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Electrosynthesized poly(pyrrole)/poly(2-naphthol) bilayer membrane as an effective anti-interference layer for simultaneous determination of acethylcholine and choline by a dual electrode amperometric biosensor

A. Guerrieri [b,*], V. Lattanzio [a], F. Palmisano [a], P.G. Zambonin [a]

[a] *Dipartimento di Chimica, Università degli Studi di Bari, Via Orabona, 4-70126 Bari, Italy*
[b] *Dipartimento di Chimica, Università degli Studi della Basilicata, Via N. Sauro, 85-85100 Potenza, Italy*

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Abstract

Several neural diseases appear related to the neurotransmitter acethylcholine (ACh) and its metabolite choline (Ch) brain levels so that their simultaneous determination is essential. A cross-talk and interference free dual electrode amperometric biosensor for the simultaneous determination of both analytes has been developed. Acetylcholinesterase (AChE) and choline oxidase (ChO) were immobilized by glutaraldehyde co-crosslinking with bovine serum albumin. A very efficient rejection of electroactive interferents has been achieved by a novel electrosynthesized polymeric bilayer membrane composed by overoxidised poly(pyrrole) and poly(2-naphthol) films. Sensitivities towards several electroactive interferents ranged from ca. 0.04% (e.g. ascorbate) to ca. 0.3% (e.g. dopamine) of those relevant to ACh and Ch (11 and 15 μA/μM, respectively). Detection limits (at S/N = 3) in flow injection analysis were ca. 100 nM for both ACh and Ch at the ChO–AChE electrode and ca. 40 nM for Ch at the ChO sensor. Biosensor performances appear more than adequate for brain tissue homogenates and cerebrospinal fluids analysis where average levels in the low micromolar range are typically found.

Keywords: Biosensor; Enzyme immobilization; Non-conducting polymers; Dual electrode sensor; Simultaneous determination; Acetylcholine analysis

1. Introduction

Acetylcholine (ACh), synthesized in nerve cells from choline (Ch) and acetyl coenzyme-A by choline acetyltransferase, plays an important role in the cholinergic synaptic neurotransmission. ACh and its metabolite Ch have received increased attention in the past decades since their brain levels seem related to various neural disorders such as Alzheimer's disease, progressive dementia (Bowen et al., 1976; Davies and Maloney, 1976; Davis and Berger, 1979; Wester et al., 1988) and schizophrenia (Meltzer and McGurk, 1999; Tandon, 1999).

The investigation of cholinergic transmitter system has been seriously hampered over the years by the lack of simple and reliable analytical methods for ACh and Ch determination. Both analytes, in fact, do not possess electroactive or chromophore groups so that well established methods typically used for other neurotransmitters (e.g. catecholamines) are useless and a conversion into more easily detectable compounds is usually required. In this respect, the most successful approach for ACh and Ch determination is based on liquid chromatography (LC) coupled to post-column enzymatic conversion using acetylcholinesterase (AChE) and choline oxidase (ChO) dissolved in a derivatizing solution (Potter et al., 1983) or immobilized on suitable enzyme reactors (IMERs) (Eva et al., 1984; Yao and Sato, 1985; Kaneda et al., 1986; Damsma et al., 1987; Tyrefors and Gillberg, 1987; Van Zoonen et al., 1987; Teelken et al., 1990; Gunaratna and Wilson, 1990; Flentge et al., 1992). In this approach, hydrogen peroxide, produced according to the following scheme:

$$\mathrm{ACh} + \mathrm{H_2O} \xrightarrow{\mathrm{AChE}} \mathrm{Ch} + \text{acetic acid} \quad (1)$$

$$\mathrm{Ch} + 2\mathrm{O_2} + \mathrm{H_2O} \xrightarrow{\mathrm{ChO}} \text{betaine} + 2\mathrm{H_2O_2} \quad (2)$$

can be revealed by chemiluminescence or, more efficiently, by electrochemical detection (ED) at a platinum electrode. Indeed,

* Corresponding author. Tel.: +39 0971 202260; fax: +39 0971 202223.
E-mail address: guerrieri@unibas.it (A. Guerrieri).

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the LC–IMER–ED method, which couples the separating power of high performance liquid chromatography with the specificity of enzymatic reactions and the sensitivity of electrochemical detection, permits a sensitive detection of both analytes in complex matrices such as brain tissues (Potter et al., 1983; Eva et al., 1984; Gunaratna and Wilson, 1990; Flentge et al., 1992), microdialysates (Damsma et al., 1987; Tyrefors and Gillberg, 1987; Flentge et al., 1992) and cerebrospinal fluids (Teelken et al., 1990; Flentge et al., 1992). Unfortunately, such an approach could suffer from some drawbacks (Marko-Varga and Gorton, 1990; Tyrefors and Carlsson, 1990) typical of IMERs, such as loss of chromatographic resolution and peak tailing or splitting.

Several amperometric biosensors, based on ChO and AChE immobilized by a variety of techniques have been described (for a review, see Larsson et al., 1998). A sensitive and fast-responding amperometric biosensor for ACh and Ch based on ChO and AChE immobilized on a platinum electrode by glutaraldehyde (GLU) co-crosslinking with bovine serum albumin (BSA) has been developed in our laboratory (Guerrieri et al., 1995). Since this simple immobilization procedure works well also on the working electrode of a thin-layer flow cell, the use of an amperometric ACh/Ch biosensor as a LC detector has been recently proposed (Guerrieri and Palmisano, 2001). In this approach, the enzymatic conversion of analytes, the production of hydrogen peroxide and its electrochemical detection are confined in the same physical device and the IMER is no more required, thus simplifying the experimental set-up. Detection limits in the order of 10–20 fmol injected have been demonstrated as well as the determination of ACh and Ch in rat brain homogenates.

The ultimate goal of the above described approach (Guerrieri and Palmisano, 2001) is the elimination of the LC separation step; this would make feasible the simultaneous monitoring of ACh and Ch, for example, through well established approaches (Rhemrev-Boom et al., 2001) that couple biosensors and microdialysis sampling. However, due to the particular detection scheme involved (see Eqs. (1) and (2)), where ACh needs to be firstly converted into Ch in order to be detected, a biosensor capable of performing (in the same physical device) a simultaneous determination of ACh and Ch is required. This could be achieved by a dual electrode (modified by ChO–AChE and ChO, respectively) amperometric biosensor provided that the ChO–AChE modified electrode gives additivity of ACh and Ch responses. Furthermore, since both sensors detect hydrogen peroxide, cross-talk effects should be absent and interferences from electroactive sample components (typically ascorbate, dopamine, 5-HT, DOPAC) should be efficiently removed.

The interference problem has been already dealt with by several approaches. A mixed linear sweep/pulsed detection technique at a carbon fibre ChO–AChE biosensor have been reported (Tamiya et al., 1991) to minimize ascorbate and albumin interference but, unfortunately, a significant enzyme desorption occurred due to the pulse application. Xin and Wightman (1997) used an enzyme-modified conducting organic salt electrode: the low operating potential (+100 mV versus SCE) and the presence of an outer Nafion membrane revealed successful in reducing the interference level. Alternatively, the working potential for hydrogen peroxide detection has been lowered using a carbon electrode modified by an osmium redox gel containing horseradish peroxidase (Garguilo and Michael, 1994); to further reduce ascorbate interference, a Nafion overlayer was used in this approach, eventually coupled to ascorbate oxidase (Garguilo and Michael, 1995) incorporated into the sensing membrane.

Electropolymerized non-conducting films with built-in permselectivity (Malitesta et al., 1990; Palmisano et al., 1995a,b, 2000; Guerrieri et al., 1998; Ciriello et al., 2000) have largely demonstrated to be able to remove, with unmatched efficiency, common interfering species in glucose or lactate determination, such as ascorbate, urate, paracetamol and cysteine. However, in the present case the problem is further complicated by the presence of a larger number of electroactive interferents (e.g. several neurotransmitters) and by the low concentration levels of the analytes to be quantified. One possibility to further improve the selectivity of the sensor could be based on electrosynthesized multilayered structures (Palmisano et al., 1995a) able to widen the spectrum of rejected species thus improving the overall anti-interfering characteristics of the membrane.

In the present paper, it will be demonstrated that the simultaneous determination of ACh and Ch can be achieved by a cross-talk free, dual electrode amperometric biosensor based on ChO and ChO–AChE enzymes immobilized onto platinum electrodes by GLU co-crosslinking with BSA. Electrode modification by a novel electrosynthesized permselective bilayer, composed by overoxidized polypyrrole (PPYox) and poly(2-naphthol) (P2NAP) (Ciriello et al., 2000), confers remarkable anti-interference properties to the biosensor, which appeared promising for brain tissue homogenates and cerebrospinal fluids analysis.

2. Experimental

2.1. Materials

Choline chloride, acetylcholine chloride, choline oxidase (EC 1.1.3.17 from *Alcaligenes* species, 14 U/mg of solid), cholinesterase acetyl (EC 3.1.1.7, type VI-S, from electric eel, 480 U/mg of solid), bovine albumin (fraction V), glutaraldheyde (grade II, 25% aqueous solution), dopamine (DA), homovanillic acid (HVA), 3,4-dihydroxybenzylamine (DHBA), 5-hydroxyindoleacetic acid (5-HIAA), 5-hydroxytryptamine (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), N_{ω}-methyl-5-hydroxytriptamine (*n*MET), 4-hydroxy-3-methoxyphenylglicol (MHPG), epinephrine (E), norepinephrine (NE), L-3,4-dihydroxyphenylalanine (L-DOPA), 3-methoxy-D,L-tyrosine (3-met-DOPA), ascorbic acid (AA) and L-cysteine (CYS) were obtained from Sigma (Sigma, St. Louis, MO, USA) and used without further purification. Choline chloride was dried under vacuum over P_2O_5 for at least 3 days and stored in a vacuum desiccator. Pyrrole and 2-naphthol (both Aldrich) were purified by vacuum distillation (62 °C) and vacuum sublimation (80 °C), respectively. 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