Stability and activity of laccase and glucose oxidase in PEI microcapsules

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Introduction

Enzyme immobilization continues to hold significant promises for a broad range of applications. Of these numerous fields, research on biosensors is one of the most actively involved in developing new techniques for enzyme immobilization because only major improvements in the actual techniques will allow future applications of biosensors to be seen.Kissinger (2005, Luong (2008) Recent developments in biosensors also show the need to spend effort to apply enzyme immobilization techniques on non-conventional support like plastics and papers. The impetus for such orientation is the application of biosensors to a large public and to regions with difficult or inexistent access to advanced measurement technologies. As a result, we and others have carried out research on bioactive papers and paper-based biosensors.Kouisni (2009, Martinez (2008, Su (2008, Zhao (2008) Paper is already widely distributed and found uses in numerous hygiene, personal protection, and packaging products, offering an unequalled potential to broaden the application of biosensors. Modifying papers with enzymes could allow for example to monitor glucose or protein levels in urine(Martinez (2008)) and to assess water quality or to develop active packaging to enhance preservation.Fernandez (2008, Nestorson (2008) These appealing applications require novel enzyme immobilization techniques that will address the problem of keeping enzyme stability while being compatible with current paper making processes for larger, industrial-scale production.

We have oriented our effort on developing an enzyme immobilization based on microencapsulation which consists in the entrapment of biomolecules in the core of hollow spheres made by a semi-permeable membrane, providing a chemically and mechanically robust system. Once prepared, the capsules can be easily incorporated into or onto paper sheets by simple mixing with pulp or by deposition of the surface of the sheet.Kouisni (2009) Microcapsules can also be coated or printed on paper to make it compatible with current high-end papermaking technologies. In this contribution, poly(ethyleneimine) (PEI) is studied as wall material to produce microcapsules containing hydrophilic cores. With respect to the development of bioactive papers, PEI also has the advantage of being already applied in paper coating formulas as a retention aid agent for negatively charged particles.Vanerek (2000) We have recently demonstrated that the microencapsulation technique based on the interfacial polycondensation of PEI offers a high entrapment efficiency (89 to 96%) for proteins and enzymes with size and charge similar to those of Bovine Serum Albumine (BSA). These values however provide no information on the activity of the microencapsulated enzymes which can be affected either by the microcapsules themselves (unfavourable interactions with membrane material), by the microencapsulation procedure (deactivation by the chemical used) or by a combination of both. Immobilization has been reported in several occasions to hinder enzymatic activity. We have therefore carried out a series of experiments to determine the enzymatic activity upon encapsulation of two selected enzymes; Laccase (Lc) and Glucose oxidase (GOx). These enzymes were selected for their utility in the development of biosensors and biofuel cells and because of their different active site structure. Lc is a blue copper enzyme which catalyses the oxidation of phenolic compounds with the concomitant oxygen reduction to water at copper-based active sites. GOx on the other hand does not possess any metal atoms and achieve the glucose

oxidation and O_2 reduction to hydrogen peroxide at its FAD cofactor–based active site. This difference in the nature of active site will be showed to have a great importance in the interaction between the enzyme and the microcapsule membrane material and consequently on the encapsulated enzyme activity.

Materials and methods

PEI microcapsules were prepared based on a procedure published by Poncelet (1994). Polyethyleneimine (PEI, Mw = 1300 Da; 50 wt% soln. in water), sebacoyl chloride (SC), cyclohexane, Span 85, Bovine serum albumin (BSA), Lc (EC 1.10.3.2, 22.4 $U.mg^{-1}$), *p*-phenylenediamine (PPD), and D-(+)-Glucose were purchased from Sigma-Aldrich. Other salts used for buffer preparation were purchased from A&C American Chemicals Ltd. Glucose oxidase from Aspergillus niger (GOx, E.C. 1.1.3.4, specific activity @25 ^{o}C >110$U.mg^{-1}$) was obtained from Amersco, Water was purified with a Milli-Q purification system to a specific resistance of 18 $M\Omega \cdot cm^{-1}$. Encapsulated Lc and GOx activity were assayed by monitoring the O_2 consumption rate by the enzyme in the presence of a substrate (*p*-phenylenediamine and glucose, respectively) using an oxygen cell. To study the location of protein in the capsules, a confocal laser scanning microscope (CLSM) was used. The signals from proteins and capsules were easily differentiated by tagging BSA and PEI respectively with sulforhodamine (Texas Red, λ_{em} = 520 nm) and fluorescein isothiocyanate (FITC, λ_{em} = 615 nm). An Ar (488 nm) and a DPSS (561 nm) laser were used for the excitation of the fluorophores.

Results and discussion

Poly(ethyleneimine) (PEI) microcapsules were prepared by the interfacial condensation of the polymer with sebacoyl chloride either by an emulsion approach or using an Inotech Encapsulator device and their characterisation reported earlier (Kouisni (2009)). Protein distribution in the capsule was studied via the conjugation reaction of BSA and PEI with Texas red and FITC respectively. Microcapsules were then prepared via interfacial condensation by crosslinking the FITC-modified PEI with sebacoyl chloride as presented above. The capsules were then analysed by CLSM by following Z-stack acquisition from the center to the top pole of the capsules. Figure 1 shows the presence of red signal, which reveals the presence of BSA, at a high density in the capsules wall where it is superimposed with the green signal related to the FITC-modified PEI. The encapsulated enzymes are therefore in close contact with the PEI membrane of the capsules which can be affecting its activity. We measured the activity of microencapsulated Lc and GOx with an oxygen cell to find that in both cases, only 35% of the initial specific activity remained. Kinetics of the enzymatic were studied for both enzymes. K_M value for laccase (PPD substrate) decreased from 400 to 65 µM between the free and encapsulated enzyme. For GOx, using glucose as the substrate, K_M decreased from 19 to 13 mM (Figure 2). V_{max} also decreased upon encapsulation as expected because of the diffusion of the substrate across the membrane of the capsules (see Rochefort (2008)). We therefore carried out experiments to study the causes of enzymatic activity decrease. We firstly demonstrated that PEI itself had a detrimental effect on the activity of free laccase by following its activity during the various steps of the microencapsulation procedure. Upon mixing of the PEI (5%) in the preparation of the aqueous phase to be encapsulated, a 78% decrease in Lc activity was observed. GOx lost no activity at this point. The emulsification of the aqueous phase in cyclohexane did not affect the activity of either enzyme. The interaction between laccase and PEI was therefore studied in more details. Figure 3 presents the native fluorescence curves of GOx (a) and Lc (b) for the free enzymes in presence and absence of 5% PEI. While the fluorescence signal

for GOx was, as its activity, unaltered by PEI, the signal for laccase was severely affected by the polymer, suggesting the existence of a strong interaction between PEI and the protein. PEI is a moderately strong binding agent that has been used on many occasions to complex metal ions like Cu. The interaction of PEI and the Cu-based active site of laccase is also suggested by the strong color change of a concentrated Lc solution which went from its famous blue color to a yellow upon addition of the polymer (see insets of Figure 3). The activity decrease of glucose oxidase upon oxidation cannot be explained by an interaction with PEI. We propose that diffusion restrictions of the substrate to the enzyme located within the microcapsule walls are leading to lower activity measurements.

The cross-linking of the proteins within the walls of the capsules has the advantage of increasing the enzyme thermal stability. The half-life of GOx at 60°C increased from 36 to about 3h between the free and encapsulated enzyme. Oppositely, the encapsulation decrease the thermal stability of Lc and the microencapsulated enzyme lost 100% of its initial activity after only 10 minutes of incubation at 60°C. Free laccase activity decreased from only 25% after 1h at this temperature. Again here, the strong interaction between PEI and copper active site of laccase are responsible for the activity loss.

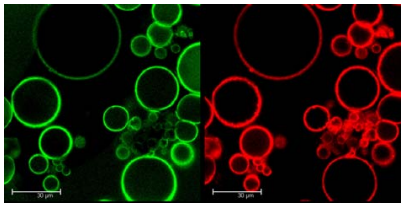

Figure 1. CLSM analysis of the protein location in the PEI microcapsules. The green signal is attributed to FITC-modified PEI, while red signal indicates BSA. Bottom image is a combination of both signals.

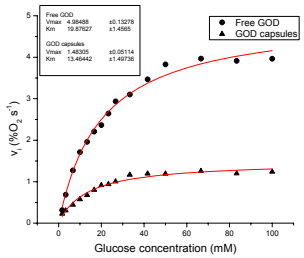

Figure 2. Michaelis-Menten plots comparing the reaction rate (%O_2/s) of free and encapsulated glucose oxidase.

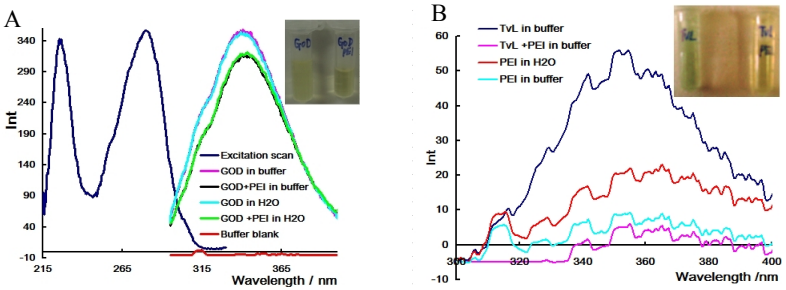

Figure 3. Fluorescence analysis of the conformation of Glucose Oxidase (a) and Laccase (b) in the presence and absence of poly(ethyleneimine). Insets show that a strong color change is only observed for Laccase upon addition of PEI.

Conclusions

The current work demonstrated the comparison of enzymatic activity of free and microencapsulated Lc and GOx, and discussed the factors that caused the loss of enzymatic activity for interfacial polymerization method. The reduction of enzymatic activity can be explained by many factors, which are dependant on the interactions between the enzyme and the polymer making the membrane. In general, diffusion through these closed shells with pores <5 nm is often a slow process. For entrapped GOx, the diffusion limitation is the key factor that led to the reduction of enzymatic activity and the mixture of GOx and PEI doesn't show significant changing of enzymatic activity. However, PEI molecules could change the conformation of Lc and decrease its enzymatic activity. In addition, the enzyme molecules do not necessarily exist as free form in the membrane and a large portion of the molecules participated in the chemical crosslinking reaction which then changed their enzymatic properties. Microencapsulation improved thermal stability of GOx, but decreased the thermal stability of Lc, since the heating accelerated the rate at which coordination between copper center in Lc molecules and nitrogen atoms in PEI molecules occurs.

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