AMPEROMETRIC MICROBIOSENSOR FOR THE DETERMINATION OF CHOLINE

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Abstract

The construction and performance of an amperometric microbiosensor for the determination of choline chloride by immobilizing choline oxidase on a cellulose acetate membrane are described. The procedure of choline determination involved the electrochemical detection of enzymatically generated hydrogen peroxide. This microsensor has a linear response between 0.038-0.1 mM choline chloride. The response time is 1-2 minutes. The optimal working conditions for the microsensor were 0.1 M TRIS-HCl, pH = 8 at 30°C.

Introduction

Choline is one of the most important metabolites in the nervous influx transmission. The knowledge of choline concentration in the human body is important in clinical analysis and diagnosis, and its *in vivo* measurement prompts considerable interest.

Since the first enzyme electrode was constructed by Updike and Hicks [1], a number of sensors have been developed based on the principle proposed by Clark and Lyons [2].

Different electrochemical transducers like potentiometric pH glass electrode [4], amperometric with O_2 [5,6] or H_2O_2 [7-10, 11] detection are used for the determination of choline.

The immobilization of enzyme is an important step in the construction of a biosensor. A wide range of techniques: physical adsorption [6], entrapment in a gel matrix [7], covalently bonding [5,8,12,13] or cross-linking with an inert protein such as albumin treated with glutaraldehyde (GA) [10,13,14] or in a polymer matrix [7], have been used for the immobilization of choline oxidase.

Recently, many research teams have begun to use a redox mediator in the chemical modification of the electrode surface. The coupling between a transducer and choline oxidase was exploited for the determination of choline-containing phospholipids in serum [5,6], bile [6], and amniotic fluids [6,8], and of choline concentration in many pharmaceutical preparations commercially available (injections and syrups) [16]. Furthermore, the coimmobilization of choline oxidase with some cholinesterase may be successfully used to measure pesticides (organophosphorus or carbamate) present in water samples [17].

The amperometric choline oxidase based sensor used in this paper consists of a teflon isolated Pt-Ir (10% Ir) wire on which a cellulose acetate membrane is deposited which acts as a support for the immobilized choline oxidase. The sensor produces a measurable current related to choline concentration.

Choline oxidase is a monomer with a molecular weight of 72000 Da and contains a flavinadenine dinucleotide group as active site [18]. It can catalyze the oxidation of choline in two steps: the first one to betaine aldehyde and the second to betaine [9].

Keywords: Amperometric biosensor; Choline oxidase; Enzyme microelectrode

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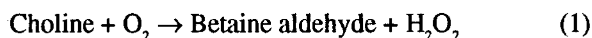
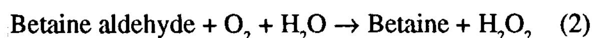


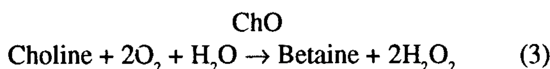
$$\text{Choline} + O_2 \rightarrow \text{Betaine aldehyde} + H_2O_2 \quad (1)$$



$$\text{Betaine aldehyde} + O_2 + H_2O \rightarrow \text{Betaine} + H_2O_2 \quad (2)$$

The first step is probably faster than the second, and both occur with simultaneous hydrogen peroxide production.

The total enzymatic reaction (3) is as follows:



$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{ChO}} \text{Betaine} + 2H_2O_2 \quad (3)$$

The enzymatically generated hydrogen peroxide is detected by anodic oxidation on a platinum anode at +650 mV vs. Ag/AgCl reference electrode according to reaction (4):

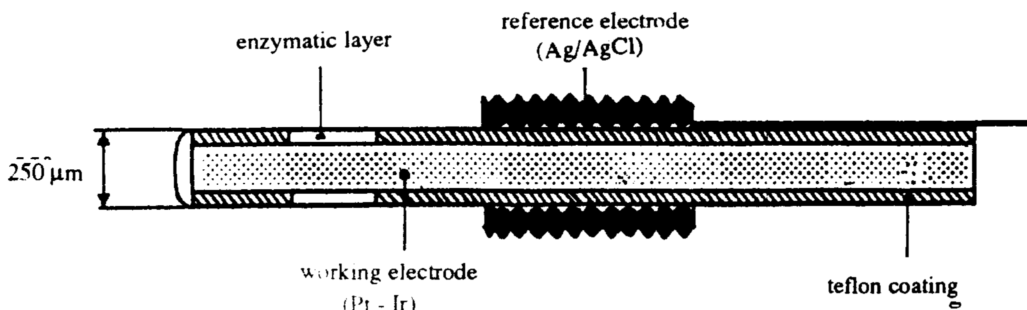
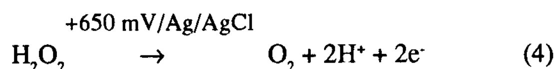


