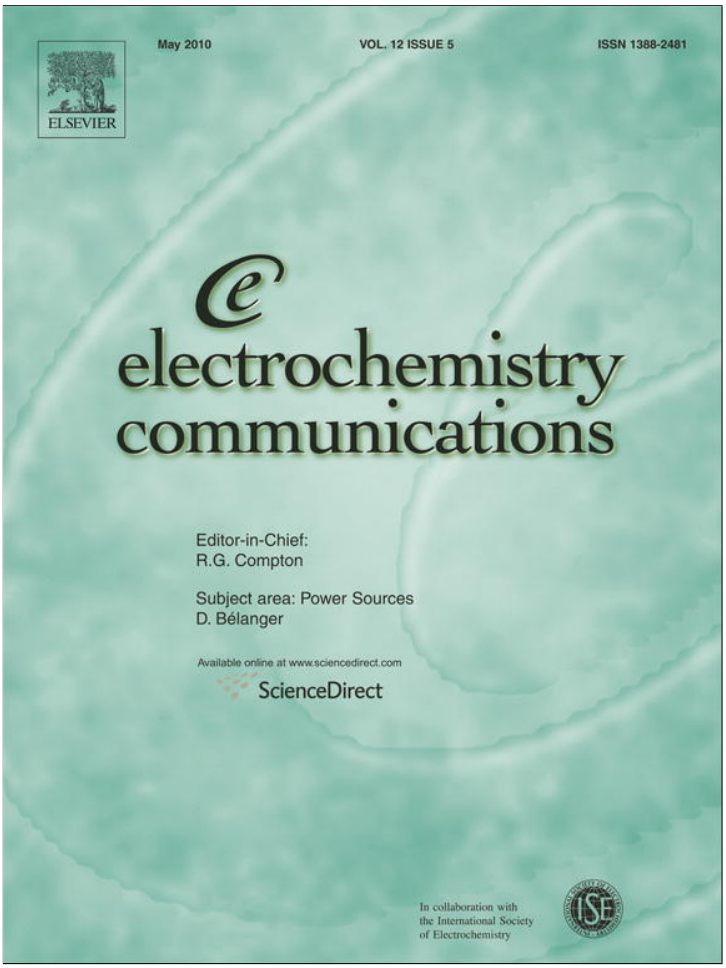

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Design of a bioelectrocatalytic electrode interface for oxygen reduction in biofuel cells based on a specifically adapted Os-complex containing redox polymer with entrapped *Trametes hirsuta* laccase

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ABSTRACT

The design of the coordination shell of an Os-complex and its integration within an electrodeposition polymer enables fast electron transfer between an electrode and a polymer entrapped high-potential laccase from the basidiomycete *Trametes hirsuta*. The redox potential of the $Os^{3+/2+}$-centre tethered to the polymer backbone (+720 mV vs. NHE) is perfectly matching the potential of the enzyme (+780 mV vs. NHE at pH 6.5). The laccase and the Os-complex modified anodic electrodeposition polymer were simultaneously precipitated on the surface of a glassy carbon electrode by means of a pH-shift to 2.5. The modified electrode was investigated with respect to biocatalytic O_2 reduction to H_2O. The proposed modified electrode has potential applications as biofuel cell cathode.

1. Introduction

Efficient four-electron O_2 bioelectroreduction at highly positive potentials using bilirubin oxidase [1–3] and laccases [4–9] "wired" with Os-complex modified polymers was described previously. Besides fundamental understanding of how O_2 is bioelectrocatalytically reduced to H_2O in nature avoiding intermediate formation of reactive oxygen species, applications in biosensors and membrane-less biofuel cells are of increasing importance [10–16]. Crucial for a membrane-less biofuel cell is that no cross reactivity between the cathode and anode reactions and moreover no reactions between the substrates used as fuels for the anode and cathode side occur. In addition, the biocatalysts have to be tightly immobilized on the electrode surface to prevent their mixing and depletion. Advantages arising from integrating the biocatalysts within three-dimensional Os-complex modified polymer layers are the significantly higher amount of the biocatalyst which is electrically connected via the polymer-bound redox centres to the electrode [17]. Recently, we have introduced a strategy for synthesizing Os-complex modified electrodeposition polymers [18–20]. Using a number of different monomers for the radical polymerization in combination with different Os-complexes, which are tethered to the polymer backbone, not only the formal potentials of the polymer-bound redox relays but also the physical properties (e.g. hydrophobicity, hydrophilicity, stability and permeability) can be tuned. This concept was already applied to the design of a biofuel cell cathode modified with bilirubin oxidase from *Myrothecium verrucaria* using an Os-complex modified anodic polymer [21]. Additionally, a complete membrane-less biofuel cell using cellobiose dehydrogenase from *Trametes villosa* at the anode and a high-potential laccase from *Cerrena unicolor* at the cathode was demonstrated [22].

