Electrochemistry of microencapsulated laccase immobilized on electrodes

D. Rochefort*, L. Kouisni and K. Gendron
Département de chimie, Université de Montréal, Montréal, Québec, Canada
(dominic.rochefort@umontreal.ca)

Introduction

Redox enzymes are powerful catalysts used to circumvent the fundamental limitations of electrochemical processes such as the lack of selectivity and poor catalytic properties of most bare electrodes. In order to use such enzymes as efficient electrocatalysts, the enzyme must be placed on the electrode surface. Although immobilized enzymes have been used for decades, the immobilization of redox enzymes on electrodes is not straightforward and must meet specific criteria: i) the enzyme must be physically retained on the electrode, along with other reagents, to avoid leaching away from the surface; ii) the immobilization method must maintain enzyme activity; iii) the enzyme must be stable over time; and iv) immobilization of the enzyme must allow rapid electron transfer with the electrode. Enzymes were designed by nature to work in specific environments such as living cells and biological fluids rather than on the surface of an electrode. Immobilizing enzymes in a way that meets these four primary criteria represents a challenge that has stimulated research in this field since 1962, with the first reported enzyme electrode.

Current methods of enzyme immobilization on electrode surfaces include covalent binding (Figure 1, left-most scheme), entrapment in polymer matrices (Figure 1, center scheme), sol-gels of silica or other materials, and entrapment by membranes, either macroscopic dialysis membranes or bilayer lipid membranes. Despite recent developments in each of these methods, a strong need remains to improve redox enzyme immobilization techniques to overcome the limitations of current methods and to stimulate the application of bioelectrocatalytic systems in the future. We therefore focussed our research on microencapsulation which is an underexploited method to incorporate enzymes on electrode surfaces.

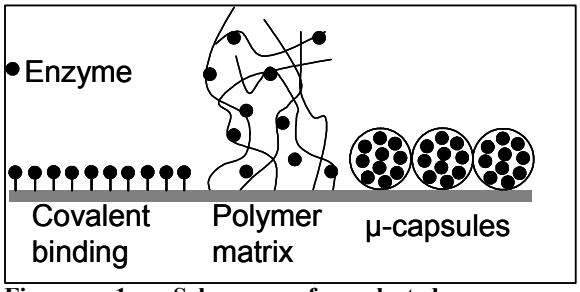

Figure 1. Scheme of selected enzyme immobilization methods.

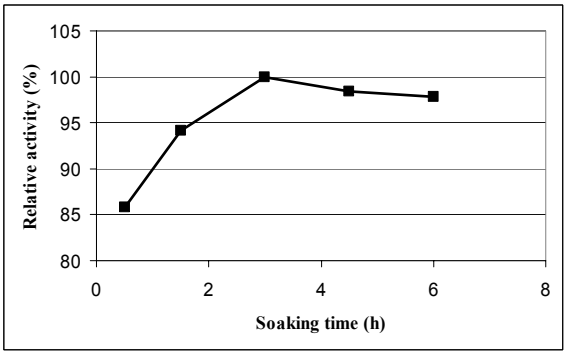

Figure 2. Hydration effect on activity of lyophilized laccase microcapsules.

Microencapsulation is under study in our group as an enzyme immobilization platform on electrodes for bioelectrocatalytic systems like biosensors and biofuel cells. We believe that microencapsulation could be a suitable immobilization method and provide strong physical retention of the enzyme at a high density, retention of enzymatic activity and achievement of an efficient electron transfer. This contribution presents the research that was carried out to study the mediated electron transfer between an encapsulated enzyme and an electrode. Microencapsulation is known to be an efficient mean to achieve stabilisation and immobilization of enzymes on inert supports for heterogeneous catalysis (Cosnier 2000, Parthasarathy 1994). The achievement of an

electron transfer between an electrode and microencapsulated redox enzymes is however not straightforward and must be studied in details. Our results demonstrate the applicability of microencapsulation as an efficient mean to immobilize redox enzymes on the surface of electrodes.

