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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.

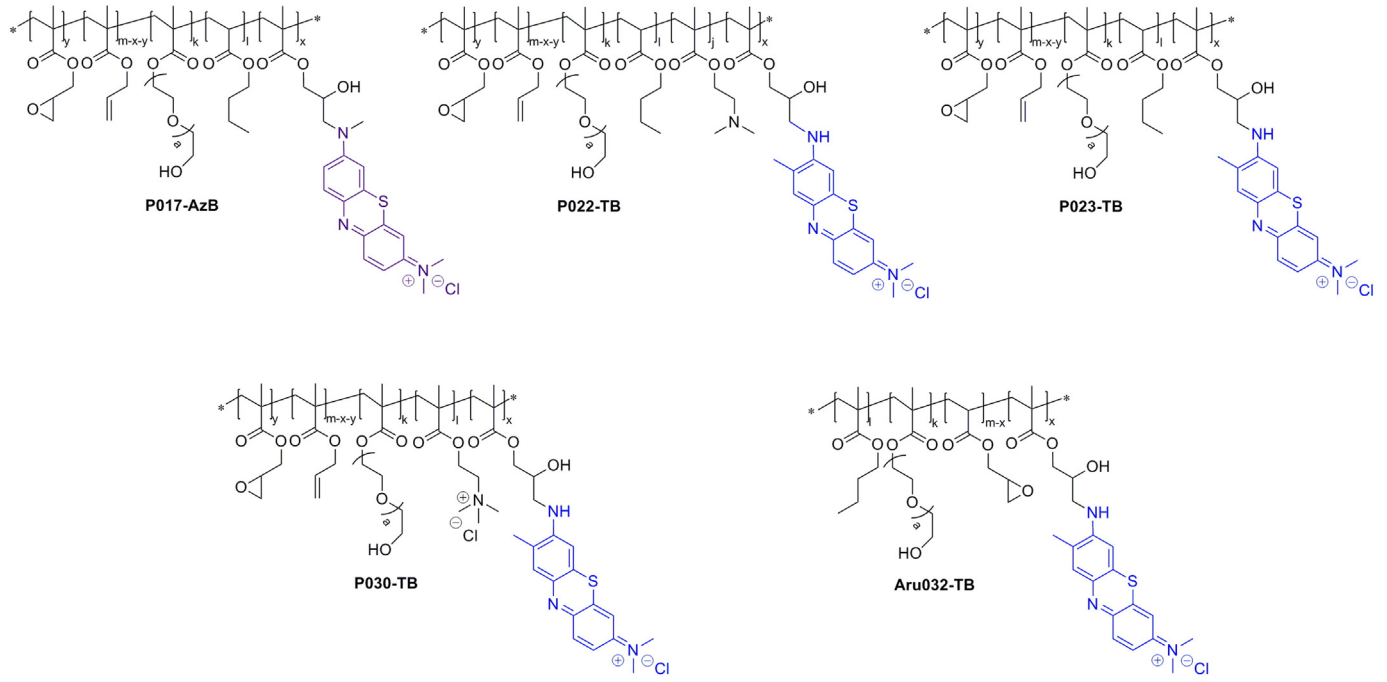
Size exclusion chromatography (SEC) measurements were performed against polystyrene standards in THF at 30 °C. Measurements were analyzed with the PSS WinGPC Unity software. Sample concentrations were around 15 mg mL^{-1}. UV–vis spectra were recorded with an Agilent Cary 60 UV–vis spectrometer in polystyrene 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.