Immobilization of *Trametes hirsuta* laccase into poly(3,4-ethylenedioxythiophene) and polyaniline polymer-matrices

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**Abstract**

The immobilization of *Trametes hirsuta* laccase (ThL) in the poly(3,4-ethylenedioxythiophene) (PEDOT) and polyaniline (PANI) matrices was carried out in order to study the catalytic effect of ThL in different biocathode structures in a biofuel cell application. By using 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) as a mediator compound, the immobilized ThL in both polymer matrices, exhibited catalytic activity for the reduction of oxygen into water. The amount of ThL was adjustable in the PEDOT matrix by controlling the working parameters, such as the charge density used in the electropolymerization of EDOT monomer and the ThL concentration used in the electropolymerization electrolyte. In the PEDOT biocathode structure, the utilization of porous material as the PEDOT supporting template was studied in order to improve the current density generated per unit area/volume. Reticulated vitreous carbon foam (RVC foam) was chosen as the PEDOT supporting template material and the biocathodes were manufactured by in situ entrapment of ThL into PEDOT films polymerized on the RVC foam. These biocathodes possessed a high cathodic open circuit potential and produced a large current density, reaching 1 mA cm⁻² at 0.45 V when 19.5 µg ml⁻¹ of ThL was used in the electrolyte. The performance of these biocathodes was extremely sensitive to variations in pH and the optimal working pH was around 4.2. The biocathode reserved 80%, 50%, and 30% of the catalytic activity after storage in a +4 °C buffer solution for 1 day, 1 week, and 1 month, respectively. The PANI matrix was prepared in a form of printable ink where ThL was in situ entrapped in the PANI matrix during the laccase activated polymerization of aniline using a chemical batch reactor method. Different amounts of the ThL-containing printable PANI ink were then applied on carbon paper and the performance of the ink was subsequently electrochemically characterized. In this way, not only two different polymer matrices, but also two different matrix manufacturing procedures could be compared.

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**1. Introduction**

Biological fuel cells (biofuel cells), are devices using enzymes as catalysts to convert chemical energy to electrical energy. These devices have been recognized as a new type of energy conversion technology and they have attracted considerable attention in the past two decades [1–3]. Due to the green way of producing energy, biofuel cells are ideal power sources for low power-demanding electronic devices, such as, radio frequency identification tags, and miniaturized implantable devices, like pacemakers and glucose sensors for diabetics. A low current output is a general problem in the present biofuel cell designs, and hence the improvement of the current output of the cell is a primary task when developing biofuel cells into a mature technology for applications. One solution to this problem is an efficient enzyme immobilization method as a means to increase the enzyme loading per unit area/volume of the matrix. To date, several different strategies for the immobilization of the enzyme in different biocathode/bioanode constructions have been developed. The most popular immobilization methods include: 'wired' enzyme immobilization in redox polymer hydrogels [4–6], enzyme immobilization in sol–gel derived silica materials [7–9], enzyme immobilization incorporated with carbon nanotubes [10–12], and enzyme immobilization in conducting polymers [13]. The different enzyme immobilization methods have particulars for the method specific benefits. The immobilization of enzyme in meso-porous silica materials, for example, can facilitate the retaining of the enzyme activity after the immobilization. The unique properties of the carbon nanotubes, on the other hand, offer the possibility to promote the electron transfer in a way that mimics 'electrical wires'. Further, enzyme immobilization in conducting polymers, allows for electrochemical deposition of films with controlled thickness and ease of manufacturing on a specific substrate.
which offers many possibilities to incorporate diversified materials with favorable features into the electrode construction.

The enzyme immobilization in conducting polymers was originally applied in the construction of biosensors [14,15]. Polyaniline, polythiophene and especially polypyrrole and its amphiphilic derivatives have been widely used as matrices for enzyme immobilization in different biosensor constructions. The enzyme can be immobilized into these polymer films by physical entrapment or covalent attachment [16]. Furthermore, conducting polymers have also exhibited electrochemical wiring ability in the electron transfer between the enzyme and the current collector for some particular enzyme cases, such as lactate dehydrogenase, where the transfer between the enzyme and the current collector for the charge in the electrode is facilitated by the charge carrier transport in the electrolyte [17]. Furthermore, conducting polymers have also exhibited electrochemical wiring ability in the electron transfer between the enzyme and the current collector for the charge in the electrode is facilitated by the charge carrier transport in the electrolyte [17].

