*Analytical Methods*

# Optimization Of "Wired" Enzyme $O_2$-Electroreduction Catalyst Compositions by Scanning Electrochemical Microscopy**

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"Wired" enzyme electrodes, comprising enzymes connected electrically through redox polymers, which swell to give electron-conducting hydrogels,[1] are already used in experimental subcutaneously implanted electrodes for the continuous monitoring of glucose in diabetics and may be used in future miniature, microwatt producing, membrane-less biofuel cells.[2] The subcutaneously implanted electrodes comprise, in addition to the "wired" enzyme film that transduces the glucose flux to a current, a glucose flux-controlling membrane, which defines the measurable glucose concentration range, and optionally, a bioinert film.[3] The membrane-less miniature biofuel cells comprise a "wired" glucose oxidase anode, and a "wired" bilirubin oxidase or laccase cathode. Optimizing the performance of these devices requires synthesis and characterization of new redox polymers,[4,5] defining the optimal enzyme–cross-linker–redox-polymer compositions,[6–9] and synthesis and optimization of flux controlling and bioinert membranes. A key parameter, which is usually explored first, is the polymer/enzyme ratio. An excessive enzyme weight fraction can decrease the current density, because the enzyme, unlike the redox polymer, is an electronic insulator.[10] Furthermore, if the enzyme weight fraction is high enough for the net negative charge of glucose oxidase to balance the positive charge of the redox polymer, an electrostatic adduct that shows poor electronic conductivity precipitates.[6,7] Moreover, when the weight fraction of the redox polymer is excessive, the flux of electrons is reduced, because of the smaller number of enzyme molecules.[11]

The effort and material expended in the optimization process[6,7,11] can be reduced by combinatorial screening techniques[12] exemplified by optimization through scanning electrochemical microscopy (SECM),[13] a technique that has been extensively used for evaluating the activity of enzymatic systems[14,15] and whose utility is demonstrated herein. Because only optimization of an exemplary parameter, the weight fraction of the multicopper oxidase bilirubin oxidase (BOD) or laccase, of a biofuel cell cathode was performed, the study does not provide as yet a fully optimized electrode, for which additional parameters, including the total loading and the weight % of the cross-linker, would have to be defined, along with the thickness of the electrode. The results obtained establish, nevertheless, that SECM screening of compositionally varying arrays of μm-size spot electrodes yields results identical with those obtained with discreet rotating disk electrodes, but much more efficiently in terms of speed and material required.

Ternary mixtures of enzyme, cross-linker, and redox polymer, with compositions ranging from pure enzyme to pure polymer, were prepared from aqueous solutions of BOD from *Trachyderma tsunodae* (8 mg mL$^{-1}$ in pH 7.2 20-mM phosphate buffer) or laccase from *Coriolus hirsutus* (8.6 mg mL$^{-1}$ in pH 5.0 20-mM citrate buffer), and PAA-PVI-[Os(4,4′-dichloro-2,2′-bipyridine)$_2$Cl]$^{1+/2+}$ (8 mg mL$^{-1}$) or PAA-PVI-[Os(tpy)(dme-bpy)Cl]$^{1+/2+}$ (8.6 mg mL$^{-1}$), respectively (PAA = poly(1-carboxy-1,2-ethanediyl, PVI = poly(1-imidazolyl-1,2-ethanediyl, tpy = 2,2′:6′,2″-terpyridine, dme-bpy = 4,4′-dimethyl-2′,2-bipyridine). A solution of polyethylene glycol diglycidyl ether at 2 mg mL$^{-1}$ (PEDGE, Polysciences Inc.) was used as the cross-linker. Arrays of spots containing these mixtures were deposited on glassy carbon (GC) plates 15 × 15 × 1 mm (Alfa) by using a piezo-based micro-arrayer, a device similar to that used by Schuhmann and co-workers for micro-patterning of enzymatic biosensors.[15] A commercial piezo-dispenser MicroJet AB-01-60 (MicroFab) with an outlet aperture of 60 μm, that dispenses on-demand picoliter-sized (≈ 100 pL) droplets by application of potential pulses (50 V, 25 μs), was installed onto the head of a digital plotter (Houston Instruments DMP-5) to control its position with a resolution of 100 μm/step. Figure 1 shows the preparation scheme. The dispenser was filled with polymer solution (3 μL), which was dispensed in a programmed number of drops at each site. To test for reproducibility, each composition was prepared in duplicate or triplicate. Thus, the arrays contained 11 rows and two columns of spots. Typically, the first two-spot row contained 10 drops per spot (pure polymer), the next row 9 drops per spot and so on down to and the tenth row with 1 drop per spot. The dispenser was

