A stable electrode for high-potential, electrocatalytic O₂ reduction based on rational attachment of a blue copper oxidase to a graphite surface. (Supplementary information)

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Additional data and figures

Compounds investigated for laccase stabilisation

Eleven different surface groups (Fig. S1) were chosen to screen for enhanced current density and stability of laccase adsorbed on PGE.

The compounds were chosen to explore a range of lengths, conjugation, functionalities, steric bulks and electron density. The surface-bound 4-aminobenzene (1) was made by cathodic reduction of surface-bound 4-nitrobenzene (8).

With the exception of 11 no enhancement or stabilisation was observed. Compound 11 gave reliable and reproducible enhancement and stabilisation.

Typical reduction scan using anthracene-2-diazonium

There are four characteristic peaks that appear in the electrochemical modification of PGE electrodes using an anthracene-2-diazonium solution (Fig. S2). The potentials are given with respect to both SCE and SHE scales. Actual experimental measurements were made with an saturated calomel electrode, but to facilitate comparison with data given in the main text which have been converted to SHE, the following results have also been converted to the SHE scale. In contrast to the scans shown in the article which were run on 2-mm diameter PGE rotating disc electrodes, scans shown in Fig. S2 were taken on a stationary PGE electrode with a rectangular graphite cross section. The first reductive scan always consists of a sharp reduction peak at 0.31 V followed by a broader reduction peak at 0.22 V. The area of these two peaks, after baseline correction and deconvolution, decreases by about 75% in the second reduction scan and by about half again in the third scan as reactive surface sites become occupied by the bulky anthracene moiety. The ratio between the areas of the 0.31 V peak and the 0.22 V peak varies; typically the former corresponds to 0.8–1.6 nmol electrons cm⁻² and the latter corresponds to 0.7–2.3 nmol electrons cm⁻². Depending on the electrode surface preparation, a third one-electron peak may be present (as it is in Fig. S2) at 0.38 V. This reduction peak develops upon repeated cycling. Scans in the oxidative direction show the growth of an asymmetric peak with a maximum at 0.39 V and a second oxidation peak centred at 0.61–0.62 V which diminished over subsequent scans.
Toyopearl DEAE 650M (Toya Soda; 1.5 cm diameter and 5 cm height; ca 10 ml resin) anion exchange column (previously equilibrated with buffer) at 4 °C and washed with buffer (ca 10 column volumes) to remove unbound material. Laccase was then released from the column by stepping salt concentration in the elution buffer to 100 mM ammonium sulphate. The fractions containing laccase were eluted as a dark green/blue band, while a brown/yellow band with no laccase activity remained bound. Fractions collected were tested for laccase activity with ABTS. Fractions showing laccase activity were combined, reapplied to an anion exchange column and eluted as previous. This led to a laccase active fraction > 95% pure by SDS-PAGE. The laccase was desalted into pH 4.0 10 mM acetate buffer and stored at 20 µl samples at –80 °C.

The PB 94 strain of *Pycnoporus cinnabarinus* fungus was purchased from the American Type Culture Collection (ATCC No. 200478). Small squares of fungus were plated onto malt extract agar and grown at 28 °C for 7 d. Washings from nine plates were used to inoculate 3 l modified Dodson media which were grown for 5 d at 28 °C with shaking at > 130 rpm and laccase expression induced after 24 h. A substrate mimic, 2,5-xylidine, is used to induce the extracellular expression of laccase. Extracellular protein was harvested by ammonium sulphate precipitation (500 g l–1), desalted into pH 4.0 10 mM acetate buffer and stored as 20 µl samples at –80 °C.
phosphate, pH 6 before addition of 3 g ammonium sulphate. These fractions were loaded onto a 1 ml HiTrap Phenyl Sepharose High Performance hydrophobic interaction column (Amersham Biosciences) previously equilibrated with 2.67 M ammonium sulphate, washed and eluted with ammonium sulphate. Fractions testing positive for laccase activity by ABTS were concentrated and dialysed back into 10 mM pH 4.6 acetate buffer, and checked for purity by SDS-PAGE. The laccase was identified as that expressed by the \textit{icc3-1} gene by N-terminal sequencing.

\textbf{Laccase labelling}

Purified TvL III was dialysed against 0.1 M carbonate buffers in a 2 ml Amicon with a YM 10 Diaflo ultrafiltration membrane, to remove ammonium ions that would interfere with the labelling and to raise the pH to facilitate the labelling. The pH was stepped from 4.0 to 7.0 in steps of one pH unit and the laccase was finally concentrated to ca 12 mg ml\textsuperscript{-1}. In a darkened container, 110 µl of the concentrated laccase solution was mixed with 40 µl of 16.9 mM fluorescein-5-EX, succinimidyl ester (F5EX, Molecular Probes) in DMSO and the mixture was stirred at room temperature for 1 h. To remove non-covalently bound dye molecules, 10 µl of aqueous hydroxyl amine (0.2 mg ml\textsuperscript{-1}, Fisher) was added, and the mixture was incubated for another hour at room temperature. The labelled laccase was separated from the unreacted dye using a PD-10 desalting column (Amersham Biosciences, contains Sephadex G-25) with 20 mM phosphate buffer (pH 7.4) as the eluent. The degree of labelling was determined spectrophotometrically based on absorption at 280 nm and the maximum visible absorption for the dye.

\textbf{Notes and references}