Figure 1. Schematic diagram of the choline oxidase based microsensor



$$H_2O_2 \xrightarrow{+650\ \text{mV/Ag/AgCl}} O_2 + 2H^+ + 2e^- \quad (4)$$

The proposed miniature needle design of the choline oxidase based microsensor is used for the amperometric determination of small volumes of choline. The sensing part of the biosensor consists of a multilayer structure including a cellulose acetate (CA) inner layer and a polyurethane (PU) outer layer. The permselective nature of CA membrane is well-known and has been utilized previously to selectively remove small interfering species (i.e ascorbic acid, uric acid, L-cysteine) from the electrode surface [20]. The inner membrane is also a support for the choline oxidase which was immobilized by cross-linking with bovine serum albumin and glutaraldehyde. The use of a polyurethane outer layer [20, 21] serves to protect the activity of the enzyme layer, to screen out macromolecules such as proteins, when the biosensor was used in *in vivo* experiments, and also to control the diffusion of choline in order to increase the upper limit of linear range. The exposure of the microsensor to human samples or waste water leads to a change in the sensitivity of the sensor [22]. Therefore, the use of an external membrane which presents a good permeability to oxygen, such as PU, becomes necessary [20]. This paper presents the sensor's design and the *in vitro* evaluation of the optimal working conditions of these sensors.

Experimental Section

Enzyme and Chemicals

Choline oxidase (ChO) (E.C. 1.1.3.17, from the *Alcaligenes* species) from Sigma Chemical Co., was used without any treatment. Choline chloride (ChCl), also from Sigma Chemical Co., was used as the substrate. The 0.1 M choline chloride solution was freshly prepared immediately before use by dissolving appropriate amounts of salts in bidistilled water. Chemicals used in the different immobilization procedures were glutaraldehyde (GA), (25%, aqueous solution: grade I), bovine serum albumin (BSA), from Sigma Chemical Co., and acetone, ethanol, cellulose acetate, tetrahydrofuran and dimethylformamide from Prolabo. Polyurethane was obtained from ENKA. All chemicals used were of analytical grade.

The buffer solution was prepared from Trizma base (tris[hydroxymethyl]amino-methane) (TRIS) supplied by Sigma Chemical Co., at a concentration of 0.1 M and pH=8 adjusted with HCl (TRIS-HCl). This buffer was used for the preparation of the enzyme solution and substrate and all experiments were carried out in this pH buffered media. All solutions were prepared with water obtained from a Millipore Q water purification system (Millipore, France).

Electrode Construction

The schematic diagram of the choline oxidase based microsensor is shown in Figure 1. The working electrode is a Pt-Ir (10% Ir) wire coated with a teflon film of 0.250 mm outer diameter.

The reference electrode is an Ag/AgCl wire of 0.125 mm diameter which forms a spiral around the working electrode. The overall diameter of the microsensor is ca. 0.5 mm. The sensing element of 1.5 mm in length is

located 3 mm from the tip of the microsensor. It consists of three superimposed membranes. The inner membrane is a cellulose acetate in acetone:ethanol 1:1 mixture. The enzyme layer is a mixture of choline oxidase (0.15 U), 25% glutaraldehyde and 4% bovine serum albumin in buffer solution. The enzyme was immobilized by cross-linking with GA and BSA on the inner cellulose acetate membrane. The outer membrane was obtained by dipping the biosensor in a 4% (w/v) polyurethane solution of tetrahydrofuran:dimethylformamide 98:2 [20].

With multiple repeated dip-coats, extended linearity was obtained more readily with the signal size correspondingly being reduced with the build-up of a less

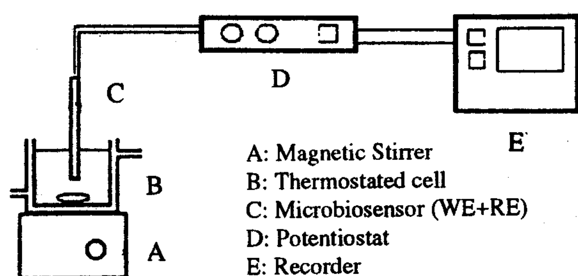


Figure 2. Schematic installation used for choline determination

permeable barrier [21]. Thus, the thickness of the outer membrane is an important factor greatly influencing the linearity of the sensor and increasing the upper value of its linear range at higher concentrations.

