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Covalently Immobilized Choline Oxidase and Cholinesterases on a Methacrylate Copolymer for Disposable Membrane Biosensors

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ABSTRACT

Bienzymatic sensors for the determination of esters of choline were prepared by covalent co-immobilization of cholinesterases and choline oxidase on polymer membranes, obtained by radiation-induced copolymerization of 2-hydroxyethyl methacrylate and glycidyl methacrylate at low temperature. Optimization of the covalent attachment of choline oxidase and acetyl- or butyrylcholinesterase to copolymer was explored. The enzyme-modified polymers were applied on platinum electrodes to form amperometric sensors, based on the electrochemical detection of enzymatically developed hydrogen peroxide. Acetyl-, acetylthio-, butyryl-, and butyrylthiocholine contents in standard solutions were measured, and linear calibration curves were determined. Temperature and pH effects on the electrochemical response are described.

Index Entries: Biosensors; acetylcholinesterase; butyrylcholinesterase; choline oxidase; covalent immobilization; radiation induced polymerization.

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INTRODUCTION

Acetylcholine is a well-known neurotransmitter both in the central nervous system and in the nerve–skeletal junctions of vertebrates, and there is considerable interest in measuring it.

The determination of acetylcholine by electrochemical biosensors was based on the amperometric measurement of hydrogen peroxide (H_2O_2) produced in the oxidation of choline, using a common bienzyme system reaction involving acetylcholinesterase (AChE) and choline oxidase (ChO). Different possibilities have been explored: ChO was immobilized on the electrode surface and free AChE was added to a substrate buffer solution *(1)*, or both enzymes were immobilized on the sensor *(2–9)*.

For the enzyme immobilization, most sensors use physical entrapment strategies, covalent immobilization, or crosslinking on the electrode surface, and only a few of them use enzyme co-immobilization on disposable membranes *(10)*.

Acetylcholine can be hydrolyzed by two classes of enzymes: AChE, which is fairly specific (K_m = 95 μM), and butyrylcholinesterase (BChE), with a rather broad specificity (K_m = 1.2 mM). AChE and BChE are also widely used for the construction of various biosensing devices monitoring the decrease of enzyme activity in the presence of inhibitors.

The inactivation of cholinesterases caused by organophoshorus and carbamate insecticides is an interesting topic *(11–17)*, in which gas chromatography is still the most common method of measurement, which is capable, when coupled to a Fourier transform infra red (FTIR) or mass spectrometer, of giving a fingerprint of pesticides. The method of determination offered by biosensors permits *in situ* analysis, and could overcome many problems, such as thermal lability or high polarity of these substances.

The realization of a reliable choline sensor, in order to determine either the amount of acetylcholine or the cholinesterase activity, still appears very attractive.

The authors' goal was to develop an effective sensor with a disposable membrane obtained through the co-immobilization of AChE/ChO or BChE/ChO couples, and responsive to different substrates.

Biocompatibility was also of interest. 2-Hydroxyethylmethacrylate (HEMA) is a well-known biocompatible polymer. Initially, the physical immobilization was considered; however, this easy method of fixing enzymes on the matrix *(18–19)* was not practicable in the case of cholinesterases, because of their sensitivity to high-energy radiation.

Epoxide groups contained in methacrylate copolymers have been reported to be reactive to protein amino groups *(20–26)*. Recently, the authors studied a covalent enzyme immobilization procedure on polymer membranes with different amounts of epoxide groups *(27)*, prepared by copoly-

merizing HEMA and glycidyl methacrylate (GMA) in the presence of trimethylolpropane trimethacrylate (TMPTM). This method used mild coupling conditions, and, in front of a low enzyme surface loading, gave a sensor with good performance, both in terms of linearity range and sensitivity.

Different immobilization methods, using epoxides as reactive groups, have been reviewed, but, although the reaction mechanism is known, a better knowledge of the procedure is essential, in order to realize a particular biosensor resulting from the immobilization of two enzymes with different specific activities (SAs).

This paper presents studies on the conditions leading to covalent coimmobilization of ChO and cholinesterases (AChE and BChE) in HEMA–GMA disposable membranes, obtained by γ-radiation-induced polymerization. The immobilized enzymes were used to assemble amperometric sensors measuring H_2O_2 produced in the enzymatic reactions. The performances of the obtained electrodes to different substrates were evaluated, and the electrochemical characteristics of the systems are also described.

EXPERIMENTAL

Materials

Choline oxidase (ChO, EC 1.1.3.17 from *Alcaligenes* sp 10 U/mg), peroxidase (POD, EC 1.11.1.7, type VI-A from horseradish, 250 purpurogallin U/mg), AChE (EC 3.1.1.7., type V-S from electric eel, 1000 U/mg), BChE (EC 3.1.1.8, from horse serum, 610 U/mg), choline chloride, acetylcholine chloride, acetylthiocholine chloride, butyrylcholine chloride, butyrylthiocholine iodide, 4-aminoantipyrine, 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB), and glycine were supplied by Sigma (St. Louis, MO).

Buffer and substrate solutions were prepared with deionized water produced by MilliQ-Plus apparatus (Millipore, Bedford, MA). Substrate solutions were stored frozen, or freshly prepared at use, to avoid chemical ester hydrolysis.

2-Hydroxyethylmethacrylate, obtained from Aldrich (Stenheim, Germany), was purified by a treatment with a $NaHCO_3$, saturated solution in a 1:1 ratio (v/v), then continuously stirred for 20 h, and salted out with NaCl. After separation from the aqueous phase, methacrylic acid-free HEMA was obtained and fractionally distilled at reduced pressure under nitrogen flushing. Glycidyl methacrylate from Fluka (Buchs, Switzerland) and TMPTM from Janssen (Beerse, Belgium) were used as received.

All other reagents were analytical grade and obtained from Merck (Darmstadt, Germany) or Carlo Erba (Milano, Italy).

Sensor Preparation

A 2-hydroxyethyl and glycidyl methacrylate copolymer with 13% GMA was prepared as described in a previous paper (*27*). A HEMA and GMA aqueous solution (polymer–water ratio 1:1, m/m), with 4% of TMPTM as a crosslinking agent, was exposed for 3 h to a ^{60}Co γ-ray source (dose rate: 1.8 KGy/h) in polyethylene vials (5 mm id) at −78°C. The sponge-like material thus obtained was sliced with a microtome to give membranes of 200-μm thickness, which were carefully washed with water and then equilibrated with the buffer solution of interest before immobilization procedures.

Two basic methods for enzyme immobilization were used. The first one involved the immersion of the activated membrane in the enzyme solution, the second by membrane wetting with a drop of enzyme solution.

In the first protocol, some membranes with the same average weight (about 6 mg) were immersed in 0.5 mL phosphate buffer (0.1 *M*, pH 8.0), containing both enzymes (about 10 mg/mL as total concentration) in a ratio of ChO to AChE activity of 10:1, and incubated with shaking at room temperature for 30 min. For the BChE–ChO couple, the same activity ratio was used, but a carbonate buffer (0.1 *M*, pH 9.0) was preferred. The wet membranes were removed, and, without any washing, were placed on a glass Petri dish, dried under reduced pressure for 5 min, and left to react at room temperature for 8 h.

The second procedure consisted of dropping 20 μL of the same bienzyme solutions on one side of the membranes, and then, after 10 min, on the other side as well, and incubating for 8 h on open Petri dish.

At this point, the enzymatically activated membranes, obtained by the first and second methods, were rehydrated and incubated overnight in a glycine solution (0.1 *M* in phosphate buffer) at room temperature. Then they were extensively rinsed off with 3 mL buffer solutions containing 0.5 *M* NaCl, to help eliminate any remaining free enzyme, and, finally, they were washed with 0.1 *M* phosphate buffer at pH 8.0.

Free and immobilized ChO were assayed spectrophotometrically. The determination of the apparent activity of ChO was realized by dipping a membrane in 2 mL choline solution (30 m*M* in 20 m*M* Tris buffer, pH 8.0), and incubating, with stirring for 2 min at 25°C. After removal of the membrane, the solution was added to a mixture consisting of 4-aminoantypyrine, phenol, and POD solution; the activity was calculated from the absorption value at 500 nm.

Free AChE and BChE activities were calculated spectrophotometrically, using a kinetic method, following an increase in the absorbance value at 405 nm caused by 5-thio-2-nitrobenzoate formed in the enzymatic hydrolysis of acylcholine derivatives. An acetylthiocholine chloride solu-

tion (1 m*M* in 0.1 *M* phosphate buffer, pH 7.0, and 1 m*M* DTNB) and a butyrylthiocholine iodide solution (0.335 m*M* in 0.1 *M* phosphate buffer, pH 7.0, and 0.4 m*M* DTNB) were used for the calculation of AChE and BChE, respectively.

The amperometric transducer consisted of a platinum wire (0.5-mm diameter) sealed in a glass tube, and polished with alumina powder to ensure a flat surface. A polymeric membrane was fixed on the electrode surface with a teflon cap having a 3-mm hole. The good mechanical properties of the wet membranes allowed their easy replacement and use. When not in use, the membranes were stored at 4°C in a phosphate buffer, or in the dry state. The obtained biosensors were used in batch analyses.

Electrochemical Measurements

The determination of H_2O_2 was performed in an electrochemical cell with a working volume of 20 mL, in a three-electrode configuration. The three electrodes were connected to an AMEL (Milano, Italy) 559 model potentiostat. A saturated calomel electrode and a platinum foil with large surface area were used as the reference and counterelectrode, respectively. All measurements were carried out in a temperature-controlled cell, using a Haake (Karlsruhe, Germany) F3-C thermostatic bath.

Spectrophotometric measurements were carried out with a Hitachi (Tokyo, Japan) U-3200 spectrophotometer equipped with a 1-cm thermostated cell.

Substrate determination was performed electrochemically by measuring the H_2O_2 production as a result of the enzymatic reaction. This was done by immersing the sensor in a stirred suitable buffer and applying an oxidative potential of +650 mV against a saturated calomel electrode. When the background current had stabilized, an appropriate amount of substrate solution was introduced to give a preselected concentration. All the measurements were carried out at 25°C, unless otherwise stated. The sensitivity of each sensor was given by the slope of the calibration graphs, and was expressed in nAL/mmol.

RESULTS AND DISCUSSION

The copolymer obtained with the described procedure gave a hydrophilic, permeable, and porous material with good mechanical properties. As described elsewhere (*27*), a composition of 13% of GMA gave the best results, regarding both enzyme immobilization and electrochemical response.

The HEMA–GMA copolymer is an interesting matrix, because the glycidyl groups provide a convenient immobilization site for the enzymes through covalent linkages with nucleophilic groups of proteins. At pre-

sent, the reactivity of this matrix is hard to predict, and the optimal conditions, for the attachment of the enzyme and maximum retention activity, were not identified, although the effects of some polymer characteristics were explored. In this case, the difficulty was increased because of the immobilization procedure involving two enzymes with different SAs in a heterogeneous phase.

Thus, the factors that may influence the efficiency of the immobilization reaction and the activity of the bound enzyme, measured as the current response to substrate additions, were investigated, focusing on the effects of ionic strength and activity ratios of the used enzymes.

Immobilization of Choline Oxidase

Preliminary experiments were carried out to explore the conditions for the immobilization of ChO alone, because the determination of esters of choline is based on the monitoring of choline produced by the enzymatic hydrolysis of esters.

Generally, low and high values of pH enhance the epoxide ring opening (acid or base catalyzed), but milder conditions are preferred, in order to prevent enzyme inactivation occurring in extreme pH regions. Experimental data reported by Hall et al. *(26)* suggest that the optimal immobilization pH should be close to that of enzyme activity maximum, rather than to the epoxide reactivity maximum.

Free ChO shows a maximum activity at pH 8.0, and it is denatured at values lower than 6.5, or higher than 9.0. Choline oxidase physically immobilized in a HEMA matrix showed an activity maximum in a glycine buffer at pH 9.0 *(19)*. This buffer was discarded because of the possible interferences of its amino groups with oxirane groups, and a phosphate buffer at pH 8.0 was selected for the immobilization of ChO in a HEMA–GMA copolymer.

The immobilization procedure consisted of dropping 20 μL of a buffer solution containing 100 U/mL ChO on both sides of a membrane, and incubating at room temperature for 8 h in an open Petri dish. Then the membrane was incubated overnight in a glycine solution (10 m*M* in phosphate buffer), to disactivate any unreacted epoxide groups, and, subsequently, was rinsed off with 0.5 *M* NaCl solution, to eliminate free enzyme.

In order to examine the influence of ionic strength, immobilization was carried out by varying the phosphate concentration in the 0.1–1 *M* range. As the ionic strength diminished from 1 to 0.1 *M*, an increase of the apparent activity was observed, with a maximum at 0.1–0.2 *M*, which decreases by 25% for 1 *M* ionic strength. The use of starting buffer solutions, with different ChO concentrations in the 100–1000 U/mL range, did not show appreciable changes in the amount of fixed enzyme.

Coimmobilization of Esterases and Choline Oxidase

In the coimmobilization of two enzymes working in sequence and having different SAs, the activity ratio and the total protein concentration must be carefully taken into consideration. The SAs of ChO and AChE were very different (10 and 1000 U/mg, respectively), and the best sensitivity was obtained with a ratio of ChO to AChE activity of 10:1 in 0.1 *M* phosphate buffer at pH 8.0. With ChO:AChE ratios of 1:1, 2:1 and 50:1, decreases in the sensitivity of about 25, 20, and 15% respectively, were observed. With a 10:1 ratio, the response as a function of the protein concentration increased up to a concentration of about 10 mg/mL, and then remained practically unchanged.

Varying the ionic strength from 0.1 to 1 *M*, a considerable decrease in sensitivity was observed, as a result of a verified decrease of about 25–30% in the amount of fixed ChO. The immobilization reaction carried out in a carbonate buffer at pH 9.5, which is very good for ChO immobilization, produced a sensor of very low sensitivity, probably because AChE immobilization was prevented.

The SAs of ChO and BChE were also different (10 and 610 U/mg, respectively), and the best sensitivity was obtained under the same experimental conditions as ChO–AChE, but using a 0.1 *M* carbonate buffer at pH 9.5.

The two procedures described in the Experimental section were tested, and maximum sensitivity for both enzyme couples was obtained when the second method (membrane wetting with a drop of enzyme solution) was used. The mean sensitivity of the AChE/ChO electrode to acetylcholine in 0.1 *M* buffer, pH 8.0, was 289 nAL/mmol when the wetting method was used, compared to 192 nAL/mmol when the membrane was immersed in the coupling solution. Likewise, the sensitivity of the BChE–ChO sensor to butyrylthiocholine in the same buffer solution was 526, compared to 310 nAL/mmol.

The response time, considered as the time required to reach a 90% steady-state current, was within 2 min for sensors with enzymes immobilized by the wetting method; it was up to 4 min in the other case. This would suggest that the ratio of immobilized activities was also different.

All the following measurements were carried out with membranes obtained with enzymes immobilized by the wetting method.

Response of AChE–ChO Electrode

For the characterization of the biosensor containing ChO and AChE covalently linked to the polymeric matrix, acetylcholine chloride as a standard substrate was used, taking advantage of the satisfactory results, both in terms of linearity range and response time.

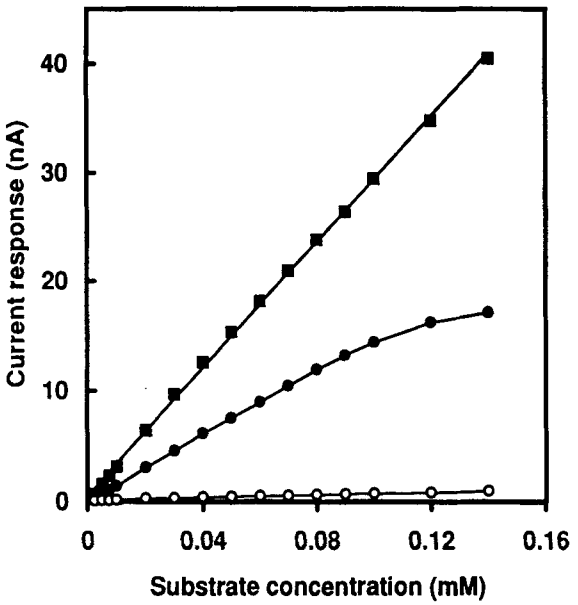

Fig. 1. Calibration graphs for different substrates obtained with AChE–ChO coimmobilized sensor at pH 8 and 25°C. (■) Acetylcholine; (●) acetylthiocholine; and (○) butyrylthiocholine.

The response to this substrate gave, at pH 8.0 and 25°C, a linear pattern in the 5×10^{-6}–1.4×10^{-4} M range, with a linear regression equation of $y = 289x + 0.5$, $R^2 = 0.9997$, where y is the electrode response (nA) and x is the acetylcholine concentration (mmol/L). A detection limit of 2×10^{-6} M was estimated with a signal-to-noise ratio of >3. Measurements repeated 5× showed a relative standard deviation of the slope <3%. The response time was 90 s.

This sensor was tested with different substrates, and a different behavior was observed, probably correlating either to relative amounts of enzymes covalently bound or to the kinetic constants for different substrates.

The sensitivities to acethylthiocholine and butyrylthiocholine were 147 and 6 nAL/mmol; the response times were 180 and 210 s, respectively.

Calibration graphs for the substrates used are reported in Fig. 1, and the results are summarized in Table 1.

The pH effect on the probe was evaluated on acetylcholine by using 0.1 M phosphate and carbonate buffers, with pH values ranging from 6.0 to 10.0. As shown in Fig. 2, the best response was found at pH 9.5. The temperature dependence of the acetylcholine sensor was studied in the 15–40°C range. The sensitivity gradually increased up to 40°C.

Table 1
Data from Calibration Curves for Different Substrates Obtained with AChE–ChO and BChE–ChO Sensors (n = 5).

Substrate	AChE/ChO			BChE/ChO		
	Sensitivity (nA/m*M*)	R^2	RSD%	Sensitivity (nA/m*M*)	R^2	RSD%
Acetylcholine	289 ± 6.9	0.9992	2.39	n.a.[b]		
Acetylthiocholine	147 ± 4.1	0.9991	2.79	—		
Butyrylcholine	—[a]			14 ± 0.4	0.9969	2.86
Butyrylthiocholine	6 ± 0.2	0.9912	3.30	526 ± 9.7	0.9997	1.84

[a] —, not determined; [b] n.a., signal not appreciable

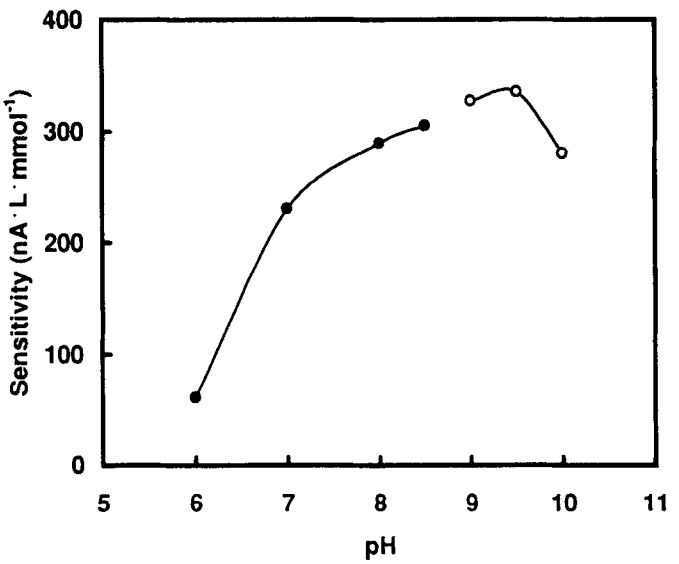

Fig. 2. Effect of pH on the sensitivity of AChE–ChO coimmobilized electrode.

The operational stability was tested over a 6-h period under the conditions defined above (0.1 *M* phosphate buffer at pH 8.0 and 25°C). After six assays, the loss of activity was 4%.

In order to examine the long-term stability of the enzyme membranes, they were stored in phosphate buffer at 4°C, or in the dry state. In the latter case, the membranes, after being rinsed with phosphate buffer, were dried at 25°C, and then stored at 4°C in a sealed Petri dish. Before being used, they were equilibrated in a phosphate buffer for 1 h at room temperature. No substantial differences in the electrode responses were

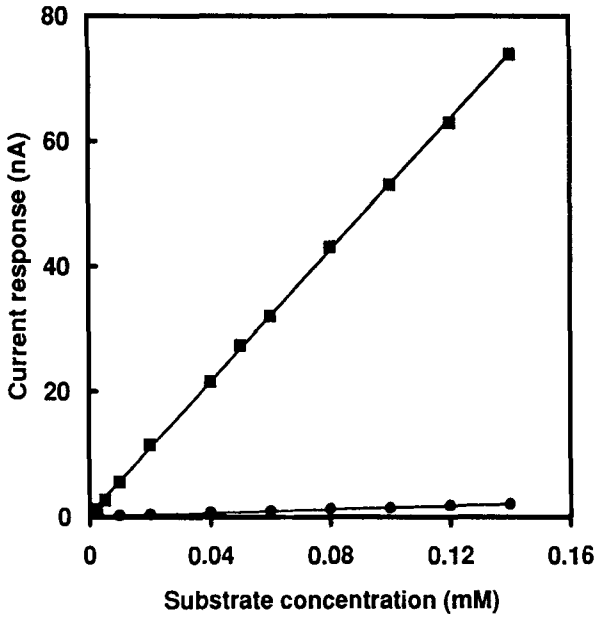

Fig. 3. Calibration graphs for different substrates obtained with BChE–ChO coimmobilized sensor at pH 8 and 25°C. (■) Butyrylthiocholine; (●) butyrylcholine.

observed over a period of 2 mo. On the other hand, when the membranes were subjected to repeated cycles of measurements, detachments, and washings, and after each assay, being stored in buffer solution at 4°C, the decrease in sensitivity was considerable. Losses of sensitivity of 50 and 80% were observed after 15 and 30 d, respectively.

Response of BChE–ChO Electrode

This biosensor was characterized using butyrylthiocholine iodide as a standard substrate, because of the high kinetic value of this esterase for its hydrolysis.

Figure 3 reports a typical calibration graph obtained in 0.1 *M* phosphate buffer at pH 8.0 and 25°C, under continuous magnetic stirring. The linearity was verified in the 2×10^{-6}–1×10^{-4} *M* range, and the linear regression equation in this range was $y = 526x + 0.5, R^2 = 0.9997$, where y was the electrode response in nA and x was the butyrylthiocholine concentration in mmol/L. A detection limit of 1×10^{-6} *M* was estimated, with a signal-to-noise ratio of >3. The response time was 120 s.

As reported in the same Fig. 3, the measurements carried out with butyrylcholine chloride showed a very low sensitivity (14 nAL/mmol), but with acethylcholine, no signal was observed. This behavior can be explained by the higher affinity of pseudocholinesterase to butyrylthiocholine than butyrylcholine and acetylcholine.

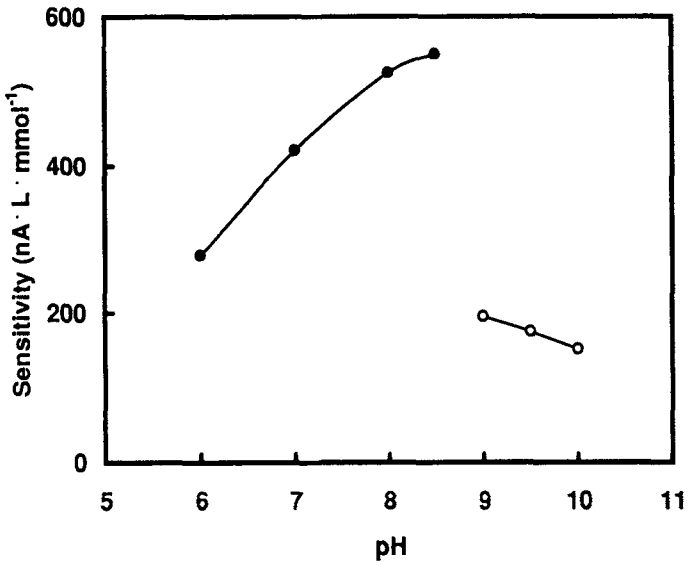

Fig. 4. Effect of pH on the sensitivity of BChE–ChO coimmobilized electrode.

The influence of pH on this sensor was also investigated, and Fig. 4 shows the response to butyrylthiocholine solution in the pH range of 6.0–10.0. It can be seen that the optimum pH was found at 8.0. Free BChE and ChO show a maximum activity at pH values of 6.0–8.0 and 8.0, respectively.

The temperature dependence was assayed, and the response of the sensor also increased with a temperature increase from 15 to 40°C.

The operational storage stability was tested under the same experimental conditions. After six running assays, no substantial differences in the electrode response were observed. When the membranes were detached, washed after each assay, and stored in buffer solution at 4°C, the electrode signal decreased by about 5% after 45 d, and then faster, reaching a decrease of about 30% after 3 mo. If the membranes were stored in the dry state for 2 mo, the sensitivity of the sensor remained unchanged.

CONCLUSIONS

This work supports the results that the material obtained from a 2-hydroxyethyl and glycidyl methacrylate copolymer is suitable for the covalent coimmobilization of cholinesterases and ChO. The immobilization procedure proposed appears to be easy to perform and needs mild conditions. The obtained biosensors allow the determination of either acetylcholine or butyrylthiocholine, and could also be used to measure cholinesterase activity. Regarding their characteristics (linear range, low

detection limit, sensitivity, storage stability in a dry state, and response time), they can be considered very attractive analytical devices.

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