Application of Polyelectrolyte Coated Alginate Microspheres for Optical Sensing

Swati M. [1#] and Srivastava R. [2 *]
[1] School of Biosciences and Bioengineering, IIT Bombay, Powai, Mumbai-400076, India
swati_m@iitb.ac.in

INTRODUCTION

Urea is known to be an important marker for evaluating uremic toxin levels. The normal level of urea in serum is from 15 to 40mg/dl (2.5–7.5mM/l). At elevated levels i.e. from 180 to 480 mg/dl, hemodialysis is required (Dhawan G. 2009). It is a method for removal of metabolic wastes from blood when the kidneys become dysfunctional (i.e. in renal failure). Blood Urea Nitrogen (BUN) estimation during pre and post dialysis is an important parameter that is used for determining the dialysis dose for each patient. But the drawbacks of this method are: they are complex, time consuming, requires blood sampling and is potentially inaccurate (Koncki R. 2000). The turnaround time for analysing these samples is long, and often the patient might be recalled for further dialysis if the percentage reduction of urea in the blood is not sufficient. Another method for estimating delivered dialysis dose that has been used is modelling serum urea but again it is an indirect technique to monitor hemodialysis and have certain disadvantages (Koncki R. 2000). Direct dialysate quantification (DDQ) is a recent method to measure total solute removal and is regarded as the gold standard for dialysis dosing (Malchesky P.S. 1982). Therefore, a sensing system integrated in line with the hemodialysis machine to monitor urea levels directly in the spent dialysate would be more convenient as compared to the conventional blood sampling methods. Although direct spectrophotometric methods are available for urea determination, biosensors employing enzymatic methods are more selective and much more extensively used.

In the fabrication of a urea biosensor, urease is commonly used as the biosensing element. Enzymatic sensors utilize biochemical reactions and in this case reaction between urea and urease result in the production of ammonium ions that can be detected by a transducer potentiometric/optical/thermal/piezoelectric. Of these, optical based (Goldfinch M.J. 1984; Hashemi P. 2006; Mascini M. 1977) urea biosensing devices are more popular due to the following advantages over electrochemical based sensors. They neither require a reference cell/electrode nor are they affected by electrical interferences (Mascini M. 1977; Koncki R. 1995). In addition to this, they are highly sensitive and display a faster response time. Optical sensing methods for determination of urea may utilize the observed color change of a pH sensitive dye after the urease mediated hydrolysis of urea to produce ammonium ions as shown in this reaction:

$$NH_2CONH_2 + 3H_2O \rightarrow 2NH_4^+ + HCO_3^- + OH^-$$

The change in pH caused due to ammonium ions can be easily detected by a pH sensitive dye. In the present work urease enzyme, which is immobilized in calcium alginate microspheres and cresol red dye (pH sensitive dye), which is encapsulated within multilayer nanofilms (assembled on the microspheres using Layer-by-Layer self assembly), has been utilized to build a microsphere based pH sensing device. This alginate based urea biosensor would be cost effective and could be used as

an alternative to the existing autoanalyzer (in dialysis) which is expensive and requires skilled professionals for operation.

MATERIALS

Chemicals

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 has been purchased from Loba Chemei while Tris buffer from SRL, Mumbai. Calcium chloride and urea were 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 calcium alginate microspheres. An optical microscope (Zeiss), 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 and urease activity studies.

METHODS

Urease/cresol red immobilization

Urease enzyme (2mg/ml) (activity of 50,000 U/gm) was mixed with sodium alginate (2 wt %) and used as the precursor solution. The microspheres were prepared by the commercially available droplet generator. It is an aerodynamically assisted jetting equipment and the product (precursor solution) enters through a central needle (diameter=0.35 mm), which is enclosed in a pressure chamber with an exit through the orifice. For gelation the alginate drops were collected in a vessel containing 250 mM calcium chloride solution under constant stirring. The microspheres obtained by centrifugation were thoroughly washed prior to LbL deposition. Multilayer nanofilms were deposited on top of the prepared calcium alginate microspheres using the polyelectrolytes sodium poly (styrene sulfonate) and poly (allylamine hydrochloride). Within these nanofilms cresol red dye molecules were encapsulated. The stability of dye within nanofilms has been studied previously (Swati M 2007; Swati M 2009).

Storage stability of urease in alginate microspheres

Storage stability studies were conducted where the activity of urease enzyme was determined in solution phase as well as in the immobilized phase. The concentration of urease taken was 2mg/ml prepared in tris buffer at pH=7.3; the enzyme was immobilized in alginate microspheres. The effect of storage temperature on enzyme activity was studied at room temperature (RT) and at 4˚C. The activity of the enzyme was determined periodically by Nessler's reagent (one of the standard colorimetric method for urea determination).

Assessment of sensor characteristics of the microspheres

Response time is a very important parameter of a biosensor for assessment of its analytical performance. In the present work, response to increasing concentrations of urea was determined with a single set of microspheres in alternation with the plain buffer. The experiment was

performed in triplicates. Reproducibility of the values obtained with the microspheres towards a single urea concentration were studied which also displayed the reversibility characteristics of microspheres.

