groups with N,N'-dicyclohexylcarbodiimide (DCC) lactate oxidase (LOD) was bond to the surface of the electrode to form a lactate sensor.

INTRODUCTION

A new and interesting method for the immobilization of biomolecules is the covalent binding to electrochemically conducting polymers by reactive groups. Heterocyclic compounds such as

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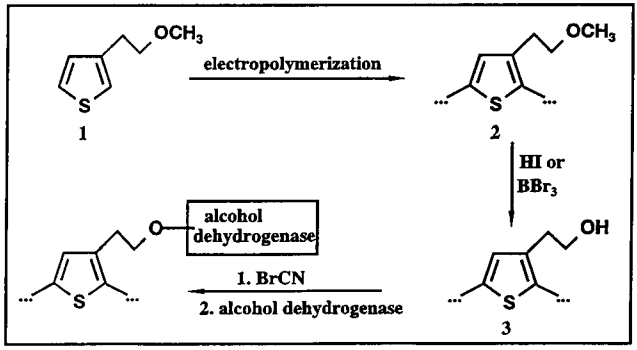
derivatives of pyrrol, furan and thiophene may be polymerized via electrochemical synthesis e. g. cyclic voltammetry [1, 2]. Suitable functionalized monomers produce polymers with reactive groups capable of immobilizing reagents. The polymer films have a reasonably high solid state order and show good electrical conductivities [3]. Examples of enzyme electrodes based on covalently bond enzymes at electrical conductive polymers are described in the case of polyazulenes [4] , polypyrroles [5] and functionalized polythiophenes [6, 7].

This paper presents new methods of synthesizing polymeric thiophene derivatives with reactive groups on the electrode surface via electropolymerization and electrocopolymerization and the subsequent conversion to special polymeric materials. The new materials may be suitable for various applications, e.g. immobilized enzymes for sensor systems.

RESULTS AND DISCUSSION

Homopolymers with hydroxy groups and immobilization reaction

3-(2-Methoxyethyl)thiophene (**1**) is a potential monomer suitable for the production of functionalized polymers. It was synthesized from 3-(2-hydroxyethyl)thiophene by methylation with diazomethane [8]. Formation of an electrical conducting polymer **2** by electrochemical polymerization of the methyl ether **1** was observed in agreement with literature [9] (see Scheme1).



Scheme 1: Polymerization of **1** and immobilization of alcohol dehydrogenase

A reducible, colored polymer film was obtained at the anode with a first broad maximum around 0.9 V. In the case of reduction, violet streaks appeared in the solution. The electrochemical characterization of this polymer film gave similar data as described by Lemaire [9].

A shiny blueish film of polymer **2** was obtained when the polymerization reaction was stopped at 1.9 V (Ag/AgCl) (oxidized form of polymer **2**) and the reddish-golden form of polymer **2** became visible when the cyclic voltammetry was completed at 0 V (reduced form of polymer **2**). The structure of polymer **2** was identified by reflection IR spectroscopy producing a strong signal at 1117 cm^{-1}(ν, C-O-C).

In order to obtain reactive groups at the surface of the polymer, the ether groups must be converted into hydroxy groups. Two practicable methods of ether cleavage were carried out: with the aid of hydriodic acid and with boron tribromide [8]. Due to the drastic conditions of ether cleavage with hydriodic acid, the polymer was expected to be destroyed during the reaction. Surprisingly, however, ether cleavage was succesful by treating polymer **2** with hydriodic acid for five hours at 126°C without destruction of the polymer layer, forming poly[3-(2-hydroxyethyl)thiophene (**3**), (see Scheme 1).

If ether cleavage is carried out with boron tribromide in methylene chloride, similar results are obtained. In the IR-reflectance spectra a broad absorption of hydroxy groups at 3479 cm^{-1} is visible and the strong ether signal at 1117 cm^{-1} disappeares completely, indicating a virtually quantitative ether cleavage [8].

As an additional indication for the presence of reactive hydroxy groups on the polymer surface and as an example for a derivatisation reaction, polymer **3** was treated with acetic anhydride in pyridine to form the correspondig acetate. A strong carbonyl band at 1735 cm^{-1} is typical of the acetate group, whereas the absorption of the hydroxy group at 3479 cm^{-1} is absent [8].