The microsensor, thus prepared, was stored in 0.1 M TRIS-HCl (pH= 8) at 20°C when not in use.

Apparatus and Procedures

The constant potential difference of +650 mV/Ag/AgCl between the working electrode (WE) and the reference electrode (RE) was supplied by a potentiostat (Tacussel, mod. PRG5). Working in two electrode mode, the experiments were performed in a thermostated cell filled with 0.1 M TRIS-HCl buffer solution. The temperature was controlled and kept at 30°C by a thermostat (Haake, mod. FE2) with ±0.1°C precision. The stirring of the solution was performed with a magnetic stirrer. The amperometric measurements were displayed on a strip chart recorder (Linseis, mod. L6514) (Fig. 2).

The current varied in the first 20 minutes and then reached a steady-state value. The enzymatic reaction was triggered by the addition of 36 µl aliquots of standard solution of choline chloride at suitable concentrations i.e. 0.038 to 0.19 mM. The current began to increase a few seconds after the addition of choline and ca. 1-2 minutes were required to restore a steady-state current. The oxidation of choline, which took place in the enzyme layer, causes an increase in H_2O_2 concentration in the vicinity of the working electrode and thus an increase in the anodic current.

Results and Discussion

The results obtained with six sensors (symbolized E1 to E6) are presented from a dozen which were measured in order to determine their sensitivity and the stability, as well as the influence, of pH and temperature on their response to choline.

Calibration of the Biosensor

A calibration curve was obtained after several additions of freshly prepared choline chloride standard solution. The final choline chloride concentration was in the 0.038-0.19 mM range (Fig. 3).

A linear relationship was observed between the current increase (the difference between the steady-state current during the assay I, and the residual current, I_0) and the concentration of choline chloride in the range of 0.038-0.1 mM. The background currents were low (2-4.6 nA).

Effect of pH

Free choline oxidase was denatured at a pH lower than 6.5 or higher than 9 [7]. The effect of pH on the response of choline oxidase immobilized on a microsensor was

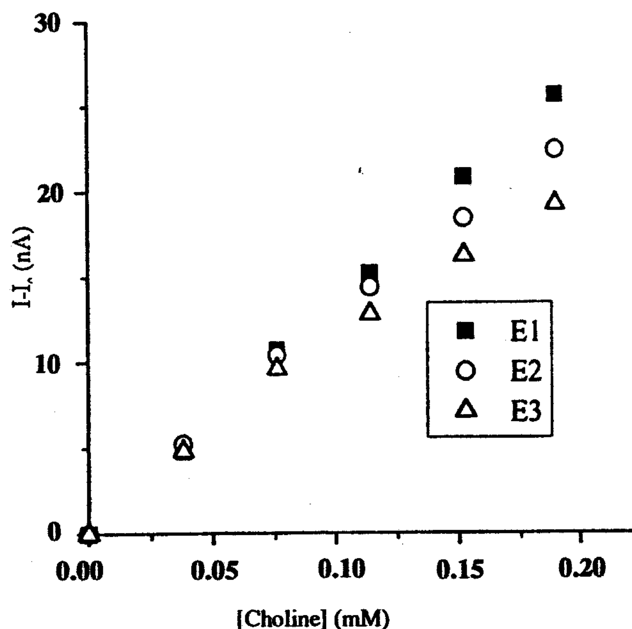


Figure 3. Calibration curve for choline oxidase based microsensor (E1-E3). Experimental conditions: applied potential +650 mV vs. Ag/AgCl, 0.1 M TRIS-HCl buffer solution, pH= 8, temperature 30°C, continuous stirring

