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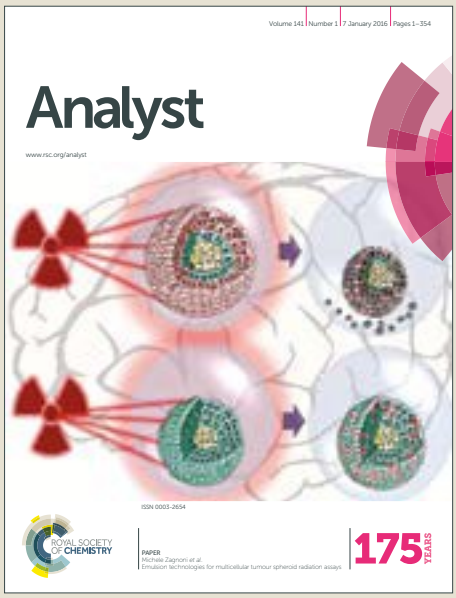

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Assay of serum cholinesterase activity by an amperometric biosensor based on a co-crosslinked choline oxidase/overoxidized polypyrrole bilayer†

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Abstract

Based on choline oxidase immobilized by co-crosslinking on a overoxidised polypyrrole modified platinum electrode, a novel electrochemical assay for cholinesterase activity in human serum has been developed. The assay was performed by adding an aliquot of cholinesterase standard solution or serum sample to a phosphate buffer containing a choline or thiocholine ester and measuring the oxidation current of hydrogen peroxide at the rotating modified electrode polarized at +0.7 V *vs* SCE. The influence of some experimental parameters like pH of the assay, mass transport at the electrode, type and concentration of cholinesterase substrate has been studied and optimised. A reversible inhibition on choline oxidase from cholinesterase substrates has been evidenced for the first time, increasing in the order acetylcholine, butyrylcholine and s-butyrylthiocholine. Wide linear range, fast response time and appreciable long term stability were assured for both acethyl- and butyrylcholinesterase assays. Allowing polypyrrole layer to efficiently remove interference from electroactive compounds in sample, the present method revealed suitable to detect butyrylcholinesterase in human serum at activities down to 0.5 U L^{-1}. The validation with a reference spectrophotometric method showed no significative differences when human serum samples were analysed.

Keywords: choline biosensor, overoxidised polypyrrole film, enzyme inhibition, cholinesterase assay, human serum

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Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) and Butyrylcholinesterase (BChE, EC 3.1.1.8), enzymes belonging to the group of cholinesterases (ChE), play a significant role in human function and health.[1,2] Despite both share a similar structure, they differs for their relative abilities to hydrolyse different choline esters. AChE (also known as *true* ChE) cleaves at high rate mainly the neurotransmitter acetylcholine (ACh) whereas BChE (or *pseudo* ChE) exhibits less substrate specificity since able to hydrolyse ACh, different choline esters, butyrylcholine (BCh) above all, and other synthetic substrates like ester-type local anaesthetics. ChE also differ for their distribution and function in human body. AChE is mainly found in the central nervous system, where it rules the cholinergic neurotransmission, but also in red blood cell membranes (where it is known also as *erythrocyte* ChE) but its role there is not well understood. Conversely, BChE is found in liver (where is synthesized), in muscle tissues and in significant amounts also in plasma and serum, from which its alternative name *plasma* or *serum* ChE.

BChE assay is of sure interest in several clinical studies. Its serum activity, for example, is used to distinguishing individuals with an atypical BChE deficiency, *e.g.* unable to hydrolyze at normal rate the muscle relaxant succinylcholine; these individuals, in fact, exhibit prolonged periods of apnoea after surgery when this drug is used in anaesthesia[3] so that the measurement of BChE activity is a prerequisite to avoid this complication. Further, plasmatic BChE is assayed also as a liver functional test since severe activity decrease is the result of liver parenchyma damage caused by pathologies like chronic liver diseases, acute hepatitis or liver cirrhosis.[4] Finally, a correlation between high serum BChE activity and metabolic syndrome, hyperlipidemia, coronary artery disease and hypertension has been also found.[5,6] Not less important, the measurement of the activity of both ChE is essential for detecting individuals exposed to organophosphate pesticides, nerve gases and similar chemicals: both erythrocyte and plasma forms are inhibited but the BChE plasma assay is the most used method since it is simpler and more reproducible.

Although several methods for ChE assay have been developed, the most widely used is still the spectrophotometric Ellman procedure.[7] Based on the enzymatic hydrolysis of thioesters like butyrylthiocholine (BTCh) or acethylthiocholine for BChE and AChE assay, respectively, in this approach the produced thiocholine reacts with the Ellman reagent dithiobisnitrobenzoic acid (DTNB) forming the yellow coloured 2-nitro-5-thiobenzoic acid which absorbance is proportional to the enzyme activity. Despite its simplicity, accuracy and relatively low cost, the Ellman procedure shows some limitations when applied to biological samples. The massive Soret absorbance of haemoglobin, in fact, interferes severely with the absorbance of the reaction product. This problem could be circumvented by measuring at a wavelength distant from the sharp

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absorption band of oxyhemoglobin: Worek and coworkers, for example, selected a wavelength of 436 nm since at this value the hemoglobin absorption is reduced to one-fourth compared to 412 nm, while the indicator absorption is still 80% of its maximum at 37 °C.[8] In addition to this limitation, DTNB is unstable, reacts with free sulfhydryl groups coming from the sample and interferes with BChE activity[9,10] causing a misinterpretation of the results when low enzyme activity is followed over prolonged times. To overcome such limitations, alternative enzyme substrates and Ellman reagents have been proposed to improve the standard assay[11,12] but other approaches to the standard Ellman method have been developed as well. As an example, a fixed time endpoint[13] method was developed, based on stopping the cholinesterase reaction after a fixed substrate reaction time using a mixture of detergent SDS and DTNB: the activity obtained by this method was higher than the activity obtained through the usual kinetic Ellman's method because DTNB interaction with the enzyme was prevented being the chromogen absent during the incubation time of cholinesterase with its substrate. Based on the self-assembly modulation of gold nanorods by enzymatic reaction, an ultrahigh sensitive assay has been recently proposed:[14] to avoid various nonspecific interactions, the procedure, however, involves long reaction times and an extreme dilution of serum samples (up to 200000 times). Unfortunately, serum dilution factor could be a crucial parameter when assaying BChE activity since, as showed by Ellman's method, the measured activity is dependent on the dilution ratio, the more diluted serum, the higher BChE activity:[15] in that case, an optimal dilution factor of 400 was recommended. A quantum dots-based fluorescence method has been also proposed:[16] this approach eliminate the color reagent because the BChE quantification was based on butyric acid detection, *i.e.* on the first step enzyme reaction. Finally, a direct assay of BChE, using a fluorescent substrate that is selectively hydrolyzed by the enzyme, has been recently developed:[17] the method is claimed as suitable for selectively monitoring BChE activity even in the presence of excess AChE.

