Fiber-optic biosensors based on covalently immobilized enzymes and thymol blue

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Fiber optic biosensors based on immobilized enzymes (urease, lipase and acetyl cholinesterase) in conjunction with immobilized thymol blue are described, and their uses in the detection of a wide range of analytes are discussed. The bioactive materials of the optical biosensors consist of enzymes covalently attached to preactivated controlled pore glass mixed with thymol blue, also covalently bound to similar matrix. A thin layer of the glass bead mixture was spread at the tip of a bifurcated fiber-optic sensor head to construct the sensor, which was then integrated with a flow-cell. The biosensors exhibit responses to different types of analytes (substrates, metal ions and pesticides), which are highly reproducible and linear over a range of analyte concentrations.

INTRODUCTION

The coupling of fiber-optic chemical sensors with immobilized enzyme systems marked a milestone in biochemical analysis. The development of optical fiber biosensors based on enzymes enhanced the capability of the optical methods of chemical analysis, which have long been a highly popular technique in enzyme assays. The integration of optical fibers in optochemical analytical systems enabled miniaturization and simplification of the assay procedures. As a result, optochemical measurements can now be performed on microliter quantities of the measurand system, with minimum pretreatment procedures. Chemical analysis can also be carried out *in situ* and in real time even in locations that are remote to the instrumentation systems or even difficult to reach. Likewise, the incorporation of immobilized enzyme systems as a molecular recognition element imparted high selectivity, increased versatility, reagent economy and improved analytical throughput.

Oftentimes, the sensing chemistry involved in optical fiber biosensors based on immobilized enzymes is characterized by the formation or consumption of low-molecular species, such of H^+, O_2, CO_2, NH_3 or H_2O_2. These species can be easily detected through suitable optical sensors that employ chromogenic or fluorogenic reagent phases. The response of these sensors can be related to the concentration of the substrate of the enzyme reaction. Reviews on this type of biosensors have been recently published [1, 2].

This paper focuses on optical fiber biosensors employing covalently immobilized enzymes and thymol blue. The immobilized enzymes catalyze hydrolysis reactions which generate acidic entities that can be detected through a change in the optical properties of thymol blue in the reagent phase. Thymol blue is a sulfonephthalein dye that changes its color in the pH interval of 5.0 to 9.0. The biosensors are useful in measuring the enzyme substrates and enzyme inhibitors.

EXPERIMENTAL

Materials. The enzymes used in this study are urease (E.C. 3.5.1.5, from jack beans, Type III, U15000, 2700 units/gram), lipase (E.C. 3.1.1.3 from *Candida cylindracea*, 60000 units/ mg solid) and acetylcholinesterase (AchE, E.E. 3.1.1.7, from electric eel, 225 units/mg solid). These enzymes were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). The corresponding substrates are urea, which was purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK), triacetin and acetylcholine hydrochloride, both of which were purchased from Sigma Chemical Co. Thymol blue as a sodium salt, morpholineethane sulphonic acid monohydrate (MES), potassium dihydrogen phosphate and tris(hydroxymethyl) aminomethane hydrochloride were also obtained from Aldrich Chemical Co.

The solid support used for immobilization are aminopropyl glass (G4643, ave. pore size = 50 nm, 200–400 mesh, 81 µmol amine/gram glass) and isothiocyanate glass (G4893, ave. pore size = 50 nm, 200–400 mesh, 39 µmol isothiocyanate/gram glass), both of which were obtained from Sigma Chemical Co. Formaldehyde (37–40%) and maleic acid were acquired from BDH-Merck Ltd. (Lutterworth, Leicestershire, UK).

All the chemicals employed were of analytical reagent grade, and were prepared using distilled deionized water.

