Confocal Microscopy Study of Polymer Microcapsules for Enzyme Immobilisation in Paper Substrates

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Received 21 March 2008; accepted 23 July 2008
DOI 10.1002/app.28997
Published online 26 September 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: The goal of this research is to develop the technology platform required for the production of bioactive paper based on enzymes as bioactive agents. The immobilization platform described here is based on microencapsulation, which consists in the entrapment of biomolecules in the core of hollow spheres made by a semipermeable membrane. The capsules containing the enzymes can be either deposited on paper or mixed with paper pulp to prepare a bioactive paper. The activity of encapsulated laccase was compared with that of free enzyme using its reaction with the *o*-phenylenediamine (OPD) substrate. Confocal Laser Scanning Microscopy (CLSM) is used to study the location of protein in microcapsules and provides explanations for differences in activity of encapsulated laccase. The location of protein in microcapsules was determined using BSA modified with the fluorescent tag sulforhodamine. Polyethyleneimine microcapsules were modified with fluorescein isothiocyanate allowing the simultaneous identification of capsule walls and of encapsulated proteins. From CLSM analysis, proteins were found to favor the wall of the capsules because of strong ionic attraction with the charged polymer. BSA was found to some extent in the core of the capsules and encapsulation of higher loadings increased the proportion of core proteins. We will also present our results on the incorporation of microcapsules in a paper substrate. CLSM was used in this section to determine the distribution and density of tagged microcapsules in the paper substrate. The response of immobilized laccase to a common substrate will also be described. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 1–10, 2009

Key words: microencapsulation; polyethylenemine; laccase; confocal laser scanning microscopy; bioactive paper

INTRODUCTION

Bioactive paper is a paper prepared by the addition of an appropriate biomolecule to bring an extra functionality to the paper. Just like chemicals that change color upon changes in pH can be used to make pH paper, enzymes could be used to prepare towels or paper tissues of common uses to detect and/or deactivate some substrates of interest. Therefore, the choice of paper as substrate in this work is due to its large application, its availability, and its easiness of production. Furthermore, paper is a good support for biological catalysts due to its opened and porous structure, its chemical and biological inertness, and its compatibility with colorimetric signal generation. On the downside, if an industrial production should be considered, the immobilization method must be efficient to limit losses and to maximize the use of expensive biocatalysts. Developing an immobilization technique that meets these criteria will be vital to the application of bioactive papers.

Two kinds of indicator paper are found in the market which either contains chromogenic organic reagents or immobilized enzymes.[1–7] Enzymes could be advantageously the best biological element used to impart an increased functionality to paper used for applications like packaging, filtration and personnel protection for the detection and/or deactivation of harmful substances present in food or water. An essential requirement for the fabrication of stable and sensitive bioactive paper based on enzyme immobilization is the loading of high amounts of enzymes with complete retention of their specific activity. An extra parameter must be taken into account when developing an immobilization technique for sensing purposes because one must get a short response time that can be provided by a small barrier to mass transport of substrate inside the capsules. Because of their catalytic properties, small amounts of enzymes can degrade a high concentration of toxic substances. Free enzymes, however, cannot be immobilized directly on solid support such as paper, because they are small enough to leach out from the substrate. In addition, they are fragile and need a favorable environment to be active and are sensitive to the external environment

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Contract grant sponsor: NSERC (through the Sentinel research network).

which can cause protein denaturation. To overcome these shortcomings, several immobilization techniques like adsorption,[8] crosslinking reaction, covalent attachment to a support,[8–11] entrapment within a membrane, film or gel,[12–15] and micro-encapsulation,[16–18] could be used. These techniques, for the most part, have some application restrictions. The adsorption technique is limited because of the weak interaction between enzyme and the support which can lead to desorption by changes in the external environment (ionic strength and temperature).[18] By using crosslinking reactions and covalent attachment of enzyme,[8–11] such desorption problem can be solved. However, the amount of immobilized enzyme remains very small and the enzymes get only little protection from the external environment, bringing stability issues. Another technique that could be used is the physical entrapment within a membrane, film, or gel.[12–15] These physical immobilization techniques can increase the stability and the enzyme loadings.

Microencapsulation-based techniques allow immobilization of high amounts of enzyme with a good physical retention of enzymes on various substrates, preservation of the enzyme's activity upon immobilization and a good retention of this activity for a suitable period of time. Microencapsulation consists in the entrapment of biomolecules in the core of hollow spheres made by a membrane,[16–18] providing a chemically and mechanically robust system.[16] 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. Microcapsules can also be coated or printed on paper to make it compatible with current high-end papermaking technologies. Microencapsulation provides a fast diffusion of small molecules (substrates) through the membrane to satisfy the short response time criteria and can be used to immobilize various types of biomolecules on different types of support. Another advantage is the use of various kinds of polymer, and a good control on capsule size and on membrane pore size which can be modulated during preparation depending on the final use of the product.

