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Short communication

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

Cobra Parsajoo, Jean-Michel Kauffmann *

Université Libre de Bruxelles, Faculty of Pharmacy, CP205/6, Brussels 1050, Belgium

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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)

* Corresponding author.
E-mail address: jmkauf@ulb.ac.be (J.-M. Kauffmann).

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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

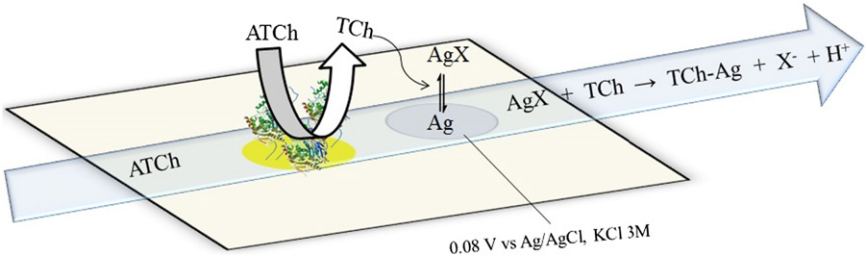

Fig. 1. Schematic view of the flow-through sensing block with immobilized enzyme on gold substrate and TCh detection at silver electrode. ATCh: acetylthiocholine, TCh: thiocholine, AgX: (AgCl, Ag(OH),…).

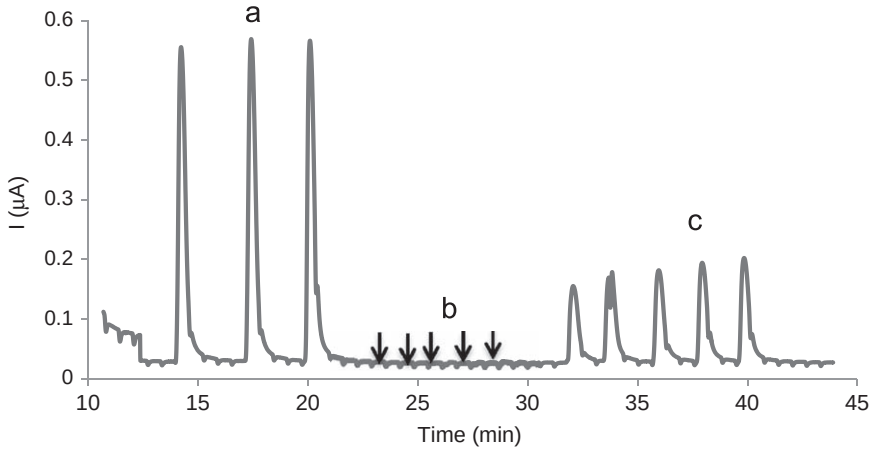

Fig. 2. Typical inhibition experiment: (a) three injections of 1×10^{-3} M ATCh, (b) five injections of 10^{-6} M neostigmine and (c) five injections of 1×10^{-3} M ATCh. 0.01 M PBS, pH 7.0, flow rate 200 μL/min, E_{app} 0.08 V.

follows:

$$I\% = \frac{I_0 - I_i}{I_0} \times 100$$

I_0 is the amperometric response of TCh before the inhibition of AChE and I_i is the amperometric response of TCh after injection of neostigmine. After completion of each inhibition study a flow of 5×10^{-4} M 2-PAM in PBS was maintained for 15 min in order to reactivate the enzyme.

Thiocholine generation in solution: the hydrolysis of ATCh by AChE to give TCh was performed in the solution phase by using an enzyme stock solution (500 U/mL in the 0.01 M PBS, pH 7). A volume of 5 μL of AChE stock solution was spiked in 5 mL of ATCh solution in the PBS 0.01 M, pH 7. Different concentrations of ATCh were investigated. The mixtures were shaken for 2 h at room temperature in order to ensure maximum reaction completion between enzyme and substrate. The produced TCh was determined using the FI set-up (without immobilized AChE). A calibration curve for TCh was obtained by injection of different concentrations of the mixture of ATCh (in the range 1×10^{-6}–5×10^{-3}) and AChE (0.5 U/ml).