Material and methods

Laccase (E.C. 1.10.3.2) was produced from a fungal source (*T. versicolor*) and purified accordingly to a procedure published before (Bourbonnais 1995). Laccase is a blue multicopper oxidase that catalyses the oxidation of a broad range of substrates (mostly phenolic compounds) with the concomitant four-electron reduction of O_2 to H_2O. Laccase was selected for our initial studies for its well characterized reactions and its possible application as cathodic biocatalyst in biofuel cells. Poly(ethyleneimine) (PEI) microcapsules containing laccase were prepared by interfacial cross-linking of the polymer by sebacoyl chloride in an aqueous microemulsion in cyclohexane, accordingly to a published procedure (Poncelet 1994). The preparation yielded microcapsules with a large size distribution, the average diameter being 30 μm. In some cases, the laccase microcapsules were lyophilized to improve the enzyme stability for longer storage periods. In this contribution, the laccase substrates are designed as mediators to emphasize their role of electron carrier in the system under study. We almost exclusively used paraphenylenediamine (PPD) as the mediator. Our electrochemical measurements were conducted in a conventional three-electrode cell, using a glassy carbon working electrode, an Ag/AgCl reference, and a Pt wire as the auxiliary electrode. The solutions were stirred during the potentiostatic experiments (described later) and were left open to atmosphere. The potentiostat was an Epsilon from Bioanalytical Systems.

Results and Discussion

The microencapsulated laccase activity was firstly studied in solution with the PPD mediator before moving on to the immobilization step. To do so, an amount of microcapsules was carefully suspended in a 50 mM phosphate buffer, pH 5. This pH value was initially selected to match the optimal pH for the PPD oxidation by free (not encapsulated) laccase. Upon addition of the PPD substrate, the increase in absorbance with time was monitored at 520 nm and the activity was calculated from the slope obatined. It has to be noted at that point that spectrophotometry provides only a rough analysis of microencapsulated laccase activity due to the turbidity of the suspension. The increase in absorbance could nevertheless be followed and yielded sufficiently reproducible results to express the activity on a relative scale. The graph in Figure 2 presents the relative activities of lyophilized laccase in PEI microcapsules after various rehydration periods during which the capsules were suspended in the buffer. Freeze-dried capsules were inactive towards PPD without a rehydration step and the activity measured reached a plateau after 3 hours. Therefore, all lyophilized capsules were let to stand in the buffer solution for this time lap before doing any further measurements.

Next, we studied the effect of pH on the activity of microencapsulated laccase. The pH-dependency of the activity exhibited a bell-shaped curve (see Figure 3) typical of most laccase-based systems, with a maximum value at pH 5.75. In comparison, the PPD oxidation catalyzed by free laccase reaches a maximum rate at pH 5 (Quan 2004). Such shifts in optimal pH are common for enzymes that are embedded in polymers and have been ascribed, at least in part, to a very different enzyme ionic environment from that in free solution (Yinghui 2002). If we apply this logic to our system, this could mean that the laccase sits not in the aqueous core of the capsules but close to or even in the PEI capsule wall. To clarify this point, we have recently undertaken fluorescence microscopy work to locate the enzyme in the microcapsules (results not available yet). However, the proton-exchange capabilities of the PEI must also be taken into account. The fraction of

protonated nitrogens in the PEI changes from 50% at pH 5 to 30% at pH 6 (Suh 1994). Protonation of PEI becomes more difficult as the pH is lowered, due to increased electrostatic repulsions with the increase in protonation level. This could yield to a pH for the solution in the capsules that is different from the "bulk" pH value and explain the 0.75 pH unit shift for optimal activity.

