

Paper modified with microencapsulated enzymes: towards bioactive paper

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Introduction

What is bioactive paper? In its broadest definition a bioactive paper is obtained by modifying a cellulosic substrate with biomolecules to add functionalities to a simple and common paper. Paper is ubiquitous, cheap and produced in various grades for many applications. It could therefore serve as very convenient substrate to bring biosensors to a large number of users. Face masks for example are used in clinics and hospitals to prevent users from contacts with airborne pathogens that *could* be in their environment. The modification of such simple device with biomolecules like antibodies, phages and enzymes could transform the mask into an intelligent device that would capture and deactivate pathogens and, by a suitable signal generation procedure, warn the user of their presence. Additionally, affordable biosensors are currently being developed as a mean to provide fast and quantitative methods for the diagnosis of several diseases or conditions and that could be used by persons with a difficult access to clinical analysis. Developing such bioactive paper is the goal of a Canadian research network named Sentinel¹ that was created in 2005 and which regroups industrial and academic researchers throughout the country. Our effort in this network is focused on the immobilisation of enzymes on paper for biosensing applications. Only a few examples of papers modified with enzymes can be found in the literature. The Whitesides' group recently demonstrated a paper-based biosensor to measure glucose and proteins levels in urine (Martinez (2007), Martinez (2008)). In their device, the enzymes glucose oxidase and horseradish peroxidase as well as the signalling reagents were simply adsorbed on paper, leading to elution of all species with the solvent front. To circumvent this problem, they rely on channels made from a hydrophobic polymer that were created by photolithography. While this strategy was showed to efficiently maintain the enzymes and reagents in test areas, it imposes a high level of complexity to the device fabrication. One would ideally like to incorporate enzymes in paper making process in a way that will allow a high physical retention of the biomolecules while maintaining their activity. Microcapsules can fulfill all these requirements and could advantageously be used to immobilise enzymes on paper-based substrates.

In this contribution we report on the use of poly(ethyleneimine) microcapsules to immobilise enzymes into paper. Microcapsules were either mixed with paper pulp prior to sheet formation or coated on formed papers by conventional coating techniques compatible with paper-making processes. The size of the microcapsules affects their distribution within the sheet. Laccase was selected as a model enzyme and its activity was determined in the capsules and after immobilisation on paper. Confocal laser scanning microscopy (CLSM) was used to determine that, under the conditions used, the encapsulated proteins favour the membrane rather than the core of the capsules, which can explain some of the differences observed in the activity upon encapsulation. CLSM was also used to evaluate the distribution of microcapsules within the paper sheet. Microencapsulation is showed here as an efficient method to maintain the activity of enzymes on paper-based substrates and appears to be a promising immobilisation platform for the development of bioactive paper.

Materials and methods

PEI microcapsules were prepared based on a procedure published by Poncelet (1994). Laccase and bovine serum albumine (BSA) capsules were obtained by the addition of the proteins in the aqueous

phase along with PEI. Laccase (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 (Bourbonnais (1995)) and BSA was obtained commercially. Encapsulated laccase activity was assayed by monitoring the O₂ consumption rate by the enzyme in the presence of a substrate (*o*-phenylenediamine) using an oxygen cell. This was showed to provide a more reliable measurement of encapsulated laccase activity when compared to spectrophotometric assays (Hébert (2008), Rochefort (2008)).

To study the location of protein in the capsules and the distribution of capsules in paper, 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 ($\lambda_{em} = 520$ nm) and fluorescein isothiocyanate ($\lambda_{em} = 615$ nm). An Ar (488 nm) and a DPSS (561 nm) laser were used for the excitation of the fluorophores. For the paper analysis by CLSM, samples of paper containing FITC-modified microcapsules were impregnated in liquid paraffin for 16h, allowed to settled and sectioned with a microtome. The 8 μ m thick layers of the cross-section of the paper were mounted on microscope slides and analysed by CLSM to evaluate how the capsules were distributed across the sheet.

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. 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 via an aqueous phase emulsion without any proteins. The signal visible on the image comes from the poly(ethyleneimine) capsule walls that was modified by FITC prior to encapsulation procedure and allows us to measure the size and membrane thickness of the microcapsules.

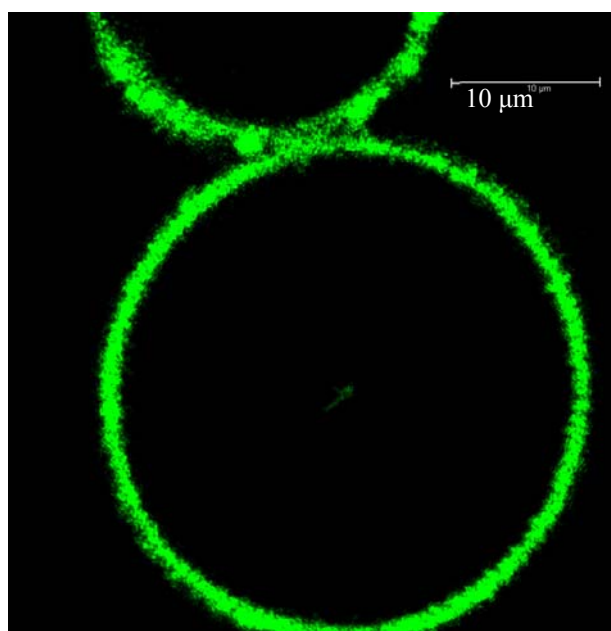


Figure 1. Confocal micrograph of a micro-capsule prepared with FITC-modified PEI by the emulsion method.