Validation studies with dialysate samples

The sensitivity of the biosensor was evaluated by comparing the biosensor performance with Nessler's reagent method. A calibration curve for Nessler's reagent was prepared with standard ammonium chloride solutions. Urea concentration was then determined in the dialysate samples from this standard curve. Thereafter, the data was used for a parallel study between the present urea biosensor and Nessler's reagent method.

RESULTS

Particle size analysis on the prepared alginate microspheres yielded results with more than 90 % of the microspheres having sizes between 50-80 µm and the deposition of polyelectrolytes was observed to be uniform on the microspheres as shown in Figure 1. Cresol red dye (immobilized within nanofilms as shown in Figure 2) stability was investigated by studying the leaching of the dye from the nanofilm coatings over a period of 4 weeks in increasing salt concentration of the polyelectrolyte solution and it was found that the lowest leaching was observed for nanofilms prepared in 500 mM calcium chloride (Swati M. 2009). Therefore, these microspheres were used for further studies.

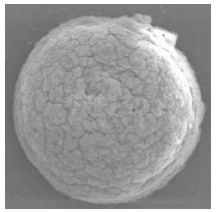

Figure 1: Polyelectrolyte coated microsphere

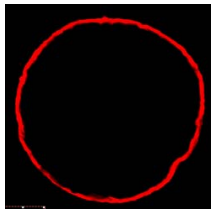

Figure 2: Cresol red within polyelectrolyte nanofilms

The urease enzyme demonstrated an enhanced activity when immobilized in polyelectrolyte coated alginate microspheres. Urease enzyme in solution phase demonstrated a residual activity of 22% and 60% at RT and 4˚C respectively by the end of 4 weeks whereas in immobilized state it retained nearly 47% and 65% activity respectively.

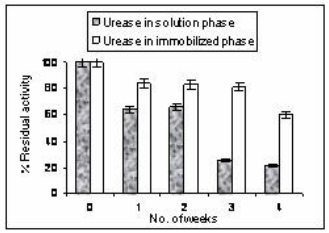

Figure 3: Residual activity at RT

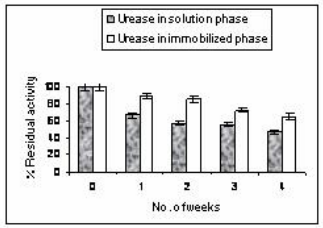

Figure 4: Residual activity at 4˚C

The particles displayed temperature stability proving the efficacy of the alginate matrix as well as demonstrated the ability of polyelectrolyte coatings to protect the encapsulant from the harsh effects of extreme temperatures as shown in Figure 3 & 4.

The sensor (microspheres) displayed a response time of nearly ≈ 2min in the range of 0.01 to 6.7 mM urea concentration and could be used ≥3 times while also demonstrated good stability over a month.

Validation with real dialysate samples proved that the estimated urea levels determined by the two methods displayed good correlation in some of the samples as shown in Table 1.

S no.	Patients	Nessler's Reagent (mM)	Present biosensor (mM)	Standard deviation
1	G	2.8	1.93	0.61
2	**S**	**2.23**	**2.1**	**0.09**
3	**M**	**1.53**	**1.69**	**0.11**
4	R	3.69	1.3	1.68
5	**Su**	**1.07**	**1.14**	**0.04**

Table 1: Comparison chart of urea levels estimated in dialysate by the Nessler's method and the optical urea biosensor

CONCLUSIONS

The present work has demonstrated the fabrication of an optical based urea biosensor that could be incorporated with the dialysis system to monitor urea levels in the dialysate samples. Urease enzyme encapsulated inside the microspheres remained stable with retention of nearly 65% activity when stored at 4˚C. The response of the biosensor to urea concentrations in the range of 0.01 to 6.7 mM was fast giving reproducible results. With proper storage (Tris buffer pH=7.3, 4 °C), the sensor can be stored for a month.

REFERENCES

- Dhawan G. et al. (2009) *Recent developments in urea biosensors*. Biochemical Engineering Journal 44(1) 42-52
- Koncki R. et al. (2000) *Potentiometric determination of dialysate urea nitrogen*. Talanta 52(1) 13-17
- Malchesky P.S. et al. (1982) *Direct quantification of dialysis*. Dialysis& Transplantation 11(1) 42-49
- Goldfinch M.J. et al. (1984) *Solid-phase optoelectronic sensors for biochemical analysis*. Analytical Biochemistry 138(2) 430-436
- Paymen H. et al. (2006) *Preparation of a novel optical sensor for low pH values using agarose membranes as support*. Sensors and Actuators B 115(1) 49-53
- Mascini M. et al. (1977) *Urease coupled ammonia electrode for urea determination in blood serum.* Analytical Chemistry 49(6) 795-798
- Koncki R. et al. (1995) *Enzyme biosensor for urea based on a novel pH bulk optode membrane.* Biosensors and Bioelectronics 53(8) 653-659
- Swati M. et al. (2007) *Nanoengineered alginate microspheres comprising multilayered assemblies of cresol red and polyelectrolytes for an optical pH sensor.* Journal of Bionanoscience 1(2) 1-6
- Swati M. et al. (2009) *Stabilization of sensing assay within polyelectrolyte coated alginate microspheres for optical urea sensing*. Analytical Letters 42(5) 1-17