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Immobilization of the urease on eggshell membrane and its application in biosensor

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ABSTRACT

Eggshell membrane is a natural material, essentially made up of protein fibers having flexibility in the aqueous solution and possessing gas and water permeability. It is used as a biomembrane for immobilization of urease for the development of a potentiometric urea biosensor. Eggshell membrane was treated with polyethyleneimine (PEI) to impart polycation characteristics. Urease was immobilized on the PEI treated eggshell membrane through adsorption. SEM study was carried out to observe the changes in surface morphology after immobilization. FTIR study of membrane was carried out to observe the changes in IR spectra after immobilization of enzyme. Immobilized membrane was associated with ammonium ion selective electrode. Biosensor exhibited sigmoidal responses for the urea concentration range from 0.5 to 10 mM. The response time of the biosensor was 120 s. A single membrane was reused for 270 reactions without loss of activity. The urease–eggshell membranes were stable for 2 months when stored in buffer even at room temperature.

1. Introduction

A large number of techniques and materials have been used for the immobilization of biocatalyst for biosensor application [1–12]. The choice of materials and the technique, for the preparation of membranes, has been dictated by the low diffusion resistance of the membrane coupled with its ability to incorporate optimal amount of enzyme per unit area. The techniques, used for immobilization, should lead to minimal inactivation of the enzyme. Also, for a successful biosensor, the immobilized enzyme membrane should be stable for multiple use as well as storage. A variety of synthetic as well as natural polymeric materials have been used for the immobilization of enzymes for biosensor preparation. Among these natural materials, especially of proteinic origin, have shown promise uses. One of these natural materials, onion membrane, has already proven its application for biosensor [7,9]. Other such membrane which has currently gained importance is the eggshell membrane. Eggshell membrane is also a natural material, essentially made up of protein fibers, having flexibility in the aqueous solution and possessing excellent gas and water permeability. It is a light pink double-layered membrane inside the eggshell composed of highly cross-linked proteins similar to keratin, collagen and elastin [13]. Some reports on biosensors have been published using eggshell membrane as a supporting matrix for immobilization of enzymes such as D-amino oxidase, catalase, myrosinase, tyrosinase and glucose oxidase [12,14–19]. The technique, which mostly used for immobilization of enzyme on eggshell membrane, involved adsorption followed by cross-linking with glutaraldehyde. Glutaraldehyde has been used for fixing the enzyme to the support. However, glutaraldehyde cross-linking often reduces the enzyme activity after immobilization [20]. This can be obviated by immobilization of the enzyme through adsorption on charged polymeric supports. Extensive studies have been carried out in our laboratory for imparting polycationic characteristics to a variety of polymeric materials using polyethyleneimine (PEI) for the immobilization of enzymes or cells [21–28].

Urea is a by-product, which is often monitored in blood to obtain information on kidney disease. It is generally accepted to be the best marker for evaluating the level of uremic toxins [29].

Urease (E.C. 3.5.1.5) promotes the reaction involving the decomposition of urea to give ammonia and carbon dioxide.

$$NH_2CONH_2 + 3H_2O \xrightarrow{urease} 2NH_4^+ + HCO_3^- + OH^-$$

Guilbault and Montalvo were the first to fabricate a potentiometric urease enzyme electrode for the estimation of urea through its enzyme-catalyzed hydrolysis [30,31]. In one review, Dhawan et al. [32], reported the recent developments of urea biosensor. Many potentiometric urea biosensors based on the detection of ammonium ion produced by the above enzymatic reaction have been reported [33–36]. Glutaraldehyde cross-linked urease has shown only 15 times operational reusability for biosensor application [37]. In one report, a potentiometric urea biosensor was developed based on modified electrodes with urease immobilized on PEI films that exhibited sigmoidal responses for the urea concentration working range from $1\times10^{-2.5}$ to $1\times10^{-1.5}$ M and a lifetime of 4 weeks [36].

The aim of the present work was to prepare a stable urease–eggshell membrane through adsorption using PEI for biosensor application.

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Reusability, reproducibility and stability of the immobilized membranes were evaluated.

2. Materials and methods

2.1. Materials

Polyethyleneimine (PEI) and jack bean urease (E.C. 3.5.1.5) (910 μM units per tablets) were obtained from Sigma Chem. Co. USA. All other chemicals used were of analytical reagent grade, obtained from standard source.