Activation of the hydroxy groups of polymers with cyanogen bromide is a classical method already applied for the immobilization of enzymes [10]. Hydroxy polymer **3** reacts in the first step with cyanogen bromide at pH 11.5 in aqueous solution to produce the isocyanate polymer, which can form covalent bonds with hydroxy, mercapto and amino groups of enzymes. By applying this method to conducting polymer films, alcohol dehydrogenase was successfully immobilized to the polymer (see Scheme 1). In the FT IR spectrum two strong bands are visible at 1657 and 1545 cm^{-1} (amide I and II). This enzyme-coated electrode is under research as a redox sensor system (see Figure 1).

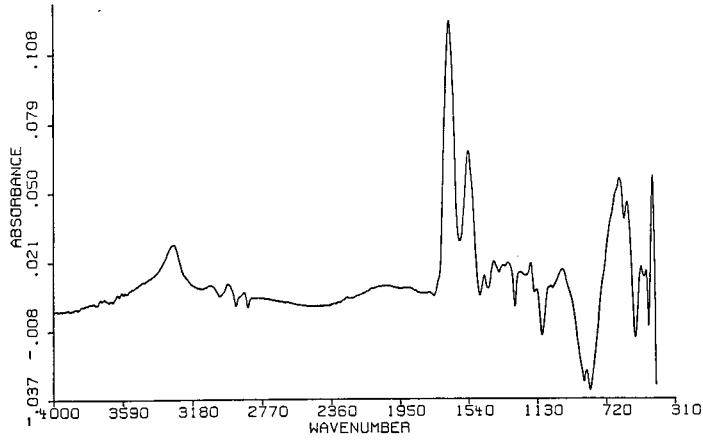


Fig. 1: Reflection IR spectrum of immobilized alcohol dehydrogenase

A cyclic voltammogram of the polymer film in acetonitrile after the immobilizing step with alcohol dehydrogenase shows the electrochemical activity of the electrode (see Figure 2), (E_p: 1.17 V for oxidation, E_p: 1.07 V for reduction).

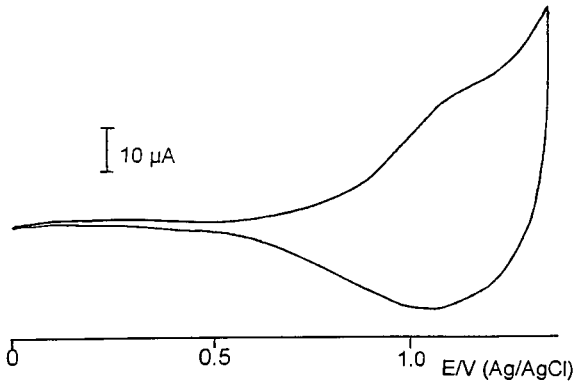


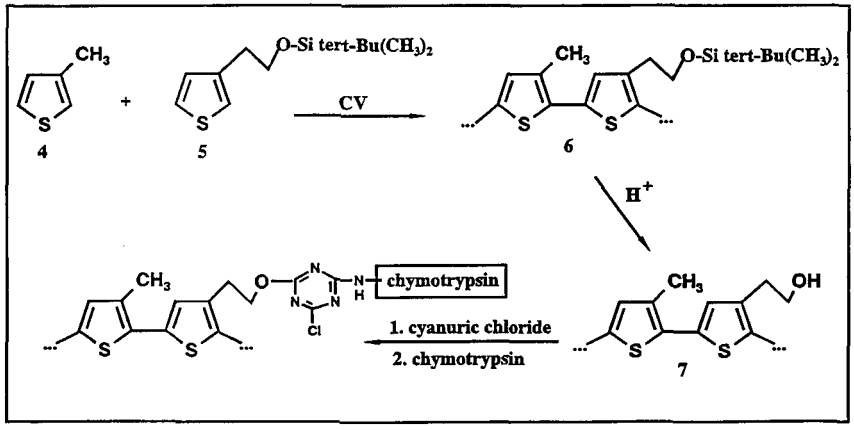
Fig. 2: Cyclic voltammogram of immobilized alcohol dehydrogenase in acetonitrile

Unfortunately this polymer on which immobilized alcohol dehydrogenase is showed no enzyme activity. But on acidic hydrolysis of the deposited protein the quantity of the enzyme was estimated to be 2 nanomoles via aminoacid chromatographic analysis.

Copolymers with hydroxy groups and immobilization reaction

Another method to protect 3-(2-hydroxyethyl)thiophene is the synthesis of the silylether **5** by reaction with tert-butyldimethylsilylchloride/imidazole [11] (see Scheme 2).

