Triacylglycerol Biosensor Based on Conducting Polypyrrole

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A polypyrrole-based potentiometric triacylglycerol biosensor was developed and characterized. The biosensor was fabricated by the galvanostatic polymerization of polypyrrole (PPy) onto a platinum wire from a 0.1 M KHP buffer at pH 5.0 containing 0.1 M monomer and 3 mg lipase (Lip). The biosensor exhibited high linearity (r = 0.9980), with a working range to triacetin of 25 to 150 mM. XPS spectra of the biosensor verified the presence of the enzyme with the sulfuhydryl groups. With SEM, black polymer film showed nonuniform, well-adhering fern-like surface morphology.

INTRODUCTION

The determination of triacylglycerol is in heavy demand in food analysis and in clinical diagnostics [1-3]. Various methods have been used for the analysis of triglyceride in food samples. Liquid, gas, and supercritical fluid chromatographic techniques are commonly used for quantitative lipid analysis [4-6]. There are also several enzymatic assays based on the spectrophotometric determination of the glycerol or of the fatty acid liberated from the enzymatic hydrolysis of the lipid [7,8]. The enzyme reaction is shown in Figure 1.

The advent of biosensors provided a basis for the development of simple and miniaturized assays of triacylglycerol [9-12]. Among the biosensors for triacylglycerol that have been reported are a lipase thermistor biosensor [9], a pH electrode-based lipid biosensor [10], a field effect transistor with immobilized lipase in its sensitive area [11], and a fiber-optic triacylglycerol biosensor [12].

Another interesting method for determining triacylglycerols is through the use of a pH-sensitive polypyrrole membrane. Lipase (Lip) could be incorporated in polypyrrole (PPy) by electrochemical polymerization to produce a lipase-doped polypyrrole (PPyLip). The enzyme reaction will induce a pH variation near the biocatalytic region and affect the electrical property of the pH-sensitive polypyrrole membrane.

Electrochemical enzyme immobilization combines the specificity of the enzyme with the advantages of electrochemical detection. Some of the notable advantages of this particular
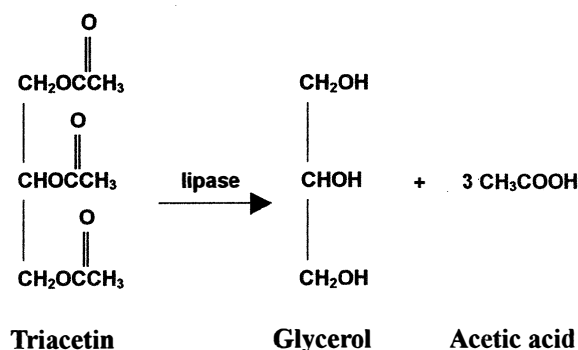

Triacetin   **Glycerol**   **Acetic acid**

Fig. 1. Enzymatic hydrolysis of triacetin

approach include the ease of polymerization, the accurate control of the amount of enzyme, the fabrication of enzyme arrays, miniaturization, and simple measurement procedure. Consequently, it has become an attractive approach to enzyme immobilization [13-18].

This paper presents the fabrication of a potentiometric triacylglycerol biosensor based on conducting polypyrrole with triacylglycerol acylhydrolase E.C. 3.1.1.3 as the dopant. Cyclic voltammetry is used to monitor polymer growth and polymer evaluation. Scanning electron microscopy (SEM) is used to investigate surface morphology of the polymer film, while X-ray photoelectron spectroscopy (XPS) is used to verify the presence of lipase in the polypyrrole film.

EXPERIMENTAL

Materials. Pyrrole monomer from Sigma Chemicals Co. (St. Louis, MO) was refrigerated and distilled prior to use. The enzyme used was triacyl glycerol acylhydrolase E.C.3.1.1.3 (Sigma Chemicals Co.) from *Candida cylindracea*. The analyte used was triacetin (Chemika).

The buffer solutions employed in the polymerization were: potasssium dihydrogen phosphate from Merck (Darmstadt, Germany) and potassium acid phtalate (KHP) from Ajax Chemicals (NSW, Australia). All buffer solutions were prepared using distilled water. All other reagents were analytical grade, unless otherwise stated.

