Monolayer anthracene and anthraquinone modified electrodes as platforms for *Trametes hirsuta* laccase immobilisation

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Surface modification techniques are essential to the construction of enzyme based elements of biofuel cells and biosensors. In this article we report on the preparation and characterisation of modified carbon electrodes which were used as supports for the immobilisation of laccase from *Trametes hirsuta*. The electrodes were electrochemically modified with diamine or diazonium linkers followed by attachment of either anthracene or anthraquinone head groups using solid phase chemical methodology. These well defined surfaces were found to effectively bind laccase and to provide direct electrical contact to the enzyme active site, as evidenced by XPS, EIS and voltammetry, respectively. The influence of the type of linker and head group on enzyme binding and bioelectrocatalytic activity are evaluated.

Introduction

Laccases (E.C. 1.10.3.2) are enzymes found mainly in plants and fungi that oxidise a range of phenolic and other organic and inorganic compounds using oxygen as the electron acceptor. Structurally laccases are members of the group of blue multi-copper oxidases and they contain four copper atoms in each laccase molecule. The general structure of various laccases has been recently reviewed and high resolution crystal structures are available for several examples. The four copper atoms in each laccase molecule are located on three different sites, one each on the T1 and T2 sites and the other two on T3 sites. These three different copper coordination environments are distinguished on the basis of their spectra and have different redox properties. The T1 copper site is located on one side of the enzyme, Fig. 1, at the base of a hydrophobic pocket which acts as the binding site of the enzyme substrate. The remaining three copper atoms are bound on the T2 and T3 sites in a triangular cluster approximately 12 Å away towards the other side of the enzyme where oxygen binds. The enzyme’s substrate is oxidised by sequential single electron transfer at the T1 site and the electrons are sequentially transferred through the protein to the trinuclear T2/T3 cluster where oxygen is reduced in a four electron process to water.

The ability of laccases to catalyse the four electron reduction of oxygen without the production of intermediates, at mild pH and at high potentials triggered enormous interest in these enzymes for possible application at the cathode in biofuel cells. Significantly, immobilised laccases are capable of direct electron transfer (DET) between the T1 copper and conducting supports. This is a very attractive property for a fuel cell biocathode since, if the process can be made efficient, it avoids the necessity of soluble or co-immobilised redox mediators.

The first example of DET was reported by Tarasevich *et al.* using fungal laccase adsorbed on carbon black. Since then various other carbonaceous materials have been shown to enable adsorption of laccase and DET to the T1 copper site. Under anoxic conditions T1 voltammetry has been observed on edge plane pyrolytic graphite and superdispersed colloid graphite. With oxygen present in solution reduction waves have been recorded for laccases adsorbed on pyrolytic graphite, carbon black, pyrocarbon, carbon aerogel and spectrographic graphite. A number of modified carbon electrodes have also been employed successfully to obtain DET to physically adsorbed laccase. Carbon fibre and glassy carbon modified with carbon nanotubes (CNT) and CNT noncovalently modified with cellulose derivatives were shown to give DET-based oxygen cathodes upon laccase adsorption and cross-linking. Liquid crystal cubic phases of carbon fibres and a range of other matrices (e.g. lecithin, hydrophobin) containing CNT or carbon microcrystals, silicate composite films with pyrene functionalised CNT or carbon nanoparticles as well as poly-l-lysine/CNT composites have also been shown to provide suitable environments for DET to laccase incorporated into the matrix.