2.2. Immobilization of urease on eggshell membrane

Eggs were broken and the eggshell membrane was peeled off carefully from the fresh eggshell. The membrane was washed with milliQ water. Circular pieces (2 cm diameter) were cut and were dried in an incubator at 35 °C for 1 h and stored at 4 °C in the refrigerator. The membranes were soaked for 2 h in 5% aqueous solution of PEI (pH 7.0) at room temperature. The PEI-soaked membranes were rinsed with milliQ water, air-dried for 1 h and were stored in a petri dish at 4 °C. An aliquot of 100 μL of jack bean urease (100 units activity) was adsorbed on each membrane. The membranes were air dried at room temperature for 6 h. The immobilized membranes were washed and immersed in buffer (50 mM phosphate pH 7.0) and stored at 4 °C.

2.3. SEM study of enzyme immobilized membrane

An environmental scanning electron microscope (ESEM) (Quanta 200 ESEM, FEI, USA) was employed to observe the surface structure of the eggshell membrane. For SEM study, pieces of the membranes were mounted on stubs and the SEM micrographs of the eggshell membrane (immobilized and unimmobilized) were studied at magnifications 5000×.

2.4. FTIR study of enzyme immobilized membrane

Membranes were studied by FTIR spectra and scanned in the range of 4000–400 cm^{-1} on Jasco (Model FTIR-660 plus) FTIR Spectrometer. The membranes were pressed directly onto the attenuated reflectance with the sampling unit.

2.5. Biosensor operating condition

An ammonium ion selective electrode (Thermo Orion No. 9512 Ammonia, Orion Research Expandable Ion Analyser EA940, Boston, Massachusetts, USA) was placed in a reaction vessel (working volume 10 mL) having provision of flow through tubes as per previous report [9]. Schematic diagram of the operating system has been shown in Fig. 1. The membrane (2 cm diameter) was attached to the tip of the electrode with the help of an O-ring and then placed in the reaction vessel. The baseline potential of the sensor was achieved with flow of buffer solution (50 mM phosphate pH 7.0). The solution was stirred during the measurement on a magnetic stirrer. The membrane electrode was allowed to stabilize for 10 min before recording the reading. Between measurements, the electrode membrane was washed with buffer and used for the next reaction. Data acquisition and analysis was carried as per previous report [9].

2.6. Calibration of biosensor using immobilized eggshell membrane

The standard solution of urea (0.5, 2.5, 5, 10, 25, 50 and 100 mM) was prepared in milliQ water. Ammonium ion selective electrode was attached with urease eggshell membrane. It was first stabilized with flow of buffer as indicated by a stable baseline potential.

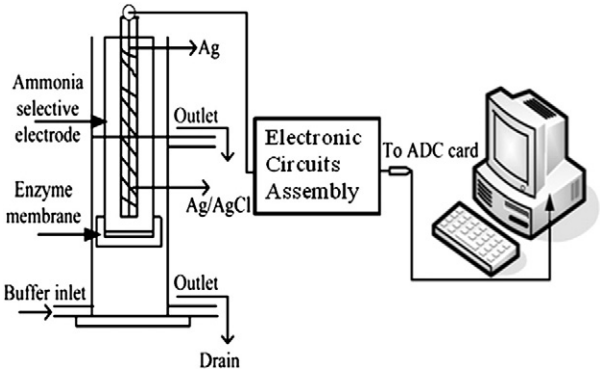

Fig. 1. A schematic drawing of the experimental setup has been included.

Subsequently, 1 mL solution of standard concentration of urea was injected in the vessel and changes in potentials in mVolts were recorded.

3. Results and discussion

3.1. SEM study of the urease immobilized eggshell membrane

SEM study of immobilized eggshell membrane showed a network-like structure without any aggregation on the surface. Membrane consists of highly cross-linked protein fibers and cavities and the observation was similar to the previous report [38]. Surface morphology of eggshell membrane showed the fibers and cavities of the eggshell membrane being occupied with PEI and urease enzyme after immobilization (Fig. 2). SEM micrographs indicated that enzyme was successfully immobilized on the surface of eggshell membrane using PEI.

