Construction of a Choline Biosensor Through Enzyme Immobilization on a Poly(2-hydroxyethyl methacrylate)-Grafted Teflon Film

Deniz Yucel,[1*] Nazmi Ozer,[2] Vasif Hasirci[1]

[1]*Biotechnology Research Unit, Department of Biological Sciences, Middle East Technical University, 06531 Ankara, Turkey*
[2]*Faculty of Medicine, Department of Biochemistry, Hacettepe University, 06100 Ankara, Turkey*

Received 1 August 2006; accepted 4 January 2007
DOI 10.1002/app.26124
Published online 8 March 2007 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: An amperometric choline biosensor was constructed by immobilizing choline oxidase (ChO) on poly(2-hydroxyethyl methacrylate) (PHEMA)-grafted Teflon (polytetrafluoroethylene, PTFE) film. Grafting was achieved by γ irradiation. PHEMA-grafted Teflon films were activated with epichlorohydrin or glutaraldehyde to achieve covalent immobilization of enzyme onto the film. To decrease the diffusional barrier caused by the enzyme-immobilized film, the film was stretched directly on the electrode. The PHEMA-grafted Teflon film, therefore, had to have appropriate mechanical properties. Glucose oxidase (GOD) was used in the determination of optimum immobilization conditions, then these were applied to ChO. With GOD, the effect of activation type and film position in electrode on enzyme activity was studied and the highest catalytic activity was obtained when the enzyme was immobilized using glutaraldehyde and the film was stretched over the electrode surface. Further studies revealed that the films activated with glutaraldehyde, immobilized in 2 mg/mL ChO concentration, and stretched directly on the electrode were suitable (specific activity, 0.427 $\pm$ 0.068 U mg^{-1}) for use in the choline biosensor. The linear working range of this biosensor was found to be 52–348 μM, with a 40 $\pm$ 5 μM minimum detection limit. The response of the sensor, however, decreased linearly upon repeated use. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 104: 3469–3477, 2007

Key words: choline biosensor; choline oxidase; Teflon film; PHEMA; immobilization

INTRODUCTION

A biosensor is an analytical device consisting of a biorecognition element connected to, or integrated with, a transducer system to detect and quantify a specific analyte by producing a reliable signal. Biosensors are of growing importance because of their inexpensive, rapid, accurate, sensitive, and selective detection of analytes. In amperometric, enzyme-based biosensors, the biologically active component, generally an enzyme, needs to be brought in close contact with the transducer for detection without loss. For ease of application, the enzyme is either attached directly onto the electrochemical transducer or to a membrane, which is then draped or stretched over the transducer. The main requirements are that the enzyme must be securely attached so that it is not lost during measurement, and the rate of transference of the analyte and the products must be high for rapid detection.

A variety of techniques such as covalent bonding, ionic bonding, entrapment, etc. are used to attach the enzyme to the transducer. For covalent bonding, some functional groups of the enzyme that are not essential for its catalytic activity can be used. This method is advantageous over ionic bonding or adsorption in that the enzyme is not lost during measurement due to the irreversibility of the bonding. It is also advantageous because an enzyme bonded on the surface of a film is rapid and more accessible than an enzyme entrapped in a polymeric film.

Choline is a very important molecule as a component of important tissues and also as a metabolite of reactions. Its rapid and sensitive detection is important because as a constituent of phosphotidylcholine it is very important for cell membrane structure. It is a precursor of acetylcholine that is involved in the signal transmission among nerves, muscles, and organs. In neurodegenerative diseases such as Alzheimer's and in the neuromuscular disease myasthenia gravis, monitoring the levels of acetylcholine and choline is important to detect the impaired cholinergic neurotransmission.[1]

Choline oxidase (ChO) is a cytosolic enzyme that catalyzes the oxidation of choline to betaine and

Correspondence to: V. Hasirci (vhasirci@metu.edu.tr).
**Present address*: Bioengineering and Biotechnology Center, Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, MA 02155, USA.

Contract grant sponsor: METU Scientific Research Grants Program; contract grant number: BAP: 2002-07-02-00-71.

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hydrogen peroxide in the presence of oxygen and as a result it is used in the detection of choline. ChO was used in the biosensors after immobilization directly on transducers, or immobilized in the bulk or on the surface of polymeric membranes and then brought into contact with the transducers. For example, choline was detected in human bile and blood sera using ChO immobilized-Nylon net and a Clark oxygen electrode, in the amniotic fluid using an amperometric–enzymatic method and in brain extracts with the enzyme entrapped in a crosslinkable, redox polymer.[2–4]

