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Biological Science

PROTOTYPE OF OPTICAL FIBER BIOSENSOR FOR THE REAL TIME DETECTION OF UREA

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ABSTRACT **Background & Objective:** In recent years, there has been a growing impetus in the development of biosensors for medical and biological applications. Various gastrointestinal and urinary tract infections and renal disorders lead to increased blood urea concentrations which require immediate follow-up. This paper reports a low-cost urease sensor for rapid urea detection by immobilizing the enzyme urease on optical fiber by covalent attachment using poly vinyl alcohol (PVA) with glutaraldehyde.

Method: The enzyme was partially purified from Dolichos biflorus seeds and further immobilized for biosensor construction. Two techniques viz. entrapment and covalent attachment were employed wherein the later was found to be a better method. The response of the biosensor for different concentrations of urea was monitored by measuring optical losses by using a power meter.

Result: The prototype constructed displayed a linear decrease in the reflectance measured in decibel with increasing urea concentration. The response time of the constructed biosensor was 30 secs and the lower detection limit was observed to be 2 x 10-6M. A linear response for a wide range of urea concentrations viz. 2 µM to 50 mM was obtained.

Interpretation & conclusion: The highlight of this biosensor is that it works on partially purified enzyme urease and a minimum volume of 10 µl is required for the detection of urea in sample.

KEYWORDS : Urease, Dolichos biflorus, covalent immobilization, SEM, Urea biosensor

INTRODUCTION:

Urea is the end product of nitrogen metabolism and has great significance in clinical chemistry since blood urea is an important indicator of possible kidney malfunction[1]. The normal level of urea in serum is 15 – 40 mg/dl and that in blood is 10 – 50 mg/dL[2]. While in patients suffering from renal insufficiency the urea concentrations in serum vary from 180 to 480 mg/dL[3]. Many gastrointestinal or urinary tract pathogens produce urease, enabling the detection of urease to be used as a diagnostic to detect presence of pathogens such as *Proteus vulgaris, Helicobacter pylori, Ureaplasma urealyticum*; *Nocardia*, *Klebsiella* spp., *Morganella*, *Providencia*, *Brucella, etc.* Urease activity is involved in urolithiasis, catheter encrustation, pyelonephritis, ammonia and hepatic encephalopathy, hepatic coma, urinary tract infections, and is known to be the major cause of pathologies (including cancer) induced by gastroduodenal infections like *H. Pylori*[4]. *H. Pylori* is considered the most important risk factor in the frequency of peptic ulcer diseases (PUD). A recent study by Dutta et.al indicated the frequency of infection was 45.7% *for* men and 33.2% for women amongst 1000 patients with dyspepsia included in the study[5].

The current diagnostic methods for detection of these infections include invasive tests such as upper GI endoscopy and biopsy from stomach for bacteriologic culture and susceptibility testing, histopathologic studies and molecular diagnostics, rapid urease test (RUT) and PCR. RUT is an inexpensive, sensitive and specific technique wherein urease enzyme produced by the bacteria hydrolyzes urea into ammonia causing a change in pH which can be measured easily. The limitation of this method is it may produce false negative result if the number of bacteria is less or the bacteria colonizes in the body and fundus. Urea breath testing (UBT) is an expensive non-invasive test with high sensitivity and specificity. Yet, no single test is the gold standard for detecting *H.pylori* infections. Hence, there is an impetus to develop better analyzers that can monitor urea in clinical samples, be cost effective and also be free of disadvantages[6].

The need for simple, rapid, inexpensive and continuous *in-situ* monitoring techniques have led to the development of various biosensors. They address the need for real-time analytical measurements in the field, where traditional measuring equipment cannot be used because of their physical constraints. Also, use of biosensors minimises the time lapse which is created during traditional method of sample preparation and measurement. Biosensors can be used to measure biological effects such as genotoxicity, immunotoxicity, biotoxins and endocrine effects. Also, to measure the concentrations of specific analytes that are difficult to detect and are important contaminants of water, waste water, soil, or air e.g. surfactants, chlorinated hydrocarbons, pesticides, heavy metals. Recently, biosensors are gaining widespread use in broad range of areas like environmental monitoring, food processing industry, clinical analysis, pharmaceutical industry, brewing, etc[7].

For many years, optical techniques like endoscopy, opthalmoscopy, colonscopy have been used in clinical practice. Recently, the development of laser and low cost optical fibers has been exploited for the development of fiber optic based biosensor. Optical fibers being flexible act as a good optical interface between the spectroscopic device and the sample which is to be examined *in situ*[8]. Fiber optic biosensors have various attractive characteristics like small size, light weight, high sensitivity, environmental ruggedness, passive composition, high temperature performance and low cost[9].