Here, a new Os-complex modified anodic polymer with a formal potential specifically adjusted to the T1 Cu-site of a high-potential laccase from the basidiomycete *Trametes hirsuta* is proposed. The enzyme is active and stable even at acidic conditions [23] and the potential of its T1 site is 780 mV vs. NHE at pH 6.5 [24]. The Os-complex modified anodic electrodeposition polymer shows a pH-independent redox potential of 720 mV vs. NHE, which is optimal to "wire" high-potential laccases from different origins [24,25].

2. Experimental

2.1. Materials

K_2HPO_4, NaOH, $K_4[Fe(CN)_6]$ and citric acid were from Merck. Azoisobutyronitrile (98%), anhydrous acrylic acid, triethylamine

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(NEt_3, 99%), and n-butyl acrylate were from Fluka. 2-pyridinecarboxyaldehyde (99%), 2,2′-bipyridyl (99%), glyoxal (40% solution in water), and K_2OsCl_6 were obtained from Acros. NaH (95%) was from Aldrich and acryloyl chloride (96%) from Sigma-Aldrich. Isopropanol, ethanol, benzene, chloroform, NH_4OH, Na_2CO_3, KOH, and KCl were from J.T. Baker. 2-chloroethanol (99%) and Na_2SO_4 were from Riedel-de-Haen. Osmium-bis-N,N-(2,2′-bipyridil)-dichloride and (2-pyridyl) imidazole were synthesised according to [26,27].

2.2. Instrumentation

^{1}H NMR spectra were recorded on a Bruker DPX200 spectrometer in Methanol-D4 and evaluated using the MestRec Lite4.59 software. Chemical shifts in ppm (δ) were referenced to the residual solvent signal. A gas chromatograph (HP5890; Hewlett-Packard) connected with a mass selective detector (HP5970) using a (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness) DBxLB-mittel Polar 8–12% diphenylpolysiloxane capillary column at an oven temperature program of 60 °C for 2 min, increasing to 300 °C at a rate of 15 °C min^{-1}, 300 °C for 3 min was used with He as carrier gas at a flow rate of 1 ml min^{-1} and a split ratio of 1/90. The injector and detector temperature was 250 °C. Particle size distribution of the electrodeposition paint was measured by dynamic light scattering (DLS) using a particle sizer (Malvern).

Glassy carbon rods (∅ 1.05 mm, HTW) melted in glass capillaries (Hilgenberg) were used as working electrodes. The electrodes were cleaned by polishing with a fine emery paper (Tufback Durite, P1200), 3.0 µm diamond suspension, followed by a 1.0 µm and 0.3 µm alumina paste (LECO). The electrodes were sonicated between and after polishing for 10 min in water.

Cyclic voltammograms of the laccase/redox polymer modified electrodes and differential pulse voltammograms (DPV) of the redox polymer solution were recorded using a three-electrode system connected to a PGSTAT12 potentiostat (Eco Chemie). All potentials were recalculated to the normal hydrogen electrode (NHE).

2.3. Os-complex modified electrodeposition polymer

2.3.1. Synthesis of 2-(2-pyridin-2-yl-1H-imidazol-1-yl)ethanol (PyImEA)

21.9 g (151 mmol) of (2-pyridyl)imidazole in 80 ml ethanol were added to a solution prepared from 3.76 g (157 mmol) NaH in 250 ml ethanol. The reaction mixture was heated at 65 °C for 1.5 h and a solution of 12.2 g (152 mmol) 2-chloroethanol in 20 ml ethanol was added drop-wise. After 12 h heating at 65 °C the precipitated NaCl was filtered off and the solvent was evaporated. The structure was confirmed by ^{1}H NMR and GC/MS.

2.3.2. Synthesis of 2-(2-pyridin-2-yl-1H-imidazol-1-yl)ethyl acrylate

N-1-hydroxyethyl-(2-pyridyl)imidazole was dissolved in 200 ml $CHCl_3$. 14.2 g (157 mmol) acryloyl chloride in 20 ml $CHCl_3$ were slowly added at 2−3 °C. After 30 min 15.3 g (151 mmol) triethylamine in 40 ml ethanol was added drop-wise under continuous stirring. The reaction was kept 2−3 °C for 1 h and then stirred another 10 h at RT. The reaction mixture was washed with 150 ml saturated Na_2CO_3 solution, 3 times with 150 ml water, dried over Na_2SO_4 before the solvent was evaporated. The structure was confirmed by ^{1}H NMR.