Various other amperometric, ChO-based choline biosensors were also constructed.[5–10] Doretti et al.[8] constructed a choline biosensor through covalent immobilization of ChO on poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) films through the epoxide group of glycidyl methacrylate and amine groups of the enzyme. These films were then used with Clark-type oxygen or hydrogen peroxide electrodes. Another electrode was constructed by direct coating of photopolymerized PVA-SbQ and ChO on the transducer tip.[11] Direct coating was found to result in superior biosensor performance with high sensitivity. In another application, ChO was immobilized via an ion-exchange process.[12] Enzyme-based choline biosensor was prepared by immersing a Pt electrode coated with the redox polymer polyvinylferrocenium perchlorate in a solution of ChO. Choline oxidase was also coimmobilized with other enzymes for the construction of a choline biosensor. For example, Doretti et al.[13] designed a two-enzyme biosensor for the detection of choline esters by covalent coimmobilization of ChO and butyrylcholinesterase on a methacrylate–vinylene carbonate copolymer. To increase the sensitivity of the amperometric biosensors for choline, butyrylcholine, and acetylcholine, several enzymes such as ChO and acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) were coimmobilized on the surface of a platinum electrode by crosslinking with bovine serum albumin and glutaraldehyde.[14] In a study to determine choline released from rat submandibular gland acinar cells, ChO was retained on a horseradish peroxidase immobilized-solid carbon paste electrode surface using a dialysis film.[10]

The goal of this study was to construct an amperometric choline biosensor. In our approach, a unique immobilization approach was developed where a Teflon membrane was grafted with PHEMA by γ irradiation and then activated by attaching epichlorohydrin or glutaraldehyde before finally attaching the enzyme. Use of the Teflon protector/membrane of the electrode as immobilization surface eliminates the need to use another membrane to carry the enzyme, thus decreasing the diffusional restriction. An electrode with this construction is expected to have a more rapid response and to be more compact. In this study, an immobilization approach was developed for enzymes for use in amperometric electrodes and also discusses the possibility of using for the biosensing of choline oxidase. In the initial optimization studies, glucose oxidase (GOD) was used as an economical, high specific activity and highly characterized biosensing element, and once the optimum conditions were established they were used in the experiments with ChO. Two spacers epichlorohydrin and glutaraldehyde were tested. The determination of the effect of the activation procedure, the location of the catalytic film, and the input enzyme concentration on the activity of the immobilized enzyme was carried out in the oxygen electrode and was based on depletion of oxygen in the reaction medium upon addition of the substrate. After optimization of the working conditions, the performance of the choline biosensor, reusability, linear working range, and sensitivity were studied.

EXPERIMENTAL

Chemicals

Teflon film (12.5 μm thick, 25 mm wide) was purchased from Hansatech Instruments (Helmut Saur, Germany). 2-Hydroxyethyl methacrylate (HEMA) from Sigma Chemical (St. Louis, MO) was distilled under vacuum and stored at +4°C until use. The crosslinker, ethylene glycol dimethacrylate (EGDMA), (Sigma, USA) was purified by extraction with a 20% NaCl–5% NaOH solution. Ferrous ammonium sulfate (FAS) [$FeSO_4(NH_4)_2SO_4{\cdot}6H_2O$, Analar grade, BDH, England] was used for prevention of HEMA homopolymerization. The coupling agents epichlorohydrin (1-chloro-2,3-epoxypropane) (Epi) and glutaraldehyde (50% (aq)) (Glut) were obtained from Sigma (USA).

The enzymes choline oxidase (E.C. 1.1.3.17, from *Alcaligenes* species) and glucose oxidase (E.C. 1.1.3.4, from *Aspergileus niger*, Type VII-S) and the substrates choline (chloride salt) and β-D-glucose were purchased from Sigma (USA) and used as received.

PHEMA grafting on Teflon films

PHEMA grafting on Teflon (PTFE) films was achieved by modification of Mohy Eldin et al.[15]

PTFE film was placed in a solution of HEMA (15% aqueous, v/v) and FAS (0.1%, w/v) in a vertical two-glass plate system separated by a spacer that was varied in thicknesses to form thin and thick films. Grafting was achieved by exposure to γ radiation for 24 h in air at ambient conditions in a Co^{60} source (Gamma Cell 220, Canada) with the average dose rate of 0.15 kGy/h. The PHEMA-grafted films (200–550 μm) were extensively washed with distilled water to remove the ungrafted PHEMA and then stored in it at 4°C

until use. In the preparation of one of the batches, the crosslinker EGDMA (0.15%, v/v) was added.

Film topography

PHEMA-grafted Teflon films were vacuum-dried and coated with gold before scanning electron microscopy (SEM) (JSM-6400, Japan).

Mechanical properties of film

The tensile properties of the film samples (1×4 cm^2) were determined in a wet state using MTS Mechanical Tester (MTLQ, Stable Micro Systems, England) at room temperature with a 0.2 mm/s test speed.

Covalent immobilization of enzyme on film

Activation of films (300 μm in thickness) with epichlorohydrin and glutaraldehyde was carried out according to Kok[16] with some modifications.

For epichlorohydrin activation, a single wet PHEMA-grafted Teflon film (2.5×2.5 cm^2 or 1×2.5 cm^2) was placed in a solution (5.4 mL) containing NaOH (4.3 mL, 2.0*M*) and epichlorohydrin (0.1 mL), allowed to react at 40°C for 30 min, and washed with distilled water to remove unreacted epichlorohydrin.

For aminohexyl-glutaraldehyde activation, a single wet PHEMA-grafted film was placed in polyethylene bottles containing $NaIO_4$ (4 mL, 0.2*M*) and gently shaken for 2 h at room temperature to achieve aldehyde formation. The film was washed with distilled water, placed in hexadiamine (4 mL, 2.0*M*, pH 5.0), and gently shaken for 6 h at room temperature. After washing with phosphate buffer (PB) (50 mL, 0.1*M*, pH 8.5), the film was placed in a glutaraldehyde solution (4 mL, 2.5%, v/v) and gently shaken for 10 min at room temperature. Excess glutaraldehyde was removed by washing with PB (250 mL, 0.1*M*, pH 8.5).