The current work aims the fabrication of urease based biosensor for detection of infections by urease positive pathogens. Urease enzyme was partially purified from *Dolichos biflorus* seeds using ammonium sulphate fractionation method. The enzyme was further immobilized using entrapment and covalent attachment method on optical fiber. These optic fibers were then used to fabricate a prototype biosensor to measure urea levels for diagnostics purpose.

MATERIALS AND METHODS:

Urease isolation and partial purification:

10 g of *Dolichos biflorus* seeds were soaked in 100ml of 0.2 M potassium phosphate buffer at 4°C overnight with constant stirring. The suspension was then filtered and the filtrate was then centrifuged at 10,000rpm for 30mins at 4°C to remove the impurities.

The urease enzyme was isolated by Ammonium sulphate fractionation. Differential precipitation was performed at 30%, 40%, 50% and 60% salt saturation. The pellets of each saturation level were then dialysed against 0.2 M potassium phosphate buffer to remove the ammonium sulphate. After each precipitation step, the pellets obtained after centrifugation were evaluated for urease activity and the total protein

content[10]. Urease activity was measured by using Berthelot assay[11].

Characterization of the free enzyme:
The optimum pH and optimum temperature of the partially purified enzyme was determined by measuring its activity in buffers of varying pH and over a varying range of temperature. Also the kinetic parameters viz. the K_m, and V_{max} values were calculated. The storage stability was determined by evaluating the enzyme activity at regular intervals.

Immobilization:
Immobilization was attempted using two methods viz. entrapment and covalent attachment. Entrapment was done by immobilizing urease in 3% polyvinyl alcohol (PVA) matrix. Covalent attachment of urease was carried out on pre-activated optic fibers[12] using two different immobilizing agents 1% polyethylene glycol (PEG) and 5% glutaraldehyde by using the protocol reported previously[13].

Characterization of immobilized enzyme:
The kinetic parameters of the enzyme immobilized by both methods i.e. entrapment and covalent attachment were studied. The immobilized enzyme was characterised by checking its optimum pH, optimum temperature, Km, and V_{max} values. Also, the percentage retention of the enzyme activity and mass transfer was studied. The samples were stored at 4°C and the enzyme activity was monitored at regular time intervals to evaluate the storage stability.

Surface characterization:
Scanning electron Microscopy (SEM) was performed to study the surface morphology of the immobilized enzyme. The surface features of blank optic fiber and enzyme covalently attached to optic fiber using glutaraldehyde were observed.

Biosensor Configuration:
For construction of the biosensor, a cell having capacity of 50l was taken and placed on a solid support. Two openings were made in the cell for the optic fiber tips. Optic fiber containing urease enzyme covalently immobilized on the optic fiber tip using glutaraldehyde was used as transmitter. Laser source (1550 nm) was attached to the transmitter optic fiber and a blank optic fiber was connected to power meter which served as the reciever. The tips of both transmitter and receiver cables were perfectly aligned and a distance of 2 mm was maintained between the tips. The entire assembly was covered by placing it in a black cardboard box so as to maintain dark conditions.

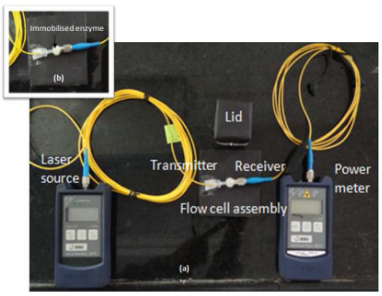

Fig.1: Biosensor prototype (a) Flow cell assembly (b) Immobilized enzyme

Calibration of the biosensor:
The calibration curve for the biosensor was obtained by measuring the signal in decibels (dBm) at various concentrations of urea. A concentration of urea in the range of 0-50 mM was used to prepare the standard graph. The response for 50 µl urea solution was measured after 1 minute.

Biosensor Performance:
The minimum volume required for the sensor response was determined by measuring the response for varying volumes of urea solution. The sensitivity of the biosensor was analysed by checking its lower detection limit and linear range. The response time was checked by recording the time in which a stable biosensor response was obtained. Repeatability and storage stability of the biosensor were also measured.

RESULTS AND DISCUSSION:
Very few reports of urease activity of *D. biflorus* are available in literature [14,15]. But since *D. biflorus* seeds are available in local markets and present throughout the year, its seeds were chosen for urease extraction. Also, urease from *D. biflorus* seeds has been used for biosensor development by our group previously[16].