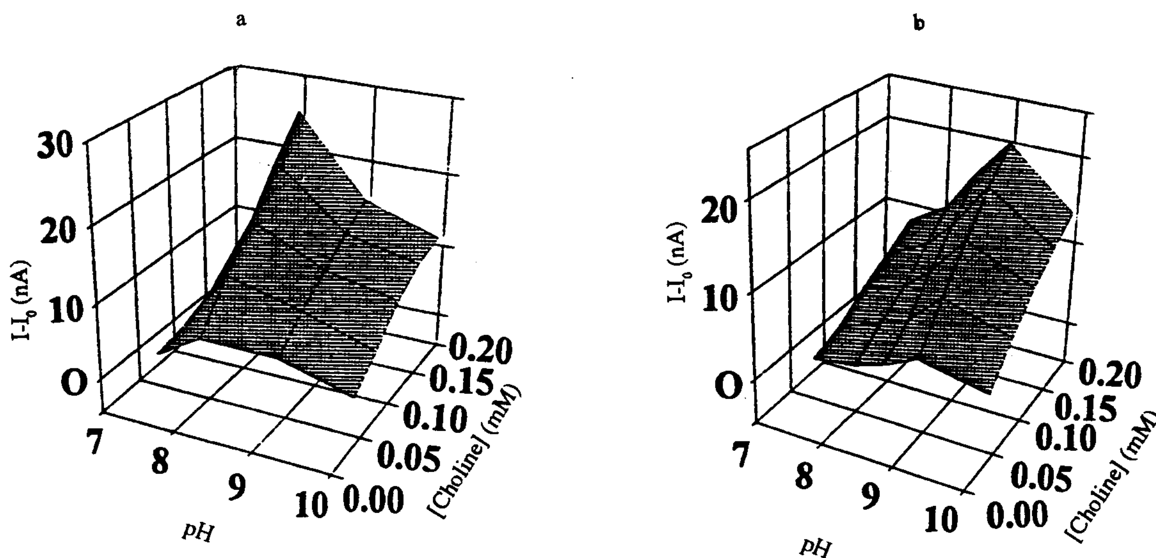


Figure 4. Effect of pH on the response of the choline oxidase based microsensor a) E1, b) E6. Experimental conditions as in Figure 3

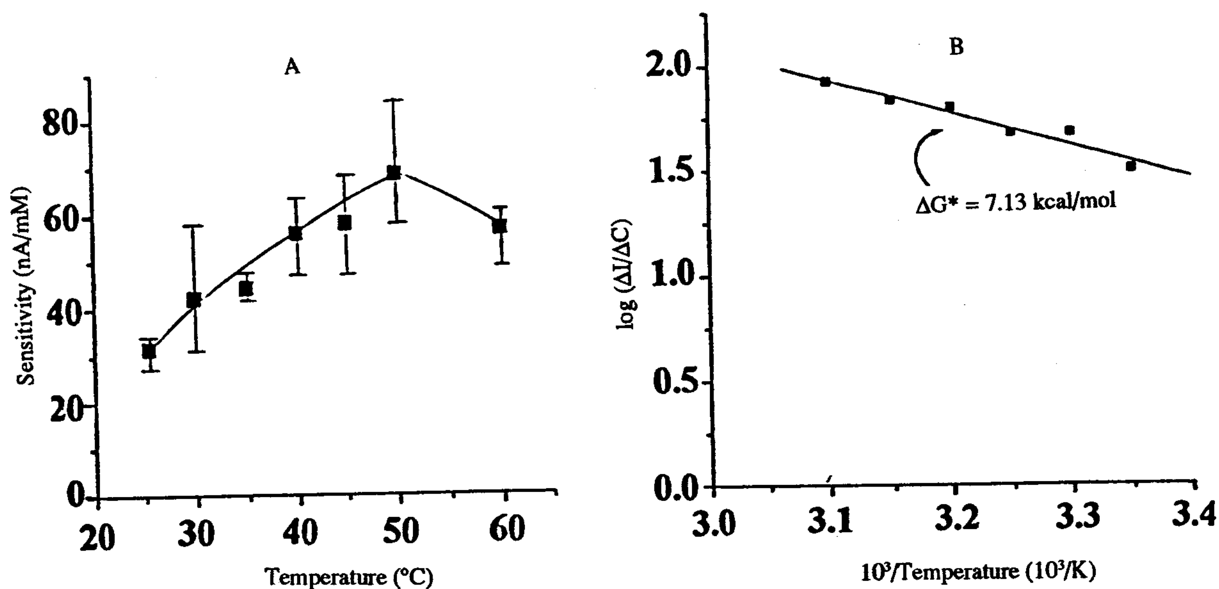


Figure 5. A. Effect of temperature on the response of choline oxidase based microsensor (E4). B. Arrhenius plot for same electrode. Experimental conditions: [choline chloride]= 0.038 mM, other conditions as in Figure 3

studied in 0.1 M TRIS-HCl buffer at 30°C, in the 7.4-10 range, by successive addition of 0.038 mM final concentration of choline chloride standard solution.

