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Freestanding redox buckypaper electrodes from multi-wall carbon nanotubes for bioelectrocatalytic oxygen reduction *via* mediated electron transfer†

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An efficient and easy way of designing free standing redox buckypaper electrodes *via* the elegant combination of multi-walled carbon nanotubes (MWCNTs) and a bis-pyrene derivative is reported. This bis-pyrene 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (bis-Pyr-ABTS) acts as a cross-linker between the nanotubes and assures the formation of a mechanically reinforced buckypaper, obtained by a classical filtration technique of a MWCNT suspension in the presence of bis-Pyr-ABTS. In addition, the ABTS derivative assures a mediated electron transfer to laccase. The electroactive buckypapers were characterized in terms of morphology, conductivity, and electrochemical properties. Two setups were evaluated. The first consisted of the immobilization and wiring of laccase enzymes *via* an inclusion complex formation between the hydrophobic cavity of laccase and the pyrene groups of bis-Pyr-ABTS that are not π-stacked to the nanotubes. The second approach was to evaluate the mediated electron transfer using laccase in solution. For this setup, the developed mediator electrodes demonstrated high performances with maximum currents up to 2 mA ± 70 μA and an excellent operational stability for two weeks with daily one hour discharges using refreshed laccase solutions.

Introduction

Glucose biofuel cells are promising candidates to replace lithium batteries in implanted devices one day.[1,2] In fact, the possibility to convert the chemical energy of glucose and oxygen into electric energy is particularly interesting for *in vivo* applications, since both glucose and oxygen are present in body fluids at relatively low but constant concentrations. Such physiological liquids generally contain about 5 mmol L^{-1} glucose and 50 μmol L^{-1} oxygen. Due to the low oxygen content, the total power output is often limited by the biofuel cell cathode and, therefore, the efficiency of the oxygen reduction reaction (ORR) and the resulting electron transfer is of particular importance. Certain multicopper enzymes possess highly favorable catalytic properties for the ORR compared to inorganic catalysts in terms of overpotentials and activity in a physiological environment. Furthermore, multicopper oxidases, such as laccase or bilirubin oxidase (BOD), are capable of transferring electrons used for the ORR with various types of electrode materials *via* direct (DET)[3] or mediated electron transfer (MET). Concerning MET, there are a wide variety of artificial mediators for efficient electron transfers. ABTS [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] is one of the most commonly used mediators for multicopper enzymes.[4]

Carbon nanotubes (CNTs) have great advantages as an electrode material in a biofuel cell design due to their high conductivity and shape which allow an enhanced DET or MET with the biocatalysts.[5] Furthermore, CNTs can be shaped to pellets,[6] fibers,[7] or films (Buckypapers, BPs)[8] which makes them easy to process into mesoporous pure CNT bioelectrodes in biofuel cell designs. Beside pellets, which are exclusively obtained by compression, and a few fiber spinning procedures,[9] there are many different ways to form CNT BP electrodes.[10–13] The most widely used method is the vacuum filtration of CNT dispersions, where the CNT quality and their homogeneous dispersion are important factors to form stable, free standing BP films.

Here, we present an original approach using ABTS, modified with two pyrene units (bis-Pyr-ABTS), as a cross-linker to form a

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free standing redox BP for efficient wiring of multicopper enzymes represented by laccase from *Trametes versicolor*.