Electrochemical Polymerization Set-up. The triacylglycerol biosensor was prepared by electropolymerization of pyrrole onto a platinum wire (99.99%; 0.26 cm^2) under constant current against a spectrographic carbon rod (Ringsdorff, Germany; 140 cm^2). The platinum wire was polished with alumina paste, sonicated and rinsed with distilled water prior to use. The anode (Pt) and the cathode (carbon rod) were faced parallel and separated by a distance of 1.0 cm. Current was passed through a current limiting device which was placed in series with the voltage source and the cell [19]. The current limiting device controls the current through the cell. The polymerization solution consisted of pyrrole monomer, enzyme dopant and buffer system. Polymerization was done for 3 min.

Instrumentation Set-up. The output voltage of the sensor was monitored against a Ag/AgCl electrode (Orion 900200) using a digital voltmeter (Metrohm). Data acquisition was done by interfacing the measurement set-up to a computer through an 8-channel, 12 bit ADC card (Innovatronix Inc).

Measurement Procedure. The fabricated pH transducer and the Ag/AgCl electrode were connected to the voltmeter and both electrodes were immersed in a beaker containing the test solution. All measurements were made at room temperature 30°C with the solution constantly stirred. The test solutions consisted of buffered triacetin solutions which were prepared by the dilution of the stock 200 mM aqueous solution with the respective buffer solutions.

The biosensor signal at a particular buffer concentration was monitored until a steady state response was reached. The indicator and reference electrodes were then washed with distilled water, dried with tissue paper and transferred to the next buffered solution of triacetin. The change in the voltage response was taken as the biosensor signal corresponding to a particular concentration of the analyte.

Cyclic Voltammetry. Votammetric experiments were conducted to monitor the growth of the polymer and were performed in a conventional (Bioanalytical Systems, Inc. BAS 100B) three-electrode, one-compartment cell (50 x 25 mm) with 20 mg freshly distilled pyrrole and 4.4 mg lipase in pH 5.0 KHP buffer solution under a nitrogen atmosphere. The electrochemical potentials were recorded versus a Ag/AgCl reference electrode (BAS) and platinum counter electrode scanning from +0.0 V to +2.0 V with a 10 mVs⁻¹ scan rate. The electrode used was platinum wire, 0.1 mm thick (99.99%). The PPyLip coated electrode was evaluated in a fresh electrolyte solution with the appropriate dopant, free of monomer, by scanning from –1.0 to +1.0 V at 50 mVs⁻¹ scan rate. Experimental procedures have been reported previously in more detail [20].

Surface Characterization. Surface morphologies of lipase dopant and PPyLip films were investigated using scanning electron microscopy (SEM). Experiments were performed using a JEOL JXA-840 Scanning Microanalyzer. The microscope chamber was maintained at a pressure below $<10^{-6}$ Torr and the instrument permitted attainment of images at a maximum magnification of 500000 times. The SEM filament voltage was operated at 3.0 kV and a current of 6 nA using magnifications of x2000 and x2500. Lipase powder was sputter coated with gold (Polaron Equipment Ltd., SEM Coating Unit E5000) prior to SEM analysis.

X-ray Photoelectron Spectroscopy (XPS) spectra were obtained using an ultra high vacuum (10^{-10} mbar) VG ESCALAB 220I XL surface analysis system. The perpendicularly mounted monochromatic $AlK\alpha$ anode X-ray source was operated at 180W (10kV x 18mA). Electron energies were determined using a concentric hemispherical analyzer (CHA) incorporating six channeltron™ electron detectors. The maximum energy resolution of the CHA was 0.5 eV operated for XPS analysis in the CAE mode with pass energy of 20 eV for the Ag $3d_{5/2}$ emission [21-23]. XPS analysis areas of 0.25 mm x 1.0 mm and 600 μm diameter or standard (wide opening) diameter were used [24]. Base pressure was maintained at 1 x 10^{-10} Torr and pass energies of 100 eV for wide spectra and 20eV for high resolution spectra were used, respectively. All experiments requiring peak deconvolution were performed at low pass energies (20 eV) to gain maximum possible degree of peak resolution. The electron binding energies (E_B) were calibrated relative to a saturated hydrocarbon C *1s* component peak at E_B = 285.0 eV [25]. Peak areas were quantified using appropriate sensitivity factors.