Table 1: Characteristics Of The Partially Purified Enzyme

Characteristics	Purified Enzyme
Total Protein	0.404 mg/ml
Specific Activity	0.0148 Units/mg
Km	0.027 mM
Vmax	0.0082 Units/min
Optimum Temperature	50°C
Optimum pH	7
Storage stability	15 days

Immobilized Urease:
Urease immobilized by entrapment method using Polyethylene glycol showed less activity as compared to covalent attachment. Also, it was observed to be less stable than urease immobilised using covalent attachment. Hence, covalent attachment method was chosen for further immobilisation of urease on the optic fiber surface. Covalent attachment of urease was carried out using PVA and glutaraldehyde (Table 2)

Table 2: Immobilization Characteristics Of Enzyme Using PEG and PVA/Glutaraldehyde

Characteristics	Immobilisation using Polyethylene glycol	Immobilisation using PVA/glutaraldehyde
% retention of activity	76.10%	93.50%
Mass transfer	0.766	0.932
Km	0.124 mM	0.162 mM
Vmax	0.03 mM/min	0.037 mM/min
Optimum Temperature	50°C	50°C
Optimum pH	Ranges between 7-8	7
Shelf life	27 days	27 days

The percentage retention of urease activity and the mass transfer was observed to be better in enzyme immobilized using PVA/glutaraldehyde rather than the using PEG. The high percentage retention of enzyme activity of the immobilised enzyme, suggests that the activity of the immobilized enzyme is not hampered due to the chemical used.

Surface Characterization:

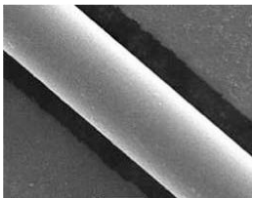

Fig. 2(a): Blank optic fiber

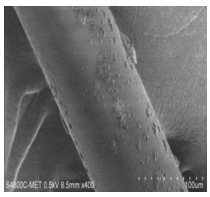

Fig. 2(b): Urease covalently attached using PVA/glutaral dehyde

Urease immobilised on the surface of optic fibers by covalent attachment using glutaraldehyde was confirmed by Scanning Electron Microscopy (SEM). Fig. 2(a) is the scanning electron micrograph of blank optic fiber. Fig. 2(b) shows deposition of immobilized enzyme covalently attached using glutaraldehyde. As compared to blank optic fiber, which has smooth appearance with no deposition, optic fiber with immobilised urease was observed to have rough appearance.

Biosensor Construction:
Working Principle:
The constructed biosensor is refractive index based biosensor. It measures the change in the refractive index of the analyte caused due to changes in its ionic concentration. When the laser source is switched on, the light passes through the transmitting cable. When an analyte is present in its path, the refractive index of the medium changes and the transmitted light is refracted. This refracted light is then collected by the receiver and transmitted to the power meter. The power meter then measures the change in the reflectance spectra. When an enzyme is coated onto the transmitting cable, the analyte interacts with the enzyme, thereby changing the refractive index of the analyte solution. This change in refractive index is proportional to the concentration of

the analyte. The change in the reflectance is thus measured in decibels (dB) by the powermeter (Fig 3). Optical power is usually measured in dBm, or decibels referenced to one milliwatt. This log scale is used because of the large dynamic range of fiber optic links, a range of 1000 or more. Power meters measure average optical power, not peak power, so they are sensitive to the duty cycle of the data being transmitted. Lower cost field optical power meters usually have a resolution of 0.1dB. Generally, the single mode fiber is tested with lasers at 1310nm and 1550nm.

Fig 3: DBC Power meter to monitor the sensor response in decibels (dB)

Reaction Occurring At The Sensing Tip:

The reaction occurring at the sensing tip of the biosensor is given in figure (Fig. 4). It can be seen that four different types of ions are formed when urease reacts with urea in aqueous solution i.e. NH_4^+, CO_3^{2-}, HCO_3^- and NH_2COO^-. Hence a polyelectrolyte solution is formed, out of the four ions formed, NH_4^+ and CO_3^{2-} ions are more active. This leads to the adsorption of the ions onto the optic fiber. Due to polyelectrolyte formation, the ionic strength of the solution increases which in turn increases the charge density of the optic fiber. This changes the refractive index of the polyelectrolyte. The change in refractive index is inversely proportional to the ionic strength of the solution[17].