Immobilization of thymol blue on aminopropyl glass beads. Thymol blue was immobilized on aminopropyl controlled pore glass by mixing the indicator and formaldehyde solution in a weighed amount of the glass material. In a typical batch preparation, about 200 µL of 10 mM formaldehyde solution, prepared in 0.1 M MES solution, was first added to 10 mg portion of the glass microbeads, kept in a small stoppered glass vial. This was then followed by the addition of 200 µL of 5 µM aqueous thymol blue solution. The mixture was gently swirled, and then stored in a pre-heated oven at 50–60°C for at least 48 h to effect the covalent binding of the indicator. At the end of this period, the excess indicator solution was carefully decanted. The glass beads were subsequently washed thoroughly, first with 2 M NaCl then alternately with copious amounts of pH 10.0 and pH 12.0 buffer solutions, followed by distilled water to remove any weakly bound or unbound indicator molecules. The glass beads were subjected to intermittent (3 to 5 times) and brief (10 s) ultrasonic agitation to facilitate a more thorough

removal of the excess indicator molecules. The dye-modified glass beads were stored as a suspension in a pH 7.0 phosphate buffer (0.1 M).

Immobilization of enzymes on isothiocyanate glass. The enzymes were immobilized by direct covalent coupling on preactivated isothiocyanate glass beads via thiourea linkages. The process simply involved interfacing a buffered solution of the enzyme with a given amount of the activated solid support. The enzyme solutions were prepared by dissolving a small amount of the enzyme reagent in a pH 9.0 borate buffer (0.05 M): urease (1.5 mg/1.0 mL buffer); acetylcholinesterase (0.5 mg/1.5 mL buffer) and lipase (3.5 mg/350 µL buffer). Aliquots of these solutions were then pipetted into separate 10 mg portions of isothiocyanate glass, kept in a small glass vial and mixed with pH 8.5 borate buffer (0.05 M): 100 µL urease solution +200 µL buffer; 50 µL acetylcholinesterase solution + 350 µL buffer; and 100 µL lipase solution + 200 µL buffer. The mixtures were then kept in a refrigerator for at least 2 h to allow the covalent binding of the enzyme. Then, the glass beads were washed thoroughly with cold distilled water, and pH 7.0 phosphate buffer (0.1 M) to remove the unbound enzyme molecule.

Biosensor construction. The sensitive reagent phases consist of the glass-immobilized enzyme intimately mixed with the glass-bound thymol blue indicator. This was prepared by thoroughly mixing each 10 mg batch of the enzyme-modified glass beads with an equivalent 10 mg portion of the thymol blue-modified aminopropyl glass material. The glass bead mixtures were stored as a suspension in a pH 7.0 phosphate buffer (0.1 M) at 4°C, where the mixture remained stable for several months.

The optical biosensors were constructed by pipetting small amounts of the immobilized reagent into the microwell (100–150 µm deep × 1.5 mm dia.) of a bifurcated fiber-optic sensor head (see inset of Fig. 1). There were then blotted dry with an absorbent paper and carefully packed until an even and compact solid layer filled the microwell at the tip of the sensing head.
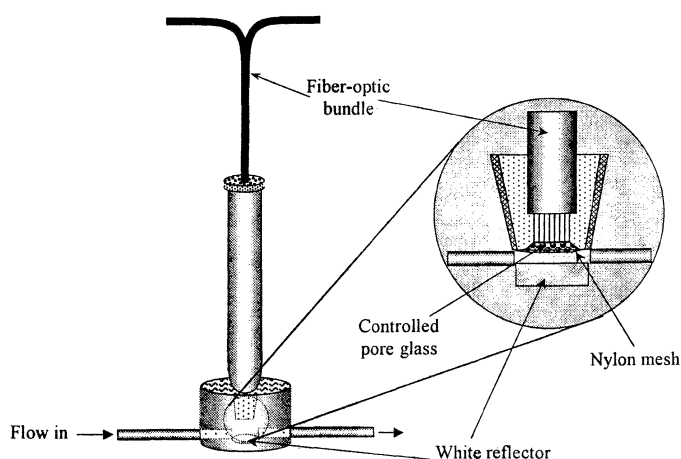

