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Wiring of the aldehyde oxidoreductase PaoABC to electrode surfaces via entrapment in low potential phenothiazine-modified redox polymers

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ABSTRACT

Phenothiazine-modified redox hydrogels were synthesized and used for the wiring of the aldehyde oxidoreductase PaoABC to electrode surfaces. The effects of the pH value and electrode surface modification on the biocatalytic activity of the layers were studied in the presence of vanillin as the substrate. The enzyme electrodes were successfully employed as bioanodes in vanillin/O_2 biofuel cells in combination with a high potential bilirubin oxidase biocathode. Open circuit voltages of around 700 mV could be obtained in a two compartment biofuel cell setup. Moreover, the use of a rather hydrophobic polymer with a high degree of crosslinking sites ensures the formation of stable polymer/enzyme films which were successfully used as bioanode in membrane-less biofuel cells.

1. Introduction

The periplasmic aldehyde oxidoreductase (PaoABC) from *Escherichia coli* catalyzes the oxidation of aldehydes to their corresponding carboxylic acid analogs in an overall two-electron-two-proton reaction according to Eq. (1). [1].

$$R - COH + H_2O \rightarrow R - COOH + 2H^+ + 2e^-. \quad (1)$$

The protein consists of three subunits, i.e. the PaoA subunit bearing two Fe2S2-clusters, the PaoB subunit containing a flavin adenine dinucleotide (FAD) prosthetic group and the PaoC subunit comprising a molybdopterin cytosine dinucleotide cofactor [1]. The aldehyde is oxidized at the PaoC moiety. The electrons are then transferred to the FAD unit via the Fe2S2 clusters in PaoA [1]. Several artificial electron acceptors (e.g. among others Os-complexes and ferricyanide) were previously employed to extract the electrons from the FAD unit within PaoABC [2–6]. The protein was successfully used in biosensor applications, i.e. the amperometric detection of various aldehydes that are present in foods, cosmetics or pharmaceuticals [2,3,7]. However, the use of this enzyme in biofuel cells and especially aiming at self-powered sensor devices was not well addressed in the literature so far [5]. The electron transfer mechanism with different electron acceptors was studied in detail and revealed different activities for negatively or positively charged mediators [4] as well as for $1e^-$ and $2e^-$ mediators [3].

Despite the fast and efficient electron transfer between a variety of electron acceptors, PaoABC shows no significant reaction rate for the reduction of molecular oxygen at high and low pH values [1,4]. This makes the protein a potential candidate for use in bioanodes in membrane-less biofuel cells in combination with biocathodes comprising enzymes that catalyze the reduction of oxygen, i.e. bilirubin oxidase (BOD) or laccase [8].

The open circuit voltage (OCV) and hence the power output of a biofuel cell is related to the potential differences between the bioanode and biocathode [8]. The high redox potentials of the O_2 reducing enzymes BOD or laccase ensure a high potential for the cathode side. For the anode, redox mediators with low formal potentials are desired.

Redox dyes based on phenothiazine derivatives are low potential electron mediators [9–14]. Polymers bearing epoxy or alcohol functions can be readily modified with phenothiazine derivatives comprising primary or secondary amino groups by a ring opening reaction between the epoxy group and the N-nucleophile [11,12] or a coupling reaction between the –NRH group of the dye and the –OH species within the polymer by the bifunctional linker oxalyl chloride [9]. Such polymers were successfully employed for the electrical wiring of glucose oxidase [11,15], cellobiose dehydrogenase [11] and photosystem II [12] as well as for the electrocatalytic regeneration of NAD^+ [9]. Moreover, direct polymerization of acrylamide based monomers bearing phenothiazine

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groups leads to redox hydrogels that are capable of wiring horseradish peroxidase [13,14].

The reduction mechanism of phenothiazine dyes follows a $2e^-/nH^+$ process (depending on the pH value $n = 1, 2$ [16]). Thus, it could be expected that this type of mediators show an efficient electron exchange rate with the FAD cofactor within the enzyme. Moreover, the activity may be controlled via a change in pH value. The low redox potential of the phenothiazine dyes should ensure high OCV values in biofuel cell applications.

Here, we report on wiring of PaoABC via the entrapment in phenothiazine-modified hydrogels and the application of these enzyme electrodes as biosensors and bioanodes in biofuel cells.

2. Materials and methods

All chemicals and solvents were purchased from Sigma-Aldrich, Alfa-Aesar, VWR, J.T. Baker or Acros Organics and were of reagent or analytical grade, except otherwise noted. The enzyme PaoABC was expressed from *E. coli* and purified as described earlier [1]. Dimethyldioxirane was prepared based on a protocol described in [17]. Toluidine blue (~80% dye content) and Azure B (~80% dye content) were purchased from Sigma-Aldrich and used as received.

Acetate Buffer (50 mM) solutions with different pH values from 4 to 5 were prepared by mixing the corresponding amounts of acetic acid and CH_3COONa in deionized water. For the pH range of 6 to 7 the corresponding phosphate buffers (50 mM) were used. For experiments in the pH range of 7.4 to 9 different Tris-buffers (Tris = Tris(hydroxymethyl)-aminomethan) were used. All buffers were prepared with deionized water.

NMR experiments were performed on a Bruker DPX 200 spectrometer with a ^1H resonance frequency of 200.13 MHz. The residual solvent peak was used as internal standard. Spectra were recorded at room temperature.