About the practical application of ChE assay with the above approaches, there is now and then some confusion in the relevant literature since many laboratories use different substrates and/or concentrations of the same substrate for the assays: a reliable evaluation between methods and their measurements is of course impracticable because every substrate behaves kinetically differently. So, there is still a lack of standardized procedures for measurement of ChE activity despite the fundamental role covered in clinical and environmental fields. This justifies the actual interest in developing assays able to assure appreciable sensitivity and accuracy without requiring expensive instrumentation or long procedure time.[18] In this context, electrochemical biosensors can play a surely innovative and quite promising role particularly because have the clear advantage, over the spectroscopic ones, that can be used even when the sample are turbid or coloured, so a minimum

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sample pretreatment is required. Not less important, electrochemical biosensors and the relevant instrumentations are cheap, easy to use and usable in field analysis.

Electrochemical biosensing of ChE by amperometric detection is generally based on the use of choline oxidase (ChO), as a secondary enzyme, which detect the choline (Ch) produced by the ChE assay: using dioxygen as cofactor, Ch sensing by ChO byproduces hydrogen peroxide which can be easily detected at a properly polarised Pt electrode. Accordingly, different ChO-based amperometric biosensors for ChE analysis have been till now described.[19–28] Even if the sensing approach is simple, when applied to real samples like serum, amperometric detection is anyway complicated by faradic interference coming from endogenous electroactive compounds in the sample which oxidise at the relatively high potential used for hydrogen peroxide oxidation, as well as by electrode fouling phenomena due to *e.g.* high molecular weight protein adsorption onto the electrode surface. In order to reduce these undesired effects, multimembranes systems were proposed[19,22] but their realization is complex and time consuming, hardly miniaturizable while producing sensing devices showing in the worst cases not-linear diffusion and long response times. The lowering of the detection potential was attempted by a screen-printed carbon electrode based on ChO incorporated in polyelectrolyte layers and MnO_2 nanoparticles acting as hydrogen peroxide oxidation mediating reagent:[23] despite the lower detection potential, the interference from the normal physiological level of ascorbic and uric acid increased the sensor response up to 394% and 129 %, respectively, so a 200-fold dilution of blood samples was necessary to reduce interference. Further lowering of the detection potential was achieved detecting thiocholine at a carbon nanotube (CNT) based sensor for assay of salivary cholinesterase activity:[24] unfortunately, compounds containing thiol in the matrix could generate interference effects and the CNT-modified electrode showed a significant response decrease after some uses, which requires an electrochemical treatment to restore its electrochemical activity. Thiocholine detection was also employed in the case of a multiwalled CNT-gold nanocomposites modified screen printed carbon electrode for assay AChE in red blood cells:[25] here an immobilised AChE specific antibody selectively captures the AChE enzyme in the sample, avoiding the problem of overlapping substrate specificity and potential interference of electroactive species in biological samples; anyway this approach, since based on a immunoassay, required two steps, one for AChE capturing in the real sample and a successive step for the electrochemical detection in buffer solution, upon careful washing of the electrode. A chitinous membrane, preventing protein adsorption onto the electrode, was employed to immobilize ChO onto a Pt electrode in a flow injection amperometric Ch biosensor:[26] anyway, when assaying serum ChE, faradic interferences from endogenous compounds at the high detection potential required for hydrogen peroxide oxidation were not mentioned and not investigated at all.

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Further, a lipid-modified ChO enzyme was used to enhance the response time of the analysis:[28] anyway, this approach requires proper modification of the enzyme while the analysis time was comparable (if not higher in some cases) to the simplest methods using the unmodified enzyme. Finally, to preserve the enzyme in its natural micro-environment, immobilized whole cells of *Arthrobacter globiformis* (containing ChO) were used on a Clarke type oxygen probe:[27] such an approach guaranteed a stable sensing up to 7 weeks but interference from glucose due to bacteria metabolism was however its main disadvantage so requiring sample pretreatment with glucose oxidase to remove glucose, thus increasing the number of steps for analysis and the cost of the entire procedure.

In the present study, we propose a novel amperometric ChE assay, easy to realize and able to overcome the limitations of the electrochemical biosensing approach till now discussed. This goal was achieved through an enzymatic co-crosslinking procedure[29] which permitted the ChO immobilization on a platinum electrode previously modified by an overoxidized polypyrrole film (oPPy), as already attempted for the analysis of Ch in food.[30] Such an immobilization procedure[29] revealed very promising, being easily adaptable to any working electrode while assuring high enzyme stability and a fast response time. Furthermore, the notable rejection behaviour of the oPPy polymer towards interferents was also demonstrated in the case of a glucose biosensor even for mild diluted serum samples[31] as well as for other enzymes and analytes.[32,33] Accordingly, the present research will show that a biosensor based on a oPPy/ChO bilayer can be used successfully yet for the ChE assay in human serum. Significantly, by a careful examination and optimization of ChE substrates and operation conditions, we have developed an assay procedure for both AChE and BChE by employing the same dispositive. Particularly, different substrates were exploited for BChE assay to allow a direct comparison with reference spectrophotometric methods thus permitting the application of the biosensor for BChE assay in human serum.

Methods

Chemicals

Choline chloride, acetylcholine chloride, butyrylcholine chloride, butyrylthiocholine chloride, choline oxidase (EC 1.1.3.17 from Alcaligenes species, 12 U mg^{-1} of solid), acetylcholinesterase (EC 3.1.1.7 from Electric Eel, 292 U mg^{-1} of solid), butyrylcholinesterase (EC 3.1.1.8 from Horse Serum, 260 U mg^{-1} of solid), bovine albumin (fraction V), glutaraldehyde (grade II, 25% aqueous solution) were obtained from Sigma (Sigma Chemical Co.; St. Louis, MO). Benzoylcholine chloride was purchased from Fluka. Before its use, choline chloride was dried under vacuum over

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P_2O_5 for at least 3 days and stored in a vacuum desiccator. The above chemicals were used without further purification while pyrrole (Aldrich, Steinheim, Germany) was purified by distillation under vacuum at 52 °C before its use. All the other chemicals were of analytical reagent grade.

Choline stock solutions were prepared in double distilled-deionized water and stored in the dark at 4°C. Acetylcholine, butyrylcholine, butyrylthiocholine and benzoylcholine chloride solutions were prepared just before use to avoid chemical hydrolysis. Acetylcholinesterase and butyrylcholinesterase solutions were prepared in double distilled-deionized water. Before use, the actual enzyme activity was assayed by an established colorimetric method using a proper reagent kit (Cholinesterase procedure n° 420, Sigma); in this method, the enzymatic reaction is carried out in the presence of an acid-base indicator, m-nitrophenol: the produced acetic acid, lowering the pH, cause a loss of colour (read at 420 nm) which is proportional to the cholinesterase activity.