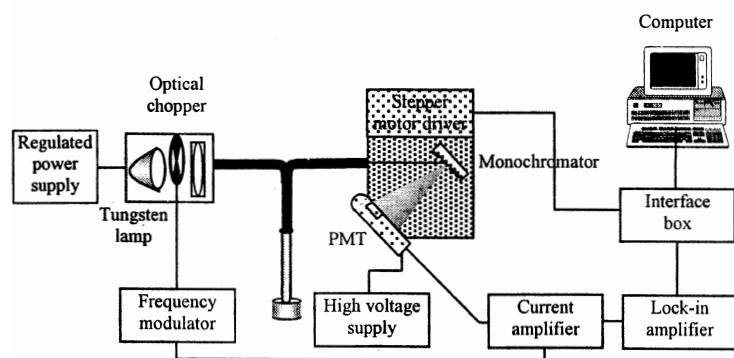*Fig. 1. Construction of the fiber-optic sensing head.*

Fig. 2. Schematic diagram of the fiber-optic instrumentation.

The solid particles were retained using a fine nylon mesh, which in turn was held in place by a plastic support tube that fitted snugly into the sensor head.

Flow cell assembly. The flow cell assembly (see Fig. 1) was machined from a Perspex cylinder (1 cm dia. × 1 cm height). A hole (ca. 6 mm deep) was bored at the center of the base of the cylinder to fit the sensing end of the biosensor probe. A white PTFE plug was set at the bottom of this hole about 1.5 mm away from the probe tip to act as a reflective surface backing. Perpendicular to this hole was bored another hole which extended across the diameter of the cylinder. The internal volume of the flow cell was approximately 25 µL. Two 19G (1 mm o.d., 20 mm length) stainless steel needles were affixed at the ends of this bore to serve as the inlet and outlet of the flow cell. The inlet was connected to an injection valve loop for the introduction of the sample, and the outlet was connected to a peristaltic pump. The flow cell was immersed in a thermostated water bath to maintain a constant temperature of 25°C.

Instrumentation. A schematic diagram of the instrumentation system used for the fiber-optic measurements is shown in Fig. 2. The set-up consists of a xenon lamp (12 V, 50 W), whose radiation was modulated by an optical chopper (Bentham 218) and focused onto the end of one arm of a bifurcated optical fiber, using simple condensing optics. The 16-polymer-fiber bundle arm directs the radiation to the common end of the bifurcated fiber, where it interacts with the reagent phase and is reflected back to the other 16 collecting optical cables. The reflected radiation is then carried into a grating monochromator (ISA Instruments, Jobin-Ivon Type H10-61) and detected by a photomultiplier (Hamamatsu R446). The photodetector signal was enhanced by a current amplifier (Bentham 286) and a lock-in amplifier (Bentham 223). The amplifier output was displayed on a digital voltmeter (Bentham 217) and recorded by a microcomputer (Viglen II) through a 12-bit A/D interface system (3D Digital Design and Development, Inc.).
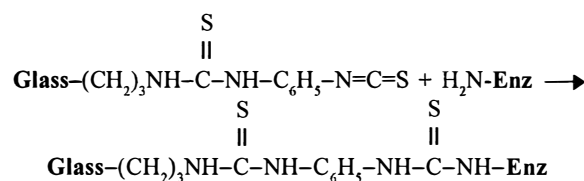
RESULTS AND DISCUSSION

Immobilization of sensing reagents. Reagent immobilization can be carried through a number of physical and chemical methods. The physical methods include adsorption, physical entrapment or electrostatic attraction of the reagent molecules on a solid support, and involve simple procedures and mild conditions. However, the binding forces in physical immobilization are weak and the reagent could leach off the solid support. The chemical methods provide a more stable means for reagent immobilization, since here covalent bonds are formed between the reagent molecules and the solid support.

A number of techniques have been employed for the chemical immobilization of reagents. A variety of solids could be used as the immobilization matrix, including inorganic materials such as glass, or organic polymers such as cellulose and nylon. In the biosensors described in this paper, controlled pore glass was employed as the supporting solid due to the ease of integrating it with an optical fiber. The large surface area of the controlled pore glass enables the binding of a large amount of reagent and its high porosity facilitates diffusion of the analyte to the immobilized reagents. Covalent bond formation is made possible by the introduction of functional groups in the silica matrix of the glass microbeads.