![Fig. 1 Copper sites of *Trametes hirsuta* laccase (protein database entry 3fp0.pdb) and a schematic representation of the catalytic reaction.](image-url)
Recently Blanford et al.\textsuperscript{33,34} used covalent modification of graphitic surfaces with a range of in situ generated diazonium salts to produce surfaces that bind laccase. The modifying groups were rationally chosen to mimic natural enzyme substrates in order to bind to the T1 pocket and provide efficient electron transfer between the electrode and enzyme’s active centre and to retain the enzyme at the electrode surface by strong hydrophobic interactions. The electrodes functionalised with diazonium compounds derived from 2-aminoanthracene and 2-aminochrysene were found to be the most effective for stabilisation of immobilised laccase and for providing direct electrical contact. In these experiments they obtained very high current densities based on the geometric areas of the electrodes for the reduction of molecular oxygen by DET with fungal laccases. Direct electrical contact to laccase covalently attached to electrodes modified with aminophenyl and aminophenol has also been reported.\textsuperscript{35} A summary of earlier work on DET between laccases and electrodes (including non-carbon materials) can be found in two review articles.\textsuperscript{36,37}

Methodologies for the functionalisation of carbon surfaces are well developed. In particular the electrochemical oxidation of primary amines\textsuperscript{38-40} or electrochemical reduction of diazonium salts\textsuperscript{38,40-42} first described by Pinson \textit{et al.}\textsuperscript{43} have been shown to be versatile methods for the attachment of a wide variety of organic species. Following the bonding of a suitable linker group to the carbon electrode using one of these two methods it is possible, in one or more subsequent steps, to elaborate the structure on the electrode surface and attach a wide range of groups using classical solid-phase synthesis methodology.\textsuperscript{44-46} This synthetic strategy is based on two key steps: first, a linker bearing a Boc-protected amine group is electrochemically attached to the carbon surface, then, following deprotection of the amine function, other organic groups are coupled to the immobilised amine under solid-phase synthesis conditions. The advantages of using the Boc protected diazonium salt or the mono-Boc protected diamine are that the conditions for covalent attachment to the carbon electrode can be separately optimised, and that the reaction is easily controlled electrochemically and monitored through the current passed. In addition the presence of the bulky Boc group discourages the formation of multilayer films and in the diamine case prevents both ends of the molecule binding to the electrode surface. The Boc protective group is then readily and cleanly removed to leave a primary free amine which can be reacted using a variety of well characterised solid phase coupling reagents that allows control over the subsequent chemical steps.\textsuperscript{46}

In the present work, we apply this methodology to the covalent immobilisation of anthracene (AC) and anthraquinone (AQ) through three different spacers to smooth glassy carbon electrodes. Previously published work by Blanford \textit{et al.}\textsuperscript{35} investigated the influence of anthracene modification of edge plane graphite on the binding and direct electrical contact of \textit{Trametes versicolor} laccase. However, under the conditions they used to functionalise the electrode it is very likely that the surface modification will go beyond monolayer coverage with the formation of branched structures containing direct C–C and azo bonds between the aromatic groups as shown in earlier studies by Doppelt \textit{et al.}\textsuperscript{47} Some evidence for this can be seen in the voltammetry of the anthracene-2 diazonium salt reported in the supplementary material of Blanford \textit{et al.}'s paper\textsuperscript{35} where an additional redox process is seen to grow in around 0.35 V vs. SCE as the surface modification proceeds. By using the approach developed in our laboratory, where the surface is first modified with a Boc protected amine linker and then, after removal of the Boc protection, an amide linkage is formed, we have much greater control over the surface modification. In previous work, using XPS and voltammetry, we have shown that the Boc protected linkers give monolayer coverage at the surface.\textsuperscript{45} Using our approach we can vary the length and type of linker arm and structure of the terminal group and investigate the impact that each part has on the overall performance as a platform for the immobilisation and ‘wiring’ of biomolecules.

In the present work the modified electrodes were characterized by XPS, impedance spectroscopy, and cyclic voltammetry.\textsuperscript{46} The activity of the immobilised laccase towards the reduction of molecular oxygen is evaluated and the different modified electrode surfaces compared.

### Experimental

#### Materials

Potassium ferricyanide (Avocado), potassium ferrocyanide (Fisher), tetrabutylammonium tetrafluoroborate (TBATFB) (electrochemical grade, Fluka), citric acid (BDH), trisodium citrate (Fisons), 2,2^′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma), N,N-diisopropylethylamine (DIEA) (Sigma-Aldrich), acetonitrile (HPLC grade, Rathburn, UK), N,N-dimethylformamide (DMF) (synthesis grade, Fisher Scientific), ethanol (EtOH) (analytical grade, Rathburn, UK), and O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) (Novabiochem) were used as received.

