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Short communication

Development of an acetylcholinesterase immobilized flow through amperometric detector based on thiocholine detection at a silver electrode

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ABSTRACT

This paper describes the use of a commercially available thin-layer flow through a detector with the sensing block customized in an original design for acetylcholinesterase (AChE) immobilization and suitable for inhibition studies by flow injection analysis (FI). AChE was chemically linked onto a gold disk substrate adjacent to a silver disk electrode. The downstream positioned silver electrode, poised at 0.08 V vs. Ag/AgCl, KCl 3 M, permitted the sensitive amperometric detection of liberated thiocholine (TCh) using acetylthiocholine (ATCh) as enzyme substrate. A typical Michaelis–Menten curve was obtained for ATCh within the concentration range 1×10^{-5}–1×10^{-2} M with a Km_{app} of 5.93×10^{-4} M. ATCh quantification was achieved with a limit of detection (LOD) of 5.3×10^{-6} M. The utility of the developed FI setup was demonstrated for AChE inhibition studies using neostigmine as model compound. The IC_{50} for neostigmine was obtained to be 1.45×10^{-7} M.

1. Introduction

The development of instrumental tools for the screening of molecules capable of inhibiting the enzyme AChE is a topic of substantial interest in the environmental, food, agricultural and biomedical domains [1]. Screening methods for identifying selective AChE inhibitors are of interest in the medical field [2–6] since the neurotransmitter acetylcholine depletion by AChE has been demonstrated to be involved in the development of Alzheimer and other neurodegenerative diseases [7].

The immobilization of AChE has been reported to be an attractive approach in biosensor and in microreactor configurations since this allowed multiple assays to be realized with eventual need for enzyme reactivation in the presence of irreversible inhibitors. Optical and electrochemical detection modes are generally employed for the detection of the product of the enzymatic reaction. Acetylthiocholine is a substrate of choice since the liberated product thiocholine can be derivatized with Ellman's reagent for optical detection and because it is readily detected by amperometric oxidation e.g. at a platinum electrode at +410 mV or at low potential at a screen printed electrode modified by an appropriate redox mediator [8–10]. Several recent review articles have clearly detailed numerous biosensing methodologies, their pros and cons, for the design and for the detection of AChE inhibitors [1,11–14]. When dealing with the search for new molecules capable of AChE inhibition, the implementation of analytical tools allowing high throughput analyses and low analyte consumption should be considered as a priority criteria. Flow systems exploiting the versatile concept of flow injection have appeared to be appropriate for such assays [15,16]. The enzyme AChE can be immobilized in-line on controlled pore glass [17] or onto the inner wall of a fused silica capillary [18].

The present work aimed to contribute to the development of an effective microfluidic tool for the monitoring of AChE activity and its inhibition. The novelty is the development and application of a customized flow through detector comprising a gold disk surface for enzyme immobilization adjacent to a silver disk electrode in a thin-layer flow cell for thiocholine detection. The amperometric detection of some aminothiols at a silver electrode poised at a potential close to 0.0 V vs. Ag/AgCl has been recently reported by our group [19] and has been exploited in the present application for the detection of thiocholine. It permitted the selective determination of thiocholine within the concentration range of 1×10^{-5}–1×10^{-2} M under the studied experimental conditions. It is worth mentioning that recently a cholinesterase inhibitor assay was developed based on the surface enhanced Raman scattering at silver colloid nanoparticles by the enzyme released thiocholine [20]. It was also reported that cathodic stripping of thiocholine adsorbed on a silver disk electrode permitted to study AChE activity in solution under batch conditions [21].

2. Materials and methods

2.1. Reagents

Acetylthiocholine chloride (ATCh), AChE (Type C3389 500 U mg^{-1} from electric eel) and pyridine-2-aldoxime methyliodide (2-PAM)

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and glutaraldehyde 25% solution were from Sigma-Aldrich and used as received. Neostigmine (3-(dimethylcarbamoyloxy)N,N,N-trimethylbenzenaminium) was from Merck, L-cysteine and cysteamine hydrochloride were from Fluka. Phosphate salts and KCl for buffer solution preparation (PBS) and other reagents used were of analytical grade. All solutions were prepared with Milli-Q quality water.

2.2. Instruments

Amperometric measurements were performed using a Bioanalytical potentiostat LC-4C (BASi, USA) with a flow through thin-layer cell (BASinc) comprising of Ag/AgCl, KCl 3 M reference electrode inserted into a stainless steel block auxiliary electrode. The opposite block was customized for housing a gold disk located upstream of a silver disk electrode (Fig. 1). The gold disk served only for AChE immobilization. The flow injection set-up consisted of a HPLC pump PM-92E (BASi), a Rheodyne injection valve (20 μL injection loop), and 150 cm PEEK tubing (250 μm ID) to the flow-through detector. Signal was monitored using a PowerChrom 280 recorder (eDAQ-Europe). All experiments were realized at room temperature 24 ± 2 °C. The data were fitted to the Michaelis–Menten equation by maximum likelihood method for curve fitting in the MATLAB software version 7.5.0.342.