In the aminopropyl glass employed for the immobilization of the sulfonephthalein dye, the amino group undergoes a condensation reaction with formaldehyde and the dye molecule. This reaction which is known as the Mannich reaction can be represented through the following chemical reaction:

$$\text{Glass--}(CH_2)_3NH_2 + HCHO + H\text{--Dye} \longrightarrow$$

$$\text{Glass--}(CH_2)_3NH\text{--}CH_2\text{--Dye}$$
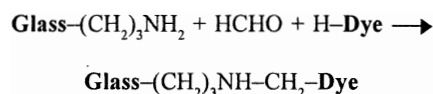
In the isothiocyanate glass used for the immobilization of the enzyme molecule, the isothiocyanate moeity forms a thiourea bond with an amino group in the enzyme molecule. This reaction can be written as follows:

$$\overset{\overset{\displaystyle S}{\|}}{\text{Glass--}(CH_2)_3NH\text{--}C\text{--}NH\text{--}C_6H_5\text{--}N{=}C{=}S} + H_2N\text{--Enz} \longrightarrow$$

$$\underset{\text{Glass--}(CH_2)_3NH\text{--}\overset{\overset{S}{\|}}{C}\text{--}NH\text{--}C_6H_5\text{--}NH\text{--}\overset{\overset{S}{\|}}{C}\text{--}NH\text{--Enz}}{}$$

Optical fiber pH sensor. Chemical transduction of the pH in the optical fiber enzyme-based biosensors was provided by immobilized thymol blue indicator. The spectral characteristics of this reagent phase was studied by loading indicator-modified glass microspheres, without the enzyme-modified glass beads, in the microwell of the fiber-optic probe. The immobilized acid-base indicator exhibited a yellow coloration in acidic pH and turned blue-violet when exposed to basic conditions. The
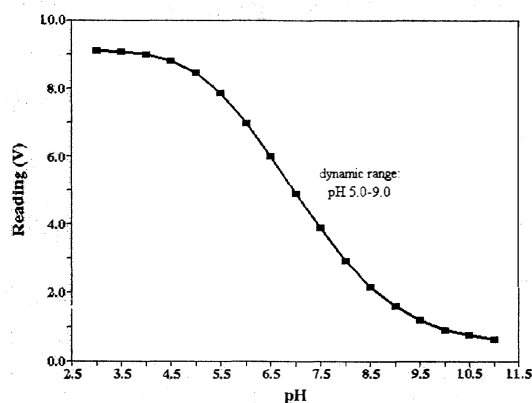
Fig. 3. pH vs. reflectance intensity profile of thymol blue covalently immobilised on controlled pore glass (measured at 600 nm).
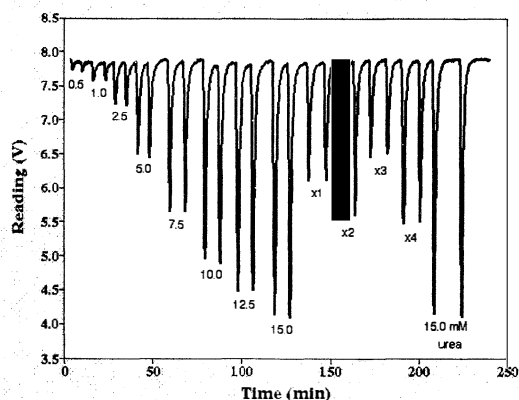

Fig. 4. Flow-injection time-response curve of the urea optrode to different concentrations of standard urea solutions and diluted urine samples. Experimental conditions: flow rate = 0.5 mL min⁻¹, carrier buffer = pH 6.0, 7.5 maleate solution, T = 25°C.

reflectance spectra obtained from this pH sensor under acidic and basic conditions showed maximum divergence at 600 nm. This wavelength was used in subsequent measurements since it ensured maximum sensor sensitivity.

The calibration curve of the pH sensor based on immobilized thymol blue is shown in Fig. 3. The dynamic range is relatively wide, covering the pH range of 5.0 to 9.0. However, the linear range lies in the pH range of 5.5 to 8.5. This response is markedly different from the behavior of the indicator in a solution phase, wherein the color change occurs between pH 8.0 and 10.0.