In the present work, the immobilization of ThL into PEDOT films was achieved through in situ entrapment during the electropolymerization of EDOT on the conductive template. The in situ entrapment of ThL was performed by the incorporation of negatively charged ThL molecules into the positively charged oxidized form of the conducting polymer. Biocathodes were manufactured through the entrapment of ThL into PEDOT films generated on RVC foam. These biocathodes were further optimized by tuning the conditions of the electrode, such as film growth rate, film thickness, and ThL concentration.

Polyaniline (PANI) based ink was chosen as the second matrix material for the ThL immobilization, as ThL can be in situ entrapped in the PANI matrix during the laccase activated polymerization of aniline using a chemical batch reactor method. The capability of laccase on catalyzing the chemical synthesis of water soluble conducting polyaniline has been reported [28,29]. This enzymatic approach is an environmental friendly alternative route for the synthesis of PANI, which is applied under much milder working conditions, than in the traditional chemical polymerization using high concentrations of strong acids. To construct the enzyme electrode, a PANI ink made from the laccase-catalyzed polymerization was developed as the matrix for the ThL immobilization. The predominating advantages of the ThL-containing PANI ink are the printability of this based conducting ink, easy scalability, and mass processability on paper or cardboard based template materials.

Furthermore, the performance of both ThL immobilization matrices towards the reduction of oxygen into water was evaluated using electrochemical characterization methods. The pH dependency of the PEDOT based biocathode was also studied in order to find out its working pH profile. Further, the storage stability of the PEDOT based biocathode was examined for the application in biofuel cells.

2. Experimental

2.1. Materials

ThL was provided by VTT Technical Research Centre of Finland as a stock enzyme solution in 20 mM pH 5.0 citrate buffer. ThL produced in its native host was purified in two chromatographic steps as described in the literature [30]. The enzyme activity of this purified protein preparation was 421 U ml⁻¹ (on ABTS at pH 4.5, 25 °C) and the protein concentration was 3.9 mg ml⁻¹, measured with Bio-Rad DC protein assay kit. The monomer, 3,4-ethylenedioxythiophene (EDOT, >97%), was obtained from Bayer AG. 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) and potassium nitrate were obtained from Sigma–Aldrich. All the other chemicals used to prepare the buffer solutions were of analytical grade. RVC foam with 100 pores in.⁻¹, abbreviated as RVC 100ppi, was purchased from EGR Materials and Aerospace Corporation (USA). Slices of RVC 100ppi, with a width of 9 mm and a thickness of 2 mm, were cut from the original piece of RVC 100ppi and were used as working electrodes in the electropolymerization.

Materials used in enzymatic polymerization of aniline were aniline (BASF), ABTS (Sigma–Aldrich), polyvinyl alcohol grades Mowiol 8-88 and Mowiol 40-88 (Kuraray), citrate buffer solution pH 4.5, laccase from fungus Trametes hirsuta, abbreviated as ThL. It has a comparatively high redox potential of the T1 center, 0.78 V vs NHE and, hence, its employment in the biofuel cell cathode construction, results in a high cathodic onset for the open circuit potential of the cell [27].

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Water was purified with Milli-Q® apparatus to a specific resistivity of 18.2 MΩ cm, which was used to prepare all the solutions.