Accutrol normal control serum was purchased from Sigma as an assayed lyophilized preparation containing human and non-human enzymes and analytes in a human serum base. The reference method used for the determination of cholinesterase activity in serum was a colorimetric procedure based on the Ellman reaction (reagent kit Cholinesterase (BTC) procedure n° 421 from Sigma); this kit uses a low DTNB concentration as 0.25 mM and a reaction time of only 30 seconds which avoided any DTNB inhibition on measured cholinesterase activity.[10]

Apparatus

Electrochemical experiments were performed by using an AMEL (Milan, Italy) Model 466 polarographic analyzer coupled to a JJ instruments mod. CR65OS Yt chart recorder or by an EG&G (Princeton Applied Research, Princenton, N.J.) model 263A potentiostat/galvanostat equipped with a M270 electrochemical research software (EG&G) version 4.23 for data acquisition. The electrochemical cell was a conventional three electrode system consisting of a saturated calomel reference electrode (SCE) and a Pt counter electrode. The working electrode was a Pt disk (2 mm diameter) embedded in a PTFE body. Rotating disk electrode (RDE) experiments were performed by a CTV101 Speed Control Unit, EDI 101 Rotating Disc Electrode (Radiometer, Copenhagen). Unless otherwise stated, rotation rate was 200 r.p.m.

Spectrophotometric measurements were performed using a double beam Cary 2300 Varian UV/visible spectrophotometer.

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Biosensor preparation

Each electrode modification has been preceded by a cleaning procedure consisting in dipping the Pt working electrode in hot nitric acid and then in polishing it by alumina (0.05 µm particles) mechanical abrasion, extensive washing and sonication in bidistilled water.

The electropolymerization of polypyrrole films (PPy) was performed at a constant potential of +0.7 V versus SCE by using a solution of 0.4 M pyrrole in 0.1 M KCl supporting electrolyte until a deposition charge of typically 300 mC cm^{-2} was achieved. The Pt/PPy modified electrode was overoxidized at +0.7 V versus SCE in a phosphate buffer (pH = 7.0, I = 0.1 M) until a steady-state background current was obtained (at least 7 h).[34] Overoxidised Pt/PPy electrodes (Pt/oPPy) were then washed and air-dried at room temperature.

Choline biosensors were prepared as follow.[29] A 300 µL volume of a phosphate buffer (I = 0.1 M, pH 6.5) solution containing 16 mg of BSA and 1 mg of ChO (or 1 mg of ChO and 1 mg of BChE for BChE-ChO electrodes) were carefully mixed with 30 µL of 2.5% glutaraldehyde solution (25% glutaraldehyde solution diluted 1:10 with phosphate buffer). 3 µL of the resulting solution were pipetted onto the Pt/oPPy working electrode surface, avoiding air bubble formation, and carefully spread out to cover completely the electrode surface. The so modified Pt/oPPy/ChO electrode was then air-dried at room temperature for few minutes and preliminarily soaked in the background electrolyte for a few minutes just to remove weakly bound or adsorbed enzyme and to permit swelling of the enzyme layer. When not in use, the biosensor was stored in phosphate buffer, pH 6.5, I 0.1 M, at 4 °C in the dark.

Electrochemical measurement

All the electrochemical measurements were performed using a detection potential of +0.7 V versus SCE, which was the lowest potential for maximal detection. ChE activities were measured in batch addition experiments by immersing the sensor in a stirred (200 r.p.m.) phosphate buffer (I = 0.1 M, pH = 7.8) solution maintained at the desired temperature by a thermostated bath. An aliquot of a choline ester solution, and then an aliquot of a standard cholinesterase solution or serum sample were added into the cell. The current variation with time was recorded.

Results and discussion

The ChE assay method described in the present paper is performed simply by immersing the Pt/oPPy/ChO biosensor, assembled as a rotating disk electrode, in a thermostated electrochemical

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cell containing a properly solution of the desired choline ester in phosphate buffer: then an aliquot of ChE standard solution (AChE or BChE) or serum sample is injected in the cell for the assay. The free Ch produced during the assay is sensed by the biosensor, *i.e.* by the immobilized ChO which catalyze the oxidation of Ch to betaine with subsequent production of hydrogen peroxide: upon the progressive formation and then oxidation of hydrogen peroxide at the electrode surface, a current signal is recorded which increases linearly with time. From the slope of such current signal increase (elsewhere here indicated as response), the ChE activity in the cell solution can be measured after proper calibration of the sensor.

The biosensor here used for ChE assay consists of a ChO layer immobilized by co-crosslinking onto the surface of a Pt/oPPy modified electrode; such a biosensor is in fact able to sense the free Ch produced during the assay through the following reaction:

$$Ch + 2\ O_2 + H_2O \xrightarrow{\text{immobilized ChO}} \text{betaine} + 2\ H_2O_2 \qquad (1)$$

where the produced hydrogen peroxide is oxidised at the Pt/oPPy modified electrode. As already reported for other enzymes,[35] ChO co-crosslinking is optimal if performed in the pH range 6-9:[29] here enzyme immobilization has been carried out at a pH 6.5 since such value assured either an appreciable co-crosslinking rate either an high biosensor sensitivity.[29] Before using the Pt/oPPy/ChO electrode for ChE assay, its electroanalytical characterization has been carried out at the same pH value used for the enzyme immobilization. Briefly, the sensor showed towards Ch a sensitivity of 0.29 μA mM^{-1} mm^{-2}, a linear range up to 0.5 mM, an apparent Michaelis-Menten constant of 2.18 ± 0.06 mM, a fast response time (3 seconds) and a limit of detection (LOD) of 1 μM (as measured for a signal to noise ratio S/N = 3). A long term stability study showed a moderate lowering of sensor sensitivity down to the 70% of the initial value after more than 30 days of discontinuous use (sensor stored in a phosphate buffer pH 6.5 at 4 °C in the dark when not in use). A quick comparison of the above data with the analytical performances already reported for the simple Pt/ChO electrode[29] shows that the presence of the underlying oPPy membrane would allow the fabrication of an interferent- and fouling-free biosensor without affecting the performances of the biosensor in terms of sensitivity, stability, response time and kinetic behaviour.

In order to optimize the sensor response towards the ChE assay, the influence of experimental variables like pH of the buffer solution, rotation rate of the electrode, ChE substrate and its concentration has been studied.