Activated films were immersed in GOD (2 mg/mL) or ChO (2 mg/mL) in borate buffer, 0.2*M*, pH 9.0 and gently shaken overnight at room temperature. The films were then washed with PB saline (PBS, 0.1*M*, 0.9% NaCl, pH 7.0) to remove any unbound enzyme. A schematic presentation of the film preparation process, film grafting, activation, and enzyme immobilization are given in Figure 1.

The amount of protein immobilized on the film was calculated through mass balance. The amount of enzyme immobilized on Epi-activated films was directly determined by Bradford Method,[17] however, on Glut-activated films, enzyme amount could not be detected properly by this method probably due to a side reaction between glutaraldehyde and the Bradford reagent. The enzyme content on Glut-activated films was successfully determined by Lowry method.[18]

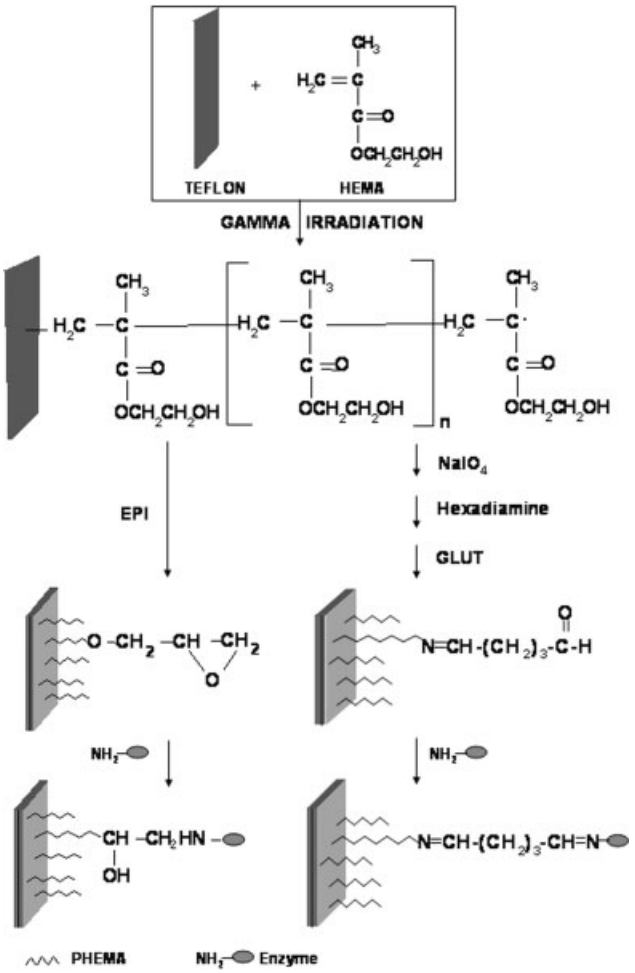

Figure 1 Schematic presentation of the film preparation steps: film grafting, activation, and enzyme immobilization.

Amperometric measurement of enzyme activity

Activity of free and immobilized glucose oxidase and choline oxidase was measured according to Kok[16] using an oxygen electrode unit (Helmut Saur, Germany). Enzyme carrying-Teflon films (300 μm in thickness) were introduced to oxygen detection unit in two different ways; (a) No Contact; by attaching to a hollow frame that was in no direct contact with the electrode, and (b) Direct Contact; by stretching across the electrode. In direct contact mode, no additional protective Teflon film covered the electrode surface. The medium (PB, 0.1*M*, pH 6.0 for GOD or pH 7.0 for ChO) was aerated for 30 min before use.

Glucose (0.066–6.6 m*M*) the substrate for GOD and choline (0.104–3.480 m*M*) the substrate for ChO were introduced to the sample unit. The oxygen depletion due to enzyme activity was expressed as the change in the potential (mV) versus time plots. From the slopes of these plots for each substrate concentration, an enzyme calibration curve of oxidation rate (mV/s)

versus substrate concentration (mM) and Eadie–Hofstee plots were constructed.

Effect of activation procedure type on GOD activity

Glucose oxidase was immobilized on PHEMA-grafted Teflon films via two different activators, glutaraldehyde and epichlorohydrin. Activity measurements for comparison were performed in no contact mode.

Effect of film position in the oxygen electrode on film performance

The activities of GOD and ChO immobilized via glutaraldehyde linkage on PHEMA-grafted Teflon films were carried out with the film in two different states in the oxygen detection unit: (a) attached to a hollow frame with no contact with the electrode or (b) stretched on the electrode achieving direct contact between the enzyme-loaded film and the sensor.

Effect of input ChO concentration on the activity of immobilized ChO

During the ChO immobilization step, Teflon films (Glut-activated, PHEMA-grafted) were exposed to a ChO solution of 2 or 5 mg/mL to study the effect of input ChO concentration on immobilized enzyme activity.