All solutions were prepared with reagent grade water (18 MΩ cm) from a Purite purification system. Pureshield argon and zero grade oxygen (BOC) were used to purge the solutions.

\textit{Trametes hirsuta} laccase (\textit{ThL}) was provided by VTT Technology (Finland). It was assayed spectrophotometrically with ABTS and the specific activity at pH 4.5 was found to be 1780 nkat mg\textsuperscript{-1} (nkat, nanokatal is defined as the amount of enzyme catalysing the oxidation of 1 nmol of ABTS per second). When not used the enzymes were stored in citrate or acetate buffer, frozen at −18 °C.

#### Electrodes and instrumentation

Working electrodes were 3 mm diameter glassy carbon (GC) rods (HTW, Germany) sealed in glass and connected to copper wires using indium (Aldrich). All electrodes were polished on silicon carbide paper (grades 600 and 1200, 3M) followed by alumina slurries (1.0 and 0.3 μm, Buehler) on polishing cloths (Buehler) before surface modification. It is difficult to precisely determine the real surface area of the electrodes prepared using the above procedure. Nevertheless it can be estimated based on the previous work by Jaegfeldt \textit{et al.}\textsuperscript{48} who reported roughness factors between 2 and 5 for graphite electrodes.
aqueous phase was extracted with ethyl acetate (2/C2 ethyl acetate (100 mL) and washed with water (50 mL). The stirred at room temperature for 16 h and then diluted with

5.2 mmol) were added dropwise. The reaction mixture was (1.26 mL, 12.96 mmol) and ethyl bromoacetate (0.58 mL, (1.0 g, 5.18 mmol) was dissolved in DMF (20 mL) and DIEA (3

concentrated. The obtained solid was washed with ethanol

(10020

1H NMR (DMSO- 50 mL) to give a light brown solid (1.33 g, 92%/C0)

300 MHz and 75.5 MHz respectively. Spectra were referenced

ESCA300 photoelectron spectrometer with AlKα X-ray source at Daresbury Laboratory, UK. 1H and 13C NMR spectra were recorded on a Bruker AV300 spectrometer at 300 MHz and 75.5 MHz respectively. Spectra were referenced with respect to the residual peak for the deuterated solvent. Chemical shifts (δ) are reported in ppm and coupling constants (J) are given in Hz.

Synthesis of (anthracen-2-ylamino)-acetic acid

Ethyl (anthracen-2-ylamino)-acetate. 2-Aminoanthracene (1.0 g, 5.18 mmol) was dissolved in DMF (20 mL) and DIEA (1.26 mL, 12.96 mmol) and ethyl bromoacetate (0.58 mL, 5.2 mmol) were added dropwise. The reaction mixture was stirred at room temperature for 16 h and then diluted with ethyl acetate (100 mL) and washed with water (50 mL). The aqueous phase was extracted with ethyl acetate (2 × 50 mL) and the combined organic phase was washed with brine and concentrated. The obtained solid was washed with ethanol (3 × 50 mL) to give a light brown solid (1.33 g, 92%).

1H NMR (DMSO-d6): δ = 1.23 (t, 3H, J = 7.3 Hz), 4.04 (d, 2H, J = 6.2 Hz), 4.17 (q, 2H, J = 7.3 Hz), 6.50 (t, 1H, J = 6.2 Hz), 6.66 (s, 1H), 7.15 (dd, 1H, J = 2.0 Hz, J = 9.0 Hz), 7.35 (m, 2H), 7.87 (m, 3H), 8.09 (s, 1H), 8.30 (s, 1H).