2.3.3. Synthesis of poly(co-(2-butylcarboxylatoethylene)-co-(2-(2-pyridin-2-yl-imidazol-1-yl) ethylcarboxylatoethylene)-co-(carboxylatoethylene))

The polymerization was carried out as described previously [19,28]. 200 µL azoisobutyronitrile (12.5% in benzene) were added to a mixture of 500 µl (1.9 mmol) of 2-((2-pyridyl)imidazolyl)ethyl acrylate, 500 µl (7.3 mmol) of acrylic acid and 2000 µl (14 mmol) of butyl acrylate. The copolymerization was initiated by heating the mixture at 90 °C for 5 h. The copolymer was dissolved in 5 ml methanol and neutralized with 10 M KOH (190 µL). The copolymer composition was determined by regressions analysis of the NMR-data to be ca. 71% butyl acrylate, 22% acrylic acid and 7% PyImEA (mol%).

2.3.4. Synthesis of poly-(co-(2-(2-pyridyl-kN)imidazolyl-kN))-bis-(2,2′-bipyridyl-k2N,N′)-dichlorido-osmium(II)-ethylcarboxylatethylene)-co-(carboxylatoethylene))-co-(2-butylcarboxylatoethylene)))

7 mg of osmium-bis-(2,2′-bipyridil)-dichloride was added to 1.517 g of the copolymer solution. The reaction mixture was heated to 90 °C and stirred for 72 h. Then methanol was slowly replaced by water. The mixture was continuously stirred for 24 h to form a stable Os-complex modified polymer suspension with a solid content of ca. 10%. The redox properties of the resulting reaction mixture were investigated by means of DPV. The major part is directly reacting via an exchange of both labile chloro ligands to the envisaged product. A small fraction of the Os-complex is bound via an exchange of one chloro ligand (redox potential about 280 mV). Particle size from DLS: 193.4 nm (z-average).

2.4. Isolation and purification of laccase

The basidiomycete *T. hirsuta*, strain *T. hirsuta* 56, was obtained from the laboratory collection of the State Research Institute of Protein Biosynthesis (Moscow). The extracellular laccase was isolated from the culture media and purified to homogeneity following [24]. The enzyme homogeneity was confirmed by HPLC and SDS-PAGE. The laccase preparation (12.5 mg ml^{-1}, 315 U mg^{-1}) was stored in 50 mM phosphate buffer pH 6.5, at −18 °C. The laccase concentration was measured spectrophotometrically at 228.5 nm and 234.5 nm using BSA as standard [29].

2.5. Laccase assay and kinetic studies

The dependence of laccase activity on the pH-value in homogeneous solution was determined by estimation of the initial rates of O_2 consumption using a Clark oxygen electrode in a sealed cell at 25 °C with constant stirring. An appropriate concentration of $K_4[Fe(CN)_6]$ was used in order to ensure a measurable linear rate for the first 40 s after addition of the laccase preparation. The concentration of O_2 was assumed to be 260 µM in air-saturated solution. Bioelectrocatalytic activity was determined from the O_2 reduction current of the laccase/Os-polymer modified electrode at a potential of 350 mV vs. NHE.

2.6. Fabrication of modified bioelectrodes

Mixtures of the laccase and the Os-complex modified polymer with compositions of pure enzyme, pure Os-complex modified polymer and a polymer-to-enzyme ratio of 1:1 were prepared using the *T. hirsuta* laccase preparation (12.5 mg ml^{-1} in 50 mM phosphate buffer, pH 6.5) and the Os-complex modified polymer (ca. 100 mg ml^{-1} in H_2O). For cyclic voltammetry 0.5 µl of the mixtures were placed on a cleaned electrode surface. After 20 min drying in air the electrodes were immersed for 30 s into a 100 mM phosphate/citrate buffer, pH 2.5, for decreasing the solubility of the electrodeposition polymer by protonation.

3. Results and discussion

In order to precisely match the potential of the T1 site of *T. hirsuta* laccase an Os-complex tethered to an anodic acrylate-based electrodeposition polymer was designed with a ligand sphere containing two 2,2′-bipyridyl groups and a bidentate pyridyl-imidazolyl ligand (Fig. 1).