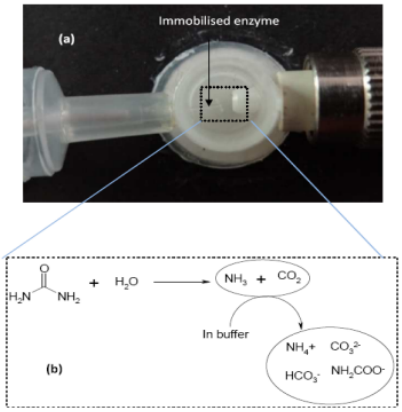

Fig. 4: Reaction Occurring At The Sensing Tip Of The Biosensor

Calibration Of The Biosensor:

The response of the biosensor was checked for urea concentrations ranging from 2 uM to 50 mM. The response was measured after 1 minute. Fig. no. 5 shows a urea calibration graph obtained with this biosensor. The power meter measures the loss in decibel values upon addition of urea solution. Increasing loss in the decibel values was found upon increasing the urea concentration. The loss in decibel values was found to be inversely proportional to the urea concentration.

A blank optic fiber coated only with glutaraldehyde without the enzyme was checked for activity at varying concentrations of urea. No change in the refractive index was observed at any urea concentration (data not included).

The biosensor was calibrated by measuring its response over the urea concentration range of 2 uM – 50 mM exhibiting a linear response. Thus the concentration range of the biosensor constructed was found to be broad. Also, the sensitivity of the constructed biosensor was found to be comparable to earlier reports, with the lower detection limit of 1 x 10^{-6} M [15].

Response Time, Repeatability And Minimum Volume Required:

Response time of a biosensor is the time required by the sensor from the addition of an analyte to the sensor to achieve stable output. The response time of the constructed biosensor was found to be 30 seconds. Hence, rapid detection of urea can be done using the biosensor. Also, the output signal was measured every 5 minutes and the readings were observed to be stable for 30 minutes after the addition of urea solution. Thus, fast and stable response is observed for the fabricated biosensor.

The minimum volume required was determined by checking the response of the biosensor for decreasing volumes of urea solution. A stable response of the biosensor was observed for 10µl of urea solution. Thus, the biosensor can be used for detecting urea in samples with low volumes.

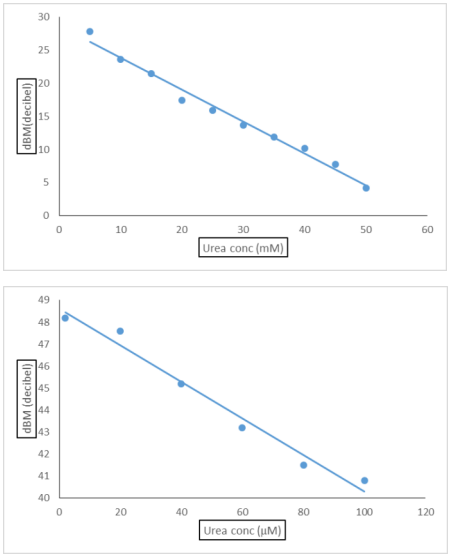

Fig. 5: Calibration curve of the constructed biosensor(a) 5 to 50 mM (b) 2 to 100 M

The biosensor was checked for its response for 25 cycles. A stable output was observed for 21 cycles. Thus, the repeatability of the biosensor is high. After 21 cycles, a sudden drop in the response was observed. As the ionic concentration of the electrolyte increases, the adsorption of the ions on to the optic fiber surface occurring due to electrostatic interactions decreases. Due to this decrease in the adsorption of the ions, the surface charge present on the optic fiber does not change and hence a drop in response is observed[16].

Table 3: Biosensor Performance

Biosensor characteristics	Performance
Minimum Volume required	10 µl
Response time	30 secs
Linear range	1 µM – 50 mM
Lower Detection Limit	1 µM
Repeatability	21 cycles
Storage stability	27 days

CONCLUSIONS:

The prospect of making a cost effective prototype device for urea detection was achieved by fabricating urease based optic fiber based biosensor. Urease was partially purified from a local source, viz *Dolichos biflorus* and immobilised on optic fiber surface using covalent attachment and entrapment. Urease immobilisation by covalent attachment was better than entrapment. The characteristics of free enzyme as well as the immobilised enzyme were studied and compared. The kinetic properties of the immobilised enzyme were observed to be comparable to free enzyme. Also, the shelf life of the immobilised enzyme was found to be better than free enzyme. The biosensor constructed can be used for simple and rapid detection of urea. The biosensor constructed was observed to have very short response time i.e. 30 seconds and the response is stable for 30 minutes after the addition of urea. Thus, it can be used where rapid detection of urea is necessary. The biosensor has a very broad linear range of 2 µM to 50 mM. The lower detection limit of the biosensor is 2 µM. Hence, the sensitivity of this biosensor is high and is comparable to the biosensors reported. The biosensor can be useful for low volume sample as the minimum volume required by the biosensor is 10 µl. Further studies are ongoing to develop a commercial device. Thus a device has been constructed for rapid detection of urea which can be used detection of urea in milk, clinical samples etc.

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