A stable electrode for high-potential, electrocatalytic O_2 reduction based on rational attachment of a blue copper oxidase to a graphite surface†

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Attachment of substrate-like anthracene based units to the surface of pyrolytic graphite greatly enhances the adsorption of high-potential fungal laccases, 'blue' Cu enzymes that catalyse the four-electron reduction of O_2, providing a stable cathode for enzymatic biological fuel cells and electrochemical studies.

Blue copper oxidases known as laccases[1] catalyse the clean, four-electron reduction of O_2 to H_2O using phenolic lignin degradation intermediates as one-electron donors. The enzymes produced by certain fungi operate under weakly acidic conditions and display high rates of catalysis at high potential (within a few tenths of a volt of the pH-corrected thermodynamic potential for the O_2–$2H_2O$ redox couple). Laccases are therefore of interest for technological applications, not only in bioremediation but also as the cathodic electrocatalyst in novel, enzyme-based H_2–O_2 fuel cells.[2,3] An important issue here is how to construct a laccase-modified electrode that combines good electroactivity, stability, and ease of production.

The various approaches to using laccases as electrocatalysts are represented by (a) attaching laccase to modified precious metals such as gold,[4] (b) immobilising laccase in a network of high-potential electron mediators such as ABTS‡ or Os–pyridine complexes,[2,5] (c) simple, direct adsorption on relatively cheap carbon materials.[6] If it could be optimised, the much simpler, latter course ought to be preferred for technological applications.

We started this research by examining the electrocatalytic behaviour of three different fungal laccases (from *Trametes versicolor*, *Trametes hirsuta*, and *Pycnoporus cinnabarinus*), each simply adsorbed on a freshly polished pyrolytic graphite 'edge' (PGE) electrode. After numerous experiments we concluded that even with chromatographically and electrophoretically pure enzyme samples, it was not possible to obtain a stable response without the presence of excess laccase in solution; moreover the electrocatalytic response for O_2-saturated solution was generally below 100 μA cm^{-2} (see later). We therefore sought a rational method for modifying the electrode surface to bind laccase tightly and allow fast, direct electron transfer.

The crystal structure[7] of laccase III from *Trametes versicolor* (TvL III, Fig. 1) features a trinuclear Cu active site (Type 2 and Type 3 Cu) at which O_2 is reduced to two H_2O molecules, and a separate, mononuclear Type 1 Cu near a wide, hydrophobic binding pocket, rich in π electron density, to which a range of organic substrates can bind and undergo rapid, one-electron oxidation to radical products that dissociate before further reaction.[1] In electrochemical applications such as protein film voltammetry (PFV) or fuel cells, the electrons for O_2 reduction that would be provided by the oxidation of phenolic substrates instead