Choline oxidase biosensor performance studies

Reusability

The reusability of ChO-immobilized films (Glut-activated, 2 mg/mL input ChO concentration, tested in direct contact form) was studied for 45 successive runs within 8 h using choline (0.348 m*M*) as the substrate.

Linear working range

Linear working range of ChO-immobilized film (Glut-activated, 2 mg/mL input ChO concentration, tested in direct contact form) was studied with the choline concentration range of 0.026–3.480 m*M*. Linear range was determined from the change in the oxidation rate (mV/s)/change of choline concentration (m*M*) plotted against choline concentration (m*M*).

RESULTS AND DISCUSSION

Characterization of films

Film topography

The changes in the Teflon film topography caused by a series of consecutive preparation steps were studied by SEM. Compared to the smooth SEM of the pristine film [Fig. 2(a)], the SEM of PHEMA-grafted film [Fig. 2(b)] reveals a porous, thick coat. This is observed better when both sides of a film are seen simultaneously [Fig. 2(c)] or when partially detached coat [as seen in the enlarged portion of Fig. 2(c)] reveals the underlying smooth Teflon [Fig. 2(d)]. During activity testing, the PHEMA-grafted side of the film was placed to face the assay medium and the PHEMA-free side faced the electrode. Figure 2(c) shows that it was possible to graft PHEMA mainly on one side of the film. The thickness of the grafted film could be determined from the SEM of the cross section of the film as 300 μm [Fig. 2(c)]. There was no significant difference upon activation of films but the lace-like structure on the surface seemed to be more porous in Epi-activated film with respect to Glut-activated film (data not provided).

Mechanical properties of the films

In this study, the enzyme-grafted film of the biosensing element of the biosensor had to have appropriate mechanical properties because it was designed for use in direct contact mode, stretched over the electrode surface.

It can be seen in Table I that simple γ irradiation increases the Young's modulus of the Teflon film by 27%, indicating the films became stiffer upon exposure to radiation. PHEMA grafting with γ irradiation, however, made the films substantially softer. The presence of EGDMA, a crosslinker for HEMA, made the films slightly stiffer but still the rigidity was much lower than the pristine Teflon. The presence of crosslinker increased the strength of PHEMA-grafted films, however, decreased the performance of enzyme-immobilized films in terms of catalytic properties (data not provided). Further studies, therefore, were carried out with crosslinker-free PHEMA-grafted Teflon films. The thickness of the HEMA film was a parameter that influenced the mechanical properties. The stiffnesses of the thinner films (200–300 μm) with and without EGDMA were approximately threefold higher than the thicker films (500–550 μm); thus, it was observed threefold that the thinner films were stiffer. A further step of treatment, Epi and Glut activation, decreased the Young's modulus from $8.577 \times 10^6 \pm 5.29 \times 10^5$ N m^{-2} to $3.824 \times 10^6 \pm 5.07 \times 10^5$ N m^{-2} and to $4.458 \times 10^6 \pm 3.73 \times 10^5$ N m^{-2}, respectively.

The difference in the toughness between the pristine and treated films study was not as significant as in the Young's modulus (Table I). The results showed that, contrary to the stiffness data, activation via epichlorohydrin improved the toughness of PHEMA-grafted Teflon films. Even though the Epi-activated film appeared to be the best in terms of toughness, the high enzyme activities observed (Table II) led us to choose the Glut-activated, EGDMA-free, thin