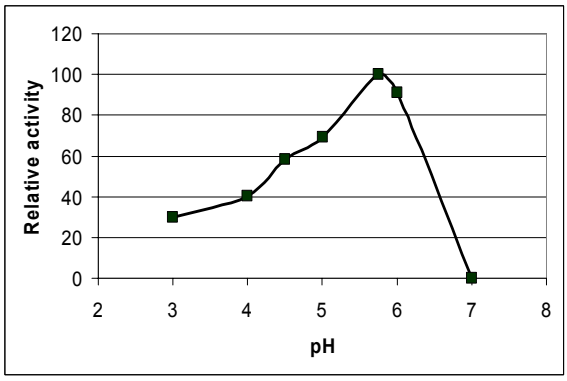

Figure 3. Activity profile of laccase in PEI microcapsules.

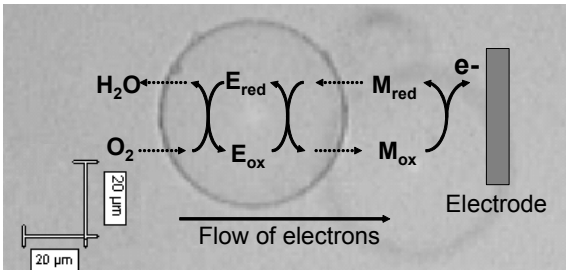

Figure 4. Kinetic scheme of electron transfer with encapsulated laccase (E) using a redox mediator (M).

We then moved to the electrochemical characterization of laccase microcapsules. On Figure 4 is depicted the reaction scheme for the laccase (E) oxidation of the mediator (M) and its subsequent reduction at the electrode. This scheme is superimposed over an actual image of a PEI microcapsule. As mentioned earlier, as long as there is oxygen available to laccase in the solution, the mediator will be oxidized by the enzyme and then be subsequently reduced at the electrode upon application of an appropriate potential. The potentiostatic study was therefore done by poising the glassy carbon working electrode at + 0.085 V (vs. Ag/AgCl), a potential at which the reduction of PPD is mass-transport limited. This means that all oxidized mediator molecules reaching the electrode surface will be reduced to generate a current proportional to the bulk concentration of oxidized PPD, which is in turn affected by the laccase catalytic properties towards the mediator. The chronoamperometric response of free laccase obtained for successive additions of PPD mediator (in 1 µM concentration increments) is presented in Figure 5. From such curve it is possible to determine a response time which represents the delay of the system to reach a steady state after the perturbation brought by the addition of an aliquot of the mediator solution in its reduced form. With free laccase, the response time, which we defined as the time to reach 95% of the current at steady state, is about 10 seconds. The red trace in Figure 6 is the response obtained for a suspension of the laccase microcapsules in a buffer solution, using 5 µM PPD increments. The first observation to be made is the much slower response of the encapsulated laccase (about 80 s), which could be due to a slow diffusion of the mediator in and out of the capsules (from size or electrostatic hindrance of the capsule wall) or to a much lower laccase amount present in the suspension vs. in free solution. The interactions with the capsule wall that would slow the mediator diffusion could originate from size (if the pore size is too small and/or mediator is too large) or from electrostatic interactions. When oxidized, PPD forms a radical cation and its diffusion out of the capsule could be hindered by electrostatic repulsions with the positively charged PEI. The occurrence of interactions between the mediator and the PEI microcapsules was evidenced with the use of two anionic mediators ABTS (2^-) and octacyanomolybdate (4^-). These mediators showed negligible oxidation rates with the encapsulated laccase, while they reacted promptly with free laccase (results not presented). Furthermore, the white PEI capsules became coloured in the presence of these anionic species, showing strong adsorption. Electrostatic interactions between PEI and PPD, which is neutral in its reduced state and positively charged in an oxidized state, does not seem to affect the system as much, based on the occurrence of a stable signal proportional to mediator concentration and a visual inspection of the suspension.

