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Laccase wiring on free-standing electrospun carbon nanofibres using a mediator plug

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Electrospun carbon nanofibres (CNFs) containing CNTs were produced by electrospinning and subsequent thermal treatment. This material was evaluated as bioelectrode for biofuel cell applications after covalent grafting of Laccase. Bis-pyrene-modified ABTS was used as plugs to wire laccase to the nanofibres leading to a maximum current density of 100 µA.cm\textsuperscript{-2}.

Electrospinning is an efficient technique to produce submicron sized fibres under high voltages\textsuperscript{1}. The resulting electrospun membrane is a web like structure composed of continuous fibres with diameters in the nanometer range\textsuperscript{2}. Due to the tremendous evolution of this technology enabling to spin fibres out of a large variety of materials\textsuperscript{3}, such nanosized fibres are envisioned for many different applications\textsuperscript{4}. By using conductive fillers\textsuperscript{5}, coatings\textsuperscript{6}, or annealing processes\textsuperscript{7}, such macroscopic fibre mats can be used as electrodes with high surface area\textsuperscript{8, 9}. Especially the formation of carbonized fibres at high temperatures is of particular interest due to their high mechanical properties and conductivity\textsuperscript{9}. Such carbon nanofibres are promising candidates for biosensors\textsuperscript{10, 11} and (bio) fuel cell or energy related devices\textsuperscript{12}. For this type of bioelectrochemical applications, the immobilization of bioreceptors\textsuperscript{13} or enzyme wiring\textsuperscript{14} is a constant challenge. Laccase from Trametes versicolor is a widely used enzyme for biocathodes because direct electron transfer can be achieved by simple adsorption on carbon electrodes thanks to a hydrophobic domain close to the T1 centre which serves as electron supplier for the catalytic reduction of oxygen to water at the T2/T3 centre\textsuperscript{15}. This particular structure of laccase enables efficient wiring by oriented immobilization using small hydrophobic functional groups such as Anthraquinone\textsuperscript{16}, naphthalene\textsuperscript{17} or pyrene\textsuperscript{18}.

Here, we studied the electrochemical performances of carbonized polycrylonitrile-multiwalled carbon nanotube (PAN/MWCNT) fibres as biocathode material using laccase as catalyst for the reduction of oxygen. These carbon nanofibres (CNFs) were functionalized with carboxyl groups via reduction of 4-carboxyphenyldiazonium (4-CPD) for laccase immobilization using amide coupling.

The amount of wired enzymes could clearly be enhanced using a bis-pyrene modified mediator ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as a bridge between MWCNTs and laccase. One pyrene group attaches to laccase’s hydrophobic pocket while the other pyrene moieties interact with the carbon nanofibre by π-stacking. In addition, this bis-pyrene derivative acts as an electric plug for the enzyme by establishing mediated electron transfer via ABTS.

\textbf{Fig. 1:} Schematic presentation of the production of CNT containing carbon nanofibres and the covalent grafting of randomly oriented laccase enzymes which were finally wired using Bis pyrene ABTS.
Polyacrylonitrile (PAN, Mw = 150,000), N,N-dimethylformamide (DMF), Laccase (3.10 Umg⁻¹ solid) from Trametes versicolor, tetrabutylammonium tetrafluoroborate (NButBF₄), acetonitrile, hexaammineruthenium(II) chloride, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and N-(3-dimethylaminopropyl)-N’-ethyl-carbodiimide hydrochloride (EDC) were used as received from Aldrich. Commercial-grade thin multi-walled carbon nanotubes (MWCNT, 9.5 nm diameter, purity > 95%), obtained from Nanocyl were used as received, Phosphate buffer solutions were prepared with mono and dibasic phosphate (pH 5; 0.1M). Bis-pyrene 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (bis-Pyr-ABTS) and 4-carboxyphenylidiazonium salt (4-CPD) was synthesized as described.