Silylether **5** was investigated by cyclic voltammetry and characterized by its peak potential for oxidation E_p at 1.88 V [12]. The peak for anodic oxidation shifts to lower potentials in the multisweep experiment, their height decreases and the cyclic voltammogram approximates the base line in the cycles thereafter. No reduction peak and no visible polymer film was obtaind on the electrode, which means that homopolymerization was not possible. From the decreasing current it was concluded that a thin nonconductive film was deposited on the surface of the electrode [12]. Because no homopolymerization could be achieved, the copolymerization should be studied. With previous investigations we showed that the dominant step of the electropolymerization of thiophene derivatives is the radical cation dimerization [13]. Our copolymerization experiments were carried out with 3-methylthiophene (**4**) with an oxidation potential E_p of 1.94 V which lies in the same range of the oxidation potenial of silylether **5**. Copolymerization experiments were carried out at a monomer concentration of 0.05 M of both monomers, 3-methylthiophene (**4**) and silylether **5** (see Scheme 2).



Scheme 2: Copolymerization of **4** and **5** and immobilization of chymotrypsin

Copolymerization experiment of **4** and silylether **5** gave an electroactive copolymer **6** which was stable up to the 10th cycle (see Scheme 2 and Figure 3) and which could be identified by its IR spectrum ($\nu_{c\text{-}o\ ether}$ 1108 cm^{-1}, further bands see [12]).

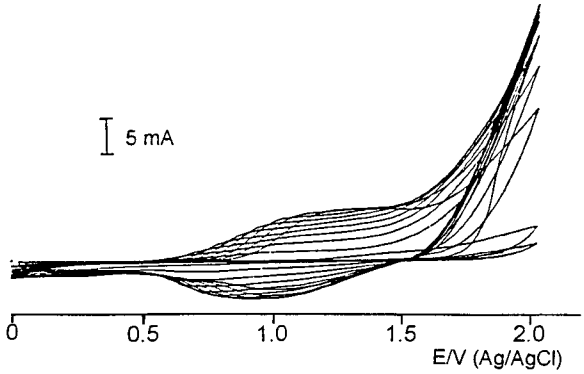


Fig. 3: Cyclic voltammogram of the copolymerization of **4** and **5**

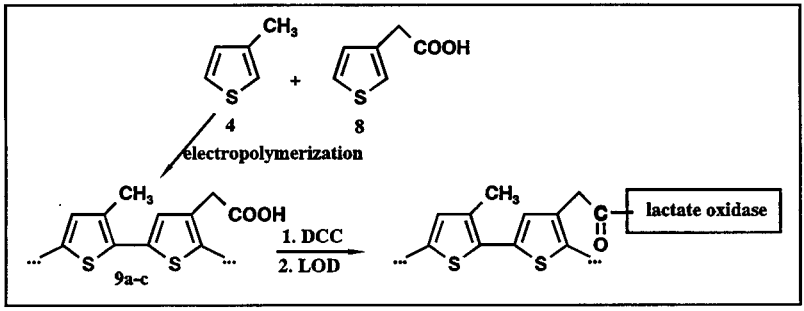
Cyclic voltammetric investigations of copolymer **6**, washed with acetonitrile and ether to remove monomers and oligomers and dried in vacuo, was carried out in a monomer free solution of 0.1 M TBAP in acetonitrile at a potential range of -0.143 to 1.4V to show a stable redox behaviour under these conditions (peak potentials for oxidation E_p 0.97 V and for reduction E_p 0.80 V), [12]. Because of the very low quantities of copolymer **6** elemental analysis was not possible.

In order to proof the activity of the functional groups in the copolymers model reactions with the copolymer **6** were carried out to show the reactivity of the hydroxy groups on the surface of the polymer film. The protecting group of **6** was removed by acidic hydrolysis. The resulting copolymer 7 with reactive hydroxy groups was treated with acetic anhydride/pyridine. Acetate groups of the resulting polymer gave characteristic IR bands at 1735 cm^{-1}.

Immobilization of chymotrypsin was started from copolymer 7 (see Scheme 2). The hydroxy groups of 7 reacted immediately with cyanuric chloride to give an acid chloride which reacted with the amino groups of chymotrypsin [14]. Reflection IR spectrum showed the amide bands at 1696 cm^{-1} (amide I) and 1523 cm^{-1} (amide II). The amount of immobilized chymotrypsine was determined after acidic hydrolysis of the enzyme from the peak height of prolin and valin to be 6 nanomoles enzyme on an electrode with an area of 4 cm^2.