RESULTS AND DISCUSSION

Optimization of Immobilization Parameters

The optimum conditions were investigated as those which gave the highest response/response time ($\Delta R/t_R$) ratio. This criterion was used because of the desire to produce a sensor which gives a relatively high response (ΔR) at a reasonably short response time (t_R).

Table 1. Effect of Amount of Enzyme. *Polymerization condition: 0.1 M phosphate buffer at pH 7.5 at 10 mA/cm² for 3 min. Measurement condition: 0.01 M phosphate buffer at pH 7; 100 mM triacetin.*

Amount of enzyme (mg)	ΔResponse (mV)	Response time (min)	$\Delta R/ t_R$
3	12	4.5	2.7
6	10	2.5	4.0

Table 2. Effect of Polymerization Time. *Polymerization condition: 3 mg lipase, 0.1 M phosphate buffer at pH 7.5 at 10 mA cm². Measurement condition: 0.01 M phosphate buffer at pH 7; 0.1M triacetin.*

Polymerization time (min)	ΔResponse (mV)	Response time (min)	$\Delta R/t_R$
3	12	4.5	2.7
6	10	40.0	0.2

Effect of Enzyme Loading. The amount of lipase enzyme immobilized in the polypyrrole membrane was varied in the immobilization solution. The $\Delta R/t_R$ ratio of the resulting membrane was found to increase as the amount of enzyme in the immobilizing solution was increased (Table 1).

The enhancement of sensor response time indicates an increase in the enzyme activity as the amount of enzyme entrapped in the polymer is increased. The polymer could have incorporated a fixed amount of enzyme dopant, in a ratio of 1:4 to 1:3 [26], but it is likely that some of the enzymes could be incorporated in its free form [27].

The increase in the ratio of $\Delta R/t_R$ is not very significant considering that the amount of enzyme was doubled. Enzymes are expensive and the smallest possible amount of enzyme incorporated that would give a relatively high $\Delta R/ t_R$ ratio is considered. Consequently, the amount of enzyme chosen for further polymerization is 3.0 mg.

Effect of Polymerization Time. The $\Delta R/t_R$ ratio was found to decrease with an increased in polymerization time (Table 2). This behavior is due to a thicker polymer film formed as polymerization time is prolonged [28]. In effect, there is slower diffusion of the H⁺ ions through the membrane and the response time of the sensor is increased significantly.

Effect of Buffer Type and pH. Figure 2 shows the effect of the pH and nature of buffer on the polymerization process. The sensitivity of the biosensor response at a certain pH is dependent on the buffer type. The KHP buffer at pH 5.0 gave a high $\Delta R/t_R$ ratio and thus, was chosen as the pH and buffer species for the polymerization process.

Optimization of Measurement Procedure

Effect of Buffer Type and pH. The response of the sensor was determined under different pH values using two buffer systems, namely the Tris and phosphate buffers. The sensor response under these conditions are given in Figure 3. The greatest value of $\Delta R/t_R$ was obtained using Tris buffer at pH 7.5. The behavior of the biosensor is a compromise between the enzyme activity and the pH of the buffer solution. Lipase shows optimum activity at pH 7.2. At this pH value, maximum change in potentiometric response is expected. For the buffer, a small sensor response change is shown at pH values nearest the pKa of the buffer solutions: phosphate buffer, pka ~7.2 and Tris buffer, pKa ~8.1). The buffer system chosen was therefore Tris buffer at pH 7.5.