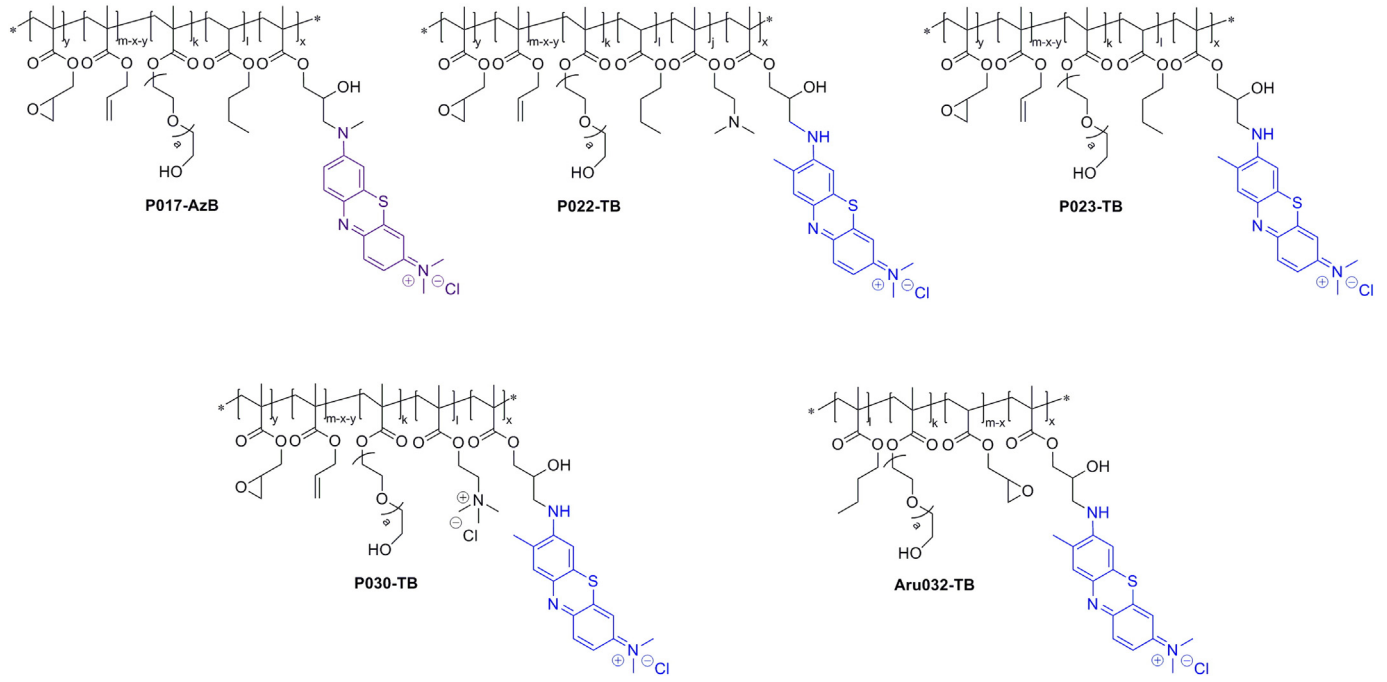
Size exclusion chromatography (SEC) measurements were performed against polystyrene standards in THF at 30 °C. Measurements were analyzed with the PSS WinGPC Unity software. Sample concentrations were around 15 mg mL^{-1}. UV–vis spectra were recorded with an Agilent Cary 60 UV–vis spectrometer in polystyrene cuvettes (340–900 nm, optical path length = 1 cm). Polymers were purified with 5 kDa membranes (Vivaspin500, 500 μL, Sartorius) using a micro centrifuge (Eppendorf).

2.1. Synthesis

The synthesis of polymers P017-AzB and P022-TB was described earlier [11,18]. Preparation of P023-TB was reported in [12]. Monomers for copolymer preparation were allyl methacrylate (AllMA), butyl acrylate (BA), poly(ethylene glycol) methacrylate (PEGMA), 2-(dimethylamino)ethyl methacrylate (DMAEMA), [2-(methacryloyloxy)ethyl] trimethylammonium chloride (TMAEMA, aqueous solution, 80 wt.%) and glycidyl methacrylate (GMA). Polymers P017-AzB, P022-TB and P023-TB were stored in aqueous solutions with a concentration of around 30 mg mL^{-1}. The nominal compositions (for molecular structures see Scheme 1) of the corresponding polymer backbones are P017: AllMA = 50 mol% (m), PEGMA = 7.5 mol% (k), BA = 42.5 mol% (l); P022: AllMA = 50 mol% (m), PEGMA = 7.5 mol% (k), BA = 32.5 mol% (l), DMAEMA = 10 mol% (j); P023: AllMA = 50 mol% (m), PEGMA = 7.5 mol% (k), BA = 42.5 mol% (l). For all polymerization reactions inhibitor-free monomers were used. The inhibitors were removed by means of the corresponding inhibitor removers (Sigma Aldrich). Inhibitor-free monomers were stored at a temperature of −20 °C or +4 °C.