Copolymers with carboxy groups and immobilization reaction

Electrochemical copolymerization of **4** and **8** produces copolymer **9** [15]. Because of the different oxidation potentials of the monomers (1.92 V for **4** and 2.11 V for **8**) a maximum of 2.2 V was used for the polymerization reaction to oxidize both monomers to the corresponding radical cations which are react to copolymer **9** [13].



Scheme 3: Copolymerization of **4** and **8** and immobilzation of lactate oxidase (LOD)

Three samples of the copolymer (**9a-9c**) were investigated for the immobilization of lactate oxidase (LOD). At first copolymer **9a** was produced by cyclic voltammetry. The charge consumption added up to 40 mC. Copolymers **9b** and **9c** were produced at a stationary potential of 2.2 V with a monomer ratio **4** to **8** of 1:1 for **9b** and **4** to **8** of 1:2 for **9c**, also up to a charge consumption of 40 mC.

In all cases during the synthesis of our copolymers (**9a - 9c**) we finished the electrochemical synthesis at a potential of about 2.2 V (oxidative form). After this synthesis the prepared copolymer on the electrode is reduced electrochemically in order to form the unoxidized (reduced) form in the polymer phase. The cyclic voltammograms of the polymer layers (**9a - 9c**) in monomer free solution of 0.1 M tetrabutylammonium perchlorate (TBAP) in acetonitrile at a potential of 0 to 1.4 V showed a reversible redox process (see Fig.4 for **9b** and **9c**). The peak potential for oxidation (doping) is observed at 1.15 V and the peak potential for reduction (dedoping) at 0.85 V. During the redox process the colours of the polymer layers of **9** change from blue for the oxidized form to red brown for the reduced form.

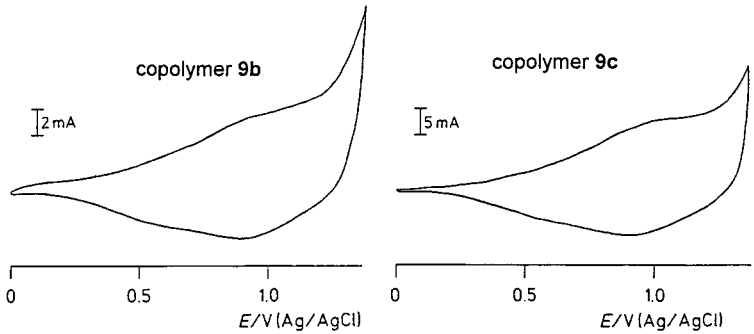


Fig. 4: Cyclic voltammograms of the copolymers **9b** and **9c**

For many cycles the same form of cyclic voltammograms was obtained, meaning full reversibility of the thiophene redox system and also meaning a practical stable structure for copolymers **9a** - **9c**. Only the reduced forms of **9** were used for immobilization experiments. FT IR spectra directly derived from the dried polymer layers of **9a** - **9c** showed the carbonyl absorption of the copolymerized thiophene-3-acetic acid (**8**) at 1714 cm^{-1} for **9a**, 1708 cm^{-1} for **9b** and 1714 cm^{-1} for **9c** (at 1700 cm^{-1} for the monomeric acid **8**) (see Fig. 5 for **9c** and the carboxylate of **9c**), [16].

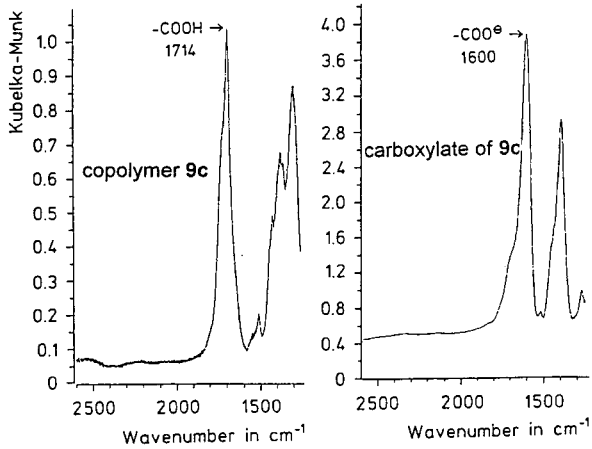


Fig. 5: Reflection IR spectra of copolymer **9c** and the corresponding carboxylate of **9c**

A further experiment to prove the activity of these carboxylic groups was the conversion of the carboxylic groups to the corresponding carboxylates (see Fig.5 for the carboxylate of **9c** at 1600 cm^{-1}; at 1560 cm^{-1} for the carboxylate of monomer **8**). In all cases of polymer **9** it was possible to receive the carboxylate by treatment with aqueous NaOH. The carboxylic groups can be restored by acidifing with hydrochloric acid. The sharp IR bands clearly show the carbonyl bands of the acid and the carboxylate.