Effect of Buffer Concentration. The response of an enzyme sensor based on pH variation is affected by the concentration of the buffer solution. The $\Delta R/t_R$ ratio of the sensor de-
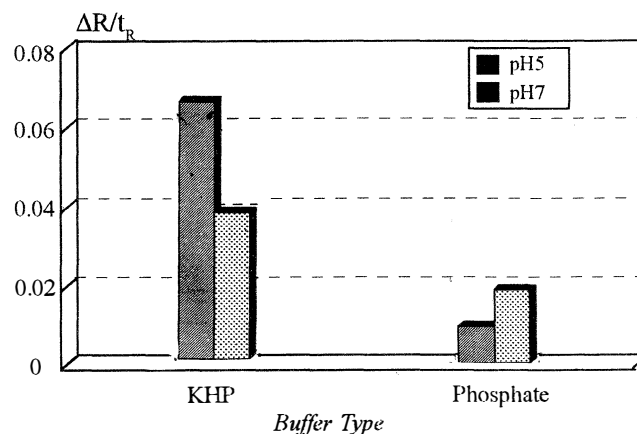

Fig. 2. Effect of the nature and pH of buffer on polymerization. Polymerization condition: 0.1 M buffer solution, current density of 10 mA/cm² for 3 min. Measurement conditions: 0.1 M phosphate buffer at pH 7; 20-100 mM triacetin.
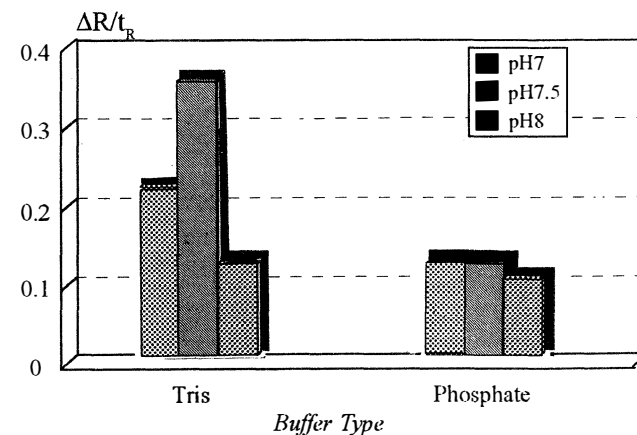

Fig. 3. Effect of the nature and pH of buffer. Polymerization condition: 0.1 M KHP buffer at pH 5; 3 mg lipase using 10 mA/cm² for 3 min. Measurement conditions: 0.1 M buffer; 20-100 mM triacetin.

creased as the concentration of the buffer increases (Fig. 4). At a high buffer concentration, the buffer solution has more capacity to resist pH changes and the observable pH change is small. Based on the results, 10 mM was chosen as the optimum buffer concentration.

Characterization of the Triacylglycerol Sensing System

Biosensor Response. Figure 5 shows the time response of the polypyrrole-based triacylglycerol sensor. The biosensor achieved a steady state signal within 7 to 10 min. When transferred to a buffered solution of triacetin, there was a rapid increase in the sensor signal. The sensor signal was highly reproducible, showing low c.v. of 8.49 % for n = 6 (Fig. 6) on repetitive exposure to 50 mM triacetin and Tris buffer solution. On the seventh trial, there was no response from the sensor which could possibly be due to the leaching out of the enzyme from the polymer.
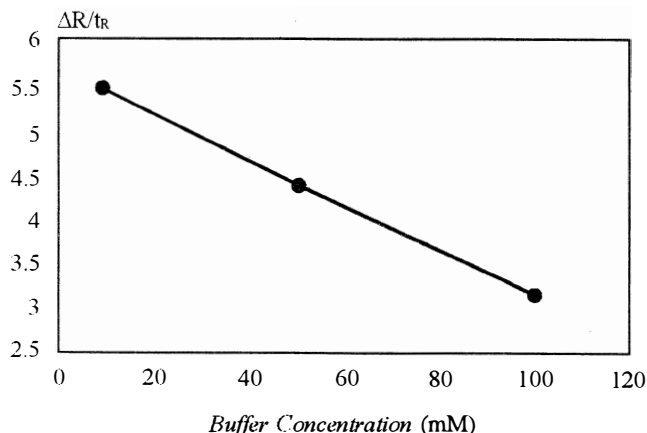

