Nanoengineered alginate microspheres towards an optical urea biosensor

R. Srivastava*, M. Swati

School of Biosciences and Bioengineering, IIT Bombay, India-400076

Centre for Research in Nanotechnology and Science, IIT Bombay
rsrivasta@iitb.ac.in

Introduction

The requirement for improved, continuous monitoring and diagnostics is rising due to the increasing demands by the health service to reduce health care costs, maximize efficiency and reduce hospital stay without compromise to patient care. There are currently more than three lakh people with end-stage renal disease in the United States and in India; approximately 11,000 people per million population (in the year 2001) undergo regular dialysis (*www.philhealth.gov.ph accessed on 12-05-07)*. Currently, most of the dialysis clinics use the procedure where the blood is drawn out to determine the levels of urea to verify the adequacy of dialysis. Turnaround time for these procedures could be quite long, and often the patient might be recalled for further dialysis if the percentage reduction of urea in the blood is not sufficient. In the absence of a blood test, the use of time of dialysis alone as a measure of completion, especially if hemodialysis is not carried out long enough, could clearly lead to morbidity and mortality.

The most convenient fluid to monitor is the dialysate, which is the capturing medium for blood contaminants during the hemodialysis process. By the use of an appropriate sensor arrangement, the dialysate could be continuously or intermittently monitored at the point-of-care. The sensor of this invention is based upon measurement of the pH change produced in an aqueous environment by the products of the enzyme-catalyzed hydrolysis of urea. The enzyme urease has been encapsulated in the alginate microspheres and on top of these, nanofilm coatings have been assembled using Layer-by-Layer (LBL) self-assembly technique. The optical method is advantageous that it does not require any reference electrode because the measurement of pH is based on the use of organic dye molecules. The loss or gain of a proton changes the electronic structure of the molecule, producing a measurable change in the manner in which the molecule interacts with light (S. F. Norman, 1999). In this sensor, cresol red (CR) dye has been utilized which is a ratiometric dye. A ratiometric pH sensitive dye is preferred over single wavelength absorbance dyes as it offers the advantage to reduce measurement uncertainty (*www.bioscienceworld.com accessed on 30-12-06*). System inaccuracies affect the concentration measurement lesser, as they tend to cancel out each other, resulting in a more stable result. Also, the calibration standards drift over time, while ratios exhibit greater stability. LbL technique has been used to immobilize the dye molecules within the nanofilms and their imaging was performed using fluorescence microscopy.

Materials and Methods

Materials

Sodium alginate (low viscosity; 250 cps, 2 wt %), sodium poly (styrene sulfonate) (PSS, MW~70,000), poly (allylamine hydrochloride) (PAH, MW~70,000) and Urease have been purchased from Sigma. Cresol Red dye has been purchased from Loba Chemei while Tris buffer from SRL, Mumbai. Calcium chloride was obtained from Merck Ltd, Mumbai. All chemicals were reagent grade and used as received.

Instrumentation
Encapsulation unit Variation J30 (Nisco Engineering AG, Zurich) and syringe pump (Multi-Phaser™, model NE-1000, New Era Pump Systems, NY) have been used for preparing uniform size alginate microspheres. Optical microscope (Zeiss) and scanning electron microscope (Hitachi S3400N) have been used for size characterization and surface analysis of blank as well as coated microspheres. Zeta plus (Brookhaven Instruments, USA) was used for the determination of surface charge. A fluorescence microscope has been used for imaging immobilized dye particles between the nanofilms. A UV/Vis spectrophotometer (Helios) has been used to measure the absorbance spectrum of cresol red solution and the dye loaded microspheres.

Preparation of calcium alginate microspheres
The alginate microspheres were prepared using the droplet generator in which a solution of 2 mg/ml urease enzyme was mixed with 2% w/w sodium alginate and filled into the syringe of 60 c.c. capacity. The J30 is the aerodynamically assisted jetting equipment where the product enters through a central needle (diameter=0.35 mm), which is enclosed in a pressure chamber with an exit through the orifice. The size of the drops is determined by the product flow rate and the pressure inside the chamber. The product flow rate is typically controlled by a precision syringe pump which is connected to the product nozzle. The flow rate of sodium alginate solution was fixed at 20 ml/hr and the pressure was maintained at 80 mbar. The drops were collected in a vessel containing 250 mM calcium chloride solution for gelation under constant stirring.