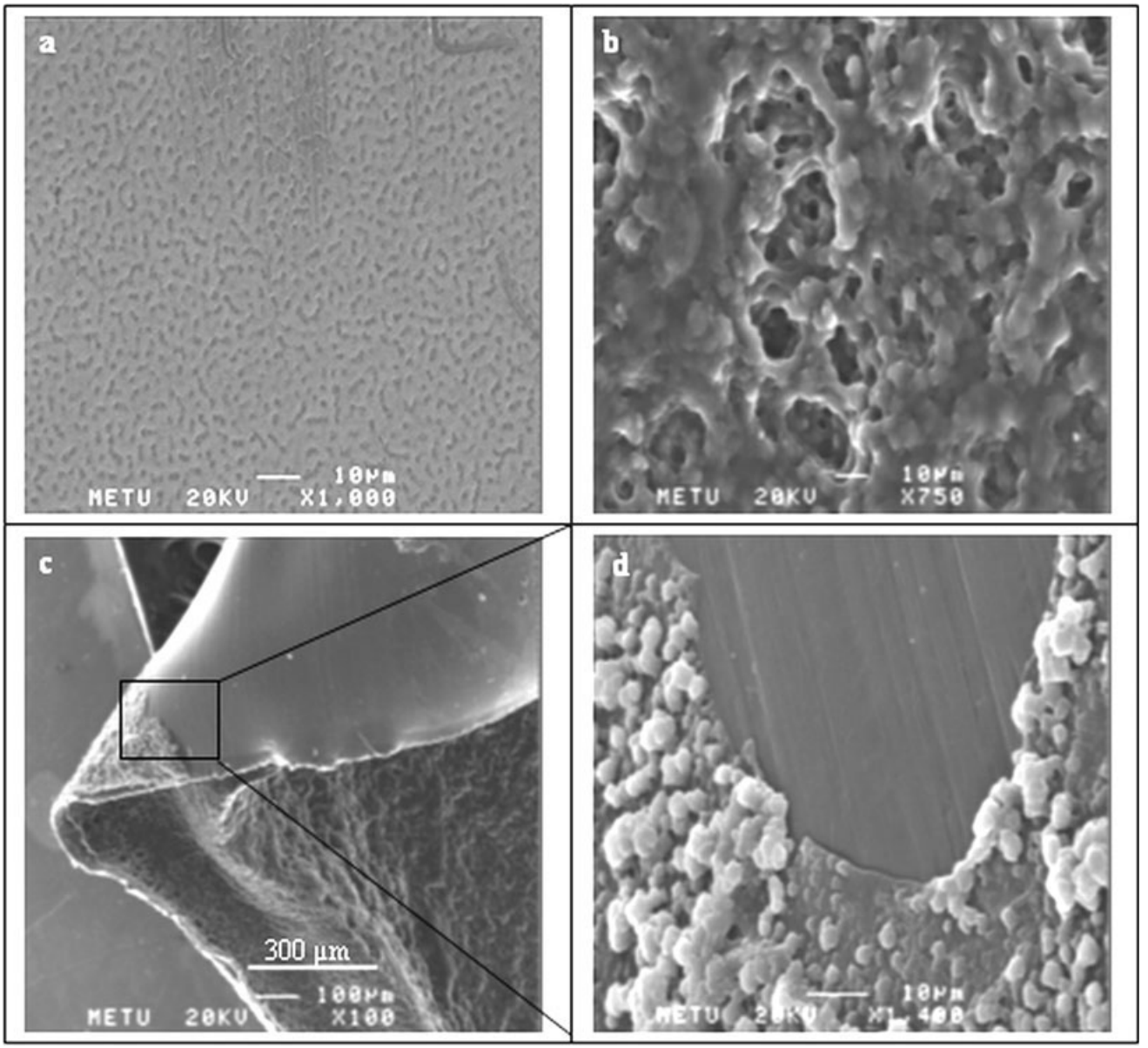

Figure 2 SEM micrographs of (a) pristine Teflon film (×1000), (b) PHEMA-grafted Teflon film (×750), (c) cross section of PHEMA-grafted Teflon film (×100), and (d) magnified (14-fold) view from the back of PHEMA-grafted Teflon film (×1400).

PHEMA-grafted Teflon films as the film type to be used in the rest of the study.

Amperometric measurement of enzyme activity

To study the effect of film activation methods, input enzyme concentration, and the mode of activity measurement, kinetic parameters K_m, V_{max}, and specific activities were determined.

The reference values for the free GOD K_m, V_{max}, and specific activity (SA) were found to be 3.334 m*M*, 15.997 mV s^{-1}, and 5.352 U mg^{-1}, respectively. These reference values for free ChO were 1.033 m*M*, 4.773 mV s^{-1}, and 1.582 U mg^{-1}.

Effect of the activation procedure type on GOD activity

The kinetic parameters are significantly different with two different activation types; glutaraldehyde activation leads to ~ 2-fold higher V_{max}, 3.7-fold higher enzyme specific activity, and 65% higher K_m. The higher enzyme activity through glutaraldehyde immobilization is probably due to the higher length of the spacer in comparison to epichlorohydrin. The enzyme immobilized with glutaraldehyde spacer is quite distant from the polymeric support and the restriction of movement of the enzyme due to immobilization is probably lesser than that with epichlorohydrin. As a result of these, glutaraldehyde coupling was chosen for further studies.

TABLE I
Mechanical Properties of Teflon Films

Films	Young's modulus (10^6) (N m^{-2})	Toughness (N mm)
Pristine Teflon	33.467 ± 1.600	135 ± 71
γ-Irradiated Teflon (in distilled water)	42.357 ± 0.814	144 ± 24
Thick PHEMA-grafted Teflon, w/o EGDMA	2.803 ± 0.337	146 ± 8
Thin PHEMA-grafted Teflon, w/o EGDMA	8.577 ± 0.529	168 ± 2
Thick PHEMA-grafted Teflon, w EGDMA	3.883 ± 1.871	166 ± 57
Thin PHEMA-grafted Teflon, w EGDMA	10.657 ± 6.550	128 ± 89
Thin PHEMA-grafted Teflon, w/o EGDMA, epi-activated	3.824 ± 0.507	210 ± 21
Thin PHEMA-grafted Teflon, w/o EGDMA, glut-activated	4.458 ± 0.373	128 ± 14

In an earlier study by our group,[16] acetylcholine oxidase and choline oxidase were coimmobilized on a PHEMA film. It was observed that acetylcholine oxidase could bind covalently to the film via glutaraldehyde or epichlorohydrin, but immobilization of ChO was not successful. Immobilization of ChO could only be achieved via complexation with a dye, Cibacron blue F36A. In the present study, however, it was possible to successfully immobilize ChO via glutaraldehyde onto PHEMA-grafted Teflon film. The success in the present study probably arises from the fact that PHEMA chains extend away from a surface making binding more feasible, whereas in the previous case the membrane itself was PHEMA possibly restraining the chains in the bulk.