Fig. 4. Effect of buffer concentration. Polymerization condition: 0.1 M KHP buffer at pH 5; 3 mg lipase using 10 mA/cm² for 3 min. Measurement conditions: Tris buffer at pH 7.5; 60 mM triacetin.
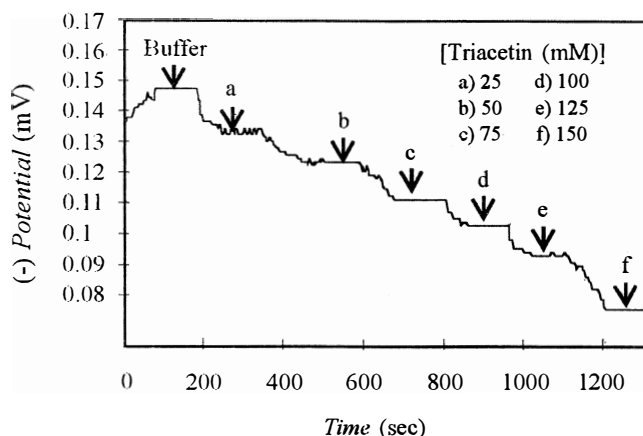

Fig. 5. Typical response of the polypyrrole-based triacylglycerol biosensor. Polymerization condition: a 0.1 M KHP buffer at pH 5; 3 mg lipase using 10 mA/cm² for 3 min. Measurement conditions: 0.1 M Tris buffer at pH 7.5; 20-100 mM triacetin.

The sensor exhibited a linear response within 25 to 150 mM triacetin with a sensitivity of 0.37 mV/ mM triacetin (n = 3). The average y-intercept obtained is 11.5 and the typical c.v. observed within this range was lower than 8.3%. The sensor has a limit of detection of 4 mM. The error bars on the calibration curve shown in Figure 6 indicate ± 1 standard deviation.

Electrode Lifetime. The sensor performance was monitored over a period of three days and a marked decreased in the sensor sensitivity was observed. The sensitivity of the sensor decreased by 60% and 74% on the second and third day, respectively. This short sensor lifetime could be due to a number of factors. One reason could be the denaturation of the enzyme itself. Another is the possible leaching out of the enzymes, since they are incorporated in the polymer only by electrostatic attraction or by physical entrapment. There is no cross-linking between the enzyme and the polymer and there is the absence of a strong bond between the enzyme and the transducer. Umaña, *et al.* [29] suggested that the leaching of the enzyme could be due to the inherent porosity of the polypyrrole membrane. Cross-linking agents like gluteraldehyde, could be incorporated during polymerization to ensure tight entrapment of the enzyme within the matrix even with a highly porous membrane.

Cyclic Voltammetry

The cyclic voltammogram in Figure 7a shows the film growth of polypyrrole with lipase with oxidation potential greater than +1.0 V (vs. a Ag/AgCl reference electrode) where voltammograms were scanned over the range +0.5 to 2.0 V. It shows successive growth of the PPy polymer film and the irreversible oxidation peaks at +1.3 V, electrolyte discharge at +1.6 V, and the gradual shifts to positive anodic potential as the film thickens [30,31]. During
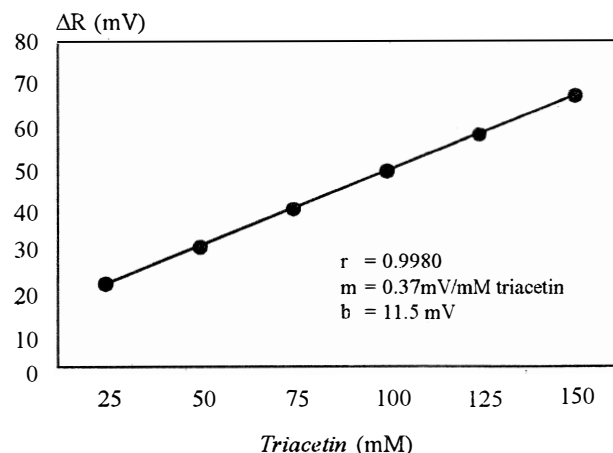