Influence of pH used for the assay

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ChE assay requires a careful control of pH, usually set at a value allowing the maximum enzyme activity. Since this method is based on the use of an auxiliary enzyme, *i.e.* ChO, it is necessary anyway to consider the pH behaviour of both the primary and the auxiliary enzyme. The present Pt/oPPy/ChO modified electrode showed its maximum sensitivity in the pH range 8.5 – 9.5, slightly higher from the pH range (7-8) reported for the enzyme in solution: pH shifts like this are common after enzyme immobilization.[36] This pH range is in agreement with the findings reported in the absence of the polymeric layer,[29] thus attesting again that the presence of oPPy film does not significantly alter the kinetic behaviour of the immobilized ChO. As concern the primary enzyme, the pH range in which AChE and BChE show the maximum activity in solution is 7.5 – 8.0 and 8.0 – 8.5, respectively, in agreement with the relevant literature.[37]

The choice of the working pH for the assay is anyway also influenced by the nature of the ChE substrate. ACh, BCh and BTCh, quaternary salts of β-aminoesters and β-aminothioesters, respectively, hydrolyze spontaneously at moderate alkaline pH values thus interfering with activity measurements. As a proof of this undesired behaviour, Figure S1 (see ESI) compares the spontaneous and the enzymatic hydrolysis of BCh as measured at pH 8.5 in the attempting to assay BChE: as can be seen, the chemical hydrolysis observed at this pH significantly bias the enzyme assay overestimating its activity of about the 25%. Also ACh showed a spontaneous hydrolysis at this pH, as elsewhere reported,[37] so that care should be used in choosing the pH of assay. BTCh, on the contrary, revealed a more stable substrate since hydrolyze spontaneously at an appreciable extent only at pH values greater than 8.5.

The above findings suggested us to perform ChE assay at a lower pH, found here optimal at pH 7.8, where spontaneous substrate hydrolysis was insignificant. While this pH value is slightly lower than the maximal pH activities found for ChE and ChO (see above), the limit of detection for Ch was 0.1 μM (S/N = 3), *i.e.* one order of magnitude lower than one previously found at pH 6.5, suggesting that assay sensitivity is not compromised by the pH lowering.

Influence of electrode rotation rate

Mass transport of substrate is known to rule the analytical performances of amperometric biosensors so that its influence was investigated. The study was performed in a phosphate buffer (pH 7.8, I 0.1 M), using several ChE/substrate couples, by measuring the slope of the current signal increase after three minutes from the injection of ChE for different electrode rotation rates spanning from 50 to 5000 r.p.m. For all the investigate ChE/substrate couples, the biosensor response decreases with rotation rate, as exemplified in Figure S2 (see ESI) for the AChE/ACh couple.

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The observed behaviour offers interesting insights about the kinetic of the bienzymatic system here involved in the assay. Since ChE are enzymes with a significant catalytic power (*e.g.*, the AChE turnover number is about 10^4 s^{-1} and its second-order rate constant almost 10^8 M^{-1} s^{-1} for ACh hydrolysis[38]) and the stirring of solution promotes the mass transfer of the relevant substrate, the rate determining step of the whole bienzymatic system is surely due to the kinetic of the ChO sensor here used for sensing the product of ChE assay (an independency from rotation rate would be expected, on the contrary, for a rate determining step due to ChE catalysis in solution). Indeed, a similar dependence with rotation rate (here not reported) was found also when using a ChO biosensor for Ch sensing, supporting the above conclusions.

Accordingly, in the following experiments a rotation rate of 200 r.p.m. has been adopted for the assay as best compromise between a high sensitivity of the sensor and an adequate controlled mass transport.

Influence of the substrate used for the assay

ChE differ in their substrate specificity and vulnerability to inhibitors. The inhibition of AChE by ACh is one of the key features that distinguishes it from BChE that, on the contrary, exhibits substrate activation.[39] Although ACh is the naturally occurring substrate of AChE, human *erythrocyte* ChE (*i.e.* AChE) is known to hydrolyze also the thion analogue of ACh, propionylcholine and acetyl-β-methylcholine.[40,41] Human *plasma* or *serum* ChE (*i.e.* BChE) catalyzes instead the hydrolysis of the thion analogue of ACh and the cleavage of propionyl and butyrylcholine and their thion analogues[42,43] being their rates of hydrolysis ACh < propionylcholine < BCh.[44] Therefore, BCh and BTCh are specific substrates for human *plasma* or *serum* ChE, whereas acetyl-β-methylcholine is a specific substrate for human red cell as it has been used as substrate for the analysis of the activity of these enzymes in whole blood; furthermore, benzoylcholine (BZCh) is also a classic substrate specific for *serum* ChE. Even if several thiocholine esters are used in various colorimetric assay, the procedure based on acetylthiocholine lacks of specificity for *serum* ChE since it also measures AChE present in red cells; further, acetylthiocholine is quite instable compared with other thiocholine esters. On the contrary, BTCh is more stable than either acetyl or propionylthiocholine, is more rapidly hydrolyzed and is specific to BChE. Thus BTCh seems to be the substrate of choice in the assay of *plasma* or *serum* ChE.

Accordingly to the above discussion, substrates specificity is the key for a correct enzyme assay so that it was reinvestigated with the present method by evaluating the response of the ChO biosensor upon the hydrolysis of ACh, BCh, BZCh and BTCh by AChE or BChE. Experimentally, the sensor was dipped in the supporting electrolyte solution where one of the above esters was

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injected at a fixed substrate concentration of 1 mM; after that, an aliquot of ChE standard (AChE or BChE) at different activities has been introduced into the cell and the slope of current increase measured. For each enzyme/substrate couple, a calibration line has been derived as a function of the enzyme activity whose slope gives evidence of the relevant sensitivity. The higher sensitivities were observed for the couples AChE/ACh (0.140 nA L U^{-1} s^{-1}) and BChE/BCh (0.0785 nA L U^{-1} s^{-1}) (see Figure S3 in ESI) in agreement with other findings.[22] As far as BChE, sensitivities were 0.0453 and 0.0284 nA L U^{-1} s^{-1} when BTCh and BZCh were used as substrates, respectively, while the couple BChE/ACh diplayed the lowest sensitivity (0.0193 nA L U^{-1} s^{-1}). Finally, in agreement with literature data, AChE didn't display any affinity towards BCh, BZCh and BTCh.

Accordingly, the subsequent experiments were performed using the enzyme/substrate couples showing the highest sensitivities, *i.e.* AChE/ACh and BChE/BCh. Since BTCh was the substrate used for the cholinesterase reference method adopted in the present paper and ACh is largely diffused in the commercial kits for *serum* ChE assay, BTCh and ACh were also investigated as possible BChE substrates.

Optimization of substrate concentration on the assay

For each of the enzyme/substrate couples listed above, the optimal substrate concentration to use in the assay was determined. These experiments have been carried out realizing in solution (phosphate buffer pH 7.8 I 0.1 M) an enzymatic activity of 10U L^{-1}.

Figure 1.(a) shows the normalized sensitivities of a typical Pt/oPPy/ChO biosensor as a function of acetylcholine concentration at the selected AChE activity. A bell-shaped dependence, with a maximum at a ACh concentration of about 1.5 mM, was observed, in agreement with literature data that reports a maximum at about 1 mM[45] and explained this behaviour in terms of an inhibiting effect above this value.[46]

Usually, for an enzyme affected by substrate inhibition, the initial rate v of an enzymatic reaction can be expressed by the following equation:[47]

$$v = \frac{v_{max} \cdot s}{\left(K_M + s + s^2 / K_i \right)} \qquad (2)$$

where s is the substrate concentration, v_{max} the maximum velocity, K_M the Michaelis-Menten constant and K_i the inhibition constant. The fitting of the experimental data of Figure 1.(a) with equation (1) allowed to evaluate a K_i value of 7.19 ± 0.24 mM and a K_M value of 0.40 ± 0.01 mM,

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in good agreement with the value reported for this enzyme (K_M = 0.46 mM[45]). Similar experiments carried out on an immobilized bienzymatic system AChE/ChO[29] gave a K_M value of 0.24 ± 0.06 mM, a K_i value of 15 ± 2 mM and a maximum for a ACh concentration close to 1.5 mM. Comparing these latter data with those obtained in the present work for AChE but in solution, it is evident that substrate inhibition is less marked upon enzyme immobilization; anyway, the effects of ChO inhibition by ACh, even if the lowest (see the below discussion about), should be also considered as well.