In this work, microcapsules were prepared by interfacial reticulation of polyethyleneimine (PEI) using sebacoyl chloride as the crosslinking reagent. Laccase was used as model enzyme system to evaluate the effect of encapsulation on the enzymatic activity. OPD (*o*-phenylenediamine) and PPD (*p*-phenylenediamine) were selected as substrates to evaluate laccase activity. To carry out our fluorescence studies, bovine serum albumin (BSA) was used as a model protein to determine the location of the biomolecule in the microcapsules. In this particular case, BSA has been chosen instead of laccase because it is easy to tag and has similar characteristics compared to laccase in its molecular weight and isoelectric point (pI). For Confocal Laser Scanning Microscopic (CLSM) study, PEI was labeled with fluorescein isothiocyanate (FITC), and BSA was labeled with Texas red (Sulforhodamine 101 acid chloride). Then, labeled proteins were encapsulated in FITC-modified PEI membrane. The interaction between PEI and BSA and the effect of this interaction on protein distribution inside capsules is discussed in details. Modified PEI microcapsules were also used to determine the distribution of microcapsules within the paper substrate.

EXPERIMENTAL

Chemicals

Polyethyleneimine (PEI, $M_n = 1200$ g mol^{-1}; 50% in water), sebacoyl chloride (SC), cyclohexane, Span 80, *o*-phenylenediamine (OPD), *p*-phenylenediamine (PPD), Texas red (Sulforhodamine 101 acid chloride), and FITC (fluorescein isothiocyanate) were purchased from Sigma-Aldrich. All salts used for buffer preparation were from A and C American Chemicals. Deionized water (18$M\Omega$·cm) from a MilliQ water purification system was used for all solutions.

Laccase

Laccase II (E.C. 1.10.3.2) from Trametes Versicolor was produced and partially purified by ion-exchange chromatography on DEAE-Bio-Gel as described previously.[19] The encapsulated laccase activity was measured in 50 mM of McIlvaine buffer at pH 4.5 by using OPD as the substrate. In this case, absorbance increases were recorded at 420 nm ($\varepsilon_{420} = 19.6$ mM^{-1}cm^{-1}(pH = 4.5)).[20] The colorimetric response was studied by using PPD as substrate.

Preparation of PEI microcapsules

PEI microcapsules were prepared according to the procedure published by Poncelet et al.[21] In short, an aqueous phase consisting of PEI (5% w/w in water), laccase (2280 units, measured with ABTS), and milliQ water was emulsified by mechanical agitation in an organic phase of cyclohexane containing Span 80 as surfactant. Alternatively, microcapsules were prepared with BSA (0.2 mg/mL) for confocal microscopy studies for the reasons mentioned in the introduction. Sebacoyl chloride, dissolved in cyclohexane, was added to the emulsion and allowed to react for 10 min. An additional volume of cyclohexane was added to gradually stop the reaction. The suspension was then allowed to settle before discarding the supernatant. The microcapsules were

then rinsed with 50 mL of cyclohexane followed by 300 mL of water, recuperated by filtration and washed with three additional volumes of water.

Enzyme activity measurements by oxygen cell

Oxygen consumption by laccase in the presence of a substrate was monitored with a dissolved oxygen electrode apparatus (Rank Brothers). The water jacketed oxygen cell was thermostated to 27°C using a circulating bath. The O_2 concentration in the cell is measured by the current generated by the reduction of O_2 at a Pt electrode poised at a potential of -0.6 V (vs. Ag/AgCl). The cell was calibrated before each experiment by saturating the buffer solution containing the enzyme with air ($[O_2] = 7.95$ mg L^{-1} at 27°C).

CLSM

To study the location of protein in the capsules and the distribution of capsules in paper, a confocal laser scanning microscope (Leica TCS SP or Leica CLSM SP2) was used. The signals from proteins and capsules were easily differentiated by tagging BSA and PEI with sulforhodamine ($\lambda_{em} = 520$ nm) and fluorescein isothiocyanate ($\lambda_{em} = 615$ nm), respectively. An Ar (488 nm) and a DPSS (561 nm) laser were used for the excitation of the two fluorophores. In all CLSM images presented here, the red signal has been attributed to the proteins, whereas the green signal indicates the location of PEI (capsules).

To tag BSA with sulforhodamine 101 acid chloride (Texas red), 0.2 mg of dry Texas red was added directly to the BSA solution (2 mg/mL) and was allowed to react with the amino groups on proteins. To minimize the hydrolysis of the dye which can decrease coupling efficiency, the protein solution was kept on ice during the reaction time (4 h). Unbound Texas red was removed from protein by size exclusion chromatography on a Sephadex G-25 column, according to the procedure published by Titus et al.[22] Buffered saline solution containing 2% BSA was passed through the column, and the column was washed extensively with 0.02*M* Tris-buffered saline solution at pH 7.8. Labeled proteins were then applied to the column and eluted with 20 m*M* Tris-saline solution at pH 7.2.

To modify PEI with fluorescein isothiocyanate, FITC was solubilized in DMSO and PEI was dissolved in carbonate buffer 20 m*M* at pH 9.2. The FITC solution (25 m*M*) was then added to the PEI solution (25% w/w). Samples were protected from light, gently vortexed every 20 min for 2 h then allowed to react overnight at room temperature.