2.1.1. Synthesis of the polymer backbone P030

To a solution of isopropyl alcohol and water (1.5:1, 25 mL) containing the monomers AllMA (1.39 g, 11.0 mmol, 60.0 mol%, m), PEGMA (1.05 g, 2.00 mmol, 10.0 mol%, k) and TMAEMA (1.25 g, 6.00 mmol, 30.0 mol%, l) 30 mg of AIBN (= azobisisobutyronitrile, re-crystallized from hot toluene prior to use) were added. The reaction mixture was heated to 80 °C to initiate the free radical polymerization. After 3 h of vigorous stirring the mixture was cooled down to room temperature and the solvent was removed under reduced pressure. The residue was re-dissolved in MeOH (20 mL) and quenched with 200 ml of Et_2O. The formed precipitate was separated by means of a centrifuge



Scheme 1. Proposed molecular structures of the azure B (AzB, violet) and toluidine blue (TB, blue) modified redox polymers P017-AzB, P022-TB, P023-TB, P030-TB and Aru032-TB. The nominal compositions of the backbones are given in the experimental part. Note, that for clarity reasons in case of the TB modified polymers only the structures resulting from the reaction between the primary amino group in TB and *one* epoxy group within the polymer backbone are shown.

(3500 rpm, 10 min) and the polymer was again dissolved in a mixture of isopropyl alcohol and water (1.5:1) to obtain the final polymer suspension (19 wt.%).

2.1.2. Modification of P030 with toluidine blue — P030-TB

The polymer suspension (131.6 mg, 19 wt.%, mass of P030 = 25 mg) was cooled to 4 °C and 2.5 mL of DMDO dissolved in acetone (≈0.1 M) was added. The mixture was stirred at 4 °C for 16 h. Then the solvent and the remaining DMDO (dimethyldioxyrane) were removed under reduced pressure at room temperature and the epoxidized polymer backbone was re-dissolved in 3 mL of MeOH. Toluidine blue (30 mg) was added and the slurry was stirred for 3 days at 50 °C. After cooling down to room temperature the polymer was precipitated by adding Et_2O. The polymer was separated by centrifugation (3500 rpm, 5 min) and the residue was re-dissolved in MeOH. The polymer was again precipitated with Et_2O and the washing step was repeated until the MeOH became colorless. Finally the polymer was dissolved in water (30 mg mL^{-1}). The redox potential (in phosphate buffer, pH = 7, drop coated on a graphite electrode) = −0.19 V (vs. Ag/AgCl/3 M KCl).

2.1.3. Synthesis of the polymer backbone Aru032

The co-monomers GMA (0.710 g, 5 mmol, 49.5 mol%, m), PEGMA (0.4 g, M_n = 500, 0.8 mmol, 8 mol%, k, an isopropyl alcohol solution with c(PEGMA) = 0.2 g mL^{-1} was used) and BA (0.545 g, 4.3 mmol, 42.5 mol%, l) was dissolved in isopropyl alcohol under argon atmosphere in a Schlenk tube and 3 mg of AIBN was added. The mixture was deaerated by argon bubbling, the reaction vessel was sealed and the mixture was heated to 80 °C and kept at this temperature for around 35 min. The turbid mixture was quenched with water (40 mL) and the polymer was separated by centrifugation (4000 rpm, 20 min). The crude polymer was washed successively with Et_2O (120 mL), water/MeOH (40 mL, 1:1) and Et_2O (120 min) again. After each washing step the polymer was separated by centrifugation (4000 rpm, 10 to 20 min). The colorless material was dried in vacuum at room temperature to yield 1.45 g of a foam like material. Finally the polymer was dissolved in 14.5 mL of acetonitrile (100 mg mL^{-1}) and stored at +4 °C.

^{1}H-NMR (200.13 MHz, acetone-d_6) δ/ppm: ≈0.96 (broad, CH_3); 1.39 (m, CH_2); 1.63 (m, CH_2); 2.70, 2.83 and 3.26 (all s, epoxide), 3.60 (s, CH2 PEGMA), 3.85, 4.04 and 4.34 (all broad, CH_2-O moieties); SEC (in tetrahydrofuran, against a polystyrene standard): M_n = 14 kDa, D = 2.8.

2.1.4. Modification of Aru032 with toluidine blue — Aru032-TB

The use of the GMA monomer circumvents the epoxidation of the polymer backbone with DMDO. Thus, Aru032 was directly used for the modification with toluidine blue. To a solution of 5 ml of Aru032 in acetonitrile (total mass of polymer = 500 mg) toluidine blue (510 mg, dye content = 80%) and 1 ml of NEt_3 were added. The blue reaction mixture was diluted with 3 ml of EtOH and heated to 60 °C. After 3 days at 60 °C the slurry was quenched with approximately 110 mL of Et_2O. The precipitate was separated by centrifugation (4000 rpm, 15 min) and washed with 400 mL of Et_2O. The ether was decanted off and the blue residue was air dried to yield 920 mg of crude product.

Samples for electrochemical experiments were purified (removal of remaining toluidine blue and organic solvents) by centrifugation using 5 kDa membranes (Vivaspin 500, 500 μL, Sartorius): the crude product was first dissolved in water. Small aliquots were centrifuged at 8000 to 9600 rpm for 10–30 min. The eluent was discarded and the residual polymer was diluted with water (Millipore) and centrifuged again. The purification was stopped when the eluent became nearly colorless. The polymer was concentrated by centrifugation using the same membrane to yield a blue polymer suspension (32 mg mL^{-1}).

UV–vis (water) λ/nm: 270, ≈610 (broad); free toluidine blue: 289, 597, 633 (sharp signals); redox potential (in PB buffer, pH = 7, drop coated onto glassy carbon, 1,3-dimercaptopropane crosslinker) = −0.20 V (vs. Ag/AgCl/3 M KCl).