All electrochemical experiments were performed with a conventional three-electrode configuration by using an Autolab pgstat100 potentiostat. The obtained fibre films were studied as working electrode with an initial geometric surface of 1 cm². A platinum wire was used as counter-electrode and a saturated calomel electrode (SCE) served as reference. Electrochemical measurements were performed using 0.1 mol.L⁻¹ phosphate buffer (pH 5) as electrolyte aqueous media at room temperature. Chronoamperometric measurements were performed at +0.2 V (vs SCE) in phosphate buffer (pH 5), saturated with nitrogen or oxygen. The electrospinning setup is a dual high voltage power supply ± 25 kV, iseq GMBH Germany using a syringe pump (KD Scientific series 200, USA). FE-SEM images were recorded using ULTRA 55 FESEM based on the GEMINI FESEM column with beam booster (Nanotechnology Systems Division, Carl Zeiss NTS GmbH, Germany) and tungsten gun with an accelerating voltage of 3 kV. The texture properties were analyzed using advanced data processing software (ADP version 5.1, Thermo Electron Corporation). The electrical conductivity was measured using a four-probe ohmmeter (Jandel Universal Probe System with RM3-AR Test Unit) BET surface area measurements were done using a Micromeritics ASAP 2020- serial 157 (700VA).

Nanosized fibres containing carbon nanotube (CNTs) were produced by electrospinning from a DMF solution containing CNT and polyacrylonitrile (PAN). In a second step, the electrospun nanofibres were carbonized at temperatures ranging from 700 to 850°C for 1 h in a high-purity hydrogen atmosphere (heating rate, 1 °C min⁻¹) PAN/CNT (850°C; 1 hour) films were studied as working electrode with an initial geometric surface of 1 cm² and a conductivity of 25.63 S.cm⁻¹. The specific area was measured by the BET method. The value was 156 m²·g⁻¹, with a pore diameter from 10 nm to 55 nm.

Four types of modified CNFs (850°C, 1h) (geometric area: 1cm²) were separately tested in term of electrocatalytic oxygen reduction: the unmodified nanofibres with adsorbed laccase (CNFs-Lacc), diazonium-functionalized fibres followed by reaction of mixture of laccase and EDC,(CNFs-CPD-Lacc), (CNFs-CPD-Lacc) coated with Bis-Pyrene-ABTS, (CNFs-CPD-Lacc-Bis-pyr-ABTS) and finally (CNFs-CPD-Lacc) tested in PBS solution containing ABTS,(CNFs-CPD-Lacc -ABTS).

For the CNFs-CPD-Lacc electrode, the diazonium salt was electrodeposited on the CNFs after n cycles [n=1, 3, 5, 10, 20] from 0.4 to -0.4 V vs SCE at 5 mV.s⁻¹. The carboxyl groups on electrode surface were activated by incubation with EDC (10 mol.L⁻¹) and laccase (5 mg.mL⁻¹) in 0.1 mol.L⁻¹ phosphate buffer pH5 for 1 h. For the wiring of laccase, the CNFs-CPD-Lacc electrodes were incubated in a DMF solution containing Bis-pyr-ABTS (1 mol.L⁻¹) for 1 hour. These treatments were accompanied by colour change which turn from bright grey (before) to black (after).

The morphology of the treated electrospun nanofibres was observed by SEM. Figure 3 shows the electrospun PAN and PAN-CNT fibres before and after heating at 850°C. During the chemical conversion of PAN and PAN/CNT to CNFs, the average diameter of the fibres decreases from 280 nm to 180 nm as determined using Mestrec 2.3 software. This is due to material loss during annealing. While the texture of PAN fibres seems to be not affected by this heating step, the PAN-CNT composite fibres show more kinks and the surface appears slightly coarsened.

Fig. 2 shows photographs of as-prepared PAN/CNT fibre mats; before (a) and after (b) heating treatment.

Fig. 3: SEM images of PAN nanofibres (A) before and (B) after heating treatment.
The produced CNFs were cut by a razor blade into rectangular strips of 1 cm width. For all electrochemical investigations, these fibres were connected with an alligator clip and placed in a conventional three-electrode electrochemical leading to an immersed geometric area of 1 cm² for the electrode in the electrolyte. Figure 4 presents cyclic voltammograms (CV) of 1 mmol.L⁻¹ [Ru(NH₃)₆Cl₃]²⁺ in aqueous KCl solution (0.1 mol.L⁻¹) at sample electrodes. Taking into account the capacitance of the carbon electrodes, the cyclic voltammograms were recorded at low scan rates (2 mV.s⁻¹). The current intensity of carbonized PAN/CNT fibres was five times larger than that of annealed PAN. This result from the electrical double layer expansion region related to an increased surface area and average pore diameter of the PAN-CNT nanofibre felts. This clear current increase demonstrates the beneficial effect of the CNT fillers in terms of conductivity of the resulting nanofibre electrodes.