Fig. 6. Calibration curve of the polypyrrole-based triacylglycerol biosensor. Polymerization condition: 0.1 M KHP buffer at pH 5; 3 mg lipase using 10 mA/cm² for 3 min. Measurement conditions: 0.1 M Tris buffer at pH 7.5; 20-100 mM triacetin.
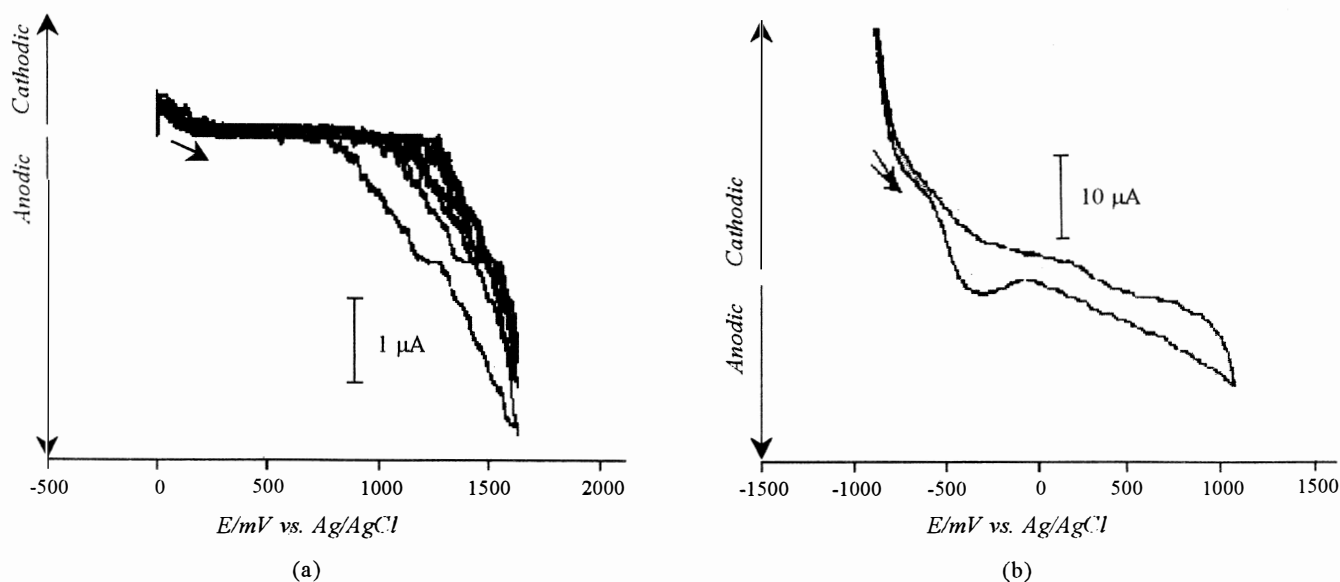
Fig. 7. (a) *Cyclic voltammogram growth of polypyrrole on platinum wire electrode doped with lipase enzyme.* (b) *Evaluation of the polymer film in fresh electrolyte and dopant solution.*
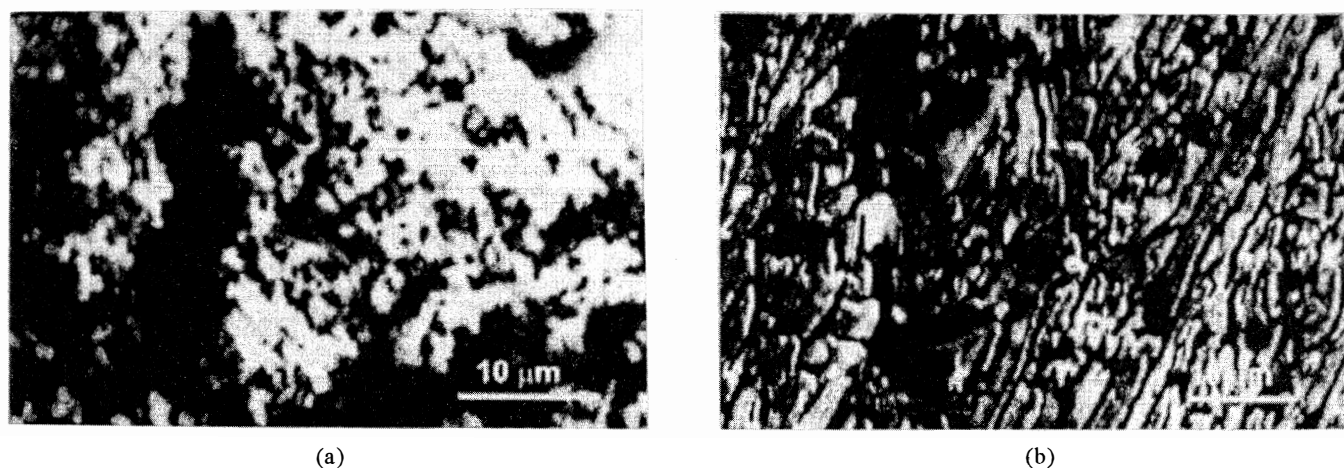