2.2. Electrochemical measurements

All electrochemical experiments were carried out with a CHI1030 potentiostat (CH Instruments) or an Autolab PGSTAT12 potentiostat (Metrohm) in a standard three electrode electrochemical cell at room temperature except when otherwise noted. As working electrodes modified graphite electrodes were used. The counter electrode was a Pt wire, the reference electrode was an Ag/AgCl/3 M KCl system. All potentials are reported vs. Ag/AgCl/3 M KCl. All chronoamperometric and differential pulse voltammetric experiments were conducted under Ar atmosphere (solutions were deaerated by argon bubbling).

2.2.1. Electrode preparation

Graphite electrodes (nominal diameter = 3.05 mm, SGL Carbon) sealed in glass were successively polished with different emery papers (P400, P800 and P1500). The polished electrodes were successively sonicated in water and ethanol (3 min each) prior to modification. Enzyme electrodes were prepared via a drop-casting process from mixtures of 1 μL of the corresponding redox polymer solution, 1 μL of PaoABC dissolved in buffer (13 mg mL^{-1}) and 1 μL of an ethanol solution containing the crosslinker 2,2′-(ethylenedioxy) diethanethiol (EtOH:crosslinker 50:1 *v*/*v*). The mixture was placed on the electrode surface by means of an Eppendorf pipette and left to dry under ambient conditions for at least 2 h. The modified electrodes were rinsed with the corresponding buffer solution prior to use to remove loosely bound enzyme, redox polymer and crosslinker.

Diaminoalkane modified electrodes were prepared based on a protocol reported in [19]. For the anodic grafting, two potential cycles (scan rate = 50 mV s^{-1}) between 0 and 1.2 V (vs. Ag/Ag^+) were performed in 0.1 M $LiClO_4$/absolute EtOH containing 4.7 mM 1,7-diamino hexane (see, Fig. S1 in the Supporting information). After modification the electrodes were thoroughly rinsed with EtOH and water.

2.3. Biofuel cell experiments

2.3.1. Preparation of bilirubin oxidase based biocathodes

Bilirubin oxidase (BOD, from *Myrothecium verrucaria*, Sigma Aldrich) based biocathodes were prepared with graphene screen printed electrodes from Dropsens (nominal surface area = 0.12 cm^2). First the graphene electrodes were decorated with 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBSE) according to a procedure described in [20]. Then 3 μL of a phosphate buffer solution (0.1 M, pH 7) containing BOD (10 mg mL^{-1}) were drop-coated onto the modified graphene electrode. The enzyme electrodes were stored at 4 °C.

2.3.2. Biofuel cell measurements

Biofuel cell experiments were conducted with an Autolab PGSTAT12 potentiostat (Metrohm-Autolab) by combining PaoABC based bioanodes and BOD biocathodes in a two compartment cell connected via a KNO_3 salt bridge. The compartment for the anode side was filled with Ar saturated Tris-buffer (pH 8) containing vanillin (1 mM) acting as fuel. The cathode compartment was filled with air-saturated PBS (= phosphate buffered saline) buffer pH 7.4. Measurements in a one-compartment cell were conducted in O_2 saturated PBS buffer (pH 7.4) containing 1 mM vanillin. After equilibration of the OCV, catalytic currents for calculating the power density profiles of the biofuel cell were measured by applying potential pulses 50 or 100 mV against the measured OCV value.

3. Results and discussion

3.1. Redox polymers

Electrical communication between PaoABC and the electrode was demonstrated using different mediators. The FAD subunit within PaoABC undergoes a $2e^-/2\ H^+$ electron transfer process [1]. Hence,

the use of a $2e^-$ mediator should enhance the electrical communication with the prosthetic group of the enzyme and the mediator itself and, thus, increase the electron transfer rate between the electrode and the enzyme under catalytic conditions.

Recently we have shown that polymers bearing the $2e^-/nH^+$ azure B (P017-AzB) and toluidine blue (P022-TB and P023-TB) mediators indeed show efficient electron transfer with FAD dependent enzymes, i.e. glucose oxidase and cellobiose dehydrogenase [11,15]. Consequently, these polymers (Scheme 1, top) were used to study the electron transfer with PaoABC. In addition, two new toluidine blue modified hydrogels (P030-TB and Aru032-TB, Scheme 1, bottom) were synthesized to examine the influence of a variety of different polymer backbones on the electrical communication of the enzyme and mediator. The molecular structure of the two mediators (azure B; AzB, and toluidine blue; TB) differs only in the position of one $-CH_3$ group, which in the case of the TB species is located at the aromatic core, in *ortho*-position to the primary amino group. In the case of AzB a secondary amino group (−NMeH) is present. The $-CH_3$ group at the aromatic core is absent. Both mediators reveal similar redox potentials: −0.19 V for both TB and AzB in phosphate buffer at pH 7 (Fig. S2).

The polymer backbones are functionalized with epoxy functions that allow for a covalent attachment of toluidine blue or azure B via a ring opening reaction of the epoxides with the N-atoms of the primary (TB) and secondary (AzB) amino groups that are attached to the aromatic core of the phenothiazine dyes (see Fig. S3 for the reaction scheme of the complete synthesis of Aru032-TB as an example).

While polymers P017-AzB, P023-TB and Aru032-TB comprise a hydrophobic backbone, polymers P022-TB and P023-TB bear a rather hydrophilic backbone due to the incorporation of tertiary amino functions (P022-TB) and the charged tetraalkylammonium groups (P030-TB).