Effect of film location in the oxygen electrode (measurement mode) on enzymatic activity

Ultimately, the enzyme-immobilized PHEMA-grafted Teflon film would be used stretched onto the electrode in place of the gas permeable Teflon film of the oxygen electrode to increase performance of the biosensor through better gas and solute transference.

The change in enzyme activity with the mode of measurement was first studied with GOD and then with the target enzyme ChO. The significant changes in the specific activities and in the K_m and V_{max} values upon change of mode are presented in Table II.

It could be seen from the table that, stretching the film directly on the electrode instead of attaching to a hollow frame away from the electrode surface increased the specific activity of GOD by 12.7-fold and ChO by 9.7-fold.

The increase in enzyme activity of the stretched film could be due to that the enzyme reaction was carried out directly on the electrode as was originally aimed, and by this placement the decrease in oxygen concentration in the medium and electron transfers involved could be sensed more rapidly by the electrode.

Moreover, the increase in activity of direct contact mode is probably due to the following reason: upon stretching, the immobilized enzymes become more distant to each other, the crowding effect is decreased,

TABLE II
Enzyme Immobilization and Kinetic Parameters

Film type	SA (10^{-3}) (U/mg)	V_{max} (10^{-3}) (mV s^{-1})	K_m (10^{-3}) (mM)
Epi-GOD, no contact	50 ± 9	901 ± 47	596 ± 165
Glut-GOD, no contact	185 ± 13	1918 ± 730	985 ± 24
Glut-GOD, direct contact	2343 ± 136	1823 ± 204	2565 ± 229
Glut-ChO, no contact	44 ± 9	214 ± 38	224 ± 46
Glut-ChO, direct contact	427 ± 68	230 ± 18	474 ± 40
*Glut-ChO, direct contact	109 ± 15	202 ± 49	595 ± 59

In all cases, except the (*) marked sample, the enzyme concentration in the immobilization medium was 2 mg/mL. In the (*) sample, it was 5 mg/mL.

Epi-GOD: Film with Epichlorohydrin activation and GOD immobilization.

Glut-GOD: Film with Glutaraldehyde activation and GOD immobilization.

Glut-ChO: Film with Glutaraldehyde activation and ChO immobilization.

Direct contact: During measurement of activity, enzyme-immobilized film is stretched over the electrode.

No contact: During measurement of activity, enzyme-immobilized film is attached to a hollow frame.

and the probability of the enzyme interaction with the substrate is increased.

Effect of concentration of ChO in the immobilization medium on the activity of ChO immobilized films

The effect of concentration of ChO used during immobilization on the activity of the resultant immobilized enzyme was investigated using 2 and 5 mg/mL concentrations. The K_m and V_{max} values, specific activities determined are presented in Table II. The 2.5-fold higher input concentration led to a 12% lower V_{max}, 75% lower specific activity, and 26% higher K_m. The significantly higher amount of enzyme on the film probably leads to the crowding of the microenvironment of the enzymes resulting in the restriction of the conformational changes of the enzyme needed in the presence of the substrate. Similar results were obtained by Cremisini et al.[19] who immobilized ChO on a polymeric film starting with polyazetidine prepolymer. They observed an increase in the sensitivity of the biosensor upon an increase in enzyme density up to a certain enzyme content, after which a sharp decrease was observed. In another study, the thickness of the enzyme film on the electrode affected the response.[20] GOD and xanthine oxidase (XOD) were coimmobilized on a modified glassy carbon electrode with a thin Nafion film to form a glucose (Glu)/hypoxanthine (Hx) sensor. Glu or Hx current responses at the electrodes coated by treating with various amounts of enzyme solution showed a maximum at a certain amount of enzyme solution after which the enzyme layer became too thick and led to a reduction in response. Thus, the decrease in ChO activity upon increase of enzyme content indicates that the 5 mg/mL enzyme level leads to a crowding effect also reported with the other enzymes upon exceeding a certain enzyme level. The immobilization process leads to a change in the kinetic behavior due to either conformational alteration within enzyme resulting in deactivation or to restriction in the enzyme mobility, resulting in a decrease in V_{max} and the specific activity of the enzyme. Upon immobilization, an increase in K_m value was expected because of the increased diffusional resistance (limited rate of mass transfer of the substrate from the bulk of the solution to the surface of the support material). However, the activity results obtained in this study show that the V_{max} values for the immobilized enzymes were lower than that of the free enzymes as expected but their K_m values were also lower than that of the free enzymes. The apparent values of K_m and V_{max} may differ due to the changes in the properties of the solution in the immediate vicinity of the immobilized enzyme or due to the effects of molecular diffusion within the local environment. K_m value of an enzyme can be reduced when the substrate concentration in the vicinity of enzyme active site is higher than that measured in the bulk of the solution due to the influence of charge and hydrophobicity of the surface.[21] It was reported that the K_m values of immobilized enzyme films used in direct contact form were closer to those of the free enzyme compared to those of films used in the no contact position. The possible reason was the decrease in the influence of charge and hydrophobicity of the surface in the direct contact form.