Fig. 8. *Micrograph images of* (a) *lipase enzyme powder* (mag x2500) *and* (b) *PPyLip polymer film* (mag x2000).

the cyclic voltammetric experiments, a black, continuous film was deposited on the Pt wire electrode after the first scan. This implies that oxidized PPy is formed readily during the early stages of electrodeposition. After removal of the PPy polymer film from the solution and replacement of the electrolyte solution, scanning of the system in the range -1.0 to +1.0 V showed anodic potential Ea ~ -0.5 V and cathodic potentials Ec ~ 0.100 V and 0.800 V (Fig. 7b). The use of a system based on aqueous buffer solution showed peaks from the reduction of water.

Surface Properties of the Polypyrrole Biosensor

Scanning Electron Microscopic Analysis. SEM studies of surface morphology (Fig. 8a) show that the powdered lipase enzyme has nodular structures in a mesh-like configuration. This shows that the lipase is highly porous. The PPyLip (Fig. 8b) has a nonuniform, well-adhering fern type sur-

face. There is no evidence of the lipase structure on the surface, indicating that the enzyme is imbedded within the polymer matrices.

X-ray Photoelectron Spectroscopic Analysis. Lipase is an enzyme or more exactly a group of enzymes belonging to the esterases which contains sulfuhydryl (S-H) groups. The structure of polypyrrole, a poly(five-membered heterocycle), corresponds to the coupling of monomeric pyrrole units in 2,5-positions with preservation of the aromatic nucleus [32]. The polymer film surface of PPyLip sensor was analyzed using XPS. Figure 9 shows the typical wide scan spectrum of PPyLip film on Pt wire showing the expected elemental composition: C $1s$, N $1s$, O $1s$ and S $2p$ peaks. The presence of the small S $2p$ peak is indicative of lipase and the characteristic N $1s$ peak is indicative of the polypyrrole film. The N $1s$ and S $2p$ regions were submitted to curve fittings and the deconvoluted spectra are shown in Figure
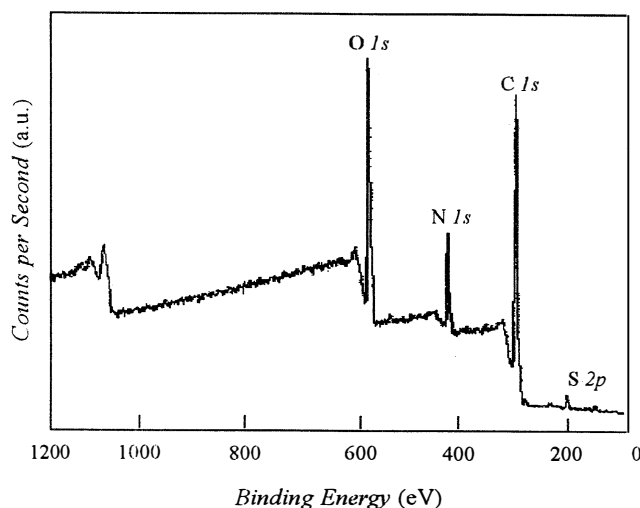
Fig. 9. XPS wide scan spectrum of PPyLip over binding energy range 1200 to 0 eV. Peaks identified as carbon (1s), oxygen (1s), nitrogen (1s) and sulfur (2p).
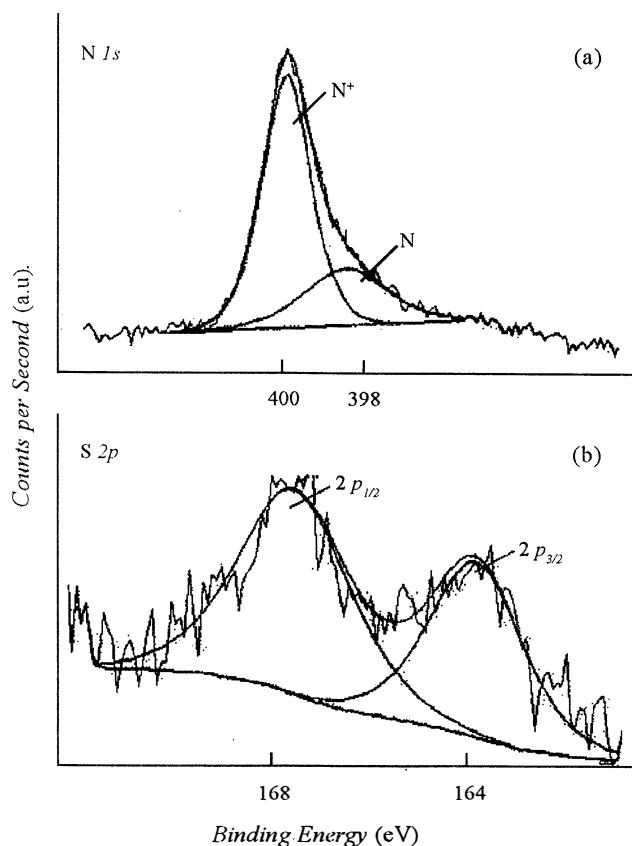