2.2. Methods

2.2.1. Manufacture of biocathode

Glassy carbon (GC) electrode (projected area 0.07 cm²) was polished with 0.3 μm and 0.05 μm aluminium oxide powder to a mirror-like finish and subsequently rinsed with water and ethanol and cleaned in an ultrasonic bath for at least 5 min before use. Biocathodes were manufactured on the first matrix material (RVC foam) through in situ entrapment of ThL into PEDOT films during the electropolymerization. The electropolymerization was performed in a one-compartment, three-electrode cell with a GC electrode (d = 3 mm) or a slice of RVC 100ppi as the working electrode, a glassy carbon rod as the auxiliary electrode and with a Ag/AgCl/KCl (saturated) electrode as the reference electrode. A 0.05 M pH 6.0 succinate buffer solution, containing 0.01 M EDOT, 0.05 M KNO₃, and ThL with different concentrations (3.9, 7.8, and 19.5 μg ml⁻¹), was typically employed as the electrolyte for the electropolymerization. The electrolyte was thoroughly purged with N₂ for 15 min before electropolymerization and N₂ supply was kept to blanket the electrolyte during the electropolymerization. PEDOT films with ThL entrapped in the polymer films as dopant, were generated on the corresponding working electrodes under a constant potential of 0.915 V. The passed charge was varied by controlling the duration time of the electropolymerization.

In situ entrapment of ThL during the laccase activated polymerization of aniline using chemical batch reaction method and simultaneously preparing a printable ink for cathode construction was made in the following way. In the first stage the binder mixture was prepared using a mixture of succinate buffer solution with pH 4.5 (15 ml) and polyvinyl alcohol Mowiol 40-88 and Mowiol 8-88 in 10% dry material content in succinate buffer. The amount of polyvinyl alcohol was 4% of the mixture and it was used to enhance the adhesion to the printed surface. 2% of carbon nanotubes were added and mixed over night with magnetic stirrer and 8 min with ultrasonic treatment. Carbon nanotubes were used to enhance the ink porosity and to improve the conductivity of the ink. 0.568 g of ABTS was dissolved in the mixture and 125 μl of aniline was added. Mixing was continued in ice-bath for 1 h. ThL was added in amounts of 102 U (PANI ink 1) and 138 U (PANI ink 2). Mixing was continued for overnight and the temperature was let to rise slowly to room temperature. The final product was a black coloured, slightly greenish ink like dispersion. This ThL containing PANI ink was, further, applied on a disc of carbon paper CDL35 AA with a diameter of 5 mm. For the electrochemical evaluations, the CDL35 AA disc with PANI ink applied was then inserted in an electrode structure, where the surface facing the electrolyte solution was covered with a doubly folded cellophane membrane.

2.2.2. Electrochemical measurements

Both cyclic voltammetric and chronoanaperomeric measurements were performed using an IviumStat potentiostat (the Netherlands) in a conventional three-electrode cell equipped with an Ag/AgCl/KCl (saturated) reference electrode and a glassy carbon rod as the auxiliary electrode. The working electrodes were the PEDOT film based biocathodes generated on a GC electrode or RVC 100ppi foam and the PANI ink based biocathodes drop-cast on a carbon paper material and sealed within the electrode structure with the double layered cellophane membrane. The working electrolyte in all cases was 0.05 M pH 4.5 succinate buffer containing 0.2 mM ABTS. The electrolyte was purged with O₂ for half an hour before performing each measurement, and the O₂ supply was kept to blanket the electrolyte during the entire measurement.

Polarization curves (j–E) for the different biocathodes were plotted by extracting the steady-state current from the chronoanaperomeric measurements.

To investigate the pH dependence of ThL immobilized in PEDOT films, the chronoanaperomeric measurements of the biocathodes were performed under 0.45 V in a conventional McIlvaine buffer containing 0.2 mM ABTS (pH 4–7).

To evaluate the storage stability of the biocathode with ThL immobilized in the PEDOT film, chronoanaperometric measurements of the biocathode were performed under 0.45 V after different storage times. The biocathode was kept in a pH 4.5, 0.05 M succinate buffer in +4 °C during the storage.

2.2.3. Scanning electron microscopy

The morphology of the PEDOT film with ThL entrapped in the PEDOT film structure, generated on RVC 100ppi, was imaged using a scanning electron microscope, SEM model LEO 1530 (LEO Electron Microscopy Ltd., Germany). The sample was mounted with a conductive tape to a metal plate for direct observation without any coatings.