For all polymers the maximal loading of redox mediator, assuming 100% conversion in every reaction step (epoxidation and modification with the redox dye), is between 49.5 to 60 mol%. For polymers P017, P022, P023 and P023 the epoxy function was introduced to the backbone in a polymer-analog reaction using the mild epoxidation reagent dimethyldioxirane (DMDO). This step could be avoided by using glycidyl methacrylate (GMA) as monomer during polymerization with the co-monomers butyl acrylate (BA) and poly(ethylene glycol) methacrylate (PEGMA). Polymer backbone Aru032 was synthesized following this approach (see, experimental part and Fig. S3). The high number of epoxy functions in combination with a bi-functional crosslinker should ensure the formation of stable polymer films on the electrode for this type of polymer backbone. The crosslinker reacts with the remaining epoxy functions in the polymer chains and thus a dense and insoluble network is formed on the electrode surface.

After modification with TB or AzB all polymers show an intense blue-violet color. The UV–vis spectrum of a purified sample of Aru032-TB in water shows rather broad signals located at 270 nm and ≈610 nm (Fig. S4a). These values are in good agreement with the sharp absorption bands of the freely diffusing TB: 289 nm, 597 nm and 633 nm (Fig. S4b). The broad signals that were observed in the case of the polymer-bound dyes were attributed to the variation in the electronic environment of the mediator due to agglomeration effects in aqueous solutions and/or to the broad molecular weight distribution of the polymer (polydispersity index = 2.8, M_n = 14 kDa, revealed by size exclusion chromatographic measurements (SEC) with the pristine backbone). Nevertheless, the spectral signature of the polymer bound mediator indicates that the electronic structure of the phenothiazine dye does not seem to be affected during anchoring to the polymer backbone (note, that the high background in the spectrum of the polymer hampers quantitative analysis of the absorption bands).

The redox behavior of the polymer films drop-coated onto glassy carbon electrodes was investigated by means of differential pulse voltammetry in buffer solutions at pH 7 or 7.8 (see Fig. S5a and b for the *I-E* curves of P030-TB and Aru032-TB, respectively). Potentials of maximum current (E_{max}, vs. Ag/AgCl/3 M KCl) in differential pulse voltammograms of the polymers are −0.19 V (P022-TB, P023-TB, P030-TB), −0.20 V (Aru032-TB) and −0.22 (P017-AzB). These values are consistent with those obtained for the freely diffusing molecules and previously reported results for P017-AzB and P022-TB [15,18] as well as P023-TB [12]. Cyclic voltammograms (Fig. S5c) of Aru032-TB in Tris buffer (pH 8) drop coated onto a graphite electrode at different scan rates show a chemically reversible redox couple with a mid-point potential of −0.21 V with a rather high peak potential separation of 0.17 V (at a scan rate of 50 mV s^{-1}). The latter is further increasing with increasing scan rate indicating that the overall electron transfer is within the time scale of the cyclic voltammetric experiment. The analysis of the peak currents (I_{peak}) as a function of scan rate (v) shows that I_{peak} is directly proportional to $v^{1/2}$ (Fig. S5d). Thus, the electron transfer within the hydrogel seems to be diffusion controlled most probably caused by the diffusional segmental movement of the polymer chains and the diffusion of counter ions within the redox hydrogel.

3.2. Enzyme electrodes

Enzyme electrodes using PaoABC and the corresponding phenothiazine-modified polymer were prepared via a drop-coating process by mixing the enzyme dissolved in buffer and the polymer suspension in the presence of a dithiol based crosslinker (2,2′-(ethylenedioxy)diethanethiol). In the following, these enzyme electrodes are designated as PaoABC/P017-AzB, PaoABC/P022-TB, PaoABC/P023-TB, PaoABC/P030-TB, and PaoABC/Aru032-TB. The biocatalytic activity of the films was first tested in 50 mM Tris-buffer at pH 8 with vanillin as substrate. Indeed, chronoamperograms of all polymers (Fig. 1a) recorded at an applied potential of −0.1 V (vs. Ag/AgCl/3 M KCl) reveal a pronounced current response upon addition of vanillin (1 mM). Obviously, the phenothiazine dye modified polymers are able to shuttle electrons from the enzyme to the electrode. Current densities (J) in the range of 2.7 to 17.8 μA cm^{-2} were observed. Polymer Ar032-TB shows the smallest current density (around 2.7 μA cm^{-2}). Polymer P023-Tb shows the highest J value (around 17.8 μA cm^{-2}). Both, hydrophilic and hydrophobic polymer backbones are able to form active films and significant differences could not be observed. The rather slow increase of the current in the case of PaoABC/Aru032-TB is striking. We attribute this effect to the presence of a very dense polymer/enzyme film (high degree of crosslinking) and thus a hindered diffusion of the substrate into the active layer.

Since the mediator and the enzyme possess a proton coupled electron transfer mechanism, the catalytic current may be altered by changing the pH value of the buffer solution as it was observed for mono cationic and mono anionic mediator species [4]. Chronoamperometric experiments with PaoABC/P023-TB modified graphite electrodes were performed at different pH values starting from pH = 4 to pH = 9 and with different applied potentials values, i.e. −0.1 V and 0 V. The observed current response increases with increasing pH value. The highest catalytic activity was observed at high pH values (>7) and an applied potential of 0 V (Fig. 1b). The peak potential in cyclic voltammograms of the mediator reduction (E_{red}) decreases with increasing pH value from −79 mV to −219 mV (Fig. 1c, this effect was also observed for other phenothiazine dyes incorporated in nafion films [21]) which leads to an absence of a current response at pH 4 since the applied potential is below the formal potential of the mediator.