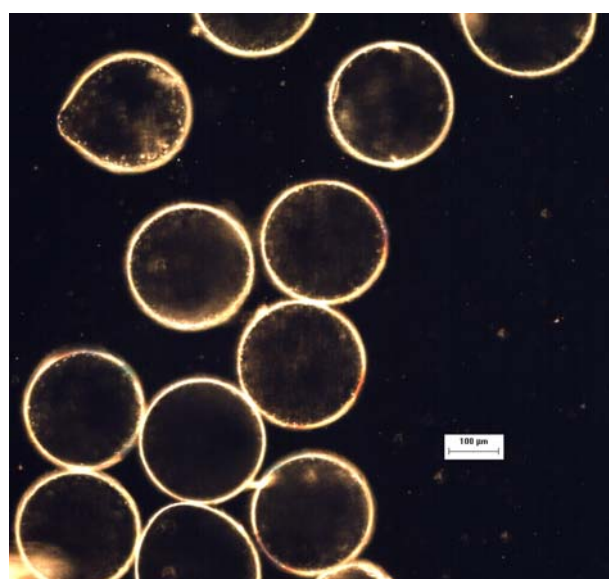


Figure 2. Optical micrograph of PEI microcapsules prepared with the Inotech encapsulator. Scale bar is 100 μ m.

From microscopy, the microcapsules are showed to be spherical, with their size ranging from below 10 μ m and up to 50 μ m. A more precise evaluation of their size was obtained from light scattering

particle size distribution analysis (Horiba LA-950). The geometrical mean size is determined to be 36 μm with a wide distribution ($\sigma = 24 \mu\text{m}$). 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. Alternatively, we were able to prepare larger PEI microcapsules with a much narrower distribution with the use of an encapsulator (Figure 2). A membrane thickness of about 2 μm was found for all capsules made from the emulsion technique, while encapsulator yielded membrane about 5 μm thick.

Proteins 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 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. Strong ionic attraction between oppositely charged BSA and PEI can explain this observation.

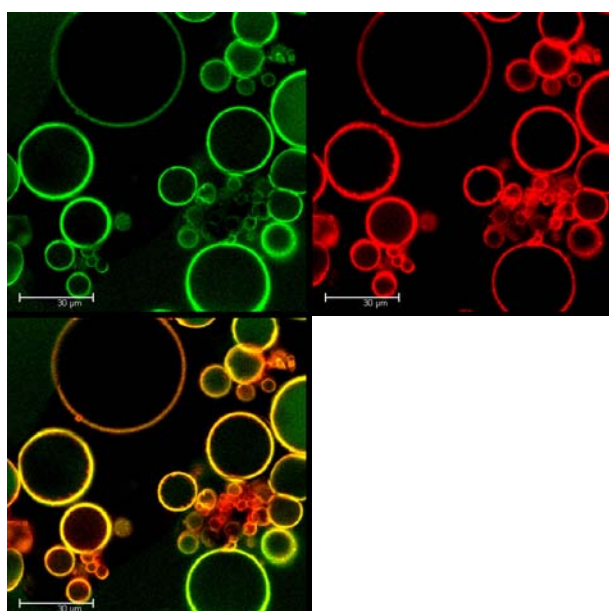


Figure 3. CLSM analysis of the protein location in the PEI microcapsules. The green signal is attributed to FITC-modified PEI, while red signal indicates BSA. Bottom image is a combination of both signals.

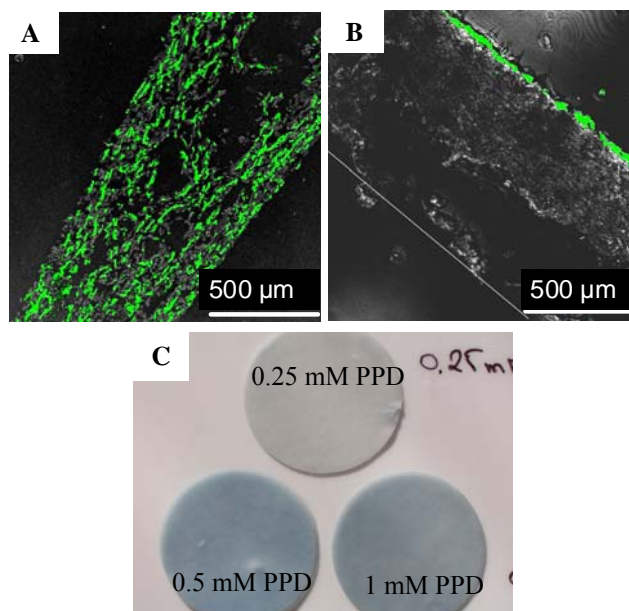


Figure 4. Microphotographs of a cross-section of the paper prepared by mixing FITC-modified PEI capsules with pulp (5 wt %) (A) and by coating of capsules on a plain handsheet (B). Oxidation of *p*-phenylenediamine at various concentrations by laccase immobilised on paper after 5 min incubation (C).