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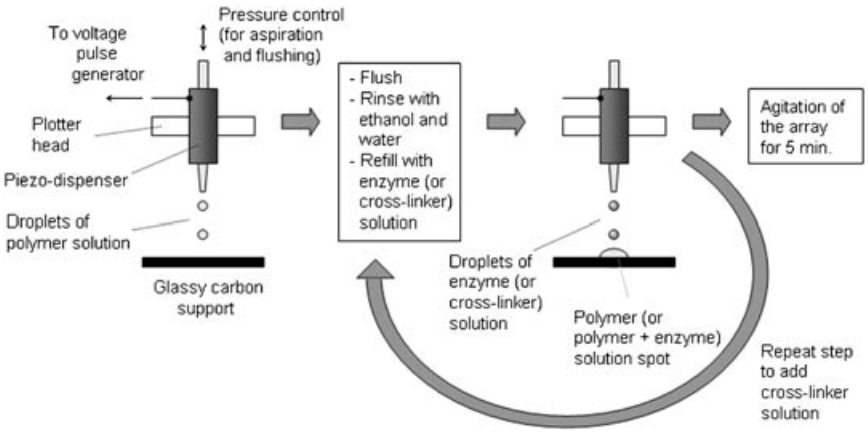


***Figure 1.*** Schematic diagram of the preparation of the wired enzyme spot arrays.

emptied, thoroughly rinsed first with ethanol and then with water, and refilled with 3 μL of enzyme solution. Then the plotter positioned the dispenser exactly over the previously prepared polymer spots, and a number of drops of enzyme solution, sufficient for the sum of the enzyme drops and the polymer drops to always equal 10, were dispensed. About 80% of the polymer or enzyme solution used to fill the dispenser can be recovered. After polymer and enzyme were deposited, 3 drops of cross-linker solution were dispensed on each spot. The substrate was maintained under a water-saturated $N_2$ stream during the complete preparation procedure to avoid the premature evaporation of the spots. To mix the components, the array was then agitated under the water-saturated $N_2$ stream for 5 min in a Vortex Genie 2 agitator (Fisher), and the array was dried overnight in ambient air. Figure 2 shows an SEM picture of typical arrays and an optical micrograph of an individual spot. The center-to-center distance between the 150–200 μm diameter spots was about 400 μm. Before testing, the arrays were washed with Milli-Q water. The thickness of the spots, estimated by negative feedback mode SECM[13] by using $O_2$ reduction on Au tips, over a spot and then over the neighboring GC, was 4–5 μm. The weight percent of enzyme (wt.%) at each spot was calculated from the number of drops (or volume fraction) and the concentrations of the polymer, enzyme, and cross-linker solutions used. The wt.% of the cross-linker was fixed at 6.9 for the BOD electrodes and at 6.5 for the laccase electrodes.