(Anthracen-2-ylamino)-acetic acid. Ethyl (anthracen-2-ylamino)-acetate (279 mg, 1.0 mmol) was suspended in methanol (10 mL) and an aqueous solution of NaOH (1.0 M, 10 mL) was added. The reaction mixture was stirred at 100 °C for 16 h and was then cooled down and poured in an aqueous solution of HCl (1.0 M, 10 mL). The resulting precipitate was filtered, washed with toluene and petroleum ether, and dried under vacuum to give a light brown solid (234 mg, 93%).

1H NMR (DMSO-d6): δ = 3.61 (s, 2H), 6.61 (s, 1H), 7.18 (dd, 1H, J = 2.0 Hz, J = 9.0 Hz), 7.31 (m, 2H), 7.83 (m, 3H), 8.05 (s, 1H), 8.26 (s, 1H).

Electrochemical modifications of GC electrodes

Electrodes 1 and 2. The grafting of the Boc-protected diamine spacer45 (see Scheme 1) was carried out from a solution containing 15 mm Boc-protected diamine and 0.15 m TBATFB in acetonitrile by electrochemical cycling (6 cycles) the electrode potential in potential range from 0.8 to 2.1 V vs. Ag/AgCl at a scan rate of 50 mV s⁻¹.

Electrode 3. The covalent attachment of C6H4CH2NHBoc to the GC surface was performed by electrochemical reduction from a solution containing 5 mm BocNHCH2C6H4 diazonium salt and 0.25 m TBATFB in acetonitrile. The modification was carried out by cycling the electrode potential from 0.6 to −0.9 V vs. Ag/AgCl (1 cycle) at a scan rate of 50 mV s⁻¹.

Synthetic modifications of GC electrodes

General procedure for the Boc-deprotection of modified GC electrodes. A Boc-protected modified GC electrode was suspended in a solution of HCl in dioxane (4.0 M, 0.5 mL) at room temperature for 1 h. The electrode was then washed thoroughly with water and absolute EtOH and dried before further modification.

General procedure of the coupling reaction of carboxylic acid at the GC surface. Carboxylic acid (1.0 mmol), HBTU (450 mg, 1.2 mmol) and DIEA (0.7 mL, 4.2 mmol) were dissolved in DMF (1.0 mL). The mixture was heated at 100 °C for 15 min under magnetic stirring in order to obtain a homogeneous solution. A Boc-deprotected modified GC electrode was then suspended in this solution which was allowed to cool down at room temperature and stirred for 16 h. The electrode was then washed thoroughly with DMF and absolute EtOH and dried.

Enzyme adsorption on AC and AQ modified electrodes. Modified electrode surfaces were incubated for 24 h at 4 °C with ThL solution (10 μL of 3.9 mg mL⁻¹ in 20 mm citrate buffer, pH 5). Following the incubation the electrodes were rinsed with plenty of water and washed in pH 5, 100 mm citrate buffer for 30 min. In between experiments the electrodes were kept in the same buffer.

Results

A total of six different surface modifications were prepared and tested for the binding of laccase and for direct electron transfer (DET) to the enzyme active site. These were the combinations resulting from attachment of three different
linkers and two terminal groups containing either anthracene (AC) or anthraquinone (AQ) moieties.

The linkers were attached by potentiodynamic electro-oxidation of N-Boc-ethylenediamine (Boc-EDA) or N-Boc-2,2'- (ethylenedioxy)diethylamine (Boc-EDDA) and electroreduction of 4-(N-Boc-aminomethyl)benzene diazonium tetrafluoroborate. Typical corresponding voltammetric curves are shown in Fig. 2 and the structures of the linkers are shown in Scheme 1.

The presence of bulky Boc protecting group is believed to block attack of the diazonium derived radical on the ortho positions of the aromatic rings already attached to the electrode and hence prevents the formation of multilayers of linker. In the case of the alkyl diamine linkers, the Boc group prevents the formation of bridge structures where both ends of the molecule are bound to the carbon surface. The Boc protecting groups on electrodes 1, 2 and 3 were removed by reaction with 4.0 M HCl solution in dioxane and the resulting electrodes were treated with (anthracen-2-ylamino)-acetic acid or anthraquinone-2-carboxylic acid in the presence of HBTU as coupling agent, in the presence of DIEA in DMF at room temperature for 16 h (Scheme 2). (Anthracen-2-ylamino)-acetic acid was synthesized in two steps: 2-aminoanthracene was first reacted with ethyl bromoacetate to give ethyl (anthracen-2-ylamino)-acetate in 92% yield and the ester group was then converted to a carboxylic acid group by a saponification reaction in 93% yield. The six different electrode modifications are shown schematically in Fig. 3. Following the attachment of AC or AQ the electrodes were incubated with enzyme solution.