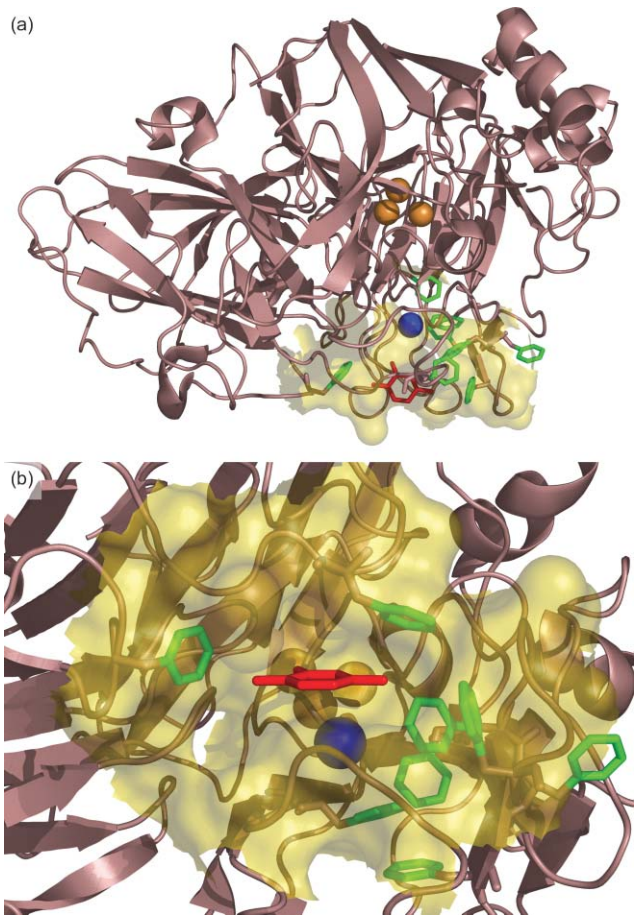

Fig. 1 Representations of *Trametes versicolor* laccase III (PDB code: 1KYA) (a) with the hydrophobic binding pocket oriented towards the bottom of the page and (b) with the binding pocket pointing out of the page. Phenyl groups near the binding pocket are shown in green. Shown in red, a co-crystallised molecule of 2,5-xylidine sits close to the mononuclear type 1 copper (blue). The trinuclear Cu site for O_2 reduction is shown in bronze. The binding pocket is the proposed target for anthracene units projecting from the electrode.

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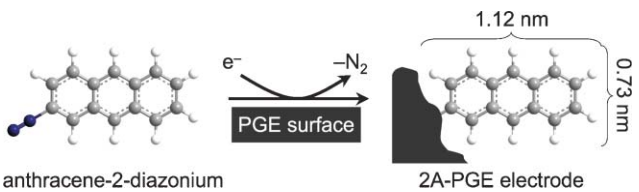
tunnel to the Type 1 site from the electrode. The aromatic binding pocket is therefore a target for rational electrode-surface design.

Diazonium coupling, the electroreduction of aryl diazonium salts, was used to create specific functional groups on the electrode surface.[8] The process involves using an aryl amine which is converted to a diazonium salt $Ar–N_2^+$ by treatment with acid and nitrite at ice temperatures. The Ar-group is then covalently attached to the electrode surface by a simple electrochemical reduction, releasing N_2.[8] This technique has already been used as the starting point for direct, covalent attachment of proteins to surfaces.[9,10] Here, we sought a linker able to penetrate and remain in the hydrophobic pocket and thus provide the equivalent of an 'electric plug' to achieve fast, direct electron transfer to the Type-1 Cu.

A library of aryl amines was selected for investigation, exploring length, hydrophobicity, functionality, flexibility and steric bulk (see ESI†). The corresponding diazonium salts were formed by established methods and all could be reductively attached to the graphite surface.§ The abilities of the resulting modified electrodes to bind laccase were assessed by observing the electrocatalytic reduction of O_2 and by epifluorescence microscopy with fluorescently tagged TvL III.¶ From these studies we identified the electrode (2A-PGE) modified by anthracene-2-diazonium (Scheme 1) as having special properties.

Fig. 2 compares the voltammetry for electrocatalytic O_2 reduction by *Pycnoporus cinnabarinus* laccase lcc3-1 (PcL) at a 2A-PGE electrode with that obtained at an unmodified PGE electrode. PcL has 84.1% sequence identity to TvL III (ESI†). Immediate observations upon introducing laccase to the electrode are that the current density on 2A-PGE (below 0.5 V *vs*. SHE this is >0.5 mA cm^{-2}) is *always* at least twice that measured for laccase adsorbed on an unmodified electrode and the electrode potential at which catalysis begins remains very high (>0.8 V at pH 4). Continued measurements reveal even more significant results: first, whereas the unmodified electrode requires the presence of laccase in solution to retain electroactivity, the 2A-PGE electrode retains its activity after transfer to enzyme-free buffer: 30 min after transfer there is less than 2% attenuation of catalytic current compared to 35% for the unmodified electrode. Second, and most importantly, the 2A-PGE electrode shows unprecedented *long-term* activity: in a typical study 57% of the original current density was retained after 8 weeks and significantly, most of this loss (40%) occurred during the first week, in other words the system *stabilises* at a high level of activity (Fig. 2, inset). (For comparison, >95% of the activity of the unmodified electrode is lost after 4 weeks.) Consistent, similar results were obtained with TvL III, but another kind of enzyme, the hydrogenase of *Ralstonia metallidurans* showed no enhancement of electrocatalytic activity compared to a PGE electrode that had simply been polished. A voltammogram



Scheme 1 Proposal for creation of 2-anthracene-modified PGE surface showing van der Waals contact dimensions of the attached molecule.