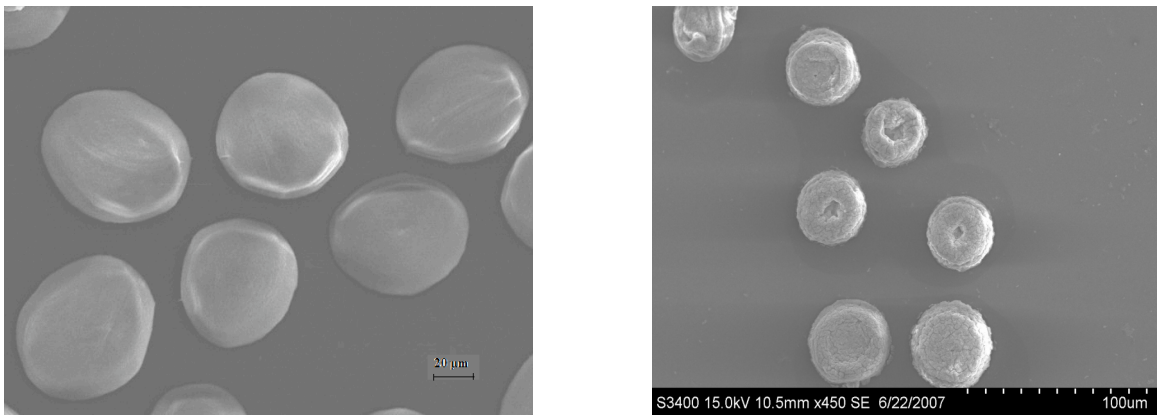
Immobilization of cresol red
The microspheres were thoroughly washed with deionized water before coating with nanofilms via the LBL technique (G. Decher, 1992; R. Srivastava, 2005). The alginate microspheres were coated with a bilayer of PAH and a mixture of cresol red and PSS (CR+PSS) $\{(PAH)/(CR+PSS)\}_2$.

Surface charge
Zeta potential represented by ζ is a function of the surface charge of the particle, any adsorbed layer at the interface and the nature and composition of the surrounding medium in which the particle is suspended. In the present work, zeta potential was determined after the deposition of each layer of polyelectrolytes to confirm the surface coating of alginate microspheres using the LBL technique.

Urease activity studies
The assay procedure was adapted from the method by Chandler (M. H. Chandler, 1982). The range of urea concentrations that were studied was 0.01- 6.7 mM (i.e. 0.1 mg/dl – 40 mg/dl). All the experiments were conducted at pH=6.0 and at room temperature. The increase in pH (due to production of ammonia) was monitored with the pH sensitive dye, cresol red at its two characteristic wavelengths, 434 nm and 572 nm. Similar experiments were performed with urease enzyme encapsulated microspheres and the results were compared with that of urease enzyme in solution.

Results and Discussion

Using the droplet generator, the size of microspheres obtained was in the range of 50-80 μm as shown in figure 1a). The spheres were mostly spherical in shape. Maximum number of particles was of size 60μm. The deposition of polyelectrolytes was observed to be uniform on the microspheres as shown in figure 1b). The advantage of using LBL technique is to reduce the leaching of urease enzyme from the microspheres and in this work it has been used to immobilize CR dye molecules within the PAH/PSS nanofilms.



(a) **(b)**

Figure 1: SEM micrographs of (a) uncoated and (b) coated microspheres

The deposition of polyelectrolytes $\{(PAH)/(CR+PSS)\}_2$ was observed as a thin film on top of the microspheres as confirmed from the fluorescent images in figure 2 and zeta potential was observed to be in the range of -28 mV (standard deviation SD= ±0.89) for uncoated microspheres. After the deposition of PAH the zeta potential value was observed to be +37.29 mV (SD= ±0.51) whereas (CR+PSS) deposition displayed a value of -24.6 mV (SD= ±0.76) as shown in figure 3.