Handsheets preparation and colorimetric response

Capsules were mixed to dried bleached pulp and suspended in 1 L of deionized water before agitation. The suspension was poured in a handsheet maker, homogenized before quickly draining the water from the bottom of the apparatus. Handsheet was recovered, pressed and dried in a controlled humidity room. Samples of 2.7 cm of diameter were cut out from the handsheet and used to study the colorimetric response of encapsulated laccase immobilized in paper as function of time and substrate concentration. Three solutions of *p*-phenylenediamine (PPD) at different concentration (0.25, 0.5, and 1 m*M*) were added and the allowed to react with the laccase.

Sample preparation for confocal microscopy

For the paper analysis by CLSM, samples (1.8 × 0.4 cm) of paper containing FITC-modified microcapsules were impregnated in paraffin liquid for 16 h. They were embedded in a paraffin cassette and sectioned with a Leica model 2155 microtome. The 8-μm thick layers of the cross section of the paper were mounted on microscope slides and analyzed by CLSM to evaluate how the capsules were distributed across the sheet.

RESULTS AND DISCUSSION

Microencapsulation

Microcapsules were prepared as described in the experimental section. A fast interfacial condensation of the polymer was achieved with a diacid chloride. During the crosslinking reaction, primary and secondary amines on highly-branched poly(ethyleneimine) react with the acid group at each end of the sebacoyl (decanedioyl) chloride through nucleophilic substitution reaction to covalently links PEI chains together. The capsule is that way build up around the aqueous phase which contains PEI. BSA and laccase microcapsules are prepared by adding the desired protein in the aqueous phase, along with PEI. In Figure 1, we show an example of the microcapsules prepared without any proteins. The signal visible on the image comes from the poly(ethyleneimine) capsule walls that was modified by FITC before encapsulation procedure and allows us to measure the size and membrane thickness of the microcapsules. From microscopy, the microcapsules are showed to be spherical, with their size ranging from below 10 μm and up to 50 μm. When looking at one particular capsule, the focus of the microscope was varied until the diameter was at a maximum corresponding to the center of the sphere. Figure 2 shows a more precise evaluation of their

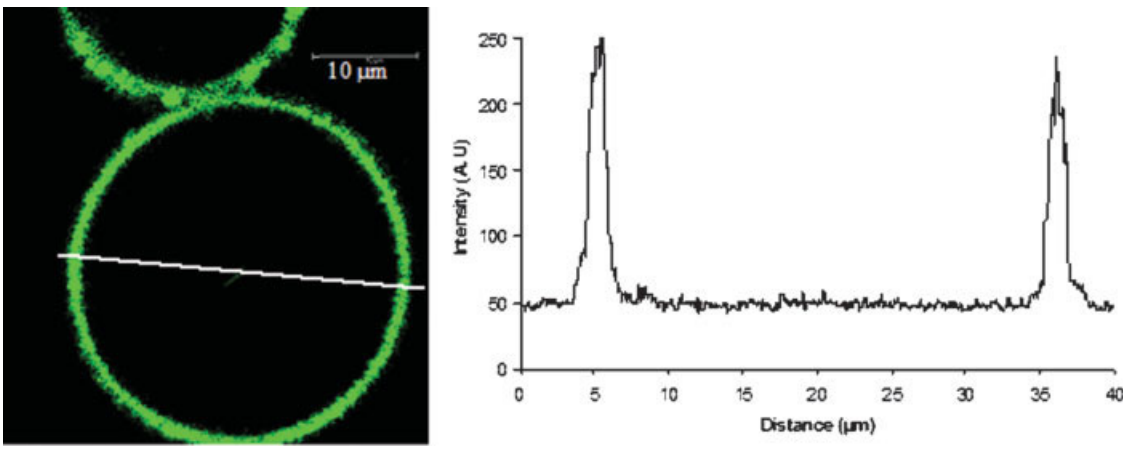

Figure 1 CLSM image taken at the center of a PEI microcapsule modified with FITC (left). The signal intensity histogram (right) corresponds white bar on the image. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

size as measured from light scattering particle size distribution analysis (Horiba LA-950). The geometrical mean size is determined to be 36 µm with a relatively wide distribution in accordance with the less accurate size analysis done by microscopy. The wide size distribution observed is a direct consequence of the capsules preparation procedure during which the size of the capsules is controlled by the size of the water microdroplets in the emulsion. Membrane thickness can be evaluated from the histogram presented in Figure 1. A thin membrane of about 2 µm was found for all capsules. The capsules size, the shape and the yield are similar to those obtained without modified PEI showing that the derivation reaction does not affect the microcapsules formation. This is because FITC reacts mainly with primary amines representing 25% of total N atoms in PEI,[23] whereas the crosslinking reaction of PEI with sebacoyl chloride is favored at the secondary amines (50% of total N atoms in PEI), so PEI is still reactive toward sebacoyl chloride despite its conjugation reaction with FITC.

Protein location in capsules

The distribution of proteins in the capsule was studied via the conjugation reaction of BSA and PEI with Texas red and FITC, respectively. Tagged BSA and PEI were incorporated in the aqueous phase before the capsules formation. Microcapsules were then prepared via interfacial condensation by crosslinking the FITC-modified PEI with sebacoyl chloride as presented above. The capsules were then analyzed by CLSM by following Z-stack acquisition from the center to the top pole of the capsules. Figure 3 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 capsules in this CLSM image appear to have a broader size distribution than the results of Figure 2. However, since only a small quantity of the capsules is showed in Figure 3, the size distribution measured by light scattering, which measures thousands of capsules, is more reliable.