After each stage of the surface modification the electrodes were characterised using electrochemical impedance spectroscopy in ferrocyanide/ferricyanide solution. Based on the simple Randles equivalent circuit we expect the Nyquist plot to show a semicircle at high frequency and a 45° Warburg line at low frequency. The high frequency intersection of the semicircle on the real axis then gives the uncompensated solution resistance (R_u) while the diameter of the semicircle gives the charge transfer resistance (R_{ct}). Typical results are shown in Fig. 4.
shown for electrode 5a in Fig. 4. Starting from the bare GC electrode we can see that $R_{ct}$ increases significantly (from $\sim 90 \ \Omega$ to $\sim 5.5 \ \kOmega$) upon electrochemical attachment of the Boc-protected linker, consistent with the bulky Boc group blocking access of the ferri/ferrocyanide to the electrode. Removal of the Boc protecting group then decreases $R_{ct}$ to values even lower ($\sim 65 \ \Omega$) than those observed originally for the bare GC. This is most likely due to attractive forces between the negatively charged redox probe and the protonated amine groups on the surface of the electrode. Subsequent attachment of anthraquinone leads to a moderate blocking effect ($R_{ct}$ increases to $\sim 225 \ \Omega$). Finally treatment with ThL hinders electron transfer to the ferri/ferrocyanide very significantly ($R_{ct}$ of $\sim 100 \ \kOmega$) consistent with the presence of a dense layer of enzyme adsorbed at the electrode surface.

The presence of the ThL on the six different electrode surfaces was also confirmed by XPS. A typical family of spectra for modified electrode 4c with and without adsorbed enzyme are shown in Fig. 5. Carbon, nitrogen and oxygen signals are markedly changed upon enzyme adsorption. The C 1s spectrum for the enzyme free electrode is dominated by sp$^2$ carbon from aromatic groups on the surface and the underlying GC. In contrast the surface with adsorbed ThL is found to have a much more complex spectrum with contributions corresponding to aliphatic carbon atom (250.1 eV), C=O (288.6 eV) and C–N and C–OX signals (286.6 eV). Both nitrogen (N 1s) and oxygen (O 1s) peak areas increase significantly for ThL treated electrodes as compared to the enzyme free surfaces.

Atomic percentages for all the characterised surfaces are summarised in Table 1. In every case the electrodes treated with ThL gave very similar values for C, O and N, and show much less variation than the enzyme free electrode. The values for C, O and N for the ThL treated electrodes appear to correspond to the average elemental composition of the beam penetrable enzyme layer.

The different modified electrodes were tested by cyclic voltammetry for enzyme presence and activity. Clearly defined peaks corresponding to the T1 copper redox processes in the absence of oxygen were difficult to resolve and rather variable. The charge for oxidation or reduction of the T1 copper is expected to be small due to the submonolayer coverage of correctly oriented enzyme molecules. However, the peaks’ appearance and their reversibility was found to be affected by the electrode history (e.g. number of voltammetric cycles, potential limits, exposure to oxygen etc.). Nevertheless, there is clear evidence of direct electron transfer in oxygenated solution, Fig. 6. It is important to note that neither the anthracene nor anthraquinone groups are redox active in the potential range studied here (reduction of the AQ occurs at $\sim 0.35 \ \text{V}$ under these conditions). Thus neither group is acting as a redox mediator in this reaction. The onset of oxygen reduction is found at approximately 0.55 V vs. SCE for all six electrodes, this corresponds well with the potential of the T1 copper in blue laccases ($\sim 0.54 \ \text{V}$). Control experiments either in oxygenated solutions using chemically modified electrodes without adsorbed enzyme or unmodified GC treated with enzyme give only background voltammetry. This confirms that AC and AQ surfaces are incapable of oxygen reduction catalysis in the investigated potential range and the observed current waves can be ascribed to bioelectrocatalysis originating from adsorbed laccase.