3. Results and discussion

3.1. In situ entrapment of ThL into a PEDOT film during electropolymerization

Prior to manufacturing the biocathode using RVC foam as electrode material, the immobilization of ThL into PEDOT films was performed on the GC electrode due to its well-defined surface area. Since both GC electrode and RVC foam are made of vitreous carbon, no difference related to the nature of the electrode materials on the enzyme immobilization is foreseen in these two cases. For electropolymerization, a pH 6 buffer solution was employed as electrolyte to keep ThL negatively charged (the PI value of ThL is around pH 4.7) and 0.05 M KNO₃ was added as the supporting electrolyte to increase the ionic conductivity of the electrolyte, since comparatively low concentrations of ThL were typically used. However, to avoid the total replacement of ThL to nitrate as counterions in the PEDOT film, high concentrations of KNO₃ were avoided. Furthermore, in the potentiostatic electropolymerization of PEDOT, a low electropolymerization potential, 0.915 V, was used to make a compromise between the growth of the polymer and the diffusion of ThL into the polymer network.

PEDOT films with ThL entrapped were examined by cyclic voltammetric measurements to clarify whether the immobilized ThL reserved the catalytic activity towards the reduction of oxygen. As seen in Fig. 1a, a reversible redox-wave at E₁/₂ = 0.52 V, corresponding to the redox couple ABTS⁻/ABTS⁺, appeared in all three voltammograms. The redox potential of this couple is quite close to that of ThL: T1 Cu⁺ (0.78 V vs NHE). Therefore, the combination of ThL and ABTS, as the catalyst and the mediator, respectively, offers the benefit of only a small potential loss on the cathode side. A PEDOT film, generated on the GC electrode by using 1 mC charge in the electropolymerization, demonstrated much larger redox peaks of the ABTS⁻/ABTS⁺ couple compared with the bare GC electrode, due to the increased active surface area of the electrode. This can be seen in Fig. 1a, curve 2. When ThL was present in the electropolymerization electrolyte, an even larger cathodic current was obtained in the potential range 0–0.4 V, compared with the plain PEDOT film, as seen in Fig. 1a, curve 3. It was, hence, evident that ThL was entrapped from the electrolyte into the PEDOT film preserving its catalytic activity to the reduction of oxygen. Since ThL was entrapped as dopant into the polymer film, the amount of
entrapment of ThL was dependent on the amount of PEDOT polymer generated on the support. This was verified by cyclic voltammograms of PEDOT films with entrapped ThL generated by using a variable amount of charge in the electropolymerization, as shown in Fig. 1b. The working parameters for obtaining the curves 1, 2 and 3 in Fig. 1b were identical except that the amount of charge used in the electropolymerization was varied by controlling the duration time. A larger cathodic current was obtained with increasing the amount of charge used in the electropolymerization. This indicates that more ThL was entrapped into the PEDOT films when more polymer was produced as the result of the prolongation of the electropolymerization time. These films also demonstrated larger catalytic capacities on the reduction of oxygen.

Until now, it has been shown that ThL entrapped into PEDOT films retains its catalytic activity towards the oxygen reduction into water and the entrapped amount of ThL is controllable by adjusting the experimental parameters. It is, thus, concluded that in situ entrapment of ThL into PEDOT films is applicable for the purpose of immobilizing the biocatalyst in the construction of the cathode of biofuel cells.