Urease-based biosensor. Urease catalyses the hydrolysis of urea, as expressed by the following equation:

$$H_2\text{–}CO\text{–}NH_2 + 3 \text{ H2O} \xrightarrow{\text{urease}} 2 \text{ } NH_4^+ + HCO_3^- + OH^-$$
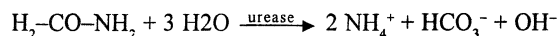
The reaction yields OH⁻, so that it causes an increase in the pH of the microenvironment of the reaction system. This change in pH is detected by the immobilized thymol blue indicator which subsequently generates a decreased optical signal.

The sensor response exhibited very good reproducibility, with a relative standard deviation of 1.9% for eight measurements. The response was highly dependent on the pH of the analyte solution, the buffer composition and the buffer concentration. These effects can be attributed to the pH dependence of enzyme activity and the sigmoidal pH characteristic of thymol blue indicator. The optimum conditions were pH 6.0 and phosphate buffer having a concentration of a 7.5 mM. The flow-rate and temperature expectedly affected the sensor response. A decrease in the sensor response occurred as the flow rate was increased due to the diminished contact of the analyte with the enzyme system.

Figure 4 show the response of the urease optrode to different concentrations of urea standards and to diluted urine samples. The response was linear between 0.5 and 10 mM urea (r^2 = 0.999) and had a sensitivity of 297 mV V per mM urea. The urea concentrations for the original urine samples were calculated to vary from 97 to 165 mM.

The response of the urease-based biosensor was found to be highly sensitive to the presence of heavy metal ions, such as Hg^{2+}, Ag^+, and Cu^{2+}. This can be attributed to the inhibitory effect of these ions on enzyme activity through their binding with the sulfhydryl group in the active site of the enzyme. The extent of inhibition varied with the coupling reagent used in the immobilization of the enzyme, with phenylene
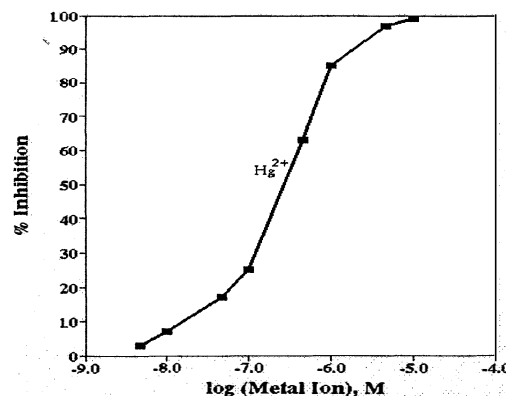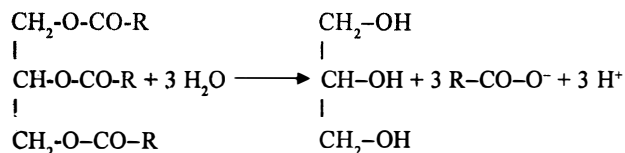

Fig. 5. Calibration curve for Hg²⁺ ion based on the inhibition of immobilised urease coupled using cyanuric chloride activation. Measurement was performed with alternate reactivation with pH 6.0, 0.75 M diethylenetriaminepentaacetic acid.

diisothiocyanate yielding an immobilized enzyme with excellent resistance to metal inhibition [3]. Thus, urease bound through phenylene diisothiocyanate, as described in this paper, can be used to measure urea even in the presence of an inhibiting metal ion. However, the bound urease (e.g., via cyanuric chloride) exhibiting sensitivity to the presence of metal ions is useful for the biosensing of the inhibitors [3]. Figure 5 shows a calibration curve for the measurement of Hg^{2+} based on its inhibitory effect on a urease-based biosensor.