Taking into account the variation in the capacitive currents for the different modified electrodes the recorded current

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Without adsorbed ThL</th>
<th>With adsorbed ThL</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>82.7</td>
<td>76.9</td>
</tr>
<tr>
<td>4b</td>
<td>85.3</td>
<td>76.7</td>
</tr>
<tr>
<td>4c</td>
<td>89.1</td>
<td>76.9</td>
</tr>
<tr>
<td>5a</td>
<td>81.1</td>
<td>N/A</td>
</tr>
<tr>
<td>5b</td>
<td>86.0</td>
<td>77.3</td>
</tr>
<tr>
<td>5c</td>
<td>87.8</td>
<td>79.0</td>
</tr>
</tbody>
</table>

Table 1 Elemental composition of modified GC surfaces as derived from XPS measurements
densities are quite similar irrespective of the linker used, with slightly higher currents for EDA. The terminal group on the electrode surface, anthracene or anthraquinone, appears to have a more significant effect on the shape and magnitude of the oxygen reduction current. Clearly, better defined reproducible waves were observed on anthraquinone terminated surfaces with the exception of the electrode with the longest linker (5b).

Of all the electrode modifications studied the combination of short linkers with anthraquinone gave the best results for direct electron transfer to the enzyme active centre.

To evaluate the total amount of active enzyme present on the electrode surface voltammetry was also performed in the presence of ABTS as a soluble redox mediator which can shuttle electrons between the electrode and the T1 copper of ThL adsorbed at the electrode surface. In all cases the bioelectrocatalytic current densities were significantly higher in the presence of mediators and at pH 5 ranged from 11 to 16 μA cm⁻² based on the geometric surface area of the electrode. The variation of MET (mediated electron transfer) currents between differently modified electrodes did not exceed 20%. Similar reproducibility was found for duplicate electrodes both in the presence and in the absence of soluble mediator. An example of the voltammetry in the absence and presence of oxygen is shown in Fig. 7. The similar current densities obtained for all six modifications strongly suggest that similar amounts of the active enzyme are adsorbed on the electrode surface irrespectively of the character of the underlying linker and terminal group. This is consistent with the XPS data showing comparable elemental composition after the electrodes are incubated with the enzyme solution.

<table>
<thead>
<tr>
<th></th>
<th>EDA-AC</th>
<th>EDA-AQ</th>
<th>C₆H₇CH₂-AC</th>
<th>C₆H₇CH₂-AQ</th>
</tr>
</thead>
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<tr>
<td>j/μA cm⁻²</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0.2</td>
<td>-0.8</td>
<td>-0.8</td>
<td>-0.8</td>
<td>-0.8</td>
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<tr>
<td>0.4</td>
<td>-0.8</td>
<td>-0.8</td>
<td>-0.8</td>
<td>-0.8</td>
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<tr>
<td>0.6</td>
<td>-0.8</td>
<td>-0.8</td>
<td>-0.8</td>
<td>-0.8</td>
</tr>
</tbody>
</table>

Fig. 6 Cyclic voltammograms for modified electrodes with adsorbed laccase in deoxygenated (dashed lines) and oxygenated (solid lines) pH 5, 0.1 M citrate buffer solution, v = 1 mV s⁻¹.