The SECM tip generation-substrate collection mode was used to image activity of the arrays.[16] This mode of operation is well suited for imaging activity of surfaces with morphological features since it is relatively insensitive to changes of the tip–substrate distance.[17] SECM images of $O_2$ reduction activity of the "wired" BOD arrays were obtained in 0.2 M pH 7.2 phosphate buffer, and of the "wired" laccase, in 0.2 M pH 5.0 citrate buffer, as previously described.[16] Briefly, a 25 μm Pt tip situated at 40 μm from the GC surface was scanned in the *xy* plane (parallel to spot rows) at step intervals of 50 μm every 0.2 s while electrogenerating $O_2$ from $H_2O$ at a constant current. Under these conditions, areas of $5 \times 2$ mm can be screened in about 1 h using the SECM (CH Instruments model 900B) with stepper-motor translators. The substrate array potential ($E_S$) was held at 0.3 and 0.4 V versus Ag/AgCl (3 M KCl) for BOD and laccase, respectively, where the reduction of $O_2$ was diffusion controlled.[6,7] The substrate current ($i_S$), measured as a function of tip position to produce the SECM image, was larger when the $O_2$-generating tip passed over a more active spot. Thus, the measure of the electrocatalytic activity of any of the spot electrodes was the magnitude of $i_S$. Figure 3 presents SECM color-map images obtained for the BOD and the laccase arrays. To establish reproducibility, triplicate experiments were performed. Because of the time-dependent processes, such as diffusion of $O_2$ into the hydrogel and charge transport through the film, the response times were slower than for metallic electrodes, and individual spots were not well resolved in the direction of the scan ($x$). As a result, the rows of the spots have a smeared, band-like appearance, particularly for the BOD films. Slower scanning can overcome the smearing, at the cost of increased imaging time.

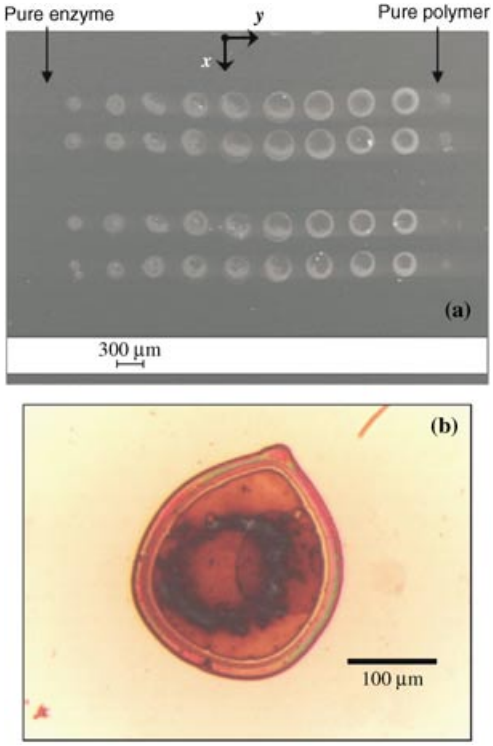


***Figure 2.*** a) SEM photograph of two arrays of redox-polymer–laccase spots made with 6.5 wt.% cross-linker. Each two-dot row corresponds to a particular composition. b) Optical micrograph of the 46.5 wt.% enzyme, 46.6 wt.% polymer, 6.9 wt.% cross-linker "wired" BOD spot, prepared by mixing the component solutions in situ.

In the BOD arrays (Figure 3a), $O_2$ electroreduction was seen for a 20–80 wt.% BOD range. The largest oxygen reduction reaction (ORR) current (close to the highest expected) was for the 46.5 wt.% BOD spots. With the laccase arrays (Figure 3b), $O_2$ electroreduction was observed for a 15–70 laccase wt.% range, with a maximum at 46.7 wt.%

  