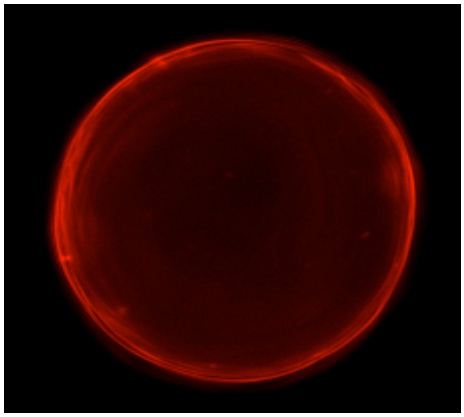


Figure 2: Microsphere coated with a bilayer of $[PAH/(CR+PSS)]_2$

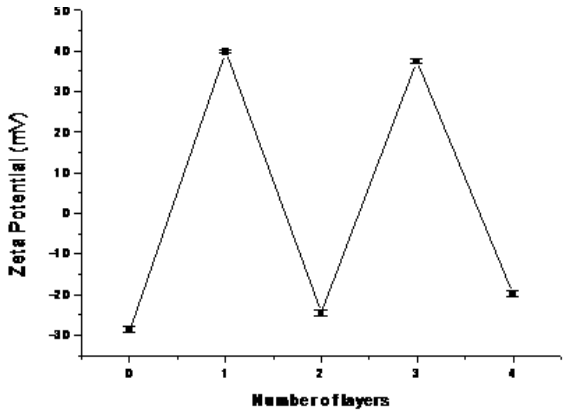


Figure 3: Zeta potential after deposition of each polyelectrolyte layer

Absorption spectrum of cresol red

The experiment was performed with an initial concentration of 2mg/ml cresol red solution dissolved in buffers of varying pH from 6 to 10. The results of the wavelength scan displayed the two characteristic peaks of cresol red, one at 434 nm and the other at 572 nm. The ratio of the two peaks (572/434) was plotted against the pH for the construction of a calibration curve of cresol red dye solution between pH 6 to 10. Similar experiment was performed with immobilized dye molecule and compared the results (M.Swati et al, 2007). The absorbance ratio curve displayed a linear response in the pH region 7.5 to 10 which is ideal for the determination of urea in the dialysate.

Calibration curve for urea

The experiment was performed to construct a calibration curve for urea Vs CR dye absorbance at its characteristic wavelengths with urease in solution/immobilized form. The absorbance ratio (572/434) curve was plotted with different concentrations of urea (0.01 mM - 6.7 mM) with urease in solution phase and the results were compared with the immobilized urease particles (results not shown).

Thereafter, a validation curve (as shown in figure 4) was prepared where urease immobilized microspheres along with cresol red dye molecules within the polyelectrolyte nanofilms were used for urea sensing using the same procedure as mentioned previously. It clearly shows that cresol red dye is very sensitive to minute changes in the pH caused due to production of ammonium ions in the solution. The rate of the enzyme-substrate reaction is highly sensitive to substrate concentration until the K_m value is reached, beyond which it becomes independent of the substrate molecules. Therefore, a higher slope was observed in the lower range of urea concentrations.

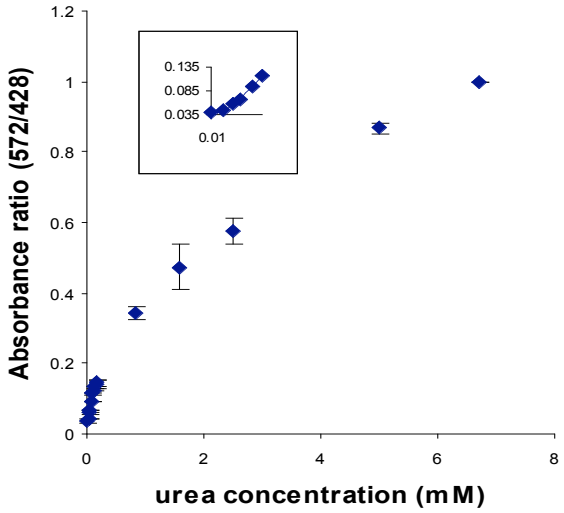


Figure 4: Absorbance ratio curve (572/428) for urease immobilized particles along with cresol red dye in multilayers (s=0.076) [Inset: ratio curve for lower range of urea concentrations where s=0.54]

Conclusion

Alginate microspheres (50-80 μm) have been prepared by the droplet generator technique and used for the encapsulation of the urease enzyme. The microparticles have been characterized for size and charge by microscopy and zeta potential analysis, respectively and 93% retention of the enzyme has been obtained in the microspheres. Cresol red dye molecules have been successfully encapsulated within the multilayer nanofilms of PAH and PSS polyelectrolytes displaying 95 % immobilization. The results demonstrate that the microspheres displayed a higher sensitivity to urea concentrations in the range of 0.01 mM to 0.16 mM that is desirable for determining urea levels in the dialysate.

References

S. F. Norman et al. (1999) *Measurement, Instrumentation and Sensors Handbook*, Edited by John Webster. CRC Press LLC.

G. Decher et al. (1992) *Buildup of ultrathin multilayer films by a self assembly process*: *III. Consecutively alternating adsorption of anionic and cationic polyelectrolytes on charged surfaces*. Thin Solid Films, 210 (2) 831–835.

R. Srivastava et al. (2005) *Stabilization of glucose oxidase in alginate microspheres with photoreactive diazoresin nanofilm coatings.* Biotechnology and Bioengineering 91(1) 124-131.

M. H. Chandler et al. (1982) *An investigation of the use of urease-antibody conjugates in enzyme immunoassays*. Journal of Immunological methods 53, 187-194.

M. Swati et al. (2007) *Nanoengineered Alginate Microspheres Comprising Multilayered Assemblies of Cresol Red and Polyelectrolytes for an Optical pH Sensor.* Journal of Bionanoscience 1(2) 90-95.