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Electrochemistry of bilirubin oxidase and its use in preparation of a low cost enzymatic biofuel cell based on a renewable composite binder chitosan

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ABSTRACT

Spherical carbon nanoparticles KetjenBlack (KB) with a high sorption capacity together with conductivity increasing single walled carbon nanotubes (CNTs) were "glued" together by chitosan for the preparation of a composite. A biocathode with bilirubin oxidase (BOD) adsorbed within the composite was characterised and its composition optimised. A renewable biopolymer chitosan present in the composite offered (1) pre-concentration of BOD within the matrix via electrostatic interactions, (2) favourable orientation of BOD for a direct electron transfer (DET) between BOD and the composite, (3) electrochemical visibility of 3 redox sites present in BOD, (4) low charge transfer resistance, (5) high proton conductivity and (6) low overpotential for oxygen reduction. Electrochemical investigation of BOD revealed interesting internal redox communication within the enzyme with some novel insights provided. At least one of tyrosines present in BOD seems to be involved in electron transfer route, as well. The composite used for the biocathode was directly applied for the preparation of a bioanode with fructose dehydrogenase immobilised, working in a DET mode of operation. Finally, integration of the biocathode and the bioanode into a biofuel cell operated in a reagentless and membraneless mode offered a power density of 50 μW cm^{-2} at 300 mV.

1. Introduction

One of the efficient ways for limiting an environmental impact of civilisation is to pay more attention to alternative energy sources. Development of biofuel cells (BFCs), which are devices harvesting an electricity from a chemical energy of a substrate, is one of the many ways to decrease our dependence on fossil fuels [1]. An enzymatic BFC consists of two electrodes (a bioanode and a biocathode), which are covered by redox enzymes [2,3]. Presence of biocatalysts is the main distinction between BFCs and conventional fuel cells employing inorganic catalysts such as precious metals. The enzymes within the bioanode catalyse oxidation of the biofuel, whereas biocatalysts of the biocathode are responsible for reduction of a depolarisator, which is mainly oxygen [1,4]. In order to use BFCs in a sustainable way highly abundant or renewable material should be used for their construction and organic compounds or organic waste should be applied as a biofuel.

A type of the biofuel to be employed determines which biocatalysts are applicable for a construction of the bioanode. Typically, aliphatic alcohols, polyols and saccharides are the first choice biofuels due to high abundance/availability or low cost of their production [5,6]. Most often oxidases and NAD-dependent dehydrogenases are used for the preparation of the bioanodes. Oxidases rely on oxygen as a co-substrate and thus oxygen has to be removed from the anodic solution to avoid loss in the oxidation performance. Moreover, hydrogen peroxide generated can have destabilising effect on the electrode and the biocatalyst. An alternative is to use NAD-dependent dehydrogenases, but an effective way of NADH regeneration must be established together with a proper immobilisation of a coenzyme for the device to operate in a reagentless mode (i.e. only substrate is present in the anolyte) [7–10]. Above mentioned difficulties can be solved by employing enzymes with a built-in cofactor such as PQQ-dependent dehydrogenases [11–15].

Enzymatic biocathodes are constructed employing laccases or bilirubin oxidase (BOD), enzymes consisted of two copper containing clusters – a T1 site binding and oxidising the substrate and a T2/T3 cluster where electrons generated at the T1 site are employed in oxygen reduction [16,17]. Moreover, these enzymes reduce oxygen with low overpotential [18].

A substantial effort has been devoted to establish immobilisation protocols allowing a direct electron transfer (DET) between an active site of an enzyme and an electrode [16,19]. As a result, there is no need to have additional reagents such as redox mediators in the electrolyte [20]. It has been proved recently, immobilisation allowing DET is possible for many oxidoreductases, including fructose dehydrogenase (FDH) [11] and BOD [16,20,21]. Integration of nanomaterials is another way to construct effective enzymatic electrode

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interfaces [22]. The most promising way for the integration of CNTs is the preparation of their dispersions in biopolymers (chitosan and hyaluronic acid [23,24]), which are supposed to provide a suitable biocompatible matrix and high active surface area, while allowing to prepare the final composite, which is electrically conductive. Spherical nanoparticles made from carbon black [8,25] were employed for highly efficient bioanodes and biocathodes, but synthetic polymers such as poly(vinylidene fluoride) (PVDF) and poly(tetrafluoroethylene) were used as dispersing agents.

The main aim of this work was to prepare a cheap and scalable composite matrix with the aid of renewable biopolymer chitosan to make a BFC in a green way, with the amount of CNTs kept at the lowest possible level, while ensuring an efficient performance of the BFC. Moreover, a DET mode of operation for both the biocathode and the bioanode was a targeted feature in order to simplify an overall construction of the BFC. A by-product of the effort to prepare BFC was an interesting electrochemistry of BOD with effective mutual redox communication of redox sites in BOD revealed.

2. Experimental

2.1. Reagents

CNTs (d = 1.1 nm, l = 0.5–100 μm, >90% purity), BOD (8 U mg^{-1}, *Myrothecium verrucaria*), PVDF (M_W = 530 kDa) and chitosan (CHI, M_W = 50–190 kDa), Nafion 117 (5% solution was diluted with ethanol in ratio 1 + 4 before use), octyltrimethylammonium bromide and 1-methyl-2-pyrrolidone) were purchased from Sigma–Aldrich (St. Louis, USA). KetjenBlack EC 600-JD (KB, Akzo Nobel Polymer Chemicals B.V., Amersfoort, Netherlands) was kindly donated by Biesterfeld Silcom s.r.o. (Prague, Czech Republic). All other reagents were of analytical grade.