Results and discussion

Bis-Pyr-ABTS was synthesized with the aim to immobilize the redox mediator for the ORR on sp^2 hybridized carbon allotropes *via* π-stacking interactions. The sulfonamides were formed using the coupling reagent benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP) in the presence of ABTS and pyrenemethylamine. Bis-Pyr-ABTS served furthermore for the cross-linking of CNTs, forming stable free standing redox active BPs with enhanced electron transfer rates with laccase. The BP electrodes were formed *via* filtration of CNTs in the presence of bis-Pyr-ABTS. As-received MWCNTs (30 mg) were dispersed in 250 mL dimethylformamide (DMF 99%, Sigma Aldrich) and sonicated 3 times for 5 min to achieve a homogeneous black suspension. Then, 20 mg of bis-Pyr-ABTS was added to the MWCNT dispersion under magnetic stirring overnight. This mixture was then filtered under vacuum through a PTFE membrane filter with a 0.45 μm pore size. Once filtration of the MWCNT suspension was complete, the resulting MWCNT film was washed with deionized water to remove the excess of the cross-linker. The bis-Pyr-ABTS-BP, supported on a PTFE membrane, was then taken out of the glass funnel and dried in a vacuum oven at 50 °C for 15 min, preventing the deformation of the film. Once dried, the supported film was gently handled and could be carefully peeled off the surface of the membrane filter with a scalpel. Bis-Pyr-ABTS-BPs with a defined thickness can be controlled by adjusting the volume of the CNT suspension. For instance, using a 35 mm filtration system, a BP of about 100 μm thickness can be obtained after the filtration of 250 mL of the MWCNT–ABTS dispersion. It has to be noted that such MWCNT samples do not form BPs under identical conditions without the presence of bis-Pyr-ABTS. The coupling reaction and the BP formation are sketched in Fig. 1A.

Fig. 1B presents photographs of the as-obtained free standing ABTS-BP with satisfying mechanical stability. Fig. 1C and D show representative SEM images of the as prepared bis-Pyr-ABTS-BPs. The MWCNTs are randomly oriented throughout the sample, but predominantly parallel to the filter membrane surface (Fig. 1D). Even at high magnification (Fig. 1C), no excess of bis-Pyr-ABTS can be observed.

The obtained bis-Pyr-ABTS-BPs were finally cut by a razor blade into rectangular strips of 1 cm width. For all electrochemical investigations, these BP strips were connected with an alligator clip and placed in a conventional three-electrode electrochemical cell giving an effective geometric area of 1 cm^2 of the electrodes in the electrolyte.

Likewise, it was observed that the fabricated BPs show thicknesses about 100 ± 2 μm for a BP weight of 23.7 g m^{-2} and a specific volume equal to 4.2 ± 0.1 cm^3 g^{-1}. This is similar to the results found for BPs produced for the ORR using bilirubin oxidase (BOD).[8]

The electric resistance of the buckypaper was measured using a four-point probe and revealed a high conductivity of an average (5 samples) 51 ± 5 S cm^{-1}.

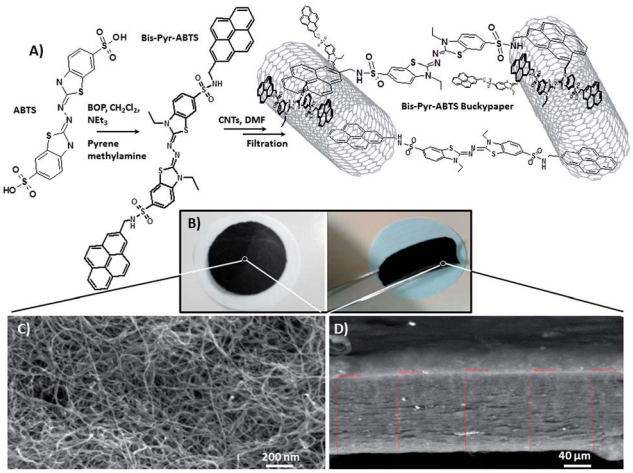

Fig. 1 (A) Reaction scheme for the synthesis of bis-Pyr-ABTS and its capacity to cross-link CNTs giving a free standing BP for mediated electron transfer. (B) Photographs of an as formed BP on a membrane filter and bent with tweezers. (C) SEM image showing the bis-Pyr-ABTS-BP surface. (D) SEM image showing the bis-Pyr-ABTS-BP cross section.