Figure 1 also shows the normalized sensitivities of the biosensor with the concentration of BCh (b), ACh (c) and BTCh (d) for a BChE activity of 10 U L^{-1}. Again but surprisingly for BChE, a bell shaped profile was observed for each choline ester with a maximum located at different concentration values, depending on the particular substrate (see Table 1). Interestingly, a analogous profile was observed for a biosensor using both ChO and BChE immobilized onto the electrode surface (Pt/oPPy/ChO/BChE, data not shown); also in this case, the current response started to decrease at substrate concentration close to the value reported for BChE in solution. With respect to the solubilized enzyme, the current depression was less pronounced in the case of immobilized BChE, as it was previously observed for AChE.

The bell shaped curves (*i.e.* an apparent substrate inhibition) observed for both soluble and immobilized BChE are not immediately comprehensible as in the AChE case since BChE catalysis often show substrate activation at high substrate concentrations while, as far as we know, an inhibition effect was reported only for BZCh.[48] In an attempt to rationalize this unknown phenomenon, several experiments were planned to verify if this behaviour depended from the electrode itself or from the auxiliary enzyme (*i.e.* ChO) immobilized on it. Incubating even for long time the Pt/oPPy/ChO electrode in several BChE substrate solutions, even in the presence of BChE itself, didn't show any electrode fouling effects since the sensor responses to Ch, before and after incubation, were almost identical. Further, even using massive amounts of Ch in solution, at concentrations well above the saturation region of ChO enzyme, any decrease of the current response was observed at a Pt/oPPy/ChO electrode; accordingly, a ChO inhibition by its substrate has been excluded in agreement with the relevant literature: neither BChE nor ChO seem to be inhibited from the proper substrate. Finally, the chance of a ChO "cross-inhibition" from BChE substrates has been also considered. For this purpose, aliquots of standard solutions of BChE substrates have been progressively introduced in a cell containing Ch concentrations in ChO saturation region, while the current responses were monitored with a Pt/oPPy/ChO electrode. Upon the injections of any esters, unexpectedly, a continuous decrease of the response has been observed which cannot be ascribed at a simple dilution effect. A ChO inhibition from BChE substrates can

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then be assumed even if, at our knowledge, there is no reference of such an inhibition in the literature. A systematic study was then carried out in order to quantify the inhibition effect exerted by BChE substrates on ChO. Figure 2 reports the results of this study showing the percentage of inhibition observed for ChO due to ACh, BCh and BTCh: clearly, ACh gave the lowest inhibition effect while BTCh the highest. For all substrates, however, a reversible inhibition was observed since biosensor sensitivity does not change before and after its incubation in ester solutions, as already pointed out above in electrode fouling investigations.

In conclusion, the present study allowed to define the optimal substrates concentration for ChE assay as reported in Table 1: the following assay experiments were performed using these concentration values. It is interesting to observe that the maximum BChE activity is reached at a substrate concentration of 1.75 mM for BCh and BTCh and 2.5mM for ACh. Under the light of ChO inhibition above described, this difference could be tentatively due to the combined effect of the stronger inhibition exerted by the first two substrate on ChO and the higher affinity of BChE for them.

Calibration of the ChE assay

The calibration curves for the assay of ChE activity have been realized by injecting in the cell the substrate at the optimal concentration listed in Table 1 and then by introducing an aliquot of an enzyme standard solution. The response has been evaluated as usual by the slope of the current signal increase as observed by the sensor. For both AChE and BChE, a deviation from linearity was observed at the highest activities, even if the assay time, *i.e.* the time required for a linear current increase, was sufficiently low to allow the classical conditions of initial rate for the enzymatic reaction. This behaviour has been attributed to the deviation from linearity of the Ch response at the ChO biosensor since a simple calculus shows that the ChE activity values corresponding to the observed deviation from linearity are able to produce, upon esters hydrolysis, a choline concentration sufficiently high to fall outside the linear range (up to 0.5 mM) of the ChO biosensor. Obviously, the different ChE activity values from which deviations are observed depended on the different enzyme affinity towards the various substrates.

The calibration graph for the system AChE/ACh was linear up to an enzyme activity of 200 U L^{-1} while the lowest measurable activity (LMA) was 0.5 U L^{-1} (S/N = 3). Interestingly, this LMA was the same also for other ChE/substrate couples (see below) suggesting that the LMA is limited by the LOD of Ch at the Pt/oPPy/ChO electrode. The present method is hence suited for AChE assay in cerebrospinal fluid with no sample pre-treatment since AChE activity values in cerebrospinal fluid are typically ca. 17 ± 4 U L^{-1} in normal people and can increase up to 40 in case

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of tumours or illnesses involving destruction of the cerebral parenchyma.[49] AChE in red blood cells can be assayed as well with the present method but require proper dilution of the biological sample since enzyme activities in typical red cell haemolysed solutions are in the range 11000-16000 U L^{-1} at pH 8, 37 °C.

For the system BChE/ACh, the lowest measurable BChE activity was 0.5 U L^{-1} (S/N = 3) as for AChE, but linearity was observed up to 600 U L^{-1}. BChE activity values usually found in serum with other assay methods using the same ACh substrate fall in the range 1000-4000 U L^{-1}, using similar experimental conditions: consequently, to determine the BChE serum activity through the electrochemical assay here proposed, it is sufficient to simply diluting the sample, even using the dilution factor of 400 recommended for BChE.[15] Interestingly, the present approach can be useful even to assay anomalous BChE values as found on serum samples from individuals exposed to pesticides or nerve gases: in these cases, BChE activity falls of the 40% before the appearance of the first symptoms of poisoning (typically 600-2400 U L^{-1}) and of the 80% upon acute intoxication (200-800 U L^{-1}).

Finally, the analytical performances of the proposed method for the system BChE/BTCh (linearity up to 600 U L^{-1}, lowest detectable BChE activity 0.5 U L^{-1} (S/N = 3)) were comparable to those observed using ACh as substrate, but linearity dropped down to 180 U L^{-1} using BCh. We decided then to focus our attention on BTCh as substrate while performing BChE electrochemical assay since this was the same substrate used in the spectrophotometric reference procedure that we adopted to compare our results in serum samples.