In an attempt to improve the enzyme binding and the electrical contact of surface bound groups to the T1 pocket the linker coverage was varied. In addition to linker attachment by cyclic voltammetry, diamines or diazonium salts were also grafted by a potential step of a predefined duration. The times were varied from 1 s down to 5 ms resulting in grafting charge densities as small as 0.25 mC cm⁻² compared to ~10 mC cm⁻² recorded with the voltammetric grafting method. Following coupling of AC or AQ head groups and enzyme adsorption the electrodes were tested for oxygen reduction activity both in the absence and in the presence of mediator. No clear trend in the dependence of the catalytic currents on the amount of AC or AQ on the GC surface was found. Both DET and MET currents were within electrode to electrode reproducibility. This suggests that the head groups are uniformly distributed and there is still more than enough of them to bind to the bulky ThL molecules.

The stability of mediated bioelectrocatalytic currents was periodically tested by voltammetry while the electrodes were stored in pH 5, 0.1 M citrate buffer at 4 °C between measurements. In all cases a similar trend was observed. A fairly rapid drop of 30–40% was recorded over the first 7–10 days, followed by stabilisation of the current at approximately 50% of the initial value. This level of activity was maintained over the following testing period of up to 2 months. Similarly the DET currents halved upon 30 days of storage in buffer solution.

**Discussion**

The bioelectrocatalytic current, \( I_{\text{cat}} \), can be estimated using kinetic parameters \( (k_{\text{cat}} \text{ and } K_M) \) from spectrophotometric assays of enzyme using a simple model assuming that reaction with the redox mediator free in solution is not rate limiting. If this is the case we have

\[
I_{\text{cat}} = \frac{nFAGk_{\text{cat}}c}{c + K_M} 
\]

where \( n \) is the number of electrons transferred, \( F \) the Faraday, \( A \) the area of the electrode, \( \Gamma \) the enzyme coverage, and \( c \) the
oxygen concentration (1.25 mM).\textsuperscript{51} Based on the crystallographic dimensions for the enzyme (protein database entry 3fpx.pdb\textsuperscript{5}), and assuming close packing of the enzyme molecules on the surface, we estimate that monolayer coverage of ThL corresponds to 4.3 pmol cm\textsuperscript{-2}. Taking $K_m = 51$ $\mu$M and $k_{cat} = 141$ s\textsuperscript{-1} determined spectrophotometrically in solution (pH 4.5) we estimate, using eqn (1), that $k_{cat}$ should be of the order of 225 $\mu$A cm\textsuperscript{-2} for a monolayer of fully active enzyme. The observed current densities with ABTS as mediator are only 5 to 7\% of this value and for DET 10–20 times less than this. Several factors can affect the magnitude of the observed bioelectrocatalytic current. The enzyme coverage is very likely less than the theoretical estimated value based on close packing due to electrostatic repulsion between enzyme molecules which are polyanions under the conditions used for adsorption (pI for ThL is 4.2).\textsuperscript{52} In addition, the activity of the enzyme may be decreased upon adsorption; this effect has been reported in previous studies of Coriolus versicolor laccase adsorbed on pyrocarbon where the activity decreased by a factor of 5 to 7 compared to the enzyme activity in homogeneous solution.\textsuperscript{19} It is also possible that the population of molecules capable of DET and connected to the electrode surface via the hydrophobic pocket next to the T1 site cannot undergo mediated electron transfer because ABTS is sterically hindered from the T1 site. In consequence this particular enzyme sub-population would be MET inactive and would not contribute to the increase in current on addition of mediator. Its contribution to the measured current would be determined by the kinetics of electron transfer through the linker-head group to T1 copper.

Taken together these factors could account for the measured bioelectrocatalytic activity when using ABTS as a soluble mediator. The values reported here are also similar to the current densities reported by Calvo et al. of 12 $\mu$A cm\textsuperscript{-2} for a monolayer of Trametes trogii laccase covalently attached to gold electrodes using 3,3′-dithiodipropionic acid di(N-hydroxy-succinimide ester) (DTSP) and using ABTS as a mediator.\textsuperscript{53}

The MET currents discussed in this work were recorded under conditions where they were independent of ABTS concentration. Therefore, the current must be limited by either electron transfer between the T1 and the T2/T3 cluster or the kinetics of oxygen reduction itself, as confirmed by nonlinear dependence on oxygen concentration (data not shown). The data derived from voltammetric experiments do not however allow us to distinguish which of these steps is rate determining.

A comparison of the direct and mediated oxygen reduction currents provides some insight into the fraction of ThL molecules undergoing DET at the electrode. For example for electrode 5a the bioelectrocatalytic current in the presence of the mediator is approximately twenty times higher when compared to the direct bioelectrocatalytic current via DET. This suggests that at least 5\% of the ThL molecules immobilised at the electrode surface are in direct electron transfer communication. The hydrophobic AC and AQ moieties can bind to the enzyme through interaction with the hydrophobic, and ideally $\pi$-rich regions, at the enzyme surface. Efficient direct electron transfer will depend on the distance between the surface terminal group and the T1 copper atom, therefore the hydrophobic cleft next to the T1 site is the ideal target binding region (Fig. 8). However, it is clear from the figure that there are several other similar hydrophobic regions on the protein surface. Attachment of the enzyme via any of these regions results in orientations of ThL that are unfavourable for DET, although these orientations electrons can still be shuttled between the electrode surface and the active site by the diffusing ABTS mediator.

Direct comparison of our results with those of Blanford et al. is not straightforward because of the differences in the experiments. Blanford et al. used edge plane pyrolytic graphite rotating disc electrodes abraded with sandpaper. This roughens the

![Fig. 8 Molecular surface of ThL (protein database entry 3fpx.pdb\textsuperscript{5}) with nonpolar aminoacid residues highlighted in yellow and aminoacids with sidechains of aromatic character (Phe, Trp, Tyr) highlighted in green (left: front view of T1 site, right: molecule rotated 180° horizontally).](image-url)
the electrode relative to the polished glassy carbon used in this work and introduces uncertainty over the real surface area of the electrodes they used. In addition Blanford et al. modified their electrode surfaces using a variety of diazonium salts formed in situ by reaction of the corresponding aromatic amines with nitrite and used without isolation or purification of the diazonium salt. Under the conditions used to modify their electrodes it is likely that the diazonium coupling goes beyond monolayer coverage. It has been shown that during electrochemical and chemical diazo-grafting polyphenyl chains containing azo bonds are formed and it is very likely that this occurs under the conditions used by Blanford et al. For Pyenomonoporus cinnabarinus laccase lec3-1 Blanford et al. obtained current densities at the rotating disc electrode under oxygen saturation of 600 μA cm⁻² in pH 4 citrate buffer based on the geometric area of the electrode. For Trametes versicolor laccase they obtained 640 μA cm⁻² (20 μA at 2 mm diameter disc) under the same conditions. These current densities are about 10³ times larger than the current densities for DET reported here but it is impossible to be sure how much of this is accounted for simply by the roughness of the electrodes used by Blanford et al. and by the possibility of multilayer adsorption on their modified electrodes.

Conclusions

Anthracene and anthraquinone were attached to glassy carbon surfaces via three different types of linker. The linkers were grafted by either electrooxidation of mono-Boc protected diamines or electroreduction of 4-(N-Boc-aminomethyl)benzene diazonium tetrafluoroborate. The resulting surface modifications were found to bind Trametes hirsuta and provide electrical contact between the underlying electrode and the enzyme active site. The presence of enzyme on the electrode surfaces was confirmed by XPS, EIS and its activity by cyclic voltammetry. While all the electrodes were capable of oxygen reduction without added mediator, the combination of ethylenediamine linker with anthraquinone head group appeared to constitute the best ‘wiring’ of the surface bound ThL. Voltammetry in the presence of soluble redox mediator increased the electrocatalytic currents 10–20 times revealing that only a small fraction of enzyme is immobilised in the correct orientation which enables DET. The constructed modified electrodes with adsorbed enzyme showed good stability and constitute a good starting point towards development of improved enzyme electrodes, particularly electrodes with higher DET/MET ratio. This goal could be achieved by the use of the optimum linkers and better terminal groups, which can be readily introduced with the combination of electrochemical and solid phase synthetic methods combined with high throughput screening.

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Notes and references