According to the literature, ABTS is an efficient electron donor in the laccase-catalyzed reduction of oxygen to water,[14] where both the oxidized and reduced species of ABTS are chemically stable and do not inhibit the enzymatic reaction. Two setups were evaluated by cyclic voltammetry. Firstly, the redox activity of bis-Pyr-ABTS used as a cross-linker was measured for the as-obtained BPs. In order to saturate the BP surface (BP_{sat}) with bis-Pyr-ABTS, the BP strips were incubated in DMF containing bis-Pyr-ABTS (5 mg mL^{-1}) in order to cover both sides of the strips. This serves, on the one hand, to increase the amount of ABTS groups in the BP structure, thus enhancing the mediated electron transfer with laccase. On the other hand, by saturating the BP with bis-Pyr-ABTS, it is very likely that a certain amount is attached to the BP with only one of the two pyrene groups. The remaining free pyrene function is then available for the oriented immobilization of laccase.[5] The electrochemical behavior of the as prepared BP and BP_{sat} was evaluated by cyclic voltammetry at a scan rate of 1 mV s^{-1} in a phosphate buffer solution (pH 5) at ambient temperature. The resulting cyclic voltammograms reveal a reversible peak system characteristic of the one-electron oxidation of ABTS at $\Delta E_{1/2}$ = 0.46 and 0.47 V *vs.* SCE for the as prepared BP and BP_{sat}, respectively (Fig. 2A). These values are similar to those previously reported for ABTS entrapped in polypyrrole (0.46 V) and for ABTS immobilized on a MWCNT (0.47 V).[16–18] This concordance indicates that the pyrenyl groups, attached to the MWCNTs, do not affect the redox potential of ABTS.

A significant difference between the as prepared BPs and the bis-Pyr-ABTS saturated BPs can be observed for the intensity of the peak current and the related charge (Fig. 2A).

The integration of the charge under the redox waves allows estimation of the amount of immobilized ABTS, *i.e.* 0.23 μmol cm^{-2} for the BP (9 wt% ABTS–MWCNTs) and 1.03 μmol cm^{-2} for the BP_{sat} (40 wt% ABTS–MWCNTs). The amount of

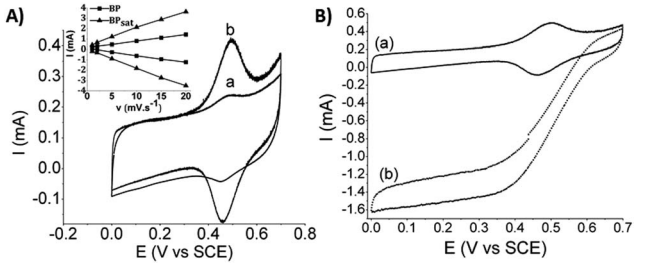

Fig. 2 (A) Cyclic voltammograms of the as formed (a) bis-Pyr-ABTS BPs and (b) BPs_{sat} in PBS (0.1 M, pH 5, scan rate 1 mV s^{-1}). The inset shows the plot of cathodic and anodic peak currents as a function of the scan rate for the BP (squares) and the BP_{sat} (triangles) under argon. (B) Electrode responses recorded in a 0.1 mol L^{-1} phosphate buffer (pH 5) solution containing 0.1 mg mL^{-1} laccase with BPs under (a) argon and (b) oxygen. Reference: SCE. Scan rate: 0.001 V s^{-1}.

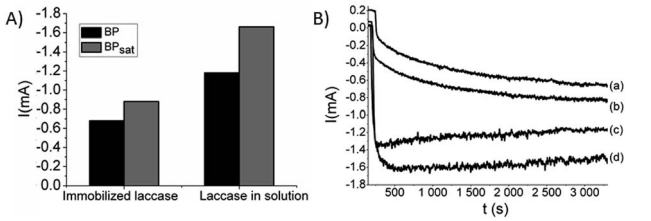

Fig. 3 (A) Diagram of the maximum currents obtained with the as prepared BP and BPs_{sat} with immobilized laccase and laccase in solution. (B) Chronoamperometric response for immobilized laccase on (a) the BP and (b) the BP_{sat}, and for laccase in solution (0.1 mg mL^{-1}) for (c) the BP and (d) the BP_{sat}. Experimental conditions: 0.1 mol L^{-1} phosphate buffer (pH 5.0) saturated with O_2; applied potential of 0.3 V *vs.* SCE.

immobilized ABTS could therefore be increased by a factor of 4.5 after further incubation in a bis-Pyr-ABTS solution giving the BP_{sat}.