2.2. Preparation of dispersions

KB was grounded in a mortar together with CHI (0.1% in 0.3% acetic acid) (KB–CHI), PVDF (1% in 1-methyl-2-pyrrolidone) (KB-PVDF) or with Nafion treated by octyltrimethylammonium bromide according to a previously published protocol [26]. KB content in these dispersions was 13 mg ml^{-1}.

CNT dispersion in chitosan (CNT–CHI) was prepared by 30 min sonication (Bandelin DT 102 H, Bandelin electronics, Berlin, Germany) of the mixture of CNTs and CHI (0.1% in 0.3% acetic acid) with a final concentration of 5 mg ml^{-1}. Consequently, KB and CNT dispersions in chitosan were mixed together in different ratio and resulted mixtures are denoted as dKB/CNT–CHI. A stock KB dispersion diluted by a chitosan solution without CNTs is referred to as dKB–CHI in the following text.

2.3. Preparation of bioanodes and biocathodes

Glassy carbon electrode (GCE, d = 3 mm from Bioanalytical systems, USA) with a geometric surface area of 0.071 cm^2 was polished using aluminium slurry (0.3 μm, Buehler, USA), rinsed with deionised water (DW) and sonicated for 30 s in DW. KB-containing dispersions were prepared by grounding of nanoparticles together with the polymer in a mortar [25] with nanoparticle concentration of 13 mg ml^{-1} and finally 10 μl of a particular dispersion was cast on the GCE surface and left to dry at laboratory temperature. In case a dispersion of KB in CHI, diluted KB–CHI dispersion and mixed diluted KB/CNT–CHI dispersion was deposited on GCE the electrodes are referred to as GCE||KB–CHI, GCE||dKB–CHI and GCE||dKB/CNT–CHI, respectively. Dispersion-modified electrodes were incubated with solutions of FDH in 100 mM acetate buffer pH 5.0 (15 U *per* electrode) or BOD in 100 mM acetate buffer pH 6.0 (0.25 U *per* electrode). After overnight incubation at 4 °C the bioelectrodes were washed with a buffer to remove non-adsorbed enzymes. The bioelectrodes are denoted to as GCE||dKB/CNT–CHI||FDH (the bioanode) or GCE||dKB/CNT–CHI||BOD (the biocathode).

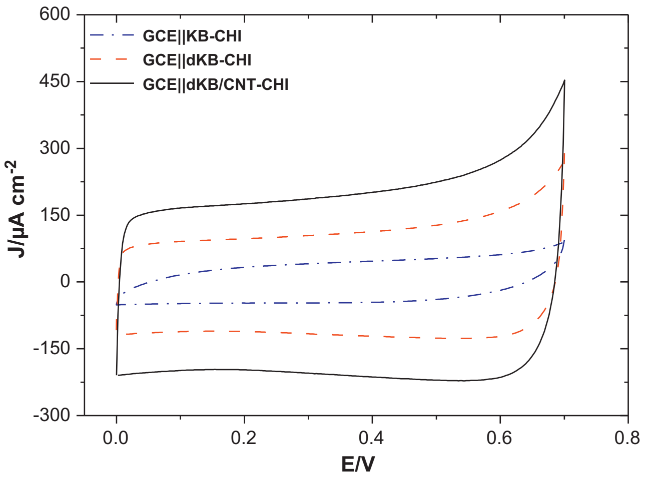

Fig. 1. CVs of GCE modified by KB–CHI, dKB–CHI and dKB/CNT–CHI dispersions. All measurements were done at a scan rate of 10 mV s^{-1} in 100 mM acetate buffer pH 6.0.

2.4. Techniques

Modified GCEs were characterised using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) run on a potentiostat Autolab PGSTAT 128N (Ecochemie, Utrecht, Netherlands) in a cell with Ag/AgCl and disc Pt electrodes as reference and counter electrodes, respectively. Bioelectrodes of the BFC were characterised in 5 ml of 100 mM acetate buffer pH 6.0 containing 200 mM D-fructose bubbled with air. Composites were also investigated by scanning electron microscopy (SEM) using ZEISS EVO 40 with an EDX BRUKER detector (Carl Zeiss, Germany).

3. Results and discussion

3.1. KB–polymer composites

KB–CHI composite was not homogeneous on the electrode surface and part of it was washed out from the surface after the electrode was dipped into the electrolyte. Obviously, chitosan with a relatively low hydrophobicity was not able to create a stable matrix capable to keep such a high amount of nanoparticles within the composite as in the case of PVDF or Nafion matrix (data not shown). Therefore one part of KB–CHI dispersion was diluted by two parts of the chitosan solution in order to decrease KB concentration and finally 5 μl of the mixture was applied on the surface of GCE (configuration GCE||dKB–CHI).

The electrodes modified with KB dispersions were tested using CV in order to compare electrochemical characteristics of their surfaces (Fig. 1). GCE||KB–CHI exhibited capacitive currents of 92 μA cm^{-2} at +400 mV, whereas an increase in an electrochemical accessible surface area for GCE||dKB–CHI (235 μA cm^{-2} at +400 mV) is observed and the composite is mechanically stable (Fig. 1). Thus, under optimal loading of KB into the CHI matrix high electrochemically accessible surface area is achieved, suggesting chitosan matrix being a good dispersant.

Chitosan is a good dispersant of CNTs with formation of an electrically conductive composite [27]. In order to connect more separated KB nanoparticles in the dKB–CHI dispersion into a highly interconnected composite, CNTs functioning as wires were