In case of the P017-AzB, P022-TB, P023-TB and P030-TB, desorption of the films from the electrode surface over time was observed indicated by a slight blue coloration of the electrolyte solution. In the case of Aru032-TB stable films were formed, most likely because of a higher amount of epoxy group and thus a higher crosslinking-ability (degree of epoxidation with DMDO for the AllMA based monomers is unknown) as well as because of the hydrophobic character (high amount of butyl acrylate within the polymer chain), and hence the lower solubility of the polymer in water.

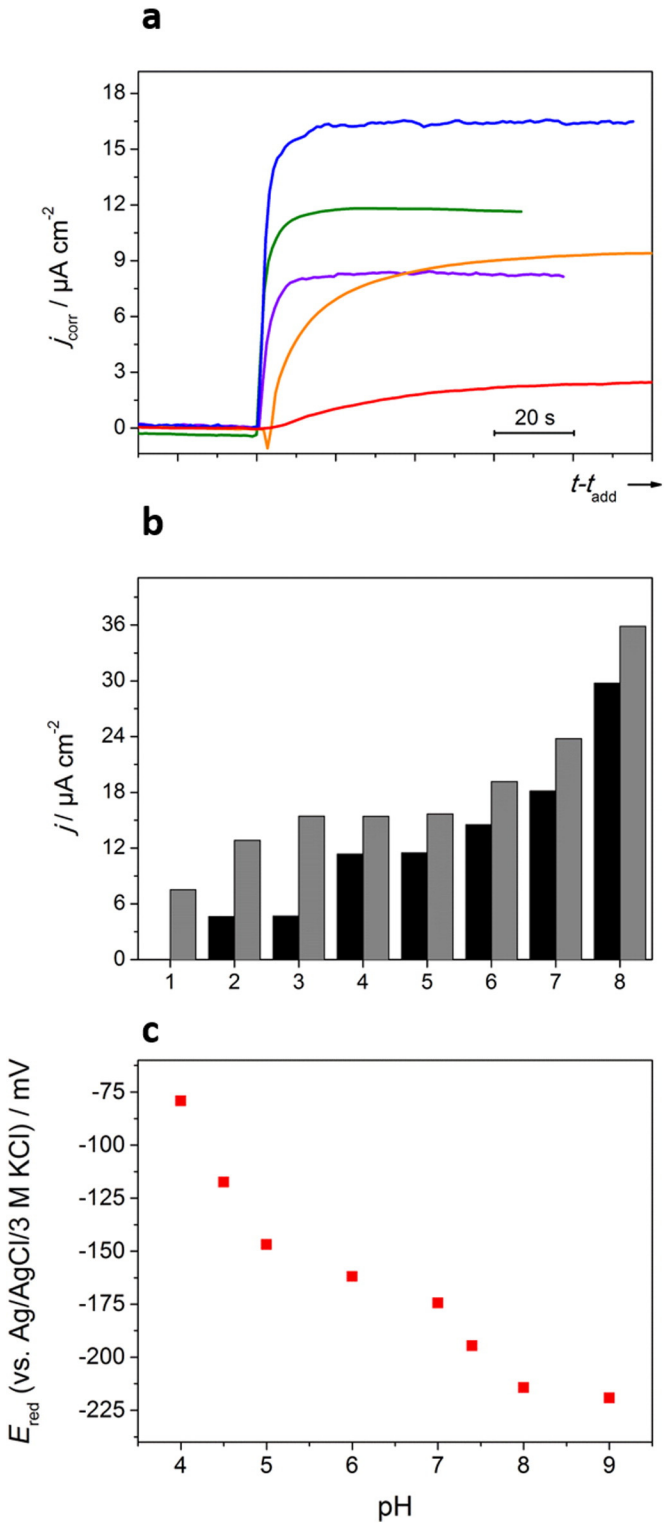


Fig. 1. a) Chronoamperometric characterization of PaoABC/P017-AzB (violet line), PaoABC/P022-TB (green line), PaoABC/P023-TB (blue line), PaoABC/P030-TB (orange line) and PaoABC/Aru032-TB (red line) films drop coated onto graphite electrodes in Tris-buffer (50 mM) at pH 8 in the presence of 1 mM vanillin and an applied potential of −0.1 V (vs. Ag/AgCl/3 M KCl); J_{corr}-t curves were background corrected for comparison reasons, the x-axis (time)was rescaled by subtracting the time at the addition of the substrate (t_{add}) from the actual time of the experiment (t); b) current densities measured for PaoABC/P023-TB films in the presence of vanillin (1 mM) at different pH values and applied potentials of −0.1 V (black columns) or 0 V (gray columns); c) plot of the potential of maximum current of the mediator as a function of pH extracted from differential pulse voltammograms of P023-TB deposited onto a graphite electrode; for all experiments a dithiol based crosslinker was used to enhance film stability. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To enhance the interactions of the polymer-enzyme films with the electrode surface, diamino compounds were grafted to the electrode surface for the anchoring of polymer chains by the reaction of –NH_2 functions with remaining epoxy groups in the polymer chains (chemisorption of the polymer). The bi-functional compound 1,7-diaminoheptane was anchored to the electrode surface by anodic oxidation of a –NH_2 group [19] (Fig. S1). These amine decorated electrodes were modified with PaoABC/P023-TB films. Compared to non-modified electrodes (physisorption of the polymer) an increase of the current response of around 40% was observed in the presence of 1 mM vanillin in Tris-buffer (50 mM) at pH 8 (bare electrode: (1.324 ± 0.28) µA; amine modified electrode: (2.131 ± 0.146) µA). We attribute this effect to the higher stability of the covalently anchored polymer film which remains on the electrode surface during the experiment. In the case of the physisorbed films a detachment of loosely adsorbed polymer seems reasonable.