3. Results and discussion

3.1. Detection mechanism

At the low applied potential, a constant background current was observed at the silver electrode. In the phosphate buffer solution, in the absence of chloride, an equilibrium existed between metallic silver and silver species namely Ag(OH) and likely some silver ions may be stripped off by phosphate anions. By addition of chloride ions to the flowing media, the equilibrium was modified due to the reactivity of silver with chloride ions [22] with the formation of a thin layer of silver halide. After a few minutes, a stable equilibrium was reached yet the background current had raised to a higher level as compared to the conditions without chloride ions. In the buffer solution containing a high concentration of chloride ions (0.01 M) it was supposed that AgCl was the dominant species and the equilibrium was essentially established between Ag and AgCl (Fig. 1). Assuming a uniform AgCl deposit, the amperometric detection corresponded to the reaction between AgCl and thiocholine (TCh) that was formed upon enzymatic reaction at the upstream gold surface. With the equilibrium between silver electrode and silver chloride species being shifted and because of constant applied potential, the electrode counteracted this change by generating Ag (I) and as a result an amperometric oxidation response was obtained. In the absence of immobilized AChE the injections of ATCh chloride standard solutions gave no response.

3.2. Michaelis–Menten plot

Typical calibration curves for acetylthiocholine (ATCh) were obtained by successive injections of the substrate within the concentration range 1×10^{-5} M and 1×10^{-2} M. Michaelis–Menten plot and its linearization by the Lineweaver–Burk plot was obtained with $y(A^{-1}) = 5.97 \times 10^{-4}\, X\,(M^{-1}) + 1.7628$, $R^2 = 0.9979$, slope RSD = 5.4% and intercept RSD = 3.2% $n = 3$ (Fig. 3). The LOD (3σ divided by the slope) and LOQ (10σ divided by the slope) obtained from the linear part of the ATCh averaged calibration curve ($n = 3$) were 5.3×10^{-6} M and 1.6×10^{-5} M, respectively. A concentration of 1 mM of ATCh was selected for the inhibition tests i.e., in the substrate saturation condition in order to obtain maximum inhibition efficiency [23]. The kinetic parameters Km_{app} and I_{max} were calculated as the average of three consecutive determinations: $Km_{app} = 5.93 \times 10^{-4}$ M (RSD = 3.9%, $n = 3$) and $I_{max} = 0.77$ μA (RSD = 1.4%, $n = 3$). The value for Km_{app} was lower than Km_{app} values reported in the literature indicating that the thin layer of immobilized AChE had high affinity to the substrate ATCh [24–26].

3.3. ATCh conversion efficiency at the AChE detector

The calibration curve of TCh generated in the solution phase (see Section 2.4 measurement procedure) gave a Michelis–Menten trend with a response quasi linear in the concentration range comprised between 1×10^{-6} and 5×10^{-5} M. It was concluded that ATCh was totally transformed into TCh in this concentration range. Subsequently, the TCh calibration curve, $y(\mu A) = 14740\, X\,(M) - 0.0155$, was used in order to estimate the efficiency of thiocholine production at the AChE detector under the selected experimental FI conditions. For ATCh injections in the concentration range of 1×10^{-5}–1×10^{-4} M, the enzyme conversion efficiency percentage was estimated to be in the range 10–20% depending on the concentration of injected ATCh.

3.4. Inhibitor assay

Neostigmine interacts with AChE in a similar manner as acetylcholine. Neostigmine is a quasi reversible inhibitor that

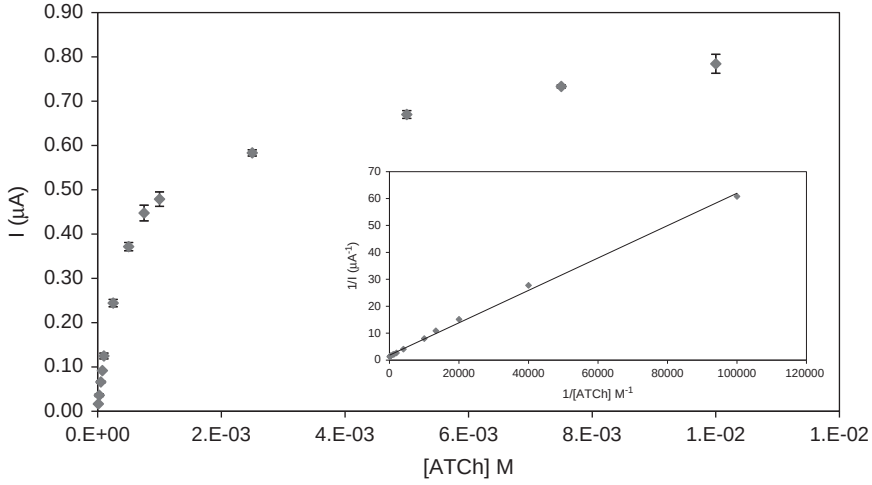

Fig. 3. Average of three calibration curves for ATCh, inset: Lineweaver–Burk plot for ATCh calibration curve. 0.01 M PBS, pH 7.0, flow rate 200 μL/min, E_{app} 0.08 V.

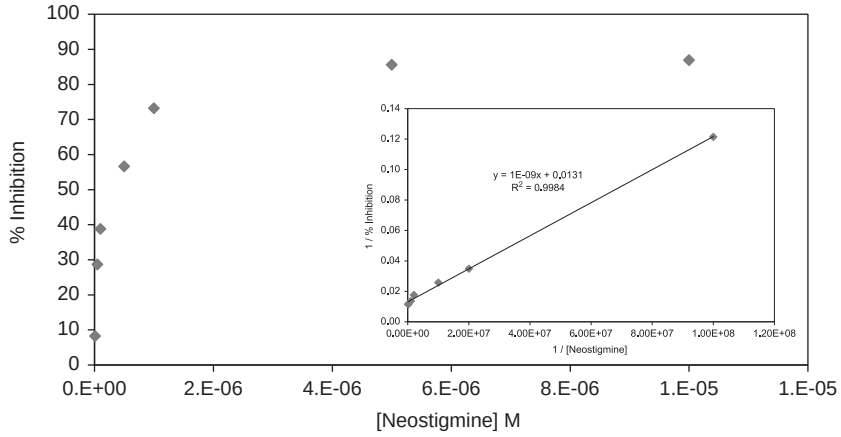

Fig. 4. Typical inhibition calibration curve for neostigmine, inset: linearized inhibition calibration curve. 0.01 M PBS, pH 7.0, flow rate 200 μL/min, E_{app} 0.08 V.

binds to both the ionic and esteric sites of AChE. The hydrolysis of the carbamylated enzyme that formed after interaction of neostigmine with AChE is, however, slower than the hydrolysis of acylated enzyme that formed after acetylcholine interaction [5,27]. Inhibition of AChE by neostigmine was monitored by measuring the decrease in the response of TCh. AChE was greatly inhibited after injection of neostigmine as observed by the decreased signal of TCh (Fig. 2). Five successive injections of 1 μM neostigmine (Fig. 2 part b) induced a diminution of the amperometric response for TCh by 73% (Fig. 2 part c) and this inhibition raised with the inhibitor concentration.

A typical inhibition calibration curve of neostigmine was obtained in the concentration range of 1×10^{-8}–1×10^{-5} M (Fig. 4). The plotting of 1/In (%) vs. 1/neostigmine concentration gave a linear trend with $R^2=0.9984$ and with an apparent IC_{50}, the concentration of inhibitor which inhibited 50% of the TCh signal, of 1.45×10^{-7} M. The low apparent IC_{50} suggested that neostigmine is a very effective inhibitor of AChE.

3.5. Enzyme reactivation

Spontaneous reactivation of AChE inhibited by neostigmine was possible in the flowing PBS since inhibition was not irreversible, but it was found to be quite slow in agreement with literature data [28]. After each inhibition study, and in order to use the FI system in an efficient manner, it was found necessary to reactivate the enzyme. This was investigated by different alternatives depending on the degree of inhibition. Complete reactivation for 80% inhibition was obtained after an overnight continuous passing of the PBS buffer at 50 μL/min. This process, however, was not useful for high throughput analyses. By increasing the flow rate to 500 μL/min incomplete reactivation for a 49% inhibition was observed after 3 h; i.e. 69% recovery was obtained. Injection of the substrate could reactivate a large part of the inhibited enzyme; 10 subsequent injections of 1×10^{-3} M ATCh at 200 μL/min for a 70% inhibition gave 63% reactivation within 40 min. In this process, substrate and buffer participated both in the displacement of inhibitor from the active site of AChE. The most common and fast investigated method for reactivation of inhibited AChE usually consisted in using oxime reagents such as pyridine-2-aldoxime methyliodide (2-PAM) and 4-formylpyridinium bromide dioxime (TMB-4) [29]. Oximes are nucleophilic compounds which can react with the carbamated center of AChE and regenerate the active enzyme. After each inhibition study, reactivation was obtained by continuously passing the solution of 2-PAM (5×10^{-4} M) at 500 μL/min for 15 min followed by 30 min in the PBS for stabilization of the response. By this method, reactivation of inhibited enzyme also depended on the degree of inhibition. For instance for 38% inhibition, the reactivation by 2-PAM gave 100% of initial response but for 73% inhibition, the reactivation gave only 85% of initial response. It was observed, especially in the lower inhibition degree, that the longer the contact time between 2-PAM and inhibited AChE, the more its activity decreased. This was probably because of interaction of 2-PAM with the active site and with peripheral sites of the enzyme. After passing the buffer or by several injections ($n=8$) of substrate, the activity was fully restored.

3.6. Repeatability

During the first injections of ATCh (1×10^{-4} M) there was a substantial increase in the electrode response (Fig. 5). After four or five injections, however, the response reached a steady state (Fig. 5). We found that this behavior was due to a surface effect at the silver electrode since sequential injections (in the 1–50 μM range) of cysteine or thiocholine (generated enzymatically) using the FI setup, but without upstream immobilized AChE, gave a similar response pattern.

After stabilization of electrode response, the RSD for six replicate injections of ATCh was 1.9% ($n=6$). Between electrode reproducibility was estimated by measuring the response ($n=6$) of 1×10^{-4} M ATCh at three different electrodes: the obtained RSD was 3.25%. For inhibition study, five inhibition tests for 1×10^{-7} M neostigmine gave a RSD equal to 4.2% ($n=5$).

3.7. Operational stability

In the selected operating conditions there are several parameters which may contribute for losing the activity of the surface immobilized enzyme such as leakage of enzyme from the surface and washing by flow of buffer. In the selected operational conditions after obtaining several calibration curves and performing inhibition tests and reactivation by using 2-PAM, the response of immobilized AChE retained 75% of the initial response for 1×10^{-3} M ATCh during 25 days.

4. Conclusions

This work showed that the implementation of a silver based detector for the amperometric detection of thiocholine permitted to monitor neostigmine an inhibitor of acetylcholinesterase under microfluidic conditions. The chemical immobilization of the enzyme upstream in the detector in close proximity to the silver electrode allows for good sensitivity to be achieved. The enzyme

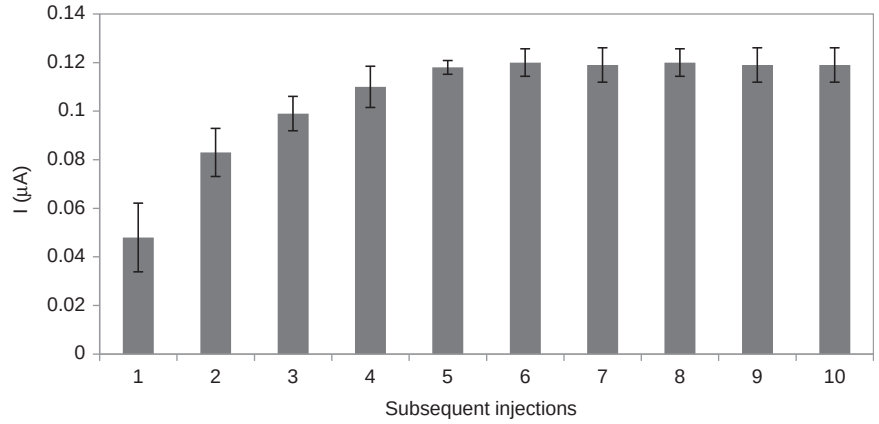

Fig. 5. Subsequent injections of 1×10^{-4} M of ATCh in the first step of measurements. 0.01 M PBS, pH 7.0, flow rate 200 μL/min, E_{app} 0.08 V. Average of three different electrodes.

was readily immobilized and renewed. The selectivity of the detection was of particular interest since the silver electrode, at the low applied potential and under the experimental flow conditions used (PBS buffer pH 7.0), was selectively sensitive toward compounds reacting with silver chloride such as sulfide or free thiols [19]. Additional detailed experimentation is anticipated for setup, physico-chemical parameters and detection optimization in order to screen for different AChE inhibitors. This newly developed detector design could be coupled to HPLC (post-column) or eventually miniaturized for nanoreactors or for capillary electrophoresis analyses.

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