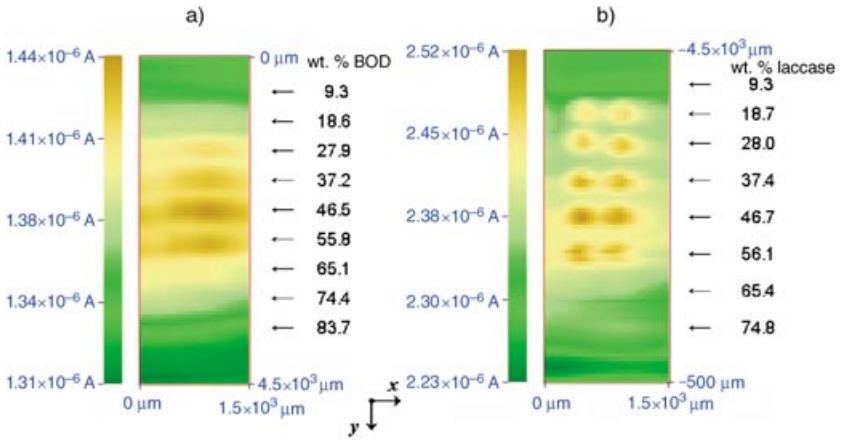


***Figure 3.*** SECM images of "wired" enzyme arrays containing spots with different polymer/enzyme ratios. a) BOD (6.9 wt.% cross-linker) in pH 7.2 phosphate buffer, $i_T = -161$ nA ($i_T$ = tip current), $E_S = 0.3$ V versus Ag/AgCl; b) laccase (6.5 wt.% cross-linker) in pH 5.0 citrate buffer, $i_T = -240$ nA, $E_S = 0.4$ V versus Ag/AgCl.

laccase. Figure 4 (solid symbols) summarizes the normalized ORR currents measured for each row as a function of enzyme wt.%. Each point is the average of three values measured from images obtained on three different arrays prepared under identical conditions.

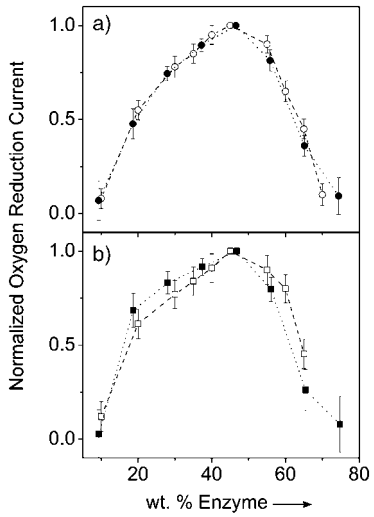


***Figure 4.*** Dependence of the oxygen reduction current (normalized with respect to the highest current) on the enzyme wt.% for a) BOD in pH 7.2 phosphate buffer and for b) laccase in pH 5.0 citrate buffer. Solid symbols = results of the SECM screening, open symbols = results of the rotating disc experiments. 1 mV s$^{-1}$, 1000 rpm, 1 atm $O_2$, total bioelectrocatalyst loading, 0.26 mg cm$^{-2}$.

To test the validity of the method, "wired" BOD and "wired" laccase GC rotating-disk electrodes were prepared, as described elsewhere, with different enzyme–polymer ratios.[6,7] Polarization curves were measured by slow potentiodynamic scans (1 mV s$^{-1}$) in $O_2$-saturated (1 atm) solutions at 1000 rpm in physiological buffer for BOD and in 0.2 M pH 5 citrate buffer for laccase. $O_2$-electroreduction limiting currents were measured five times on each composition for BOD and seven times on laccase. The mean values are plotted in Figure 4 (open symbols). The results obtained by the two methods were in good agreement. Sample preparation and testing by the SECM screening method required one day, while the preparation and testing of a rotating disk electrode required at least 10 days. The amounts of polymer and enzyme were reduced by a factor of 100000 in the SECM test.

In conclusion, SECM screening of oxygen electroreduction activity on arrays of "wired" enzyme electrode spots, allows the optimization of a single-variable more rapidly and with less material than optimization utilizing discreet rotating disk electrodes. In the next phase, all parameters of the ORR films on carbon, including total loading, thickness, and weight% of the cross-linker will be optimized.

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