Fig. 10. (a) N 1s XPS core level spectra showing the contribution from two inequivalent sites, neutral nitrogen, N and nitrogen with partial positive charge, N^+. (b) S 2p XPS core level spectrum curve fit to $2p_{3/2}$ and $2p_{1/2}$ orbitals for PPyLip membrane.

10. The two nitrogen features are assigned to two electrostatically inequivalent nitrogen environments (Fig. 10a). The two N 1s component peaks in which the main peak centered at 398.5 eV (FWHM 2.85) corresponds to neutral nitrogen, N, and the higher energy peak centered at 400.2 eV (FWHM 1.68) corresponds to pyrrole nitrogen with partial positive charge, N^+. The asymmetry at the high binding energy side of the nitrogen 1s photoelectron peak is observed due to electrostatic screening of the counterions [19]. This method of peak deconvolution was developed by Eaves, et al. [33] and Pfluger, et al. [34] to explain the non-ideality of the peak.

The lipase enzyme shows the characteristic S 2p doublet peak (Fig. 10b) corresponding to S $2p_{1/2}$ and S $2p_{3/2}$ orbitals at about ~167.9 eV (FWHM 2.97) and ~ 163.9 eV (FWHM 2.50). The surface of the polypyrrole film has the active enzyme lipase for biosensing triacylglycerol.

CONCLUSION

A triacylglycerol biosensor based on a pH-sensitive polypyrrole was fabricated showing favorable response using a 0.1 M Tris buffer at pH 7.5. Surface-sensitive analytical techniques, cyclic voltammetry, and potentiometric measurements have shown that the lipase-doped PPy can be successfully polymerized and immobilized in the polypyrrole film. The sensitive layer of the biosensor consists of the lipase incorporated in an electrochemically polymerized polypyrrole film used as pH transducer. The immobilized lipase catalyses the hydrolysis of triacylglycerol, resulting in a decrease in pH around the polypyyrole film. In turn, the change in pH induces a change in the interfacial potential of the sensor. The figures of merit of the biosensor are summarized in Table 3. The conducting PPy studied offer potential for pH sensor and biosensor device and could find wide industrial, food and medical applications.

Table 3. Figures of Merit of the Polypyrrole-Based Triacylglycerol Biosensor

Sensitivity	0.37 mV/ mM triacetin
Correlation coefficient, r	0.9980
Linear range	25-150 mM triacetin
Response time	7-10 min
Limit of detection	4 mM
Reproducibility	less than 8.49% c.v.
Lifetime	1 day

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