To evaluate these carbonized electrospun CNFs as conductive support for the immobilization of enzymes and their electrical wiring, we used the electrode, which provided the best conductivity (PAN/CNTs heated at 850°C). These CNFs were modified by electropolymerization of 4-CPD as anchor function on the fibre surface followed by incubation of the modified electrode in a mixture of laccase (5 mg.mL⁻¹) and EDC (10 mol.L⁻¹). The electrochemical reduction of aryl diazonium salts allows the controlled electrografting of functional polyphenylene on conductive surfaces. By choosing a carboxylated phenyl unit, the enzyme laccase can be covalently immobilized just by EDC coupled amide formation. In the literature, there are few studies that have been focused on the immobilization of enzyme on such fibre materials. The efficiency of these laccase-CNFs electrodes towards electrocatalytic oxygen reduction was evaluated by comparing the current intensities at different reductive scan numbers forming consequently different poly-carboxyphenyl film thicknesses. Fig. 5 shows the current intensities of these differently modified biocathodes under continuous amperometric discharge at 0.2 V vs. SCE during oxygen purging of the solution for 1h. The highest current of 135 µA was obtained for CNFs modified with 5 reductive scans for 4-CPD polymerization. Taking into account that both sides of the CNF biocathodes provide electron transfer, the corresponding current density is therefore 0.65 µA.cm⁻². The decrease of the catalytic current intensity for electrodes modified with more than 5 scans of diazonium electrografting can be explained by the passivation of the CNFs surface due to increased polycarboxyphenyl film thicknesses which reduce the electron transfer yield.

The described setup is based on an initial adsorption of laccase on 4-CPD modified CNFs via hydrophobic interaction with the concomitant covalent grafting via EDC coupling. Thanks to the mentioned hydrophobic domain close to an active centre, a high quantity of adsorbed enzymes should be favorably oriented for direct electron transfer. To estimate the ratio between immobilized and wired enzymes, these bioelectrodes were studied in presence of the redox mediator ABTS in solution in order to wire all immobilized laccases. The obtained maximum current is 150 µA.cm⁻² (300 µA; Fig. 6d) and reveals that still one-third of immobilized laccase is correctly oriented for direct electron transfer. However, the combination of laccase and ABTS in solution presents an inappropriate operational mode for oxygen reduction for practical use. To overcome this issue, we used the versatile bis-pyrene-ABTS, an original molecule which has already proven its utility as cross-linker for buckypaper formation and oriented laccase immobilization. Here, we used this molecule as extension cord for laccase wiring since it attaches to the hydrophobic domain of laccase and the hydrophobic fibre surface establishing in this way mediated electron transfer. With this setup, the maximum current density towards O₂ reduction could be increased to 100 µA.cm⁻² (200µA; Fig. 6c). This means that still one-third of the immobilized enzymes remains unconnected which is most likely due to an unfavorable orientation. Compared to the ABTS free configuration of the biocathode, relying on direct electron transfer (Fig. 6b), the amount of wired enzymes could almost be doubled. During this work, we also evaluated the possibility to orientate and wire laccase by non-covalent adsorption of annealed carbon fibres (Fig. 6a). Only a negligible current density could be obtained that shows the beneficial effect of a thin polycarboxyphenyl coating that allows covalent binding of the enzyme. Unmodified, annealed fibres also...
do not provide sufficient sp² hybridized domains for non-covalent functionalization using π-stacking interactions.

Fig. 6: Chronoamperometric responses for the formed biocathodes with laccase immobilized via a) adsorption on CNFs, b) covalent coupling on CNFs/CPD (5 scans), c) covalent coupling on CNFs/CPD (5 scans)/Bi-Pyr-ABTS and d) covalent coupling on CNFs/CPD (5 scans) in presence of ABTS in solution. Experimental conditions: 0.1 mol.L⁻¹ phosphate buffer (pH 5.0) under oxygen purging; applied potential 0.2 V vs. SCE.

Freestanding CNFs biocathodes were formed out of electrospun PAN-CNT fibers after electrochemical reduction of 4-CPD followed by laccase immobilization via amide coupling. These bioelectrodes provide a high surface area, excellent structural stability, and satisfying electrical conductivity. After evaluation of the operational modes in direct and mediated electron transfer using ABTS, the setup using the electric cross-linker bis-pyrene ABTS led to a clear increase of wired enzymes. The simple and facile procedure to produce a high amount of biocatalytic CNFs electrodes represents a promising alternative for the development of bioelectrochemical devices

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Notes and references

The enzyme Laccase was wired on a free standing electrospun carbo fiber mat using a cross-linker plug based on the pyrene modified electron shuttle ABTS.