3.2. FTIR study of urease immobilized eggshell membrane

FTIR spectra of eggshell membrane, shown in Fig. 3 and their peaks, assigned in Table 1 [39–41]. It was observed that four new peaks appeared at 3063, 1394, 980 and 843 cm^{-1} after the PEI treatment on eggshell membrane, which were corresponding to the =C-H stretch of alkenes, -C-H bending of alkanes, C-N stretch of aliphatic primary and secondary amines and N-H wag. of primary and secondary amines respectively. Results showed the occurrence of PEI on eggshell membrane. After immobilization of urease enzyme on eggshell membrane, three more new peaks at 1320, 1172 and 1011 cm^{-1} were appeared which were corresponding to the -C-N stretch of aromatic amines, C-N stretch of aliphatic amines and C-N stretch of aliphatic primary and secondary amines, amide (peptide). Results showed the presence enzyme on eggshell membrane as indicated in term of C-N amide bond in FTIR study.

3.3. Calibration of biosensor and response characteristics using eggshell membrane

The ion selective ammonia electrode was calibrated using standard solution of urea as mentioned in Section 2.6.

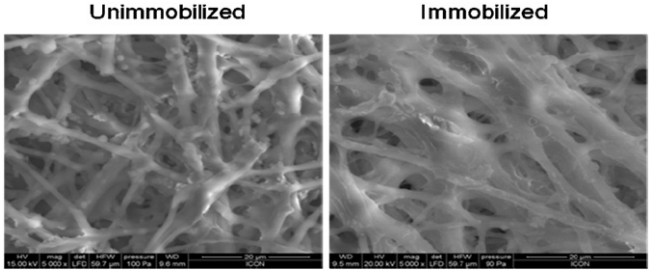

Fig. 2. SEM image of urease immobilized eggshell membrane using PEI.

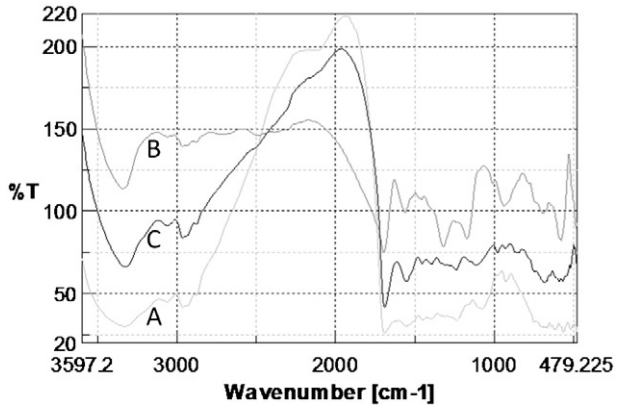

Fig. 3. FTIR study of urease immobilized eggshell membrane using PEI. (A) Only eggshell membrane, (B) Eggshell membrane treated with PEI and (C) Urease immobilized on the PEI treated eggshell membrane.

The potentiometric response was sigmoidal (Fig. 4) and governed by the following Hill equation:

$$Y = V_{max} * X^n / (k^n + X^n)$$

From the observed data, the calculated

V_{max}	2.59676
k	45.85196
n	0.58715

where V_{max} is the maximal enzyme activity of k; n is the Hill coefficient (n), and k is the dissociation constant (k).

The sigmoidal shape of calibration curves depends on the kinetic parameters of the enzyme reaction [42]. For high urea concentrations the enzyme process is a zero order reaction. It indicates that the increase of urea concentration does not raise the concentration of reaction products (causing changes of the pH at the electrode and changes of the electrode potential). However, for low concentrations of analytes it is possible to find experimental conditions for a sigmoidal response of the biosensor [43]. From Fig. 4, it was observed that the calibration curves of the urea biosensors showed a sigmoidal response for the urea concentration working in range from 0.5 to 10 mM with satisfactory regression coefficient ($r = 0.98213$) when the urease eggshell membrane was associated with biosensor. The sigmoidal response of the potentiometric urea biosensor was similar to the previous report [36,44].

Table 1
FTIR peaks assignment for urease immobilized eggshell membrane.