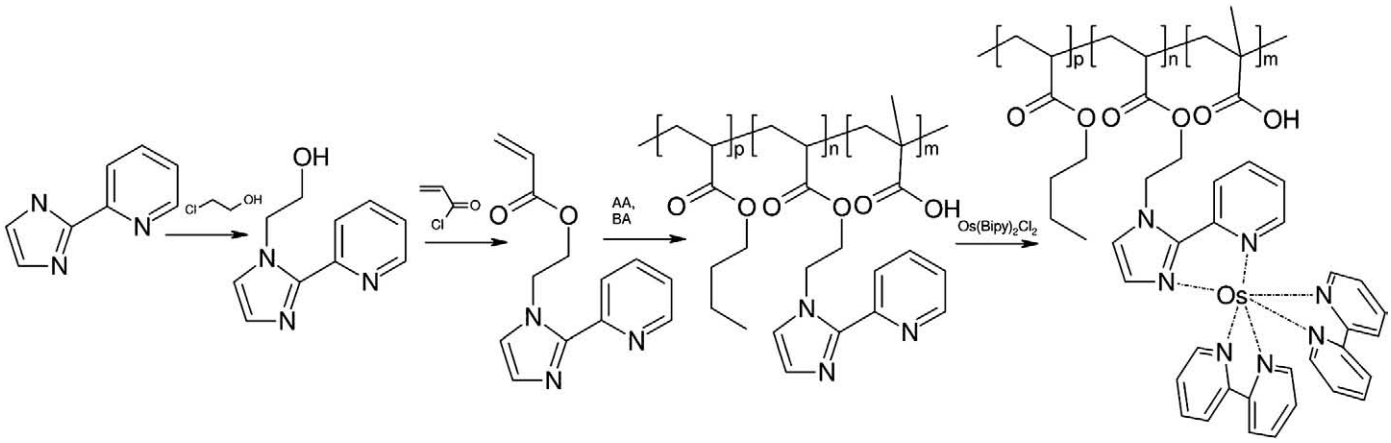

Fig. 1. Synthesis of the Os-complex modified electrodeposition paint.

The anodic shift of the redox potential upon exchanging the two labile chloro ligands against the polymer-tethered pyridyl–imidazolyl ligand is smaller then for a third bipyridyl group. A well-pronounced quasi-reversible redox conversion ($\Delta E_p \approx 150$ mV) with a midpoint potential (E_{mp}) of about 740 mV was determined by cyclic voltammetry. A second smaller redox wave was found at an E_{mp} of 280 mV (Fig. 2). The redox process at 740 mV corresponds to the redox couple of the (2-pyridyl-imidazolyl)-bis-bipyridyl-Os coordination sphere, whereas the low potential redox process is most likely due to an imidazolyl-chlorido-bis-bipyridyl-osmium complex which is formed as side product. During storage of a polymer-modified electrode almost all initially present imidazolyl-chlorido complexes transform to the high-potential 2-pyridyl-imidazolyl coordination. The redox transformation of the Os-complex modified polymer was found to be independent from the O_2 concentration (Fig. 2) which was seen as a crucial prerequisite for the evaluation of the biocatalytic O_2 reduction current in presence of both redox polymer and *T. hirsuta* laccase.

The bioelectrocatalytic activity of the laccase/Os-polymer-modified electrode with respect to O_2 reduction was investigated by means of CV (Fig. 3). The electrode was modified with a solution containing equal amounts of the laccase and the redox polymer. A well-pronounced bioelectrocatalytic response at the redox potential of the polymer-bound Os-complex was obtained in air- and O_2-saturated phosphate/citrate buffers, pH 4.0. Obviously, *T. hirsuta* laccase is not denatured during its integration into the Os-polymer as well as during the polymer precipitation at pH 2.5. As expected, neither the glassy carbon electrodes modified with either only electrodeposition paint or only laccase nor bare glassy carbon electrodes do not exhibit any electrocatalytic activity for O_2 reduction in the investigated potential range (data not shown).

Cyclic voltammograms of the modified laccase/Os-polymer electrode (Fig. 3) in an air-saturated solution showed a diffusion-limited electrocatalytic reduction of O_2 to H_2O at a current density of $-130\ \mu A\ cm^{-2}$ at about $+675$ mV vs. NHE. The decay of the catalytic current recorded in air-saturated solution indicates a mass-transport limited depletion of O_2 in the enzyme/polymer film due to the fast turnover rate of the enzyme and rapid electron transfer between the Os-polymer and laccase. Moreover, voltammograms were dependent on stirring, which is also an indicator for mass-transport limitations within the polymer film. When pure O_2 is purged into the measuring cell a limiting current density of $-325\ \mu A\ cm^{-2}$ is achieved. The experimentally obtained steady state O_2 reduction current densities in air and oxygen saturated buffers ($-80\ \mu A\ cm^{-2}$ and $-325\ \mu A\ cm^{-2}$, respectively) are in good agreement with theoretical diffusion-limited current densities assuming the angular frequency of laccase-modified electrodes to be approximately a radian per sec.