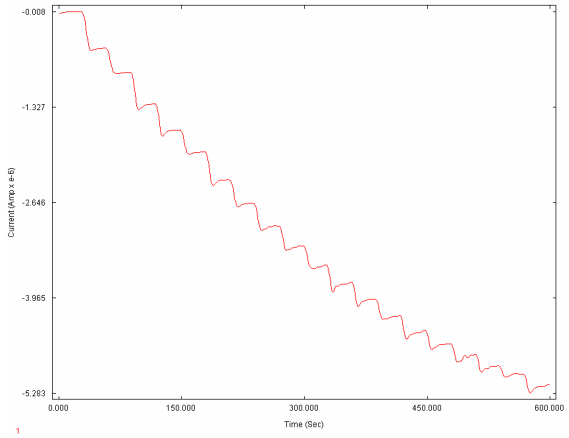

Figure 5. Current response of free laccase to successive additions of PPD (1 µM increments). See text for conditions.

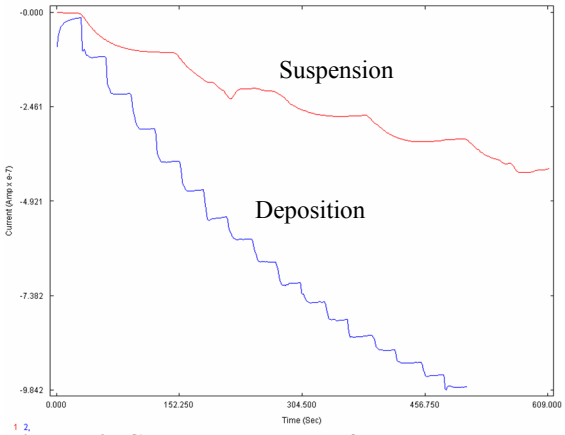

Figure 6. Current response of PEI-encapsulated laccase, either as a suspension (red) or adsorbed on the electrode (blue) to successive additions of PPD (5 µM increments). See text for conditions.

The last set of results to be presented here was obtained with the laccase microcapsules adsorbed on the electrode surface (Figure 6, blue trace). The current response obtained with adsorbed capsules is much faster (t_R = 10 s) compared to that for the capsules suspension (t_R = 80 s), even if the total amount of encapsulated laccase was 100 times superior in the latter. The adsorption of capsules on the electrode results in a very high density of bioactive molecule by formation of a dense film at its surface (SEM imaging, not shown). Clearly, this high density provides an efficient enzyme immobilization and fast electron transfer (ET), a prerequisite to design efficient enzyme electrodes for biosensors. This demonstration of mediated ET is another indication that microencapsulation could be a valuable method for enzyme immobilization on electrodes.

Conclusions

We reported on the activity of laccase in PEI microcapsules using electrochemistry to characterize the mediated electron transfer occurring between the enzyme and an electrode. While a suspension of the capsules shows a slower overall ET, adsorbing the capsules directly on an electrode resulted in a much faster transfer, giving a response time close to that of free laccase. These results suggest that microencapsulation should be considered to immobilize oxidases for the development of bioelectrocatalytic systems.

References

R. Bourbonnais et al. (1995) *Lignin oxidation by laccase isozymes from Trametes versicolor and role of the mediator 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization.* Appl. Environ. Microbiol. **61** 1876.
S. Cosnier et al. (2000) *A rapid and easy procedure of biosensor fabrication by microencapsulation of enzyme in hydrophilic synthetic latex films. Application to the amperometric determination of glucose.* Electrochem. Comm. **2** (12) 851.
R. V. Parthasarathy et al. (1994) *Synthesis of polymeric microcapsule arrays and their use for enzyme immobilization.* Nature **369** (6478) 298.
D. Poncelet et al. (1994) *Microencapsulation within crosslinked polyethyleneimine membranes.* J. Microencapsulation **11** (1) 31.
D. Quan et al. (2004) *Characterization of an amperometric laccase electrode covalently immobilized on platinum surface.* J. Electroanal. Chem. **561** (1-2) 181.
J. Suh et al. (1994) *Ionization of poly(ethylenimine) and poly(allylamine) at various pHs.* Bioorg. Chem. **22** (3) 318.
D. Yinghui et al. (2002) *Laccase stabilization by covalent binding immobilization on activated polyvinyl alcohol carrier.* Lett. Appl. Microbiol. **35** (6) 451.