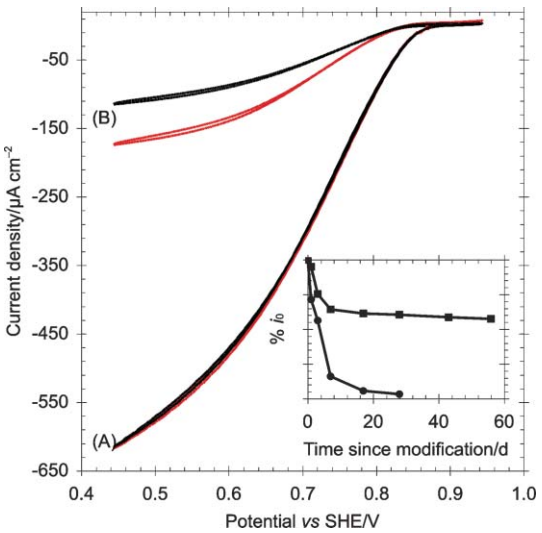


Fig. 2 The electrocatalytic activity of a film‖ of PcL on (A) a 2A-PGE electrode and (B) an unmodified PGE electrode. The black curves are the catalytic waves immediately after spotting on laccase solution; the red curves are the catalytic waves after cell solution was exchanged for fresh buffer, leaving no enzyme in solution (*ca*. 30 min after the initial enzyme application). Inset: The long-term change in catalytic current for a modified (■) and unmodified (●) electrode. The *y*-axis shows the change in current density at 0.44 V *vs*. SHE relative to the first wave with no enzyme in solution. Scan rate = 5 mV s^{-1}, rotation rate = 2500 rpm.

of the 2A-PGE electrode without enzyme showed no additional signals in the region 0.4 to 1.0 V.

Strong attachment of laccase to the electrode was observed independently by epifluorescence microscopy after attaching a fluorescent label to a lysine residue on the enzyme.¶ Fig. 3 shows images obtained for laccase on 2A-PGE and unmodified PGE. Although the enzyme molecules are too small to be individually resolved, the intensity of the fluorescence gives a semi-quantitative indication of surface coverage. In the case of the sanded electrode without diazonium modification, the surface has a low, patchy coverage of laccase "hot spots"; the 2A-PGE surface, on the other hand, has a more uniform coverage and appears much brighter despite having one-quarter of the exposure time.

The ability of 2A-PGE to bind laccase so tenaciously and display such strong electrocatalytic O_2 reduction is striking. Coupled with the lack of electrocatalytic enhancement from the other compounds (including anthracene-1-diazonium which must

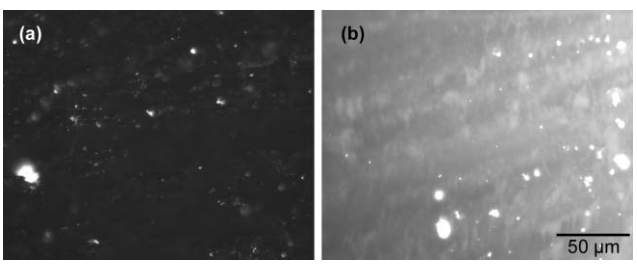


Fig. 3 Epifluorescence micrographs of TvL III tagged with fluorescein-5-EX on (a) a sanded PGE electrode surface and (b) a 2A-PGE surface.¶ Lighter shades represent greater surface concentrations of laccase. The scale is the same for both images. Exposure time: (a) 63 s, (b) 17.3 s.

become attached through the 1 position and will therefore be less free to protrude away from the graphite surface) and the lack of enhancement for another enzyme (hydrogenase), the results mean that anthracene molecules linked at the 2 position generate specific recognition sites that orient the enzyme for fast electron transfer. The first and most obvious proposal is that each anthracene unit is joined to the graphite in its native form. Each one provides a rigid, hydrophobic, π-electron conductor with the right length, width and projection angle to insert snugly into the hydrophobic binding pocket and reach close to the Type 1 Cu — almost literally a 'plug-in-socket' attachment. This proposal is extended by the intriguing possibility that the anthracene unit is itself modified by enzyme-mediated oxidation. It is reported that laccases catalyse quantitative oxidation of anthracene to 9,10-anthraquinone.[11] Although it is not clear how this happens (oxidation of anthracene itself requires potentials in excess of 1 V)[12] it is possible that a stable oxidation product becomes embedded in the substrate pocket.