Initial experiments were performed with the copolymer samples of **9a - 9c** to build up conducting layers on electrode surfaces able to covalent coupling of enzymes. The blank and the DCC activated copolymer samples of **9a - 9c** were treated with LOD under the described conditions. The measurement cell for the electrochemical determination of the activity of immobilized LOD (oxidation of lactate) is a two electrode - setup with a Ag/AgCl reference electrode [16].

The electrodes covered with copolymer **9a -9c** and treated with LOD (without activation of DCC) as well as the electrode covered with copolymer **9a** activated with DCC and treated with LOD (propably with a very low concentration of reactive carboxylic groups at the surface of the polymer layer) does not show any activity. Differing activities were found for electrodes covered with copolymer **9b** (system I) and **9c** (system II) activated with DCC and treated with LOD.

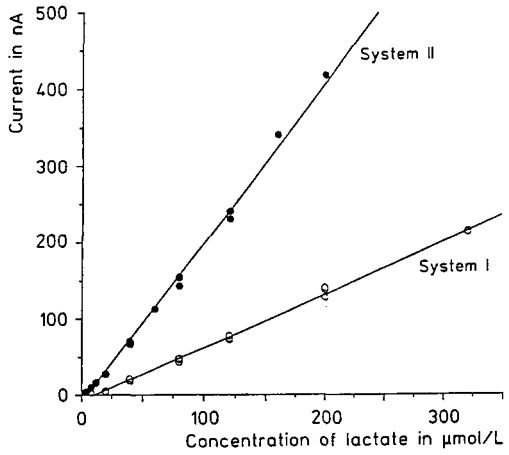


Fig. 6: Calibration graph of lactate oxidase (LOD)

Figure 6 shows the dependence of the steady-state amperometric response on the concentration of lactate. The two sensor electrodes have remarkable sensitivities. The sensor with the higher content of carboxylic groups (sample **9c**, system II) is more sensitive than system I (sample **9b**). It has a detection limit of 5 μM in contrast to 40 μM found for system I. System II seems to be able to bind a higher quantity of the enzyme. The response after substrate addition is 10 s.

The amount of immobilized LOD was calculated comparing the peak height of PTH amino acids received from hydrolysate of system I and II relative to the peak height obtained from hydrolysate of a defined amount of soluble LOD. Calculation of surface bound LOD was averaged from the peak height of 10 amino acids and determined to be 1.2 μg LOD for system I and 4.4 μg LOD for system II for an area of about 50 mm^2.

CONCLUSIONS AND OUTLOOK

Starting from poly[3-(2-methoxyethyl) thiophene] (**2**) ether cleavage was carried out. The resulting poly[3-(2-hydroxyethyl) thiophene] (**3**) contains reactive hydroxy groups on its surface. Alcohol dehydrogenase was covalently bond on the surface of the polythiophene electrode by the cyanogen bromide method. The structures of the differing polymers were characterized by FT-IR spectroscopy and the modified polymer layers analyzed by cyclic voltammetry to show their electroactivity.

In order to carry out a copolymerization experiment, 3-(2-hydroxyethyl)thiophene was protected as dimethyl tert-butyl silyl ether to give **5**. Under the chosen conditions homopolymerization of **5** was not successful, but copolymerization with 3-methylthiophene (**4**) was achieved. Cyclic voltammetric experiments showed the redox properties of copolymer **9**. Immobilization of chymotrypsin was succesful by the cyanuric chloride method.

3-Methylthiophene (**4**) and thiophene-3-acetic acid (**8**) were copolymerized under differing conditions to give the copolymer samples **9a**, **9b** and **9c**. The resulting copolymers **9b** and **9c**, produced under potentiostatic conditions, were able to bind lactate oxidase (LOD) covalently after activation with DCC. The activity of the immobilized LOD was detected by increasing current as a function of lactate concentrations. The results of the differing immobilization experiments showed the suitability of functionalized polythiophenes as carrier materials for enzyme electrodes. In the case of immobilized lactate oxidase (LOD) a lactate sensor of high sensitivity was formed.

ACKNOWLEDGMENT

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