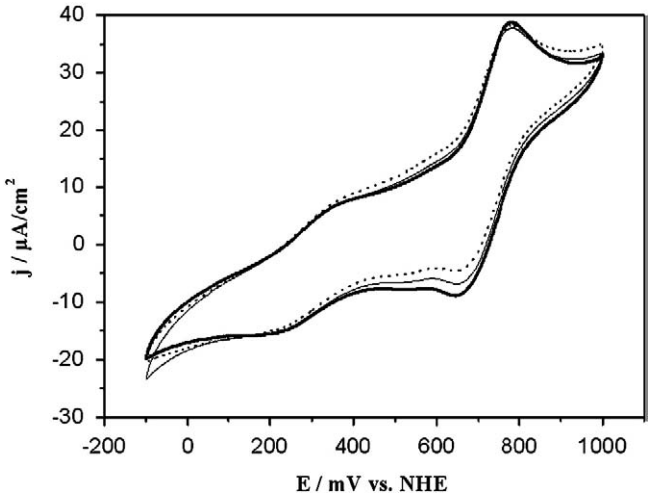

Fig. 2. Cyclic voltammograms of a glassy carbon electrode modified with the Os-complex modified anodic electrodeposition polymer (argon, air and oxygen saturated buffers – dotted, solid and bold lines, respectively). Electrolyte: 100 mM phosphate/citrate buffer pH 4.0; scan rate: 10 mV s^{-1}.

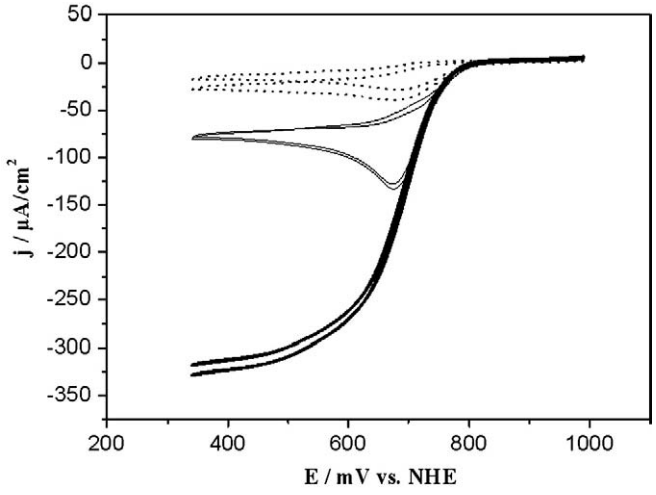

Fig. 3. Cyclic voltammograms displaying bioelectrocatalytic O_2 reduction at the glassy carbon electrode modified with *T. hirsuta* laccase entrapped within in the Os-complex modified polymer matrix (argon, air and oxygen saturated buffers — dotted, solid and bold lines, respectively). Electrolyte: 100 mM phosphate/citrate buffer pH 4.0; scan rate: 5 mV s^{-1}.

Biocatalytic oxygen reduction started at a potential of about 800 mV with a half-wave potential of about 750 mV vs. NHE (pH 4.0). This value is in good agreement with the E_{mp}-value of the Os-redox polymer (Fig. 2) suggesting that the polymer-tethered Os-centres function as electron donors for the enzyme and are hence communicating with the T1 Cu-site of the enzyme.

To further support the assumption that the Os-complex modified polymer is a pH-independent electron donor for the laccase the dependence of the electrocatalytic currents from the pH was investigated. The pH profiles for the electroreduction of O_2 by *T. hirsuta* laccase incorporated into the redox polymer matrix perfectly coincide with the pH profiles for the oxidation of an "electron-no-proton" laccase substrate (e.g. $[Fe(CN)_6]^{3-/4-}$). The redox polymer obviously acts as electron donor while the protons necessary for the reduction of O_2 to H_2O are taken from the buffer solution.

4. Conclusion

A potential biofuel cell cathode has been developed based on *T. hirsuta* laccase/Os-modified redox polymer on a glassy carbon electrode. Successful synthesis of an Os-complex modified redox polymer with a redox potential well-adapted to the T1 Cu-site of the laccase could be realized. Future work will aim on applications of the proposed bioelectrode architecture in biofuel cells namely by the optimization of the polymer-to-enzyme ratio, optimization of the Os-complex loading at the polymer, the increase of the surface area of the electrode and the stability of the enzyme/polymer assembly.

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