On an unmodified electrode, laccase molecules are active but are only weakly adsorbed. The two-phase loss of activity on 2A-PGE (Fig. 2) suggests there is initially a mixture of strongly and less-strongly bound enzyme on the surface. The poor stability of the electrocatalytic response at unmodified PGE, even when laccase is also present in solution, suggests that the anthracene units not only produce a much higher coverage but also stabilise the enzyme. The electrode kinetics, as evidenced by the catalytic waveshape, are not significantly altered. Perhaps on an unmodified PGE surface, similar sites, albeit in very low number, exist for laccase adsorption.[13]

Looking ahead, these results suggest that carbons of various types could be subjected to 2-anthracene modification for strong, stable attachment of laccases to give cathodes for clean, direct and efficient O_2-reduction. We have not discussed how this development could provide new insight into laccase mechanism, but this line looks promising and further work is now in progress.

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Notes and references

‡ ABTS = 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

§ Primary amines were purchased from Sigma-Aldrich and used as received without further purification. All water was purified by reverse osmosis and ion exchange to a resistivity of 18.2 MΩ cm. Working at ice-temperatures, 44 μl of a 7.6 mg ml^{-1} aqueous solution of anhydrous sodium nitrite was added to 956 μl of a 4.19 mM solution of aryl amine in 1 M HCl to give a 1.3-fold excess of nitrous acid to amine. The mixture was allowed to react for 5 min before 0.1 ml was added to a jacketed electrochemical cell containing 2 ml of 0.1 M HCl and held at 2–4 °C.[10] Reactant solutions were made up fresh each day and cold diazonium solutions were never kept longer than 30 min. Solutions for modifications involving the two aminoanthracene compounds were made in 50 : 50 or 75 : 25 (v/v) mixtures of ethanol–water because of low solubility in water. Electrode surfaces were modified by scanning once from 0.5 to −0.3 V (*vs.* SCE) and back again at 50 mV s^{-1}.

¶ The surface of the graphite in the rotating disc electrode was abraded with sandpaper (P400 and P800, Norton Tufbak Durite), rinsed with water, then sonicated in water for 10 min. The surface of the electrode (modified or unmodified) was spotted with 15 μM laccase in acetate buffer (50–100 μl cm^{-2} of electrode geometric area). The electrode was connected to the rotator and placed in ~2 ml of 200 mM pH 4.0 sodium citrate buffer. The cell was maintained at 25 °C. Oxygen (industrial grade, Air Products) at atmospheric pressure was flowed over the liquid while the electrode was rotated at 2500 rpm. The potential was swept from 0.7 V *vs.* SCE to 0.2 V *vs.* SCE and back again at 5 mV s^{-1}. Once the laccase wave had stabilised (typically 3–5 scans), the cell solution was replaced with fresh buffer, reoxygenated and the same potential range scanned to observe catalysis with no enzyme in solution. Potentials for laccase electrocatalysis have been scaled to the standard hydrogen electrode (at room temperature SHE = 0.2412 V *vs.* saturated calomel electrode (SCE)). During rest periods the electrode tips were kept hydrated, wrapped in paraffin film, and stored at 4 °C. For epifluorescence measurements, the laccase was labelled at Lys-174 with fluorescein-5-EX, succinimidyl ester (Molecular Probes) using the company's protocol.[14] The degree of labelling was determined to be 0.6 dye molecules per laccase molecule. The laccase-containing fraction was diluted tenfold with the eluent before it was used on graphite. Epifluorescence microscopy was carried out using a Olympus BX50 microscope using an Olympus 40× UPlan Fl objective (NA = 0.75) in air. Samples were illuminated with a mercury lamp filtered through a fluorescein filter cube (excitation 470–490 nm, barrier 515–550 nm). Images were recorded on an Optronics CCD camera. The surface was spotted with 2 μl of the dilute labelled laccase solution and the samples were left on ice for 30 min.

‖ A protein "film" is a mono/submonolayer of redox-active enzyme in direct electrical contact with the surface, in contrast to studies of enzymes either in solution or connected through an electron-transfer mediator.[15]

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