The pH buffer solution was found to have a profound effect on the response of the sensor with a maximum intensity at pH = 8-9 (Fig. 4). A shift in the optimum pH and the broadening of the pH range of an enzyme after immobilization are quite common phenomena [7,11,13,14] and may be accounted for by the change in the local concentration of hydrogen ions on the electrode surface or by the change in the structure of immobilized enzymes.

Effect of Temperature

The temperature dependence of the response of the choline oxidase based microsensor was studied in the 20-60°C range in 0.1 M TRIS-HCl buffer solution (pH = 8). The sensitivity for a 0.038 mM concentration of choline chloride gradually increased till 50°C, then decreased. The means values of 5 tests are presented in Figure 5 A.

The increase in the sensitivity of the sensor with an increase in temperature is due to the increase in the enzymatic reaction rate, substrate diffusion towards electrode and the acceleration of the electrochemical process.

The Arrhenius plot of $\log(\Delta I/\Delta c)$ vs. $1/T$ (Fig. 5 B) from equation (5):

$$\log [\Delta I/\Delta c] = \log a - \Delta G^*/2.303\, RT \qquad (5)$$

where: $\Delta I/\Delta c$ = sensitivity of sensor
a = reaction constant
ΔG^* = standard total enthalpy (cal/mol)
R = gas constant (1.98 cal/K)
T = absolute temperature (K)

A value of 7.13 kcal/mol is given for ΔG^* which coincides with the theoretical value of enzymatic reactions [23, 24].

Long-term Stability of the Choline Microsensor

The long-term stability of the immobilized enzyme on the sensor was examined. In a typical experiment, the storage stability (ability of the enzyme to retain its activity under specified storage conditions) was tested over a period of 20 days by intermittent use under the optimum conditions defined above.

As shown in Figure 6, the intensity of the choline oxidase based microsensor response decreased significantly, and 20 days after the sensors' preparation, approximately 30-40% of the response is lost. The decay of the microsensor response can be attributed mainly to the deactivation of the enzyme. Because of the inherently low specific activity of ChO and its sensitivity to its environment, it is possible that the enzyme system needs to be regenerated frequently.

Conclusion

An amperometric microbiosensor for the determination of choline chloride using the cross-linking of choline oxidase with BSA and GA on a cellulose acetate membrane and the detection of enzymatically generated H_2O_2 were realized. The dimensions of the microsensor and the technique of immobilization of enzyme are chosen so that *in vivo* experiments may be imagined.

It must be emphasized that the intrinsic kinetic parameters of an immobilized enzyme are not necessarily the same as those of the free enzyme in solution, because of conformational changes, steric effects and diffusion constraints. The optimal working conditions for the microbiosensor were 0.1 M TRIS-HCl, pH= 8 at 30°C.

In this respect, the microsensor presented in this paper for the determination of choline, as regards its performance (high sensitivity and very easy preparation of the biosensing element) appears to be a very promising tool for future environmental purposes.

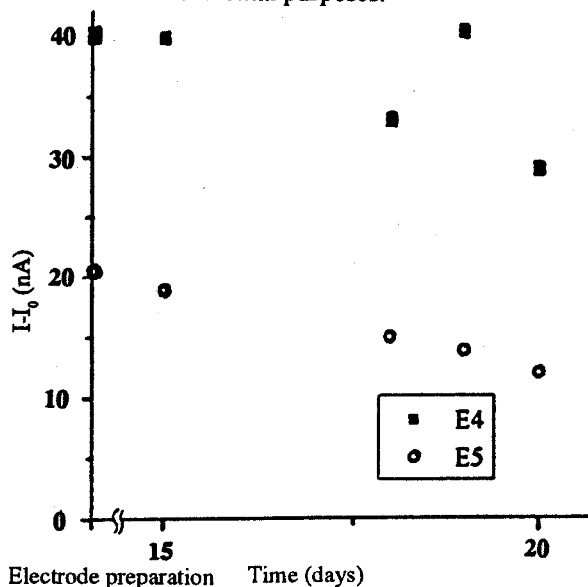


Figure 6. Storage stability curves for two (E4, E5) different sensors. Storage conditions between each calibration: 0.1 M TRIS-HCl buffer, pH = 8, temperature 20°C; experimental conditions: choline chloride final concentration 1 mM, other conditions as in Figure 3

Acknowledgements

Graziella Turdean is very grateful to the French Government for its financial support of this work at Université Paris XII, and to all those who made this paper possible.

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