Fig. 2 shows a calibration curve obtained for a PaoABC/P023-TB film drop-casted onto an amino functionalized electrode surface in the presence of different vanillin concentrations (0–1 mM). The course of the current density in the J-c(vanillin) curve shows a Michaelis-Menten type behavior. The apparent Michaelis constant (K_M) value was estimated to be K_M = 122 µM. This value is slightly smaller than the one reported for PaoABC based biosensors based on one-e^- mediators (K_M = 132 µM) [1]. This demonstrates that the phenothiazine based mediator reveals an efficient electron transfer with the prosthetic group of the enzyme. The effect of temperature on the catalytic activity of PaoABC/P023-TB films drop-coated on amino modified graphite electrodes was examined at temperatures of 20, 30, 40 and 50 °C in Tris-buffer (50 mM) at pH 8 and a vanillin concentration of 1 mM at an applied potential of −0.1 V (vs. Ag/AgCl/3 M KCl). The current increases from ≈2 µA to ≈6 µA upon increasing the temperature from 20 to 40 °C. At 50 °C a significant drop of the current to lower values (≈1 µA) is observed. We attribute this to a fast desorption of the polymer/enzyme film from the electrode (visible with the bare eye, blue colored electrolyte solution) or to a thermoinactivation of the enzyme. The current increase at temperatures of 30 and 40 °C is assigned to enhanced electron transfer kinetics.

3.3. Enzymatic biofuel cells

Fig. 3a shows the power output curve measured at different potentials for a biofuel cell built from PaoABC/P023-TB and PaoABC/Aru032-TB modified graphite electrodes (to increase the stability of PaoABC/

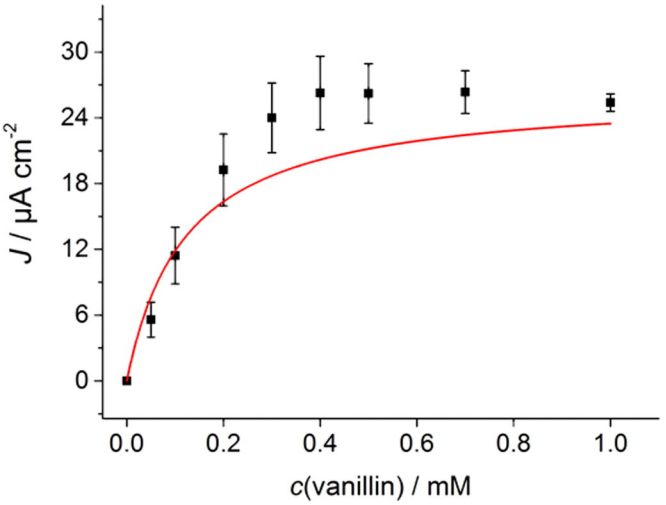


Fig. 2. Current density (black squares) for a PaoABC/P023-TB film drop-coated onto an amino modified graphite electrode in the presence of different vanillin concentrations (0–1 mM) at an applied potential of −0.1 V (vs. Ag/AgCl/3 M KCl); red line: fit of the data with a Michaelis–Menten based function; error bars indicate standard deviations.