2.3. AChE immobilization

The sensing block with the gold (3 mm) and silver (3 mm) surfaces was smoothed on a water wetted polishing cloth in the presence of alumina powder (0.05 μm), rinsed with Milli-Q water and dried at room temperature. The silver disk was covered by a parafilm tape then 20 μL of 1 M cysteamine hydrochloride was spiked onto the gold disk and left for 2 h at room temperature for the formation of a self assembled monolayer. It was subsequently rinsed with Milli-Q water to remove unbounded cysteamine molecules. The surface was allowed to dry at room temperature and then spiked with (i) 5 μL of 1% glutaraldehyde solution that was freshly prepared in PBS (0.01 M phosphate buffer and 0.01 M KCl at pH 7.0) and (ii) 5 μL of AChE (500 U/mL) in 0.01 M phosphate buffer. Care must be taken to avoid spreading of enzyme solution out of the gold disk area. It was left to dry at room temperature for 3 h. Finally, the gold surface was rinsed with Milli-Q water and dried at room temperature.

2.4. Measurement procedure

The flow injection (FI) system operated at a flow rate of 500 μL/min using PBS (0.01 M phosphate buffer and KCl 0.01 M at pH 7.0) for 15 min in order to remove unbound bio-component at the detector and to stabilize the system. Subsequent experiments were performed at a flow rate of 200 μL/min. ATCh and neostigmine standard solutions were manually injected into the system. TCh was generated upon enzymatic hydrolysis at the upstream gold surface close to the silver electrode. The amperometric response (peak height) was based on the detection of TCh at the silver disk electrode poised at a potential of 0.08 V. Inhibition measurement consisted of performing three injections of 1×10^{-3} M substrate (TCh) followed by five injections of inhibitor (in order to ensure a sufficiently long incubation time of enzyme and neostigmine) and three injections of 1×10^{-3} M substrate once more (Fig. 2). From the peak width at the base, the contact time between the injected plug of inhibitor and AChE for each injection was estimated to be 50 s, repeated five times (Fig. 2 part b). The inhibition percentage was calculated as

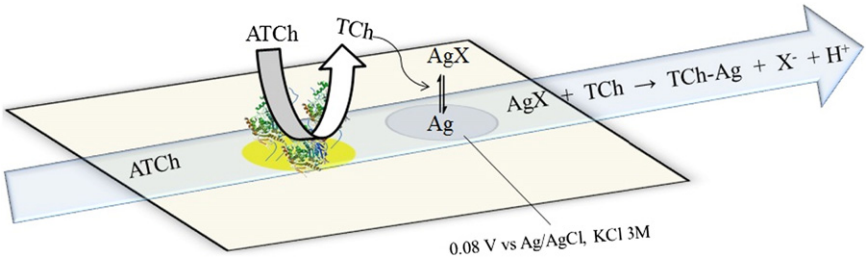

Fig. 1. Schematic view of the flow-through sensing block with immobilized enzyme on gold substrate and TCh detection at silver electrode. ATCh: acetylthiocholine, TCh: thiocholine, AgX: (AgCl, Ag(OH),…).

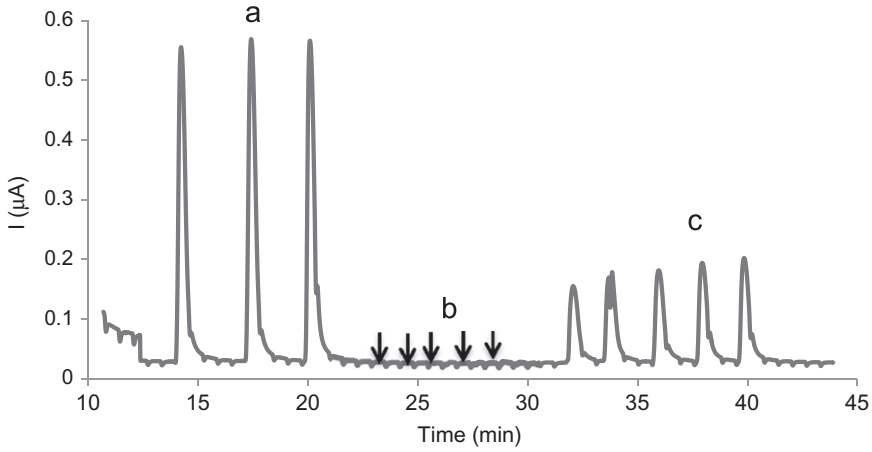

Fig. 2. Typical inhibition experiment: (a) three injections of 1×10^{-3} M ATCh, (b) five injections of 10^{-6} M neostigmine and (c) five injections of 1×10^{-3} M ATCh. 0.01 M PBS, pH 7.0, flow rate 200 μL/min, E_{app} 0.08 V.