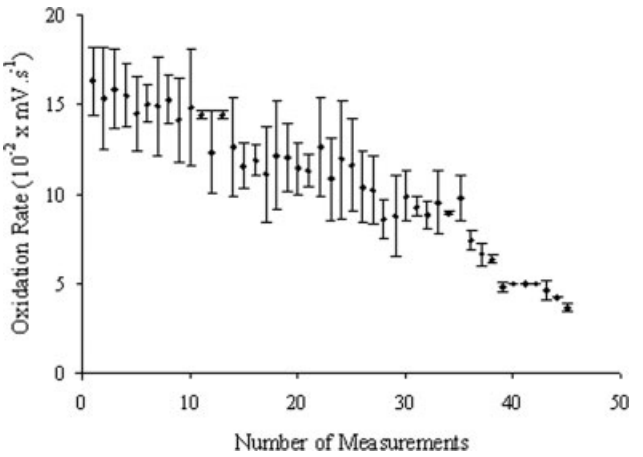

Figure 3 Repeated use of choline biosensor employing a Glut-activated, ChO-immobilized film.

A decrease in both the K_m and V_{max} values similar to our study was also reported by the immobilization of horseradish peroxidase on polyaniline with glutaraldehyde.[22] It was found that the K_m value for the immobilized enzyme (5.2 mmol L^{-1}) was lower than the K_m value for the free enzyme (9.58 mmol L^{-1}) with pyrogallol used as the substrate. Similarly, the V_{max} value decreased from 1.47 to 0.96 mmol min^{-1} upon immobilization. Although it was not as significant as in the present study, a similar K_m decrease was also reported in the study of Gulce et al.[23] In that study, galactose oxidase was immobilized in a polyvinylferrocenium matrix coated on a platinum electrode surface, and the K_m value was found to be 21.7 m*M* for the immobilized galactose oxidase, and 22.8 m*M* for the free.

As a result of the enzyme activity studies, ''the Teflon film grafted with PHEMA activated with glutaraldehyde, employing a 2 mg/mL ChO solution and used in direct contact form'' was selected as the ChO biosensor configuration used in the further studies.

Choline oxidase biosensor performance studies

Reusability

The activity of the immobilized ChO was measured using a 0.348 m*M* choline solution for 45 successive runs conducted in 8 h (Fig. 3). The fluctuations in the

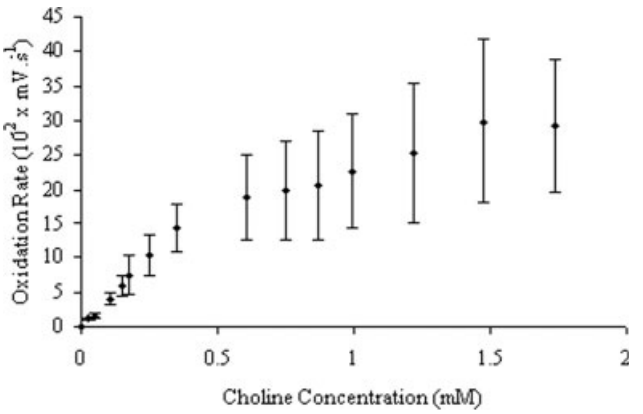

Figure 4 The change in the activity of the immobilized ChO with choline concentration.

signal appeared to be 5%. The decrease in the activity of ChO biosensor started after about five runs and it dropped linearly to 17% of the original response after 45 measurements. The loss in the activity could be due to hydrolysis of the enzyme in the test medium and under the given test conditions.

Linear working range

To determine the linear working range of the biosensor, the oxidation rate versus choline concentration was plotted (Fig. 4). Plotting Δ(oxidation rate)/Δ(substrate concentration) versus substrate concentration (data not provided), it was observed that the ChO biosensor has a linear response in the range 52–348 μ*M* choline with a correlation coefficient of 0.9925. The minimum detection limit was found to be 40 ± 5 μ*M* choline. During the linear working range studies, the average response time was determined as 22 ± 6 s and the complete analysis duration was 7 min.

In the literature, Ruiz et al.[6] reported two calibration curves constructed with two different ChO loadings and the linear range was reported as 1–10 m*M* for 0.30 U cm^{-2} loading and 1–13 m*M* for 0.382 U cm^{-2} loading with a detection limit of 50 μ*M* choline. Although their linear working range was more extended than ours, it was in a much higher concentration region, and their minimum detection limit is 25% higher than the present study.

A choline biosensor, constructed by entrapping ChO in photocrosslinkable poly(vinyl alcohol) bearing styrylpyridinum, had a linear range of 2.5–150 μ*M* choline with an average response time of 2 min.[5] In another study, ChO coimmobilized with catalase on a nylon net via glutaraldehyde and cyclohexyl isocyanide linear range was determined as 3.34–167 μ*M*.[24] In addition, Ricci et al.[25] immobilized ChO onto Prussian Blue-modified, screen-printed electrodes by crosslinking with glutaraldehyde and the linear range was 0.5–100 μ*M*. These three biosensors had a lower detection limit but a shorter linear range than the one developed in this study. Doretti et al.[7] constructed an amperometric choline biosensor immobilizing ChO on PHEMA films and obtained linear working ranges of 10–200 μ*M* and 0.005–0.25 m*M* choline concentration with an oxygen and a hydrogen peroxide probe, respectively. The reported linear working ranges indicated above are quite close to that obtained in the present study.