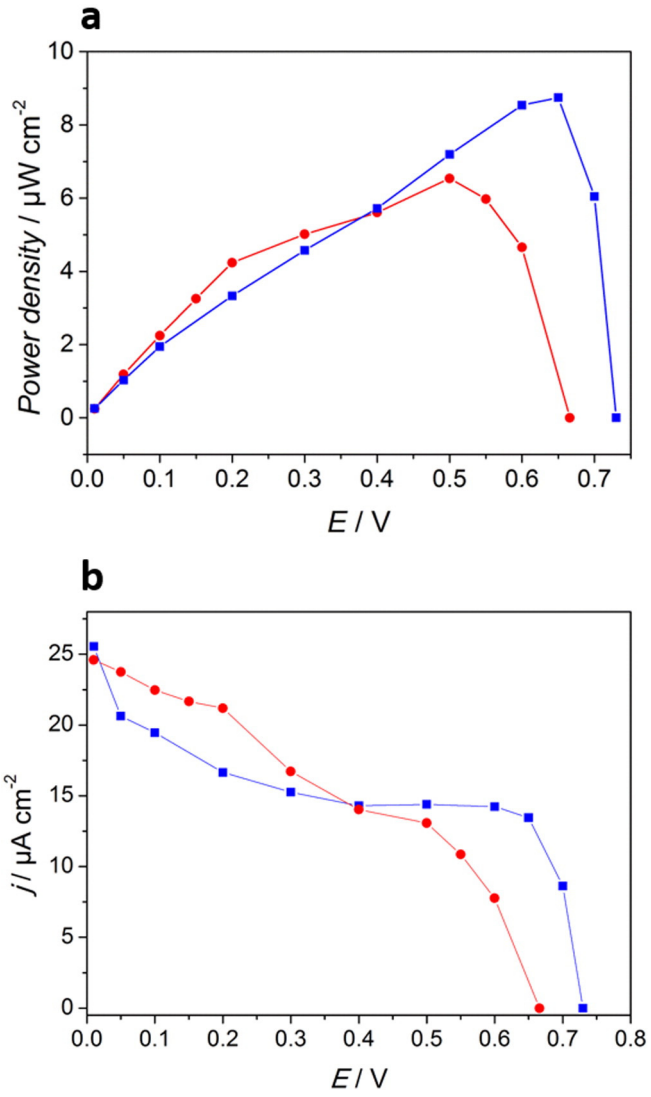


Fig. 3. Characterization of vanillin/O_2 biofuel cells comprising PaoABC/P023-TB (blue filled squares) or PaoABC/Aru032-TB (red filled circles) bioanodes in combination with a BOD based biocathode; a: power density curves obtained at a vanillin concentration of 1 mM; b: current densities (J) of the bioanodes at c(vanillin) = 1 mM; experiments were performed in a two compartment cell at room temperature under argon atmosphere in Tris-buffer (pH = 8, bioanode compartment) and under O_2 atmosphere in oxygen saturated PBS buffer (pH = 7.4, biocathode compartment), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

P023-TB film an amino decorated graphite electrode was used) and a bilirubin oxidase (BOD)-based graphene biocathode (for preparation see experimental part) measured in Tris-buffer (50 mM) at pH 8 (to avoid a hydrolysis of the ester functions within the polymer backbone at high pH values, all biofuel cell experiments were conducted at pH 8) and room temperature with a vanillin concentration of 1 mM in a two compartment cell.

Obviously, the obtained OCVs (PaoABC/Aru032-TB: ≈0.66 V; PaoABC/P023-TB: ≈0.73 V) are consistent with the OCV that could be expected in terms of the redox potentials of the bioanode (TB: ≈−0.21 V vs. Ag/AgCl/3 M KCl, Fig. 2) and the biocathode (BOD: ≈0.45 V). The maximum power output is achieved at a potential of around 0.5 V against OCV (PaoABC/Aru032-TB, power density = 6.6 μW cm^{-2}) and 0.65 V (PaoABC/P023-TB, power density = 8.8 μW cm^{-2}), respectively. Fig. 3b exhibits the current densities of the PaoABC/Aru032-TB and PaoABC/P023-TB bioanodes.

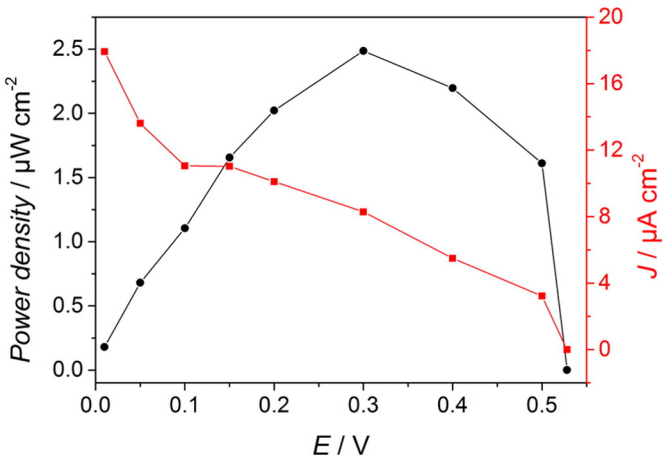


Fig. 4. Power density curve (black filled circles) and current density (red filled circles) of a membrane-less biofuel cell based on a PaoABC/Ar032-TB bioanode and a BOD biocathode in O_2 saturated PBS buffer (pH = 7.4) containing 1 mM vanillin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Both electrodes show similar behavior with maximum values for J at 10 mV of ≈25 μA cm^{-2}.

Since Aru032-TB in combination with PaoABC reveals rather high film stability, biofuel cell tests with BOD based cathodes were performed in a membrane-free one compartment cell. Fig. 4 shows the obtained power output curve recorded at different potentials. The OCV (0.53 V) and the maximum power output (2.5 μW cm^{-2} at 0.3 V) are considerably smaller than the values obtained with the two compartment cell, most likely due to parasitic O_2 reduction at the anode (via the mediator, the enzyme and/or graphite electrode). However, the use of the oxygen tolerant enzyme PaoABC allows the biofuel cell to operate in the presence of O_2.

4. Conclusion

Phenothiazine modified polymers provide a suitable immobilization matrix for the electrical wiring of the aldehyde oxidoreductase PaoABC. $2e^-$ transfer mediators toluidine blue and azure B show the envisaged efficient electrochemical communication with the FAD subunit within the enzyme. Biosensors for the amperometric detection of vanillin show comparatively low K_M values that are comparable to those using ferricyanide as free-diffusing redox mediator. The highest activity was identified at high pH values (8–9). The low formal potentials of the employed phenothiazine modified polymers (≈−0.2 V) ensure high open circuit voltages (≈0.73 V) for biofuel cells in combination with bilirubin oxidase based biocathodes. Such high OCV may open new routes for self-powered enzymatic biosensors that can be used in the detection of aldehydes (e.g. benzaldehyde) in foods or drugs. Moreover, the oxygen tolerant PaoABC allows for the design of membrane-free biofuel cell which is of particular interest for of bioelectrochemical devices that operate under ambient conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bioelechem.2015.12.005.

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