3.2. Biocathodes manufactured by in situ entrapment of ThL into PEDOT films generated on RVC foams

3.2.1. PEDOT film electrodeposited on RVC 100ppi foam

RVC foam is a sponge-like carbon material, which is solely composed of vitreous carbon and has quite a homogenous pore size distribution. RVC foam has a porous structure with a high void volume. This makes the electrodeposition of PEDOT onto RVC foam diverged from the case of a flat and smooth surface, like a GC electrode. The presence of the small voids affects the diffusion of the active species, like the monomer and enzyme, through the electrode material in the electropolymerization. This directly affects the growth of the polymer film, or in other words, the distribution of the film on the RVC foam. From the point of view of biocathode construction, an evenly distributed film over the entire porous structure is preferred, since the main purpose of utilizing the RVC foam is to make use of its large surface area per unit volume. To obtain a balance between the diffusion of monomer and enzyme molecules through the voids of the RVC foam material and the electropolymerization speed of PEDOT, an appropriate polymerization potential needs to be applied. Otherwise it is possible; for example, that the polymerization proceeds so fast that the PEDOT film preferably grows along the outermost surface of the foam to a thick film, instead of being polymerized into a well-distributed film within the entire pore structure. When the electropolymerization was performed under a comparatively low potential of 0.915 V, and a thickness of 2 mm was chosen for the RVC 100ppi electrode, the PEDOT film was able to grow evenly in the entire foam structure. When the RVC foam electrode was observed after the electropolymerization, a blush-coloured PEDOT film was visible on the foam skeleton. In Fig. 2, SEM images of the RVC foam electrode with PEDOT film polymerized on the foam surface (100 mC cm⁻³), are displayed. The average pore size of RVC 100ppi is about 230 μm. As seen in Fig. 2b, a PEDOT film was deposited on the surface of the foam skeleton. When the polymer film was observed closely, its surface exhibited a rough and ‘grain-like’ morphology.

3.2.2. Fine-tuning the electrochemical performance of the biocathode RVC 100ppi/PEDOT–NO₂/ThL, by optimizing the working parameters in the electropolymerization

ThL was entrapped into the PEDOT conducting polymer film during the electropolymerization. Therefore, the amount of polymer generated and the concentration of enzyme in the electropolymerization electrolyte are the dominating parameters affecting the performance of a biocathode using entrapped ThL as the biocatalyst.

Biocathodes were manufactured by electrochemically depositing 100 mC cm⁻³ PEDOT film on RVC 100ppi with varying concentration of ThL present in the electrolyte solution. The performance of the hereby obtained biocathodes, towards the reduction of oxygen, was examined by chronoamperometric measurements. The polarization curves for the different biocathodes can be seen in Fig. 3a. The curve 1 in Fig. 3a is the reference curve for PEDOT film generated on RVC 100ppi without any ThL entrapment. Without the presence of enzyme, there was no distinct reduction current observed except the contribution from the reduction of ABTS⁻²⁻. In curves 2, 3, and 4, variant concentrations of ThL were used in the electropolymerization. In all three cases, an apparent cathodic current was detected, resulting from the oxygen reduction catalyzed by ThL. The reduction of oxygen occurs at a potential of 0.66 V, which is quite close to the reversible O₂/H₂O Half-cell potential at pH 4.5 (0.75 V vs Ag/AgCl(s)) and the cathodic current levels off at 0.45 V. The employment of ThL in the cathode renders it possible to achieve a high cell voltage for the O₂-consuming biofuel cells, when used with a suitable anodic enzyme electrode construction.

Furthermore, the plateau value of cathodic current density increased gradually with increasing enzyme concentration in the electrolyte. In Fig. 3b, the ThL concentration used in the electropolymerization is plotted vs the steady current density generated at the potential of 0.45 V. A quasi-linear dependence was demonstrated, which indicates that the entrapped amount of ThL linearly depended on the concentration of ThL in the electrolyte in the studied concentration range. When 19.5 μg ml⁻¹ of ThL, the highest concentration studied in our experiments, was used in the electrolyte, the biocathode produced a steady current density, reaching...
above 1 mA cm\(^{-3}\) at 0.45 V. The current density is here reported vs the volume of RVC foam electrode.

As shown in Fig. 1b, when more polymer was generated, more ThL was entrapped into the PEDOT film. Additionally, the charge used for the electropolymerization was varied during the biocathode manufacturing, while the concentration of ThL (7.8 \(\mu\)g ml\(^{-1}\)) in the electrolyte was kept constant. The amount of charge used in the electropolymerization is a parameter which directly reflects the PEDOT film thickness. The different polarization curves are displayed in Fig. 4. It was found that when the used charge density was <110 mC cm\(^{-3}\), the current density generated by the biocathode increased gradually when more polymer was generated onto RVC foam. However, the polarization curves in the cases of 167 mC cm\(^{-3}\) and 550 mC cm\(^{-3}\) are almost coincident with the polarization curve for 110 mC cm\(^{-3}\). Hence, the current density did not change notably when the used charge density exceeded 110 mC cm\(^{-3}\). Therefore, the thickness, corresponding to the deposition charge density of 110 mC cm\(^{-3}\), can be considered as a critical point, within which oxygen can successfully diffuse into the film and interact with the active center of the immobilized ThL in the PEDOT film. Due to the oxygen diffusion problem, even though, the film can grow much thicker, only the immobilized ThL within the critical thickness is available for the substrate (O\(_2\)) and exhibits the catalytic activity on the oxygen reduction.