Several reasons can be invoked to explain the localization of the BSA within the capsule wall. During microcapsule formation, proteins or enzyme, could react with the membrane system (PEI) and/or the crosslinking agent (sebacoyl chloride) and therefore trap the proteins in the wall, affecting the enzyme's activity. The interactions between PEI and proteins are well known. This property was used especially for long-term delivery of peptide and protein drugs.[24] This interaction reaction was also used for proteins separation and analysis like

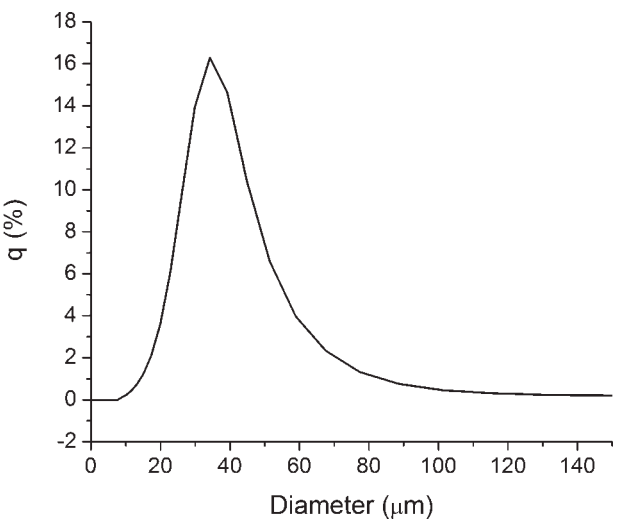

Figure 2 Microcapsule size distribution determined by laser scattering.

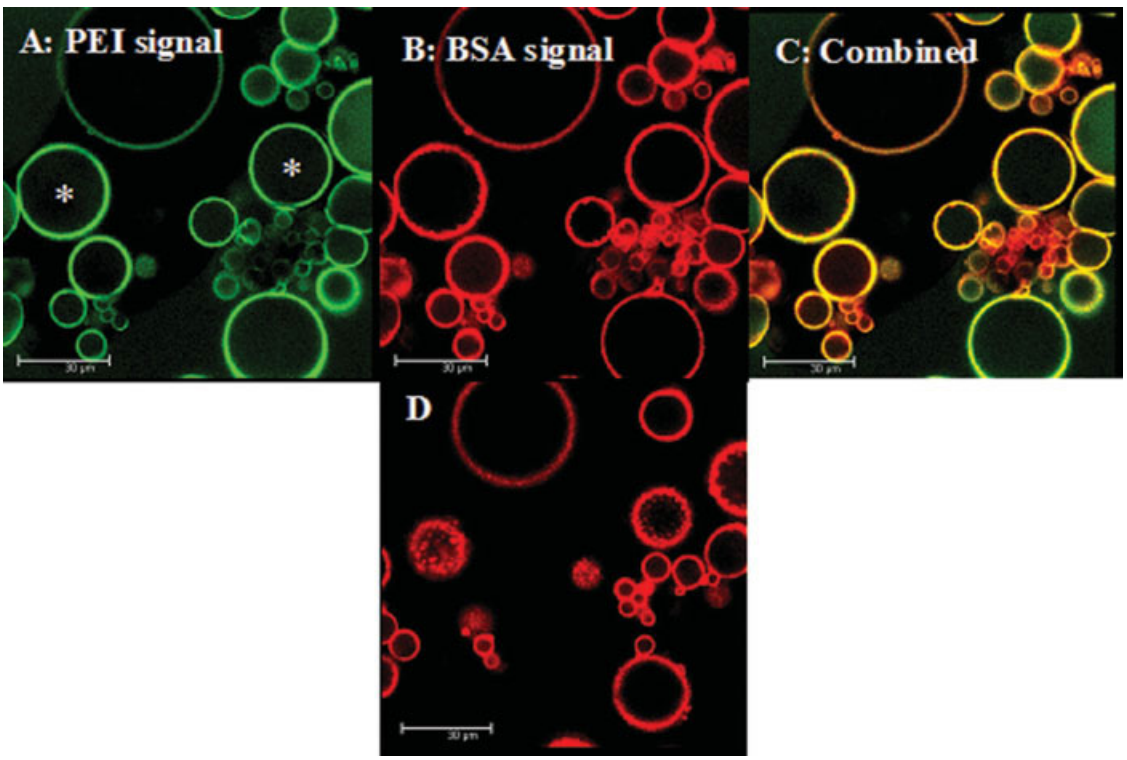

Figure 3 CLSM micrographs showing the location of the BSA proteins (B: red signal) within the PEI membrane of the microcapsules (A: green signal). Images in A, B, and C were taken at the center of the capsules marked by an *. The image in D was taken near the top end of the capsules and shows protein agglomerates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

chromatography and electrophoresis of proteins.[25–29] By comparing PEI coated supports and conventional supports, Mateo et al. and Kumar et al.[30,31] reported that proteins could be easily adsorbed on a support coated with PEI, while they cannot be adsorbed on unmodified supports. Torres et al.[25] studied the separation of proteins with conventional and PEI-modified supports. This study shows that, in a mixture of seven proteins with different isoelectric point, BSA was one of the last proteins to be desorbed from the PEI-modified agarose while a low ionic strength could release BSA from conventional DEAE-support. These are evidences of the occurrence of strong interactions between PEI and proteins that support our findings.