BChE electrochemical assay in serum

The assay of BChE activity in serum by the present ChO biosensor was possible thanks to the presence of the permselective oPPy layer onto the electrode surface. Indeed, serum is a complex matrix containing substances which can interfere during assay measurements: anyway, the notable capability of this polymer to reject interfering and fouling species[31–33] assured proper measurements. As an example, Figure 3 report the current signals, acquired in batch analysis at the same experimental conditions used for BChE assay, of common endogenous (ascorbic and uric acid) and exogenous (acetaminophen) interferents at concentrations near the upper limits of their respective physiological concentration ranges; comparing the signals acquired in absence (Figure 3.a) and in the presence of the polymer (Figure 3.b), it is possible to state that the relevant bias introduced in the measurement of BChE activity is surely negligible. It is worth noting that these rejection figures here shown comes from non-diluted serum where the concentration of interferents is at its own maximum: this is an important result considering that, as previously discussed, a

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significant serum dilution is often required to reduce interference and fouling problems with other approaches.

To validate the proposed approach, the Pt/oPPy/ChO biosensor was used to measure the BChE activity on some serum samples by using BTCh as substrate. In these assays, BChE activities have been calculated from the relevant calibration curve which showed a sensitivity value of 0.429 nA L U^{-1} s^{-1} at T = 30 °C. Serum samples have been diluted 1:200 so that the relevant enzyme activity fell within the linear range. The electrochemical assay has been compared with the results obtained by a colorimetric kit based on the standard Ellman spectrophotometric procedure using BCh as substrate and DTNB as chromofor in a phosphate buffer solution at 30 °C and pH 7.2, *i.e.* close to the experimental condition of the electrochemical assay. Table 2 reports the BChE activity values obtained by the two procedures: as can be seen, data obtained by the proposed method are not significantly different, at a confidence level of 95%, from those derived by the reference spectrophotometric assay.

Comparison of the ChE electrochemical assays

Table 3 summarizes the biosensing-based ChE electrochemical assays till now described while comparing them with the present approach. As can be seen, the analytical performances of the proposed method appear the more useful for a proper assay of both AChE and BChE in several samples of clinical interest. Particularly, the Pt/oPPy/ChO biosensor shows the best performances in terms of LMA while assuring a completely inteferent- and fouling-free assay of the enzyme. Importantly, the here proposed assay appears the unique approach showing BChE activity measurements in human serum not significantly different from standard method.

Conclusion

A novel electrochemical ChE assay procedure has been proposed by using a platinum electrode modified by a co-crosslinked ChO layer coupled to a permselective oPPy film. This sensor showed appreciable sensitivity, long term stability and notable anti-interference behaviour, thus constituting a valid alternative to ChO amperometric sensor based on other approaches. A ChE assay procedure was realized for both AChE and BChE by optimizing pH, the hydrodynamic conditions and particularly the type and concentration of the enzyme substrate. From this study, interesting evidences were derived such as a reversible inhibition on ChO by BChE substrates, never reported before.

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Calibration curves with wide linear range were obtained which allow to perform real samples analysis by simply dilution of the sample. Being the ChE activity measurable by the proposed method in samples from patients with normal and anomalous values, the achievement of a particularly sensitive detection was not our primary purpose: nevertheless, the lowest enzyme activity detectable was 0.5 U L^{-1} which represents the best LMA between the biosensing-based ChE electrochemical assays till now described. Surely, LMA surprisingly low are elsewhere reported for spectroscopic approaches but the achievement of requirements such as wide linearity, accuracy, selectivity and stability with a dispositive easy to realize constitutes surely an important perspective in the field of ChE amperometric assay. Since biosensors based on enzyme co-crosslinking/electrosynthesized permselective films as the present can be easily adopted even for screen-printed electrodes,[50] the proposed approach can surely represent the basis for the development of a portable and disposable sensor for clinical assay in the desired field.

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Tables and figures captions

Table 1. Substrate concentration values at maximum ChE activity

Table 2. BChE quantification in human serum

Figure 1. Normalized response (*i.e.* response/maximum response) of the Pt/oPPy/ChO sensor as a function of substrate concentration. (a) AChE/acetylcholine, (b) BChE/butyrylcholine, (c) BChE/acetylcholine, (d) BChE/butyrylthiocholine. Enzyme activity: 10 U L^{-1}. Supporting electrolyte: phosphate buffer pH 7.8 I 0.1 M. Temperature: 25 °C. Electrode rotation rate: 200 rpm. The continuous lines represent the best fitting of the equation (2) to experimental data.

Figure 2. Relative percentage inhibition exerted by s-butyrylthiocholine, butyrylcholine and acetylcholine on the immobilized choline oxidase under substrate saturation conditions. Supporting electrolyte: phosphate buffer pH 7.8 I 0.1 M. Temperature: 25 °C. Electrode rotation rate: 1000 rpm.

Figure 3. Current responses relevant to the oxidation of ascorbic acid (AA), acetaminophen (Ac) and uric acid (UA) in absence (a) and in the presence of oPPy (b) onto the electrode surface. Supporting electrolyte: phosphate buffer pH 7.8 I 0.1 M. Temperature: 25 °C. Electrode rotation rate: 200 rpm.

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Table 1

Substrate (ChE)	Concentration at maximum ChE activity (mM)
Acetylcholine (AChE)	1.50
Acetylcholine (BChE)	2.50
Butyrylcholine (BChE)	1.75
S-butyrylthiocholine (BChE)	1.75

Table 2

	BChE activity (U L^{-1})	
Serum sample	**Official method[c] (Spectrophotometric)**	**Developed method[d] (Electrochemical)**
1[a]	3475	3376 ± 175
2[b]	4218	4150 ± 175
3[b]	4173	4216 ± 175
4[b]	3579	3610 ± 175

[a] Accutrol normal control serum (from Sigma)

[b] from hospital patients with no particular pathologies

[c] Cholinesterase (BTC) procedure n° 421 from Sigma reported within run precision of 3%

[d] Confidence intervals ($1-\alpha = 0.95$; $\nu = n-2 = 7$)