Microcapsules were then incorporated in paper handsheets to evaluate their potential application to develop an efficient immobilisation technique for bioactive papers. To do so, two different paper samples were prepared with FITC-labeled microcapsules either by mixing dried bleached pulp with capsules (Figure 4A) or by coating capsules on a plain handsheet (Figure 4B). A uniform capsule loading can be achieved throughout or on the top of the sheet. Figure 4C presents the color response of laccase immobilized in paper by microencapsulation after a 5 min incubation period with *p*-phenylenediamine (PPD) as a laccase substrate giving a dark blue colour when oxidised. The response times obtained are relatively longer than 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, enzyme rehydration is not instantaneous.

Finally, protein retention on paper was determined for free and encapsulated laccase. Handsheets containing laccase microcapsules were prepared as described above. A solution of free laccase was also deposited on a plain handsheet. Laccase activity was measured on the paper with the O₂ cell before and after an extensive wash with water. The results from Figure 5 show that upon washing almost all laccase 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. Microcapsules retention in paper could be due to their size being larger than the average pore size of paper (1-10 μm) or from non specific electrostatic interactions between positively charged PEI and negatively charged cellulose on the paper surface, or a combination of both effects.

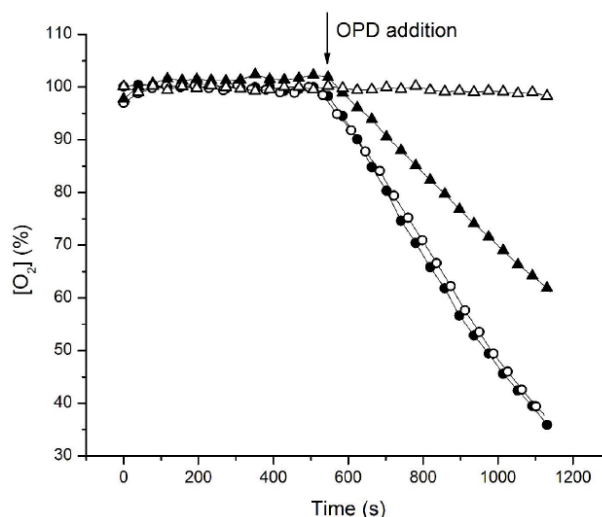


Figure 5. Oxygen consumption measured before (solid symbols) and after washing (open symbols) of paper prepared with encapsulated laccase (circles) and paper containing free laccase (triangles). The slope following addition of OPD is representative of the laccase activity.

Conclusions

Proteins were immobilised in PEI microcapsules prepared by interfacial condensation. After microencapsulation, the proteins are found mostly in the capsules wall because of attractive ionic interactions. Microcapsules were incorporated in paper by simple techniques yielding sheets with a very good dispersion within all of the handsheet depth and have been showed to be an efficient, reliable and scalable technique for biocatalyst immobilisation in paper. The enzyme activity is maintained after immobilisation by microencapsulation, as evidenced by the color transition of PPD observed on laccase-modified paper. 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.

References

1. www.bioactivepaper.caT.
- R. Bourbonnais et al. (1995) *Lignin oxidation by laccase isoenzymes from Trametes versicolor and role of the mediator 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization*. Applied and Environmental Microbiology 61(5) 1876-1880.
- M. Hébert et al. (2008) *Electrode passivation by reaction products of the electrochemical and enzymatic oxidation of p-phenylenediamine*. Electrochimica Acta 53(16) 5272-5279.
- A. W. Martinez et al. (2007) *Patterned paper as a platform for inexpensive, low-volume, portable bioassays*. Angewandte Chemie-International Edition 46(8) 1318-1320.
- A. W. Martinez et al. (2008) *Simple Telemedicine for Developing Regions: Camera Phones and Paper-Based Microfluidic Devices for Real-Time, Off-Site Diagnosis*. Analytical Chemistry Published on web, DOI 10.1021/ac800112r.
- D. Poncelet et al. (1994) *Microencapsulation within cross-linked polyethyleneimine membranes*. Journal of Microencapsulation 11(1) 31-40.
- D. Rochefort et al. (2008) *Physical immobilization of laccase on an electrode by means of poly(ethyleneimine) microcapsules*. Journal of Electroanalytical Chemistry 617(1) 53-63.