To explain this strong interaction between PEI and protein and consequently the reason of the high amount of protein found in the capsule wall, many factors need to be taken into account like surface characteristics (electric charges, roughness…) and hydrophilicity of the membrane.[32] The use of PEI results in capsules with a semipermeable membrane presenting a porous structure which will maintain the enzyme inside but allows subtrates to diffuse in and out of the capsule. It is well known that rough morphology increases the protein adsorption,[33,34] while Chang et al.[32] reported that the adsorption of fetal bovine serum protein on a membrane surface with smoother morphology was higher than the one occurring on the surface having rougher structure. Despite this contradiction, Chang et al. compared the protein adsorption on the blend membranes and the membranes containing PEI then they reported that PEI enhances the protein adsorption.[32] Clearly, surface morphology can not be the only factor affecting the protein adsorption and other factors must play a part in the protein adsorption.

The second important point explaining the presence of BSA in the capsules wall is the electrostatic interactions between the protein (pI = 4.7) and the amine groups on PEI. This finding was confirmed by other researchers[26–28] when they compared the protein adsorption on an uncoated and a support coated with PEI. They showed that proteins having a negative charge interact with PEI-coated supports that have a higher amount of positive charges, resulting in a strong adsorbtion on the modified support. A PEI solution presents a high pH of about 11. Upon addition BSA, this value drops to a pH close to neutral because of the deprotonation of the acidic moieties on the protein. At a neutral pH, PEI bears a net positive charge while the BSA is oppositely charged.[35] The strong interactions between BSA and PEI are likely maintaining these species in proximity, and reticulation of the polymer upon sebacoyl chloride addition might trap the proteins in