Table 3

Sensor Type	Ch Analysis			Operational Stability	BChE Analysis			Reference
	Linear Range (upper limit)	LOD (S/N 3)	Response Time		Linear Range	LMA (S/N 3)	Application to real sample	
dioxygen sensing biosensor								
ChO on O_2 sensor 3 membranes	0.3 mM	2 µM	120 s	2 weeks	0.5 - 60 U L^{-1} [a]	-	Human serum Difference[b]: up to 2.3% Chemical hydrolysis	[19]
Bacteria on Clark type sensor	0.2 mM	0.08 µM	200[c] s	7 weeks	up to 11 U L^{-1} [a]	-	Human serum Difference[b]: up to 2.8% Glucose interference Chemical hydrolysis	[27]
hydrogen peroxide sensing biosensor								
ChO on Pt electrode Flow analysis	70 mM	15 µM	150 s	2 months	50 - 20k U L^{-1} [a]	8 U L^{-1} [a]	Human serum No comparison[b] Precolumn to remove interference	[21]
ChO on Pt electrode 3 membranes	0.02 mM	-	-	1 month	50 - 350 U L^{-1} [d]	10 U L^{-1} [d]	Human serum Difference[b]: up to 4.46% Ascorbate interferent free	[22]
ChO/PVA-SbQ/Nafion on carbon fibre	1 mM	-	120 s	45 days	10k - 50k U L^{-1} [a,e]	-	Mice serum Difference[b]: up to 25% Ascorbate interference	[20]
Lipid-modified ChO on nafion-modified glassy carbon electrode	0.4 mM	0.3 µM	3 s	4 weeks	up to 2k U L^{-1} [a]	2 U L^{-1} [a]	None Interferent effects not shown	[28]
ChO on chitinous membrane/Pt electrode Flow analysis	5 mM	10 µM	3 s	2 months	1k - 3.8k U L^{-1} [d,f]	-	Human serum No comparison[b] Fouling - free	[26]
ChO/MnO_2 on screen printed graphite electrode	0.1 mM	0.13 µM	10 s	3 weeks	Unknown [g]	-	Mice blood haemolysates spiked with enzyme No comparison[b] Ascorbate and urate interference	[23]
ChO on oPPy-modified Pt electrode	0.5 mM	0.1 µM	3	1 month	up to 600 U L^{-1} [a,h] up to 200 U L^{-1} [a,e] up to 180 U L^{-1} [i]	0.5 U L^{-1}	Human serum No significant difference[b] Ascorbate, urate and acetominophen interferent free	this paper

-: data not reported
[a]: ACh as substrate
[b]: with ChE standard method
[c]: depended on Ch concentration
[d]: BZCh as substrate
[e]: data for AChE analysis
[f]: data inferred from figure
[g]: ChE activity erroneously reported in nM concentration
[h]: BTCh as substrate
[i]: BCh as substrate

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Figure 1

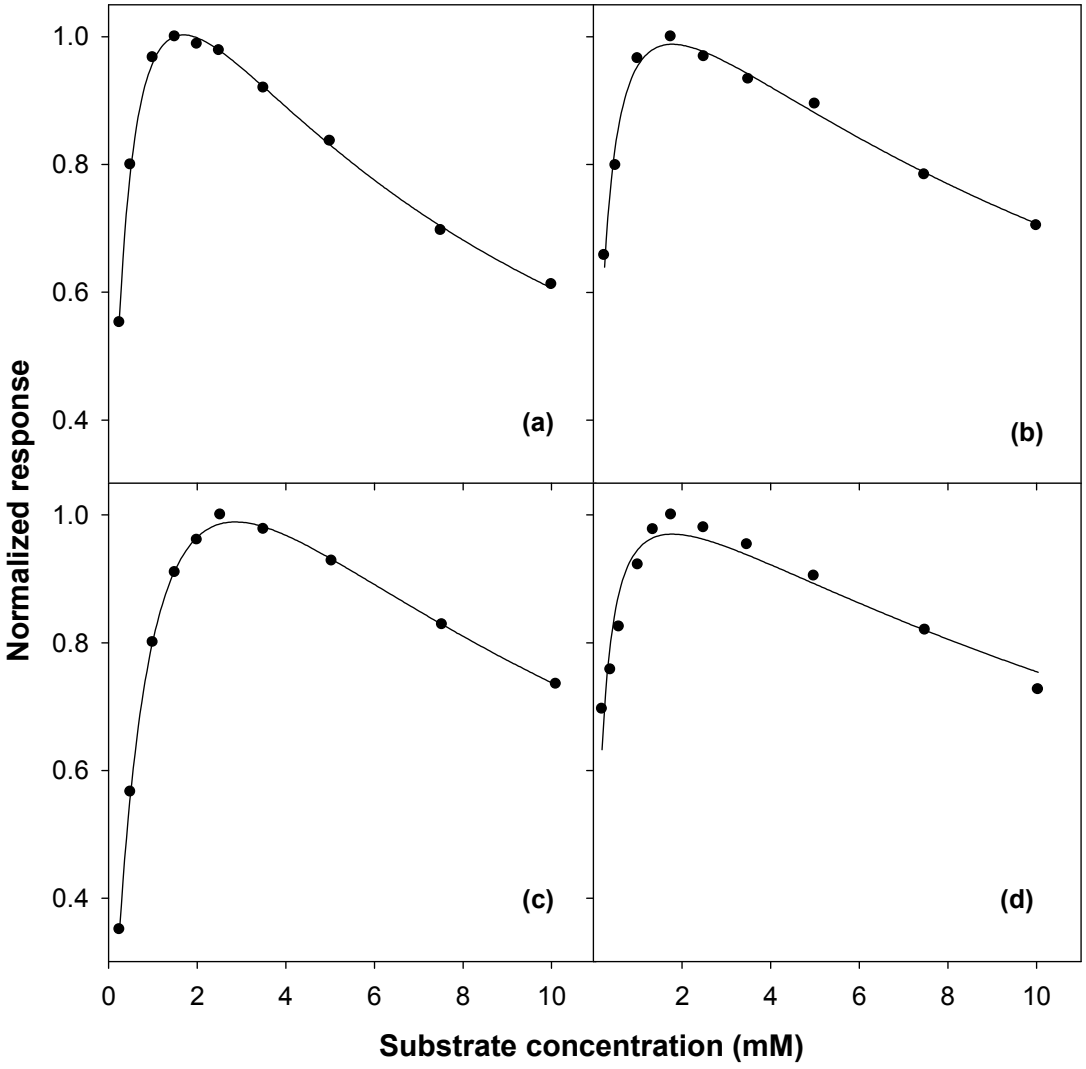

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Figure 2

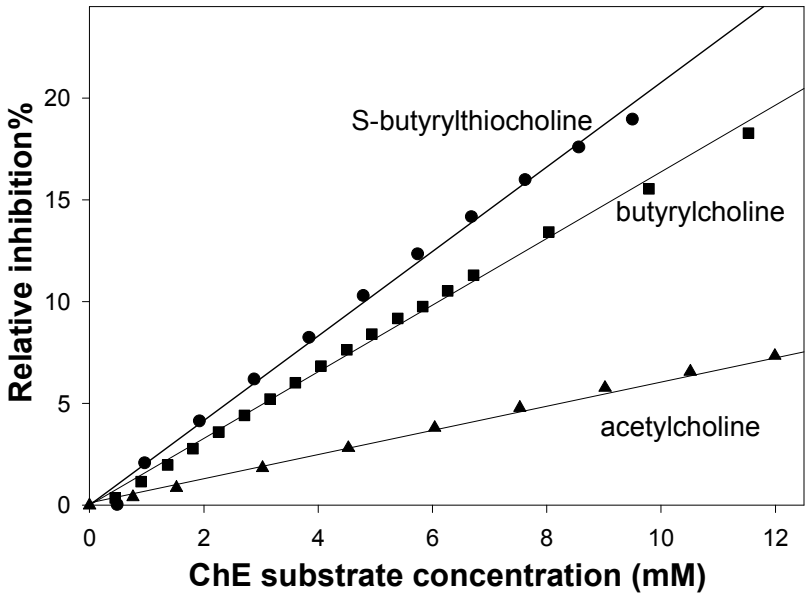

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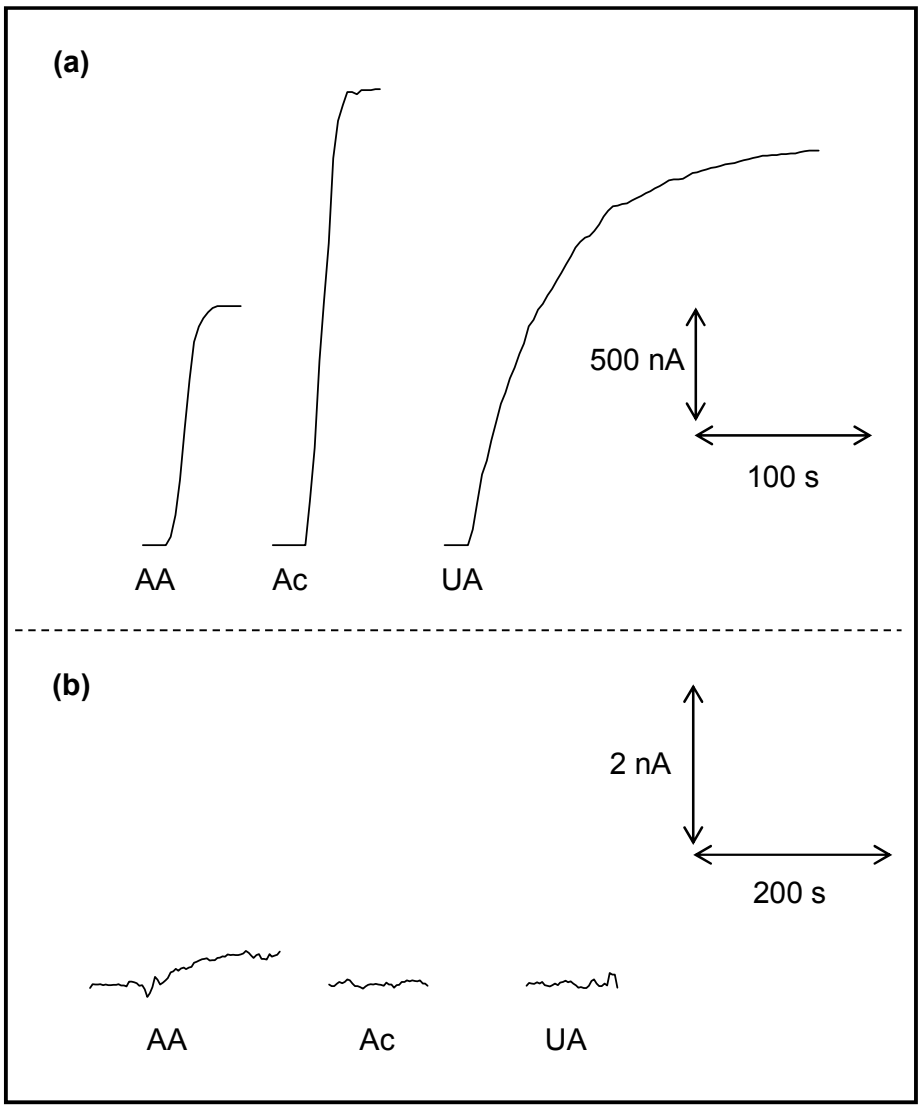
Figure 3



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Electronic Supplementary Information

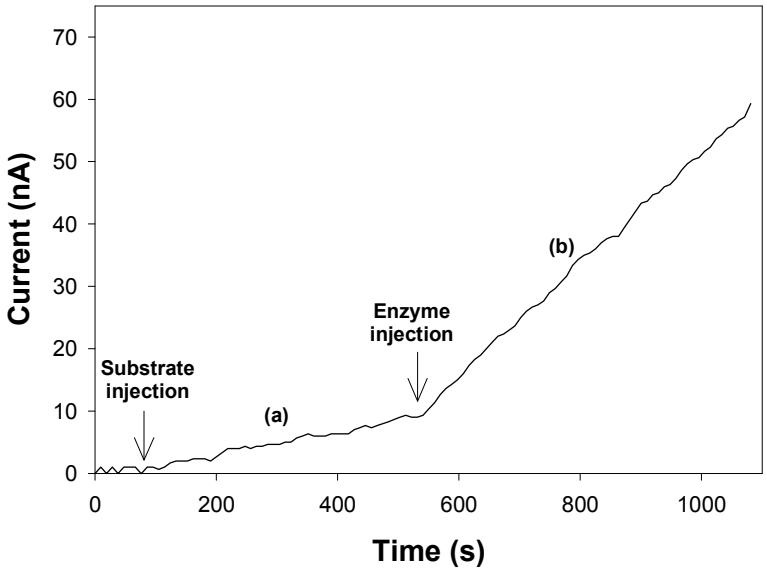

Figure S1. Spontaneous (a) and enzymatic (b) substrate hydrolysis. Supporting electrolyte: phosphate buffer pH 8.5, I 0.1 M. Substrate: butyrylcholine 1.75 mM. Enzyme: BChE 1 U L^{-1}. Electrode rotation rate: 200 rpm.

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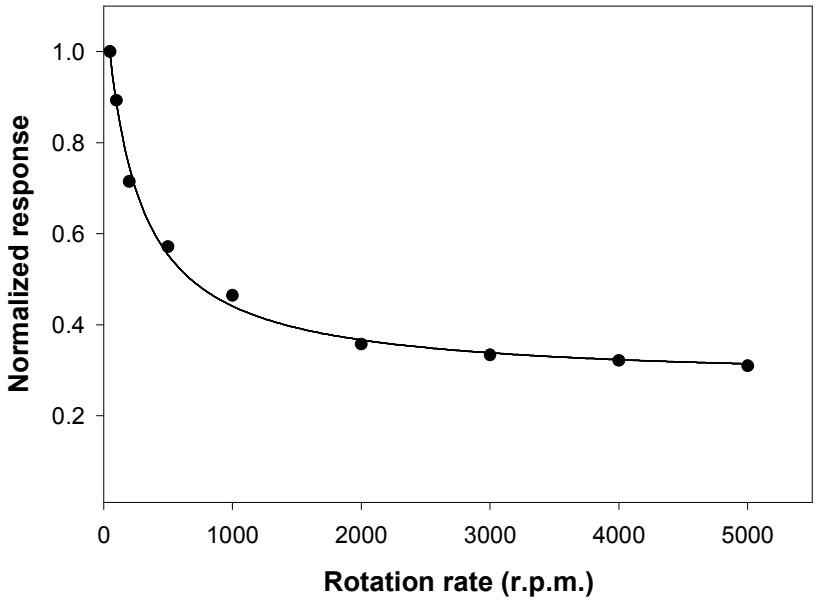

Figure S2. