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Amperometric Sensor for Choline and Acetylcholine Based on a Platinum Electrode Modified by a Co-crosslinked Bienzymic System

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A fast-response and sensitive amperometric biosensor for choline and acetylcholine based on choline oxidase and acetylcholinesterase immobilized on a platinum electrode by co-crosslinking with bovine serum albumin and glutaraldehyde has been developed. The immobilization procedure proved fast and simple, not requiring dedicated manipulation. Detection limits were in the sub-micromolar range and linearity extended over three concentration decades. After one month of discontinuous use, 70% of the initial sensitivity was still observed. The response time was around 1 s for both analytes making the developed biosensor a suitable detector for flow injection systems and high-performance liquid chromatography.

Keywords: *Biosensor; choline; acetylcholine*

Introduction

Acetylcholine (AcCh) plays an important role in the cholinergic system acting as the transmitter of impulses on cholinergic synapses. AcCh and choline (Ch), the latter being a precursor and a metabolite of AcCh, are found in the central and peripheral nervous system of mammalians. Considerable interest has been devoted to the *in vitro* and *in vivo* determination of AcCh and Ch, in connection with neuropsychological and neuropsychiatric disorders such as Alzheimer's disease and progressive dementia.[1–4]

Unfortunately, AcCh and Ch are neither oxidizable nor possess structural characteristics (*e.g.*, chromophore groups) allowing a sensitive detection. Consequently, the majority of the methods developed for their determination generally require a conversion into more easily detectable compounds. Several methods, including bio- or radiometric assay and gas chromatography–mass spectrometry, have been developed for AcCh and/or Ch determination in biological tissues and fluids (see refs. 5 and 6 for a review). HPLC coupled to post-column enzymic reaction (PER) with acetylcholinesterase (AcChE) and choline oxidase (ChO) is, by far, the most widely used method.[7] Hydrogen peroxide, released by the enzymic reaction (see Scheme 1), is detected by chemiluminescence or, more usually, by electrochemical detection (ED) at a platinum electrode. Notwithstanding some improvements,[8–10], achieved for example by enzyme immobilization in packed-bed reactors,[8] HPLC–PER–ED methods still suffer from some drawbacks.[11–13]

Amperometric biosensors, in which the enzymic conversion and the electrochemical detection of hydrogen peroxide occur in the same physical device, could represent a valid alternative to PER–ED. Indeed, several AcCh/Ch amperometric biosensors with ChO/AcChE enzymes immobilized on discrete membranes[14–18] or entrapped in photo-crosslinkable polymers,[19] cross-linked redox polymers[20] or Nafion[21] have been described. Unfortunately, most of these sensors are useless in HPLC detection because they cannot satisfy certain requirements such as response time, sensitivity and adaptability to the cell geometry usually employed in HPLC detectors.

In this paper, an amperometric biosensor based on AcChE and ChO immobilized on a platinum electrode by co-crosslinking with bovine serum albumin and glutaraldehyde is reported. The immobilization procedure can be easily adapted to the working electrode and cell geometry typically employed in electrochemical detectors. The resulting sensor is stable, highly sensitive and shows a fast response time so is to be considered a promising detector for flow injection and HPLC.

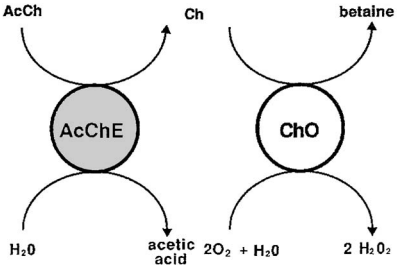
Experimental

Materials

Choline chloride, acetylcholine chloride, choline oxidase [EC 1.1.3.17 from *Alcaligenes species*, 14.6 U mg^{-1} of solid (1U = 16.67 nkat), acetyl cholinesterase (EC 3.1.1.7, type VI-S, from electric eel, 225 U mg^{-1} of solid), bovine albumin (fraction V), glutaraldehyde (grade II, 25% aqueous solution) were obtained from Sigma (Sigma, St. Louis, MO, USA) and used without further purification. Before its use, choline chloride was dried under vacuum over P_2O_5 for at least 3 d and stored in a vacuum desiccator. All other chemicals were of analytical-reagent grade. Choline and acetylcholine stock solutions were prepared in triply distilled water or buffer and stored in the dark at 4 °C. More dilute solutions were prepared just before use.

Apparatus

Electrochemical experiments were carried out using a PAR 174A polarographic analyser (EG&G Princeton Applied Re-



Scheme 1 Enzymic reaction of AcChE and ChO releasing hydrogen peroxide.

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search, Princeton, NJ, USA). The electrochemical cell was a conventional three-electrode system with a Pt rod as counter electrode and an Ag/AgCl, KCl-saturated reference electrode. The Pt working electrode was constructed by sealing a platinum disc (polycrystalline 99.95%, 4.0 mm diameter, Goodfellow, Cambridge, England) in a glass body. Solutions were stirred by an EG&G Model 305 magnetic stirrer. Signals were recorded using a Philips PM 8121 *x-y-t* recorder (Philips Scientific, Eindhoven, The Netherlands).

Enzyme-layer thickness was estimated by using an Alpha-Step 200 profilometer (Tencor Instruments, Mountain View, CA, USA). The stylus mass was 8 mg and the scan rate was 0.2 μm s^{-1}. To perform the measurements, several amounts of enzyme solution were cast on a platinum sheet as described below; before each measurement, the enzyme layer was allowed to swell by soaking in a phosphate buffer.

HPLC experiments were performed by using a Perkin- Elmer (Norwalk, CT, USA) series 10 pump module and a Rheodyne (Cotati, CA, USA) Model 7125 injection valve equipped with a 20 μl loop. The chromatographic column was a 5 μm Supelcosil (Supelco, Bellefonte, PA, USA) LC-18-DB octadecylsilica (ODS) column (250 x 2.1 mm). A 5 μm Supelguard (Supelco) LC-18-DB ODS precolumn (20 x 2.1 mm) was used to protect the analytical column. The mobile phase (filtered through a Millipore 0.45 μm filter) was a phosphate buffer (0.1 mol l^{-1}, pH 6.5) containing 5 mmol l^{-1} hexane sulfonate and 10 mmol l^{-1} tetramethylammonium phosphate. The electrochemical detector was an EG&G Model 400 including a thin-layer electrochemical cell with a platinum working electrode and an Ag/AgCl, 3 mol l^{-1} NaCl reference electrode. Two thin layer flow cell gaskets (Bioanalytical Systems, West Lafayette, IN, USA) 0.005 in thickness were used. Chromatograms were recorded on a Kipp & Zonen (Delft, Holland) Model BD 112 flat-bed recorder.

Preparation of Enzyme Electrode

Before each electrode modification, the Pt working electrode was cleaned using hot nitric acid followed by an alumina (0.05 μm particles) polishing procedure, extensive washing and sonication in triply distilled water. A 300 μl volume of a phosphate buffer (I = 0.1 mol l^{-1}, pH 6.5) solution containing 16 mg of BSA and 1 mg of ChO (or 1 mg of ChO and 1 mg of AcChE for AcChE/ChO electrodes) were carefully mixed with 30 μl of 2.5% glutaraldehyde solution (25% glutaraldehyde solution diluted 1 + 9 with phosphate buffer). Unless otherwise stated, 3 μl of the resulting solution were pipetted onto the Pt working electrode surface (avoiding air-bubble formation), carefully spread out, to completely cover the electrode surface, and air-dried at room temperature. After their preparation, sensors were soaked in a stirred supporting electrolyte to remove any weakly bound or adsorbed enzyme and for swelling of the enzyme layer itself; usually, about 15 min were necessary to obtain a stable and steady-state substrate response. When not in use, sensors were stored in a phosphate buffer (I = 0.1 mol l^{-1}, pH 6.5) at 4 °C in the dark.

Electrochemical Measurements

A detection potential of +0.650 V *versus* Ag/AgCl was used in all the electrochemical experiments. Solutions were air-saturated and the temperature was ambient. Unless otherwise specified, analyte responses were measured in batch addition experiments to a stirred phosphate buffer (I = 0.1 mol l^{-1}) having the desired pH.

HPLC experiments were performed at a flow rate of 0.2 ml min^{-1}; the detection potential was +0.650 V *versus* Ag/AgCl, the sample volume injection was 5 μl and temperature was ambient.

Results and Discussion

Glutaraldehyde (GLU) co-crosslinking of ChO/AcChE with bovine serum albumin (BSA) offers the possibility of immobilizing the enzymes simply by casting a small amount of the enzyme solution onto the Pt surface. In this way, a good compromise between sensor characteristics (*e.g.*, stability, sensitivity, response time, shelf-life time) and speed and simplicity of sensor preparation is obtained. Enzyme co-crosslinking was preferred to a simple crosslinking[18–20,22,23] procedure mainly because it allows a higher degree of intermolecular bonding,[24] and results in a lesser extent of enzyme crowding and enzyme deactivation. Moreover, no pre-derivatization of the electrode surface[23] was required for covalent binding of the enzyme to the Pt surface. In fact, AcChE and ChO co-crosslinking with BSA produces a very thin enzyme layer that is strongly adherent to the Pt surface and mechanically very stable in stirred or flowing solutions.

Influence of pH *and GLU/BSA Concentration*

Several enzyme sensors were prepared by co-crosslinking performed at different pH. A pH value of ≤5 led to sensors showing poor sensitivity and/or induced enzyme precipitation in solution. Poor responses were also observed for sensors prepared at pH levels greater than 10, probably because of irreversible enzyme denaturation (*vide infra*). A pH in the range 6–9 was optimal as already reported for other enzymes.[25]

The extent of enzyme crosslinking is mainly controlled by the amount of crosslinker employed. Hence, the GLU concentration can influence diffusivity through the enzyme layer[26] and the amount of enzyme immobilized (and deactivated) by cross-linking.[24] Fig. 1 shows the sensitivities of a series of ChO electrodes (AcChE/ChO electrodes behave in a similar way) as a function of BSA amount at two different GLU concentrations. The upper limits of both GLU and BSA concentrations were chosen such to ensure a reasonable gelation time. As it can be seen Ch sensitivity was the higher the lower the GLU concentration. A similar behaviour was observed for I_{max}, *i.e.*, the maximum steady-state current response, while the response time did not appreciably change. Since one would expect a lower yield in enzyme immobilization at low crosslinker concentrations, the observed behaviour could be explained assuming a lower extent of enzyme deactivation. Note, however, that ChO (and AcChE/ChO) electrodes prepared with GLU concentrations less than 0.1% showed low responses, poor mechanical properties and a high irreproducibility. A GLU concentration around 0.2% was then considered optimal. Fig. 1

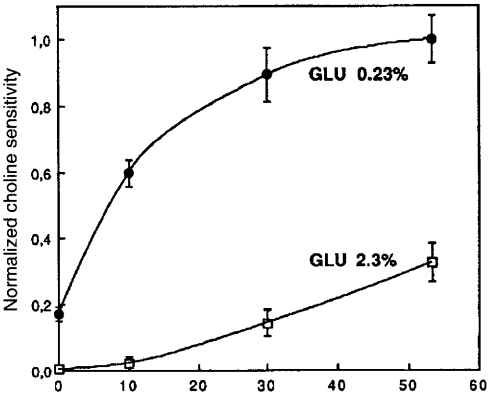

Fig. 1 Sensitivities (normalized) of ChO sensors prepared at two different GLU concentrations as a function of BSA concentration in the enzyme solution. Error bars indicate standard deviations calculated from responses derived from five different sensors. Sensor preparation and conditions as described under Experimental.

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also shows that sensitivity (as well as I_{max}) increased on increasing BSA concentration. At the lowest GLU concentration, a plateau was approached at BSA concentrations of around 50 mg ml^{-1} suggesting a lower crowding and/or deactivation of the enzymes. However, the increase in sensitivity was parallelled by an increase in the response time arising from an increase in the enzyme layer thickness and/or from changes in the diffusional characteristics of the layer itself.

Since AcCh and Ch detection depends on ChO catalysed conversion (see Scheme 1), further studies were firstly directed to a ChO sensor.

Influence of ChO Loading

Several Ch electrodes with different enzyme loading were prepared by using ChO solutions with concentration ranging from 1.5 to 15 mg of protein per millilitre (equivalent to 22–220 U ml^{-1}); it was assumed that the concentration of the active enzyme in the immobilized layer was proportional to its concentration in the solution used for sensor preparation. As can be seen in Fig. 2(a), electrode sensitivity increased on increasing the enzyme loading, whatever the pH; the same behaviour was observed for I_{max}. Moreover, an increase of the volume, V_c, of the enzyme solution cast onto the electrode surface (*i.e.*, an increase in the enzyme layer thickness, *vide infra*) resulted in an increase of Ch response, as expected[27–29] when the rate of enzyme catalysis is comparable or slower with respect to substrate diffusion. However, sensitivity and I_{max} levelled off at the higher enzyme loading, suggesting that substrate diffusion becomes the rate-determining step.[27–29]

Eadie–Hofstee plots [see Fig. 2(b)] shows that, at a given pH, the apparent Michaelis constant $K'_m{}^{Ch}$ decreased significantly on increasing the enzyme loading. For example, a ten-fold increase of ChO concentration caused a decrease of $K'_m{}^{Ch}$ from 2.6 to 0.9 mmol l^{-1}, at pH 6.5 , or to 0.1 mmol l^{-1} at pH 8 (the Michaelis–Menten constant for the free enzyme in a pH 8 solution is 0.87 mmol l^{-1} [30]). As with most flavoenzymes, ChO exerts its catalytic action through a 'ping-pong' mechanism[31] involving the two substrates choline and dioxygen (see Scheme 1). In such a case, the apparent Michaelis–Menten constant $K'_m{}^{Ch}$ for the substrate Ch when the enzyme is free in solution is[32]:

$$K'_m{}^{Ch} = K_m{}^{Ch} [O_2]/(K_m{}^{O_2} + [O_2]) \quad (1)$$

where $K_m{}^{Ch}$ and $K_m{}^{O_2}$ are the true K_m for Ch and O_2, respectively, when the other substrate is present at saturating concentration. Increasing the ChO loading, the O_2 supply becomes increasingly important and becomes the rate-determining step at high enzyme loading. In this situation $[O_2] \leqslant K_m{}^{O_2}$ so that $K_m{}^{Ch}$ results lowered by a factor equal to $[O_2]/K_m{}^{O_2}$, explaining the observed results.

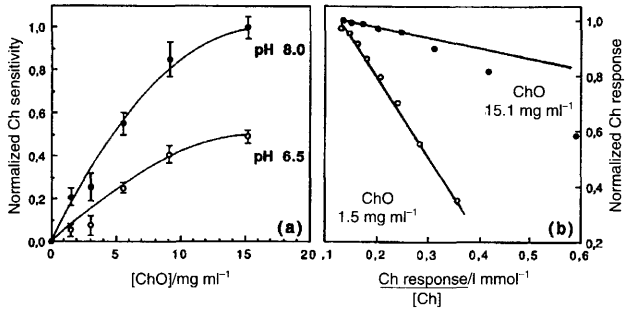

Fig. 2 (a) Normalized sensitivities of Ch sensors at pH 6.5 and 8.0 as a function of ChO concentration in the enzyme solution used for sensor preparation (see Experimental). (b) Eadie–Hofstee plots for sensors prepared from solutions at ChO concentration of 1.5 and 15 mg ml^{-1}. Working pH, 6.5. Error bars indicate standard deviations calculated from responses derived from five different sensors.

The above findings indicate that the ChO loading can be used to modulate linear range and sensitivity. Further studies were performed mainly at an intermediate ChO loading (see Experimental) where the sensor behaviour is controlled by the enzyme catalysis; in fact, this enzyme loading assured a good compromise between sensitivity and linearity of biosensor.

Influence of AcChE/ChO Loading Ratio

Since AcCh detection relies on the co-immobilization of two enzymes carrying out two consecutive reactions (see Scheme 1), a careful choice of the AcChE/ChO activity ratio is required. Several bienzyme electrodes were prepared by using enzyme solutions containing a fixed amount of ChO and variable amounts of AcChE in order to obtain different enzyme activity ratios. The upper limit of AcChE/ChO activity ratios explored was chosen according to practical considerations deriving from the different specific activities of the two enzymes and solubility limitations. Calibration experiments were then performed on each electrode for both Ch and AcCh. Fig. 3 shows the AcCh/Ch sensitivity ratio, SR, and the AcCh/Ch I_{max} ratio, IR, as a function of the AcChE/ChO activity ratio. As it can be seen, SR and IR increase on increasing the enzyme activity ratio. In particular, SR approaches its maximum value, *i.e.*, 1, at activity ratios higher than 10 whatever the pH (the IR increase was, however, pH dependent). This behaviour can be rationalized considering that SR represents the efficiency by which the AcChE/ChO system transforms AcCh into H_2O_2 with respect to the conversion of Ch into H_2O_2 catalysed by ChO. Increasing the enzyme activity ratio, the AcCh conversion rate approaches that of Ch, and SR approaches 1. The finding that an AchChE/ChO activity ratio around 10 is required for maximizing SR could be ascribed to several factors such as: a different yield in enzyme immobilization and/or extent of deactivation; different diffusivity of the two substrates and, most important, kinetic limitations of ChO. Evidence of the role played by this last factor are based on the observation that an increase of the AcChE/ChO activity ratio, obtained by decreasing the ChO loading, caused a lowering of both SR and IR values.

Influence of the Enzyme-layer Thickness

The volume, V_c, of the enzyme solution cast onto the electrode surface influences mainly the thickness of the enzyme layer. Surface inspections by a microscope revealed that using low V_c (*i.e.*, less than 100 µl cm^{-2}) a nearly flat enzyme layer was formed onto the electrode surface. In fact the enzyme-layer thickness has been found to be linearly related to V_c. In particular, the deposition of 3 µl of enzyme solution on our sensor gave an average (n = 5) thickness of 9.5 ± 2.1 µm.

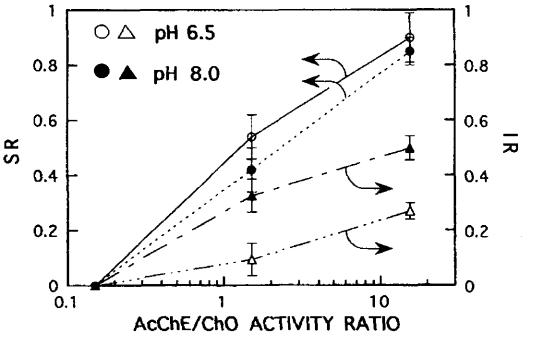

Fig. 3 AcCh/Ch sensitivities ratio, SR, and AcCh/Ch maximum steady-state current ratio, IR, as a function of the AcChE/ChO activity ratio in the enzyme solution used for sensor preparation (see text for further details). Error bars indicate standard deviations calculated from responses derived from five different sensors.

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Obviously V_c (*i.e.*, thickness) strongly influences[27] the response time; as can be seen in Fig. 4, on increasing V_c from 3 to 10 μl, $t_{0.95}$ increased from about 1 ($t_{0.95}$ for Ch 1.2 ± 0.4 s, n = 5) to about 20 s. The same behaviour was observed in the case of the AcChE/ChO sensor ($t_{0.95}$ for AcCh 1.1 ± 0.5 s, n = 5, for V_c = 3 μl) suggesting that the transient behaviour of the bienzyme sensor is still mainly controlled by substrate diffusion. Because of the linear relationship between thickness and sensitivity,[27–29] fast responses were obtained for both Ch and AcCh without a drastic loss of sensitivity (typically a 50% decrease was observed passing from 7 to 3 μl of deposited material). Response times reported for other AcCh/Ch amperometric biosensors fall mainly in the minute range.

Finally, no significant dependence of K'_m (for both AcCh and Ch) on the enzyme-layer thickness was observed, as expected in the case of an enzyme–kinetic controlled response.[27–29]

Influence of pH

Figs. 5(a) and (b) show Ch and AcCh sensitivities of a typical AcChE/ChO sensor as a function of pH. In both cases, a characteristic bell-shaped dependence was observed with a maximum located in the pH range 9–10, which, as usually observed, is slightly shifted from the value reported for the enzymes in solution: pH range 7–8 and 8–9 for ChO[30,33] and AcChE,[34] respectively.

Since Ch and AcCh are tetraalkyl ammonium derivatives, the observed activity/pH dependence could arise[32,35] from the formation of improper ionic forms of the enzymes that have lower substrate affinity and/or from changes in enzyme stability. These effects may occur in combination so that, to distinguish between them, AcChE/ChO sensors were pre-incubated, for about 1 h, at a given pH and, thereafter, their sensitivities tested at several pH values. Pre-incubation at pH values in the range 6–10 had no significant effect on sensor response (*i.e.*, on the enzyme activity) suggesting that the left branch of curves in Figs. 5 (a) and (b) is a true pH effect. This was confirmed by the analysis of the relevant Eadie–Hofstee plots in this pH range (see Fig. 6). In fact, the pH increase caused also a decrease in K'_m towards a minimum reached around pH 9: as an example, passing from pH 6.5 to 9.8, K'_m decreased from 2.4 to 0.18 mmol l^{-1}. The H^+ ion, hence, acts as competitive inhibitor[32,35] *i.e.*, as the pH decreases, the enzymes are converted into the inactive, dead-end form. At high pH values, the sensitivity increases but at the cost of a reduction in the linear range of the sensor. AcChE/ChO sensors that were pre-incubated at a pH higher than 10 showed, however, a significant loss in sensitivity [compare, for example, the two curves in Fig. 5(a)] arising from an irreversible enzyme deactivation.

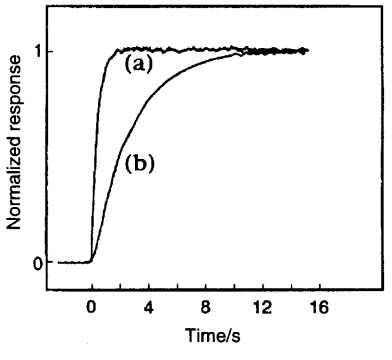

Fig. 4 Normalized current–time responses of two sensors prepared with (a) 3 μl and (b) 10 μl of enzyme solution. Ch concentration, 200 μmol l^{-1}; supporting electrolyte, 0.1 mol l^{-1} phosphate buffer, pH 6.5. Other conditions as in Experimental.

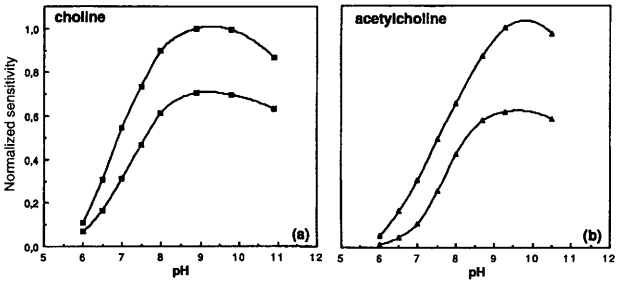

Fig. 5 Normalized sensitivities of an AcChE/ChO sensor toward (a) Ch and (b) AcCh as a function of pH. The upper and lower curve in (a) and (b) refer to sensors pre-incubated, for approximately 1 h, at pH 6.5 and 11, respectively.

Calibration Curves and Limits of Detection

Figs. 7(a) and (b) show typical calibration curves for Ch and AcCh at two different pH values. A bell-shaped curve was observed in the case of AcCh indicating inhibition of AcChE at high substrate concentrations. Enzyme inhibition at high substrate concentrations is a well known behaviour in the case of solubilized AcChE.[34] It can be shown[32] that in this case, the initial rate v is given by:

$$v = Vs/(K_m + s + s^2/K_i) \quad (2)$$

where s and V represent the substrate concentration and the maximum velocity, respectively, and K_i the inhibition constant. According to refs. 27 and 36, for a biosensor operating under conditions where the enzymic reaction is rate controlling (as in the present case, *vide ante*), the amperometric form of eqn. (2) can be written as:

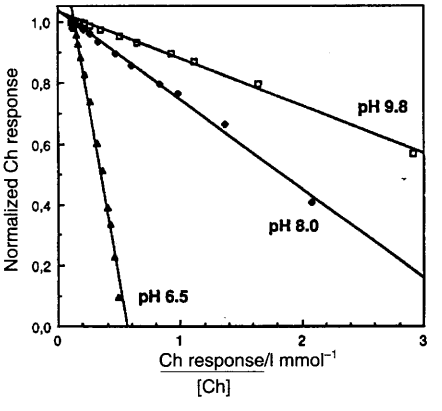

Fig. 6 Eadie–Hofstee plot (normalized) for AcChE/ChO sensors obtained at pH values of 6.5, 8.0 and 9.8.

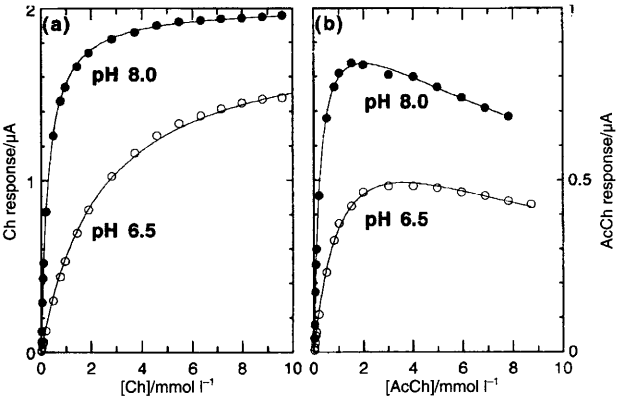

Fig. 7 Calibration curves for (a) Ch and (b) AcCh, obtained at pH 8.0 (upper plots) and pH 6.5 (lower plots).

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$$i = I_{max}\, s/(K'_m + s + s^2/K'_i) \quad (3)$$

where K'_i represents the apparent inhibition constant. Apparent K'_i values obtained through best-fitting of eqn. (3) to data in Fig. 7(b) are reported in Table 1.

Fig. 8, displaying typical Ch and AcCh batch-addition responses at concentrations near to their LODs, clearly indicates that both analytes could be easily detected at 0.1 or 0.01 μmol l^{-1} levels (see also Table 1) depending on the enzyme-layer thickness employed. As far as we know the described sensor is unrivalled in terms of detection limits achieved and response time which, in the worst situation, was around 20 s (the best figures found in the literature[16] are an LOD of 0.05 μmol l^{-1} with a response time of approximately 2 min). Finally, it is worth noting that most of the baseline noise originates from the stirrer and that improved signal-to-noise ratios could be obtained under more strictly controlled hydrodynamic conditions (*e.g.*, use of rotating disc electrodes).

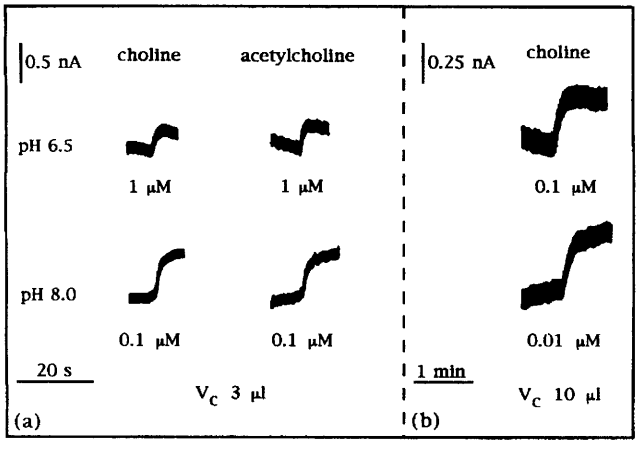

Fig. 8 Ch and AcCh responses of a typical AcChE/ChO sensor at concentration levels near their detection limits as a function of pH (see along the rows) and the amount *Vc* of enzyme solution [see along the columns (a) and (b)] used for sensor preparation. Detector time constant, 3 s.

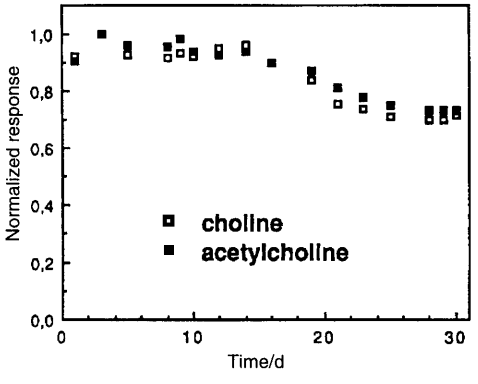

Fig. 9 Normalized Ch and AcCh responses as a function of time for a typical AcChE/ChO sensor. Ch and AcCh concentration, 200 μmol l^{-1}. Sensor storage conditions as described in Experimental.

Operational and Long-term Stability

The operational stability of the Ch/AcCh biosensor was tested by monitoring the steady-state response to 50 μmol l^{-1} Ch (or AcCh); typically after a 5 h period of continuous use the response decreased by no more than 4% showing a drift of less than 1% h^{-1}.

The long-term stability was investigated by discontinuously monitoring (see Fig. 9) the response of sensors stored in a phosphate buffer pH 6.5 at 4 °C in the dark (no particular attention was used to avoid bacterial growth in the storage medium). As can be seen, over a 30 d period, the maximum observable decrease was around 30% for both analytes. After six months the sensor was still responsive up to the 50 μmol l^{-1} level, even if 90% of the original response was lost.

Enzyme Sensor as an HPLC Detector

Since the sensor performances (*e.g.*, sensitivity and response time) proved promising for flow-through applications, AcChE and ChO were immobilized as described in this work on the platinum working electrode of a typical thin-layer electrochemical cell. The ability of such a sensor to be used as a detector for HPLC can be gathered from Fig. 10, which shows

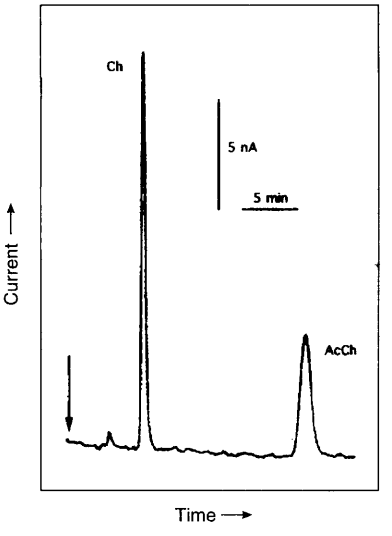

Fig. 10 Chromatogram relevant to a mixture of Ch and AcCh standards obtained by using the AcChE/ChO sensor in thin-layer cell geometry. Amounts injected, 2 nmol each. Chromatographic conditions as described in Experimental.

Table 1 Significant parameters of the AcCh/Ch sensor*

Analyte	pH	Sensitivity/ μA l mmol^{-1}	Upper linear limit/mmol l^{-1}	LOD†/ μmol l^{-1}	K'_m‡/ mmol l^{-1}	I_{max}‡/μA	K'_i‡ mmol l^{-1}
Choline	6.5	0.56 (± 0.09)	0.8	2	2.3 (±0.3)	1.9 (±0.3)	—
	8.0	4.2 (±1.1)	0.09	0.1	0.26 (±0.08)	2.0 (±0.4)	—
Acetylcholine	6.5	0.54 (± 0.10)	0.3	2	1.2 (±0.2)	0.82 (±0.16)	11 (±2)
	8.0	4.0 (±0.8)	0.08	0.1	0.24 (±0.06)	1.1 (±0.2)	15 (±2)

* Data relevant to five different AcCh/Ch sensors prepared as described under Experimental. Data in brackets represent standard deviations calculated from responses derived from five different sensors.

† Limits of detection calculated at a signal-to-noise ratio of 3. See Fig. 8 for limits of detection at V_c = 10 μl.

‡ K'_m and I_{max} for choline calculated according to a Michaelis–Menten model. K'_m, I_{max} and K'_i for acetylcholine calcula- ted according to a substrate inhibition model (see text for the equation).

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a typical chromatogram relevant to a mixture of Ch and AcCh standards. It is worth noting that analysing Ch and AcCh by HPLC typically requires coupling with post-column enzymic reactor (*vide ante*) for their detection.

In conclusion the simple enzyme immobilization procedure described here permits the construction of Ch and AcCh sensors which display remarkable sensitivity, stability and fast responses. The use of these sensors as HPLC detectors for the simultaneous determination of Ch and AcCh in biological tissues is currently being pursued in our laboratory.

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