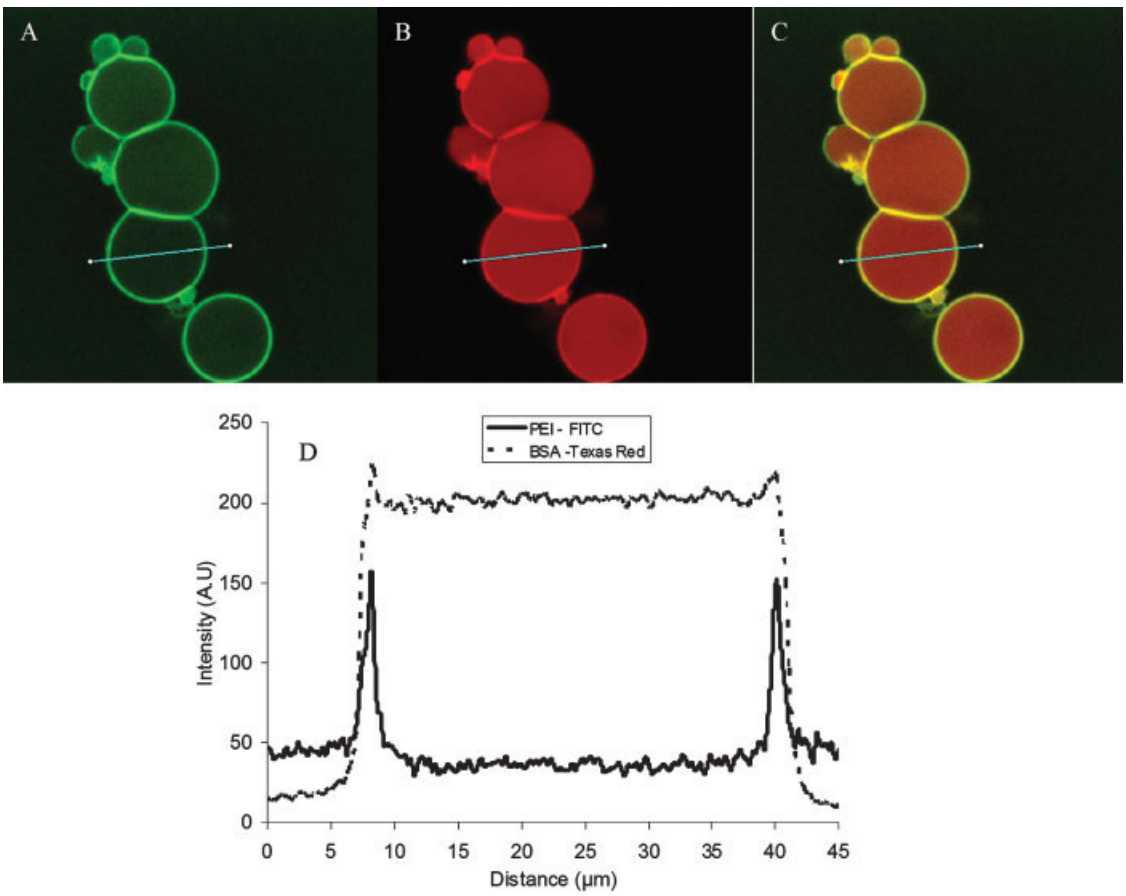

Figure 4 Fluorescence signals of concentrated BSA microcapsules. Colors are the same as in Figure 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the capsule wall. The pH of a suspension of BSA-containing microcapsules that is used for CLSM measurements is between 7 and 9, which also favors the attraction of BSA by the positive charge on the PEI wall. Park et al.[24] showed that PEI crosslinks BSA molecules by ionic interactions in poly(L-lactic acid) matrices coated with PEI. By using a neutral molecule (FITC-labeled dextran) instead of negatively charged molecule (BSA), they confirmed the crosslinking reaction between PEI and a charged BSA since only a very small amount of neutral molecule was adsorbed on the PEI-modified support.

Chang et al.[32] reported that in the case of membranes based on polyethylene vinyl alcohol containing PEI, at physiological pH, the modified membrane shows a high zeta potential, which means that the surface gets a very high density of positive charges and consequently it is supposed that the high protein adsorption is due to the hydrogen bonding between amine groups and proteins. Hydrophilicity is another membrane characteristic that could explain the BSA adsorption in the capsule membrane as Chang also showed. Reticulation reaction between sebacoyl chloride and BSA can also explain the fixation of protein in the capsule wall. Many studies showed the production of microcapsules by using proteins to form the capsule wall. For example, Tice and Meyers[36] used sebacoyl chloride to crosslink BSA to encapsulate living cells. In view of this, the observed red signal in the wall of the capsule Figure 3 could be related to the crosslinking reaction between BSA and reticulation agent which could results in protein agglomeration. This is evidenced in Figure 3D, where aggregates of BSA can be seen near the wall at the top end of the capsules.

The low signal in the core of the capsules could be explained by the low concentration (about 0.2 mg/mL) of the tagged protein used to make capsules for this experiment. To evaluate if saturation of wall with proteins is possible, a concentrated protein solution was used for encapsulation (about 4 mg/mL). The capsules were analyzed by CLSM and the results shown in Figure 4 present a uniform distribution of protein inside the capsules. The red signal is present both in the wall and in the core of the capsule but the signal is still slightly higher in the wall. This result shows that proteins will preferably be located in the wall until a certain loading is attained. In our previous work,[37] capsules with laccase were prepared with diluted enzyme solution,

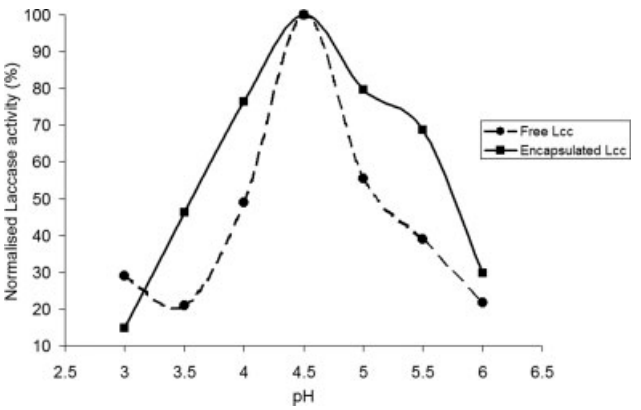

Figure 5 pH effect on the activity of encapsulated laccase. The activity was normalized to allow comparison between two different measurement methods.

and according to this finding, laccase will be most likely in the capsule wall and not in the core. This very different environment could explain the difference in the pH and K_m (constant of Michaels-Menten) values obtained for free and encapsulated laccase.[37]

Enzyme activity after encapsulation

To study the activity of encapsulated laccase, *o*-phenylenediamine (OPD) was used as the substrate. The optimum pH was determined in 50 m*M* McIlvaine buffers with pH ranging from 3 to 6 and a fixed OPD concentration of 4 m*M* to reach enzyme saturation. The enzyme activity of encapsulated laccase was measured in oxygen cell, to avoid complications due to the turbidity of the capsule suspensions, and compared with that of free enzyme which is measured by spectrophotometer. A comparison of the activities is done by setting the highest activity value to 100% and all others to a relative value (Fig. 5). The variation of the laccase activity with pH shows that microencapsulation does not affect qualitatively the enzyme activity since no difference was observed between free and encapsulated enzyme. The optimum pH was found to be about 4.5 for both encapsulated and free laccase. The only difference noted is the wider pH profile obtained for encapsulated laccase which is another evidence of the different environment created by the capsule wall, where the enzyme is located. Finally, the activity of hydrated encapsulated laccase was measured over a 1-year period of storage at 4°C. The results show that 35% of the activity is retained after such time. The lost of the activity could be explained by the diffusion of the enzyme through the membrane or by the deactivation of enzyme by the chemical components of the capsule wall. In the case of free laccase, 78% of activity was retained after 1 year. The addition of stabilizing agents with laccase microcapsules could advantageously improve the storage of encapsulated enzyme.