Wave number (cm^{-1})			Peak assignments
A	B	C	
3328	3322	3343	N-H stretch of primary and secondary amines and amides of proteins
	3063		=C-H stretch of alkenes
2964	2964	2967	-C-H stretch of alkanes of lipid and proteins
1692	1690	1698	-C=O stretch of carboxylic acids
1553	1557	1562	N-H bending of amine and C=O stretch of ketones and C=C of benzene
1448	1454		$-CH_3$/$-CH_2$ scissoring lipids, proteins
		1320	-C-N stretch of aromatic amines
	1394		-C-H bending of alkanes
1236	1240		C-O stretch of alcohols, carboxylic acids, esters, ethers
		1172	C-N stretch of aliphatic amines
1076	1108		C-N stretch of aliphatic primary and secondary amines
		1011	C-N stretch of aliphatic primary and secondary amines, amide (peptide)
	980		C-N stretch of aliphatic primary and secondary amines
928	931	938	O-H bending of carboxylic acids
	843		N-H wag. of primary and secondary amines
665	688	690	C-H bending of alkynes

(A) Only eggshell membrane, (B) eggshell membrane treated with PEI and (C) urease immobilized on the PEI treated eggshell membrane.

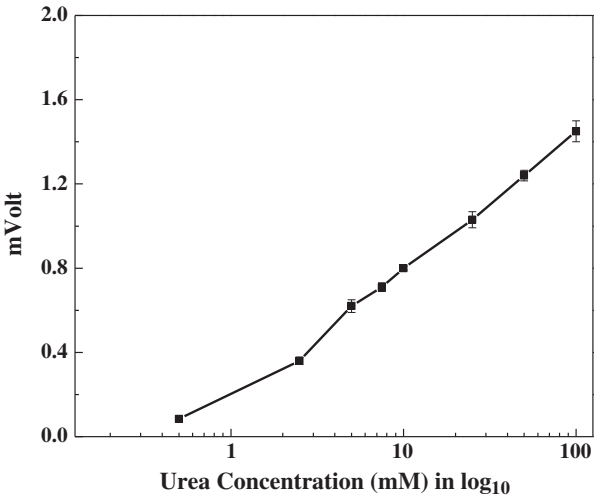

Fig. 4. Calibration of the biosensor using immobilized eggshell membrane.

3.4. Response time, detection range and detection limit

A biosensor should be having low response time, wide linear range and low detection limit. From Fig. 5 (time versus potential curve for urea), it was observed that the saturation of reaction was obtained in 120 s for the whole working range of urea concentration. Therefore, 120 s was considered as response time for the further study of the sensor. The estimated detection range of biosensor was 0.5 to 10 mM which is comparable to some earlier reports [35] or even better than few others [33,36]. A lower detection limit of 0.1 mM was estimated from signal to noise ratio (S/N = 3) in response to blank sample which was comparable to reported literature [35].

3.5. Reusability of the urease eggshell membrane

Reusability is one of the important crucial factors, when the immobilized membrane is considered for biosensor application. Reusability of the urease eggshell membrane in response to urea (5 and 10 mM concentration) on ammonium ion sensitive electrode has

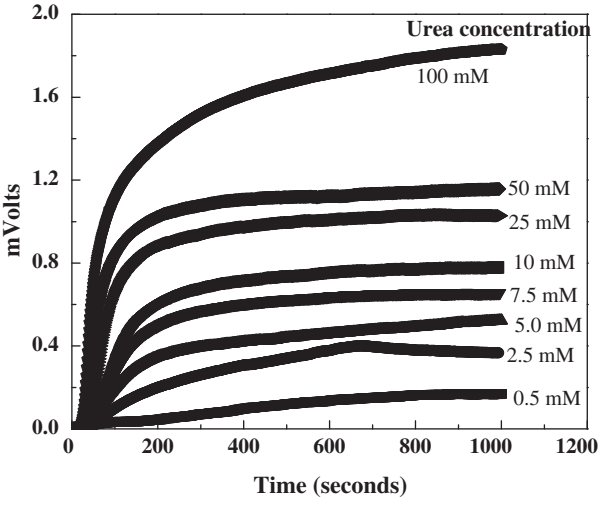

Fig. 5. Enzymatic activity of urease eggshell membrane.