3.2.3. Working pH profile of the biocathode RVC 100ppi/PEDOT-NO\(_3\)/ThL

For an enzyme electrode, its working pH profile is a crucial issue, particularly, in a biofuel cell application, as one of its potential application areas is as a power source for the implantable devices. A neutral working pH is, thus, preferable due to the physiological working conditions.

To determine the working pH profile for the biocathode and evaluate the effect of the immobilization on the working pH of ThL, the steady current density of the biocathode at 0.45 V was recorded using variant pH buffers as the electrolyte solutions. A plot of the steady current density vs the pH of the electrolyte solution is shown in Fig. 5. It was found that the biocathode performance was sensitive to the variation in pH and the immobilized ThL had a very narrow working pH range with the optimal value at pH 4.2.
current density dramatically declined when pH increased: merely 60%, 40%, 23% or 6% of the current density was reserved when pH increased to 4.5, 5, 5.5, or 6, respectively. Compared with the diffusional ThL using electron–no–proton donor, ABTS, as the mediator [30], the pH-optimum for the ThL immobilized in PEDOT film is shifted one unit more from pH 3.0–3.5 to pH 4.2. It is inferred that the micro-circumstance of ThL location is altered in its immobilization into the PEDOT film, which has an influence on the working pH profile of ThL.

3.2.4. Stability tests for the biocathode RVC 100ppi/PEDOT-NO3/ThL

The chronoamperometric response of the freshly prepared biocathode at 0.45 V was recorded for 3 h in a quiescent 0.05 M pH 4.5 succinate buffer (10 ml) with 0.2 mM ABTS as mediator. The electrolyte was thoroughly purged with O2 for half an hour before performing the measurement, to ensure the oxygen saturation at the beginning of the measurement. As can be seen in the chronoamperometric curve, shown in Fig. 6, the current density declined gradually with prolongation of the measurement time. When comparing with the result measured in the fresh electrolyte solution after 1 day, it was found out that the declination of the current density was not merely caused by the leakage or denaturation of the ThL during the test. Instead, the results indicated that the declination of the current density was mainly induced by the depletion of oxygen from the electrolyte solution.

To further evaluate the long-term stability of the immobilized ThL in the biocathode, the current density of the biocathode generated at 0.45 V is plotted vs the storage time in Fig. 7. The results show that the catalytic activity of the immobilized ThL gradually declined during the storage under +4 °C in the succinate buffer. After 1 day, the biocathode retained 80% of its original activity. After 1 week, 50% was retained. Furthermore, only 30% of the original activity was preserved after 1 month of storage. The loss of enzyme activity is mostly likely caused by the leakage of laccase molecules from the accommodation sites in the PEDOT film. The pore size distribution could account for the rapid initial enzyme loss from the largest pores, followed by slow diffusion from smaller, deeper within the layer situated pores.

3.3. The use of ThL containing PANI ink in preparing biocathodes for biofuel cells

The use of ThL in an ink-based conducting polymer immobilization matrix was additionally studied as this type of matrix would offer several benefits related to the manufacturing process and scalability of the biocathodes. PANI is an intensively studied conducting polymer, which is traditionally synthesized via chem-
in Fig. 9 that the current density of the biocathode increases with the so obtained biocathodes is thereafter measured. It can be seen 2, are applied on the carbon paper template and the response of solution. In Fig. 9, different amounts of the PANI ink from the cellophane covered electrode assembly to the electrolyte measurement in lower voltages, due to leaching of the PANI ink Ag/AgCl(s). The current, however, decreases during the prolonged a higher current density in the potential interval of 0.7–0.4 V vs the increasing amount of the carbon paper GDL35 AA (disc diameter 5 mm) by applying 2.5 nounced. Cyclic voltammograms for a biocathode manufactured on PANI ink based biocathodes, where the leakage effect is more pro-
ounced. Cyclic voltammograms for a biocathode manufactured on carbon paper GDL35 AA (disc diameter 5 mm) by applying two different compositions of the ThL containing PANI ink on the carbon paper template; (●) 10 μl PANI ink 1 on GDL35 AA disc (d = 5 mm) and (▲) 10 μl PANI ink 2 on GDL35 AA disc (d = 5 mm). The electrolyte used was pH 4.5 0.05 M succinate buffer containing 0.2 mM ABTS.

3.3.1. Electrochemical evaluation and characterization of ThL containing PANI ink biocathodes

Polarization curves of the biocathodes manufactured on carbon paper GDL35 AA (disc diameter 5 mm) by applying two different compositions of the ThL containing PANI ink (PANI ink 1 and 2) on the carbon paper template are shown in Fig. 8. As can be seen in Fig. 8, PANI ink 2, containing a higher concentration of ThL, displays a higher current density in the potential interval of 0.7–0.4 V vs Ag/AgCl(s). The current, however, decreases during the prolonged measurement in lower voltages, due to leaching of the PANI ink from the cellophane covered electrode assembly to the electrolyte solution. In Fig. 9, different amounts of the ThL containing PANI ink 2, are applied on the carbon paper template and the response of the so obtained biocathodes is thereafter measured. It can be seen in Fig. 9 that the current density of the biocathode increases with the increasing amount of the ThL containing PANI ink 2. However, the leaching effect is similarly notable during this measurement as in the previous one. It can be noted that while this electrode assembly provides a similar measurement setup as was used with the PEDOT based biocathodes, it is clearly not the optimal for the PANI ink based biocathodes, where the leakage effect is more pronounced. Cyclic voltammograms for a biocathode manufactured on carbon paper GDL35 AA (disc diameter 5 mm) by applying 2.5 μl of the ThL containing PANI ink 2 are shown in Fig. 10. The voltammograms are taken under argon (solid line) and oxygen (dashed line) atmospheres.

4. Conclusion

In the present work, we have studied the utilization of porous carbon-based materials in combination with conducting polymers as matrices for enzyme immobilization in a biocathode design to improve the generated current density.
In situ entrapment of enzyme molecules as dopants during the electropolymerization of EDOT was successfully used to immobilize ThL as the catalyst in the construction of an oxygen-consuming cathode for biofuel cell application. The immobilized ThL in PEDOT films still preserved its catalytic activity towards the oxygen reduction into water. RVC foam-based biocathodes exhibited a high cathodic open circuit potential and generated a steady-state current density with a magnitude of several hundred \( \mu \text{A cm}^{-2} \), adjustable by varying the working parameters, like the electrodeposition charge density and ThL concentration used in the electrolyte. The thickness, corresponding to the deposition charge density of 110 mC cm\(^{-2} \), is the critical thickness for successful oxygen diffusion into the polymer layer. The cathodic current density is rather sensitive to pH, and demonstrated its optima at pH 4.2. When pH increased to 4.5, 40% enzyme catalytic activity was lost. The biocathode retained 80%, 50%, and 30% catalytic activity after the storage in a +4 \( ^{\circ} \)C buffer solution for 1 day, 1 week and 1 month, respectively.

In situ entrapment of ThL during the laccase activated polymerization of aniline using a chemical batch reaction method and simultaneously preparing a printable ink product was also successfully used to immobilize ThL as the catalyst in the construction of an oxygen-consuming cathode for biofuel cell application. The immobilized ThL in PANI inks still preserved its catalytic activity towards the oxygen reduction into water. Further, the current density of the so obtained ThL containing biocathodes is controllable by adjusting the concentration of ThL in the PANI ink and the amount of PANI ink used.

Nowadays, it is commonly recognized that the removal of a diffusional mediator is a crucial step for the miniaturization of the biofuel cells; otherwise the co-immobilization of the mediator is preferred in the biofuel cell structure designing. Our ongoing and future research is aiming to the direct electrification of the immobilized enzyme and the current collector, promoted by the integration of different nanosized constructive components into the 3-dimensional enzyme electrode designs.

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