Capsules distribution in paper

This section deals with how microcapsules can be incorporated in paper handsheets to evaluate their potential application to develop an efficient immobilization technique for bioactive papers. To do so, two different paper samples were prepared with FITC-labeled microcapsules. The first handsheet was prepared by mixing dried bleached pulp with capsules (average diameter of 36 μm). The second sample was prepared simply by filtration of a suspension containing capsules on a plain handsheet. A strip of each handsheet was cut out, mounted in wax and sliced for CLSM analysis. The fluorescent images of the cross section of the two handsheet samples are presented in Figure 6. A uniform loading throughout the sheet is achieved when the capsules are mixed with the pulp before making the handsheet, while a thin layer can be deposited on the top of the paper by simple filtration. Either one of these two ways of preparation can be used depending on the targeted application of the bioactive paper. For sensing purposes, the enzymes can be immobilized only at the paper surface whereas higher loading can be achieved by having capsules throughout the sheet to maximize the conversion efficiency of substrates by the bioactive paper.

Enzyme retention on paper

Although high amounts of enzymes can be loaded in paper by microencapsulation, the physical retention of the capsules must be assessed to evaluate the extent of potential losses of enzymes. The protein retention on paper was determined for free and encapsulated laccase. First, a solution of free laccase was deposited directly on a plain handsheet (prepared without capsules). After drying at room temperature, the laccase activity on the paper was measured by placing a small piece of the sheet in the oxygen measurement cell. Next, a sample of the sheet modified with free laccase was cut and mounted in a Buchner filter through which 250 mL of water was filtered. The residual activity of the sheet sample was again measured by O_2 consumption. In these measurements, a small sheet sample (made with a common paper punch) was placed in the O_2 cell. Finally, a piece of a handsheet containing encapsulated laccase was washed with the same volume of water and the enzyme activity in the paper was measured in oxygen cell. The importance of immobilization is enhanced by the comparison of the oxygen consumption after an extensive washing of paper prepared by encapsulated laccase and the

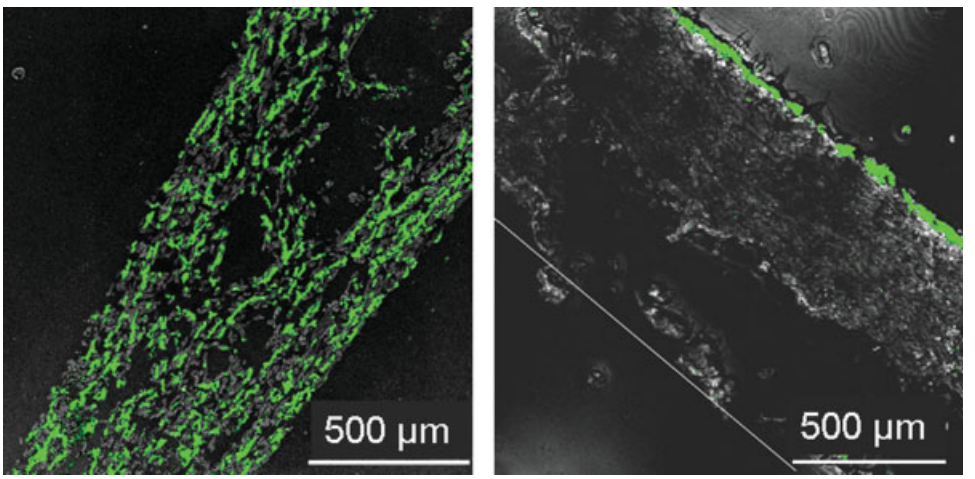

Figure 6 Microphotographs of a cross section of the paper prepared by mixing FITC-modified PEI capsules (36 µm) with pulp (5 wt %) (A) and by coating of capsules on a plain handsheet (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

one containing free enzyme (Fig. 7). As pointed out in this figure, almost all laccase (not encapsulated) that was simply deposited on the paper surface is lost (6% is retained on the paper), whereas a high retention of 94% is achieved with encapsulated laccase. This is due to the size of enzyme molecule. The enzyme size (~5 nm) is much smaller than the average pore size of paper (1–10 µm), so when enzyme is deposited directly on paper, it percolates in the handsheet and leaches out from it after washing with water. In the case of paper prepared with encapsulated laccase, the size of the capsules (average size of 36 µm) is larger than the pore size of paper which reduces the lost of enzyme after washing. Because of the chemical properties of polyethyleneimine (PEI), especially its high cationic charge density, immobilization might not only result from size but also from nonspecific electrostatic interactions between positively charged PEI and negatively charged cellulose on the paper surface. Microencapsulation appears as an efficient way to physically retain enzyme within paper structure without relying on chemical linkage, which could affect the enzymatic activity.

Colorimetric response on paper-based substrate

To evaluate the enzyme activity after laccase immobilization in paper and to check the possibility of the development of an indicator paper, the colorimetric response on paper containing encapsulated laccase was studied. A small pieces of handsheet (diameter: 27 mm) containing the same amount of capsules were prepared. To get a clear visual signal on the paper, *p*-phenylenediamine (PPD) was used here instead of OPD. PPD oxidation by laccase produces a clear color change easily detectable by the naked eye. PPD solutions at different concentrations were added and allowed to diffuse through both paper and capsules, respectively, and finally to react with laccase. Images were taken after several period of time. Figure 8 clearly demonstrates concentration-dependent color changes of the modified paper. For all samples (all periods of time) the color increased proportionally to the concentration of the PPD solution. PPD diffuses in the paper, through the semi-permeable membrane, reaches the core of the capsules and reacts with enzymes, which means that the encapsulation method could be used to produce colorimetric response with enzymes immobilized on paper. The response times obtained are, however,

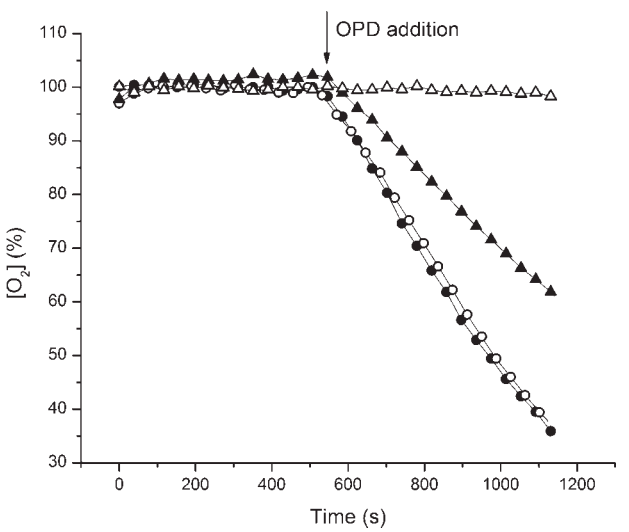

Figure 7 Oxygen consumption measured before (solid symbols) and after washing (open symbols) of paper prepared with encapsulated laccase (circles) and paper containing free laccase (triangles). The slope following addition of OPD is representative of the laccase activity.

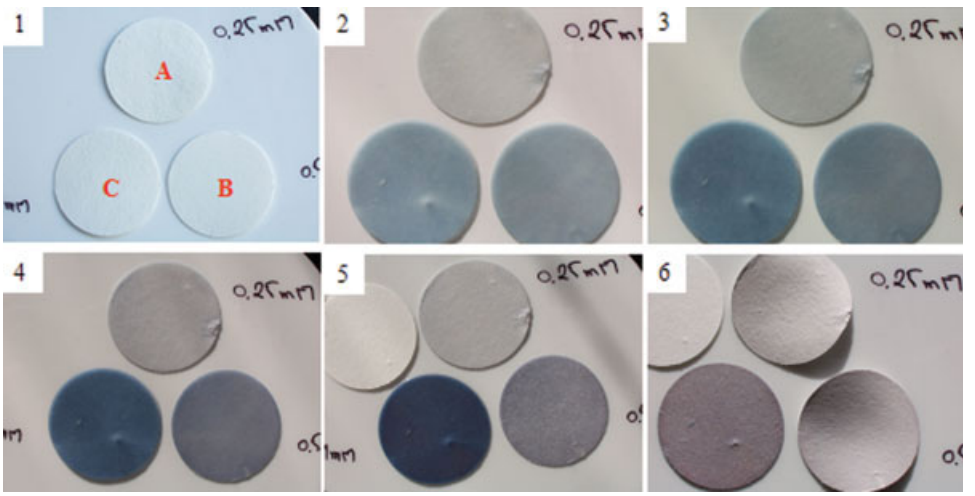

Figure 8 Following the *p*-phenylenediamine (PPD) oxidation by encapsulated laccase immobilized in paper by microencapsulation. Fixed volumes of 200 μL of the PPD solution at different concentrations were added: (A) 0.25 m*M*, (B) 0.5 m*M*, (C) 1 m*M*. Photographs were taken at different time intervals ($t = 0$; 5; 20; 30; 60; and 90 min). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

relatively longer that those obtained in for the capsules suspended in a buffer solution. This effect is due to the initially dry state of the paper and capsules and shows that upon addition of the substrate solution, the sufficient rehydration of the enzyme is not instantaneous. A noticeable signal is however detected within 2 min, a time lag that could be decreased by encapsulating higher amounts of enzyme.

CONCLUSIONS

Proteins were immobilized in PEI microcapsules that were prepared by interfacial reticulation. After microencapsulation, the proteins are found both in the capsules wall and in the core of the capsules. At low concentration, proteins are preferably located in the wall system and this very different environment affects the enzymatic activity. At high concentration, proteins are found also in the core of the capsule. Microcapsules were incorporated in paper by simple techniques yielding sheets with a very good dispersion within all of the handsheet depth. Microencapsulation has been showed to be an efficient, reliable, and scalable technique for biocatalyst immobilization in paper with a high retention of the enzymes. The enzyme activity is maintained after immobilization by microencapsulation, because encapsulated laccase immobilized in paper oxidizes the PPD with coloration change. This color transition of PPD indicates that, even after immobilization in a solid support, microcapsules could be used as a sensing material to produce a color-based response. This strategy could be extended to other types of enzyme and substrates with the aim of developing a useful bioactive paper to detect various target material.

We thank Denis Rodrigue and Michel Lauzon from Faculty of Medicine - Pathology and cellular biology for their help in paper sample preparation for the CLSM analysis.

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