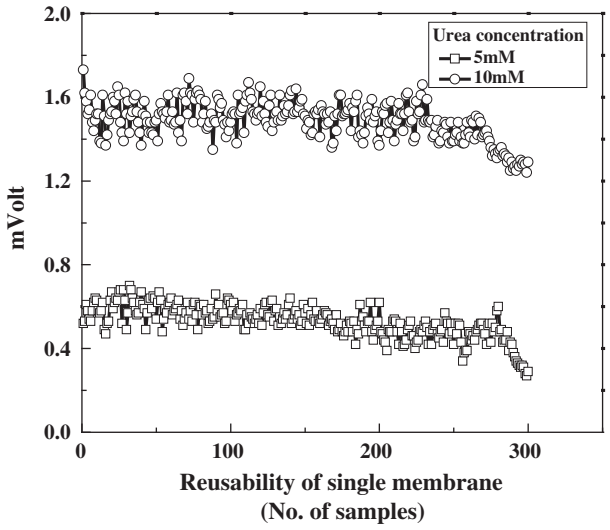

Fig. 6. Reusability of the urease eggshell membranes.

been shown in Fig. 6. Result showed that 90% of enzyme activity was retained up to the 270 repeated reactions of a single membrane, indicating the applicability of this immobilized membrane in urea biosensor study. Reusability of the immobilized membrane was better than previous reports [33,35,36].

3.6. Reproducibility and stability of the immobilized membrane

The low relative standard deviations 0.07725 ($n=6$) and standard error: 0.03154 in response of urease eggshell membrane (Supplementary Fig. 1) on ammonium ion sensitive electrode demonstrated the high reproducibility of analysis.

The urease eggshell membrane, which was prepared under optimum working conditions, was tested for storage stability (Fig. 7). The urease eggshell membrane was studied by observing the enzyme activity at certain interval of days. From result, it was observed that the immobilized enzyme was stable for 2 months when it was stored in buffer at room temperature. The stability of the membrane was comparable and better than the previous reports [33,35,36]. In one review, it was reported that, how PEI with the highest concentration of amino groups, has found acceptance as a carrier in a number of industrial immobilized biosystems [45]. In other review, Mazzaferro et al. [46] has reported the polyethylenimine–protein interactions and implications on protein stability. Polymers with amino pendant groups have been well accepted as suitable enzyme carriers. High stability in the present study may be due to the reason of availability of the highest concentration of amino groups in PEI, which has been used for immobilization of urease enzyme on the surface of eggshell membrane.

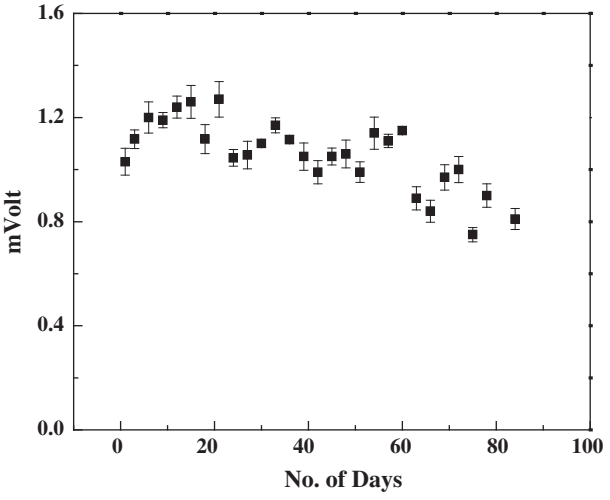

Fig. 7. Storage stability of the urease eggshell membranes at room temperature.

4. Conclusion

The present study described that eggshell membrane can be imparted positive charge by treatment with PEI and can be used for the immobilization of enzymes through adsorption. This technique has advantage over the earlier techniques described for the immobilization of enzymes through adsorption on native eggshell membrane followed by cross-linking in term of reusability. The urease eggshell membrane showed good operational and storage stability. This is also the first report on the immobilization of urease on eggshell membrane. Biosensor exhibited sigmoidal responses for the urea concentration range from 0.5 to 10 mM. The estimated response time of the biosensor was 120 s. A single membrane was reused for 270 reactions, which inferred the applicability of the immobilized membrane for biosensors application. The immobilized membranes were stable for 2 months when stored in buffer at room temperature.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2012.11.010.

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