The average response time (22 s) of a choline biosensor constructed in this study is also better than the response time of 2 min in the Doretti et al.[7] and Marty et al.[5] studies. It is also better than those of Razola et al.,[10] who developed a choline biosensor based on H_2O_2 determination and observed a response time as 62, 75, and 80 s for $4 \times 10^{-5}M$, $5 \times 10^{-6}M$, and $4 \times 10^{-7}M$ choline concentration, respectively.

CONCLUSIONS

The ultimate aim of the current study was to construct a choline biosensor with improved performance employing a single enzyme-immobilized, PHEMA-grafted Teflon film that was stretched onto the electrode instead of two separate films, an enzyme loaded film for biosensing and another for electrode protection. The effect of activation type, enzyme immobilization concentration, and the position of the enzyme immobilized film in the oxygen electrode reaction chamber on the activity of immobilized enzyme were studied. It was found that GOD enzyme immobilized with glutaraldehyde had higher specific activity (0.185 ± 0.013 U mg^{-1}) than that with epichlorohydrin. Also, it was observed that the activity of the immobilized GOD or ChO was higher when the film was stretched over the electrode in comparison to the film in no contact mode. This was probably because the metabolic products were closer to the electrode and their detection by the electrode was much more efficient due to this closeness. Influence of enzyme crowding was also studied. It was found that the films loaded with less ChO yielded higher specific activity (0.427 ± 0.068 U mg^{-1}) because of less crowding of the immediate environment. The choline biosensor had a long linear working range (52–348 μ*M*), a low 40 μ*M* minimum detection limit, and a short response time (22 s). As such, the electrode constitutes a new approach to biosensor construction and has properties comparable if not better than similar electrodes reported. The only aspect that needed further research and improvement appears to be the low number of repeated use.

References

1. Wise, D. D.; Barkhimer, T. V.; Brault, P. A.; Kirchhoff, J. R.; Messer, W. S.; Hudson, R. A. J Chromatogr B 2002, 775, 49.

2. Campanella, L.; Mascini, M.; Palleschi, G.; Tomassetti, M. Clin Chim Acta 1985, 151, 71.
3. Campanella, L.; Tomassetti, M.; De Angelis, G.; Sammartino, M. P.; Cordatore, M. Clin Chim Acta 1987, 169, 175.
4. Garguilo, M. G.; Michael, A. C. Anal Chim Acta 1995, 307, 291.
5. Marty, J. L.; Sode, K.; Karube, I. Anal Chim Acta 1990, 228, 49.
6. Ruiz, B. L.; Dempsey, E.; Hua, C.; Smyth, M. R.; Wang, J. Anal Chim Acta 1993, 273, 425.
7. Doretti, L.; Gattolin, P.; Lora, S. Anal Lett 1994, 27, 2455.
8. Doretti, L.; Ferrara, D.; Gattolin, P.; Lora, S. Biosens Bioelectron 1996, 11, 365.
9. Peteu, S. F.; Emerson, D.; Worden, R. M. Biosens Bioelectron 1996, 11, 1059.
10. Razola, S. S.; Pochet, S.; Grosfils, K.; Kauffmann, J. M. Biosens Bioelectron 2003, 18, 185.
11. Leca, B.; Morelis, R. M.; Coulet, P. R. Sens Actuat B 1995, 27, 436.
12. Gulce, H.; Aktas, Y. S.; Gulce, A.; Yıldız, A. Enzyme Microb Technol 2003, 32, 895.
13. Doretti, L.; Ferrara, D.; Lora, S.; Palma, G. Biotechnol Appl Biochem 1999, 29, 67.
14. Curulli, A.; Dragulescu, S.; Cremisini, C.; Palleschi, G. Electroanalysis 2001, 13, 236.
15. Mohy Eldin, M. S.; Portaccio, M.; Diano, N.; Rossi, S.; Bencivenga, U.; D'Uva, A.; Canciglia, P.; Gaeta, F. S.; Mita, D. G. J Mol Catal B Enzym 1999, 7, 251.
16. Kok, F. N. Construction of an acetylcholinesterase-choline oxidase biosensor for the determination of aldicarb, Ph.D. Thesis, Middle East Technical University, Ankara, Turkey, January 2001.
17. Bradford, M. M. Anal Biochem 1976, 72, 248.
18. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. J Biol Chem 1951, 193, 265.
19. Cremisini, C.; Di Sario, S.; Mela, J.; Pilloton, R.; Palleschi, G. Anal Chim Acta 1995, 311, 273.
20. Xu, F.; Wang, L.; Gao, M. N.; Jin, L. T.; Jin, J. Y. Talanta 2002, 57, 365.
21. Chaplin, M. F.; Bucke, C. Enzyme Technology; Cambridge University Press: New York, 1990.
22. Fernandes, K. F.; Lima, C. S.; Lopes, F. M.; Collins, C. H. Process Biochem 2004, 39, 957.
23. Gulce, H.; Ataman, I.; Gulce, A.; Yıldız, A. Enzyme Microb Technol 2002, 30, 41.
24. Vrbova, E.; Kroupova, I.; Valentova, O.; Novotna, Z.; Kas, J; Thevenot, C. Anal Chim Acta 1993, 280, 43.
25. Ricci, F.; Amine, A.; Palleschi, G.; Moscone, D. Biosens Bioelectron 2003, 18, 165.