The current peak amplitudes of I_{pa} and I_{pc} increase linearly with the scan rate, confirming a surface-controlled redox process without diffusion of ABTS into the solution (inset, Fig. 2A).

Fig. 2B shows the cyclic voltammograms of the as prepared BPs at 1 mV s^{-1} in a 0.1 mol L^{-1} phosphate buffer solution (pH 5) containing 0.1 mg mL^{-1} of laccase under argon and oxygen atmospheres. In the presence of oxygen, the disappearance of the anodic peak and the marked increase in the cathodic redox wave clearly reflect an electrocatalytic process.[19,20] The latter corresponds to the enzymatic oxidation of the immobilized mediator by the freely diffusing laccase, leading thus to the electro-enzymatic reduction of oxygen.

Two types of BPs (geometric area: 1 cm^2) were separately tested in terms of electrocatalytic oxygen reduction: as prepared BPs and BPs_{sat}. Furthermore, beside the evaluation of the electron transfer efficiency between the BPs and laccase (0.1 mg mL^{-1}) in solution, the performances of the BPs were also examined with immobilized laccase. For this, the BPs were incubated in a phosphate buffer solution (0.1 mol L^{-1}) containing 5 mg mL^{-1} of laccase and kept at 4 °C overnight to assure most efficient immobilization of laccase *via* hydrophobic or supramolecular interactions with some available pyrene moieties.[15] The cyclic voltammograms were recorded by scanning the potential between 0 and 0.7 V *vs.* SCE while starting the scan from the open circuit potential. Under saturation with oxygen, a stable open-circuit potential value of 0.52 V was measured for all BPs whether laccase was immobilized or present in solution, this indicates clearly that the electron transfer is dominantly realized by the ABTS mediators.

Fig. 3A shows the performance of the BP and the BP_{sat} in a phosphate buffer containing 0.1 mg mL^{-1} of laccase and in an enzyme free phosphate buffer after incubation with laccase. To compare the performance and stability of all configurations, chronoamperometric measurements were carried out to determine the catalytic current under full load discharge for about 1 hour (3300 s) at 0.3 V *vs.* SCE in an oxygen saturated solution. All values were determined after 3000 s. It appears that the catalytic current, using the laccase in solution setup, was 1.20 ± 0.07 mA for the BP and 1.55 ± 0.07 mA for the BP_{sat}. Taking into account that both sides of the buckypaper provide efficient electron transfer, the corresponding current densities are therefore 0.60 mA cm^{-2} for the BP, and 0.76 mA cm^{-2} for the BP_{sat}. It should be noted that the catalytic current for the most efficient configuration (BP_{sat}) decreased by only 6% after 1 h, illustrating the remarkable operational stability of this electrode. For the electrodes incubated with laccase, the efficient non-covalent binding and wiring of laccase lead to relatively high maximum currents that reach 44 to 50% of the preceding catalytic current values with laccase in solution. As expected, the highest electroenzymatic activity is recorded with the BP_{sat} (0.83 ± 0.03 mA; current density: 0.42 mA cm^{-2}) compared to the BP (0.67 ± 0.02 mA, current density: 0.34 mA cm^{-2}).

Due to the fact that these mediator BPs show clear advantages when the multicopper enzyme is in solution, an alternative design of future glucose fuel cells can be envisioned. By eliminating the need for immobilized enzymes, these biocatalysts, in solution, can be exchanged after the end of their lifetime.

Conclusions

In summary, the possibility to form redox active BPs with a high density of mediators for enhanced electron transfer of the ORR represents a promising alternative approach for the design of biofuel cells. The fact that high catalytic currents could be obtained in the presence of laccase in solution circumvents the need for targeted immobilization and wiring techniques. Enzyme solutions can be exchanged when the enzymes lose their activity. This principle can be envisioned for implantable biofuel cells where one of the constant issues is the lifetime of the biocatalysts. With an appropriate design, using an enzyme solution containing dialysis bags with pierceable septa, the exchange of the enzymes can be done by injections. More appropriate biocatalysts with high activities at physiological pHs and with a certain inertness to molecular inhibitors are under evaluation.

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