Lipase-based biosensor. The analytical application of the lipase-based biosensor arises from the following reaction:

$$CH_2\text{-O-CO-R} \qquad\qquad CH_2\text{-OH}$$
$$|\qquad\qquad\qquad\qquad\qquad |$$
$$CH\text{-O-CO-R} + 3\,H_2O \longrightarrow CH\text{-OH} + 3\,R\text{-CO-O}^- + 3\,H^+$$
$$|\qquad\qquad\qquad\qquad\qquad |$$
$$CH_2\text{-O-CO-R} \qquad\qquad CH_2\text{-OH}$$

The reaction produces an acid and will therefore be accompanied by a lowering in the pH of the reaction system. In the presence of immobilized thymol blue, this enzyme reaction will cause an increase in the signal obtained from the optical fiber biosensor.

The sensor response was affected by pH, buffer concentration and buffer type, typical of sensors based on the detection of pH changes. The best response was observed using 10 mM Tris buffer adjusted to a pH of 7.5. The presence of detergent, that is often added to solubilize the lipid substrate, influenced the signal generated by the sensor. The sensor response varied with the amount of the added detergent, the highest response being obtained at a 0.05% (m/v) level of Triton X-100.

The dependence of the response of the lipase-based optical fiber biosensor on the concentration of triacetin is presented in Fig. 6. The sensor responded to this acylglycerol over a wide

dynamic range covering 10 mM to 250 mM, but a linear behavior occurred only in the lower concentration range (10–75 mM). In the linear range, the correlation coefficient is 0.999 and the sensitivity is 18.5 mV/mM triacetin. The response of the lipase-based sensor changed with the triacylglycerol being measured. For tributyrin, the sensor response was lower and the dynamic range covered only a narrow range (10–75 mM). This behavior could be attributed to the solubility characteristics of the tributyrin and to the acidity of the fatty acid produced in the enzyme reaction. For an acylglycerol that has a long-chained fatty acid and very low water solubility, such as triolein, the sensor showed no sensitivity at all.

Lipase has been reported to be useful for the assay or detection of carbamate and organophosphorus pesticides [4–6]. These analytical applications exploited the inhibitory effect of these pesticides on the activity of lipase. Measurements done using the optical fiber lipase-based biosensor revealed insensitivity to pesticides such as aldicarb, carbofuran and paraoxon at concentrations of 1×10^{-5} to 1×10^{-4} M. This resistance to inhibition may be a consequence of the immobilization of the lipase wherein the preferred conformation conceals the serine active site from attack by the pesticide molecules.

Acetylcholinesterase-based biosensor. Chemical measurements employing the enzyme acetylcholinesterase (AChE) are often based on the hydrolysis of acetylcholine, as represented by the following equation:

$$\text{Acetylcholine} + H_2O \longrightarrow \text{Acetic acid} + \text{Choline}$$

The reaction releases acetic acid from the substrate and results in a lowering of the pH of the reaction medium. This pH change causes the signal of an optical fiber pH sensor based on thymol blue to increase.
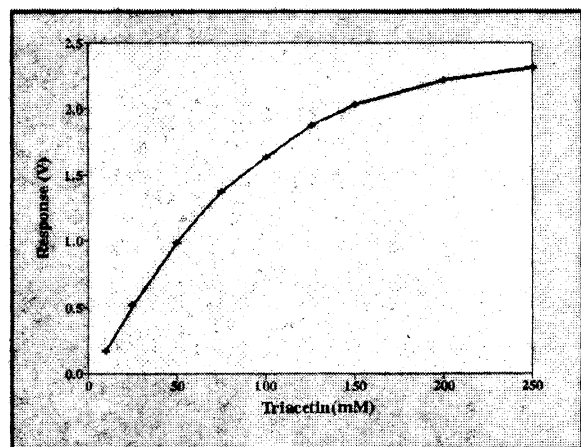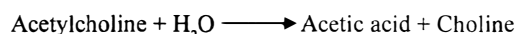

Fig. 6. Response of the lipase-based sensor to triacetin solutions. Experimental conditions: carrier solution = pH 7.5, 10 mM Tris buffer, bath temperature = 25°C, flow rate = 0.5 mL min⁻¹, NaCl concentration = 0.1 M.
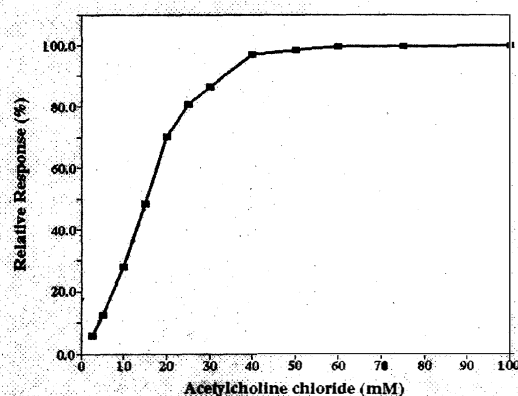

Fig. 7. Response of the acetylcholinesterase-based sensor to acetylcholine. Experimental conditions: carrier solution = pH 7.5, 7.5 mM Tris buffer, bath temperature = 25°C, flow rate = 0.5 mL min⁻¹.
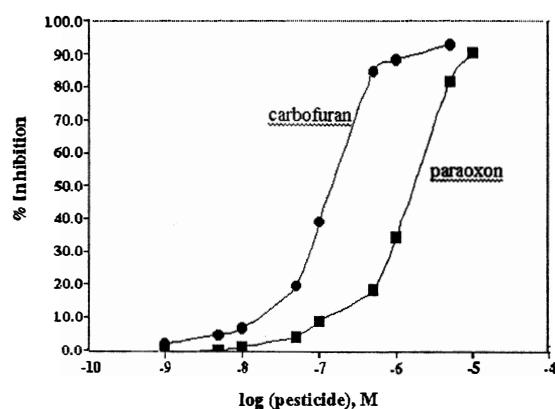
Fig. 8. Calibration plots showing the variation of percentage inhibition with pesticide concentration. Experimental conditions: flow rate = 0.5 mL/ min⁻¹, sample volume = 5 mL, temperature = 25°C, pH 7.5, 7.5 mM Tris buffer.

The sensor generated a significant response (ca 2 V) to low concentrations (ca 10 mM) of acetylcholine within a short period. The response of the sensor to a periodic exposure to 100 mM acetylcholine was highly reproducible, showing a relative standard deviation of 1.83% for 12 measurements. Similar to the other enzyme biosensors based on pH detection, the signal produced by the acetylcholinesterase-based biosensor was influenced by pH, buffer type and buffer concentration. The response profile varied almost sigmoidally with pH, the lower tailing ends being at a pH near the pKa of the buffer system. For the acetylcholinesterase-based biosensor, the highest response was obtained when the measurements were performed at a pH of 7.5 using a 7.5 mM Tris buffer.

The calibration curve for the biosensor to different concentrations of acetylcholine is presented in Fig. 7. The response of the biosensor is highly linear (r^2 = 0.992) in the concentration range of 2.5–25 mM. In this range, it exhibited a sensitivity of 84 mV/mM acetylcholine. At concentrations higher than 40 mM, the sensor response levelled off.

This biosensor can also be used to measure carbamate and organophosphate pesticides based on their inhibitory effect on acetylcholineseterase [7]. The sensor response decreased if the measurements were conducted in the presence of pesticides such as paraoxon and carbofuran. The change in the sensor response, expressed in terms of % inhibition, showed great dependence on the pesticide concentration over a limited range (Fig. 8). The detection limit was found to be 1.5×10^{-8} M (3.1 ppb) for carbofuran and 1.1×10^{-7} M (27.1 ppb) for paraoxon).

CONCLUSION

An optical fiber sensor for pH based on thymol blue immobilized on aminopropyl glass can be coupled with immobilized enzymes such as urease, lipase and acetylcholinesterase for the measurement of the enzyme substrates (urea, acylglycerol and acetylcholine), heavy metal ions and pesticides.

The enzymes were immobilized on isothiocyanate-modified glass through the formation of thiourea linkages. The coupling agent employed in immobilization contributed in some cases, such as in urease and lipase, to an increase in the resistance to enzyme inhibition. Protection against inhibition is achieved through chelation with heavy metal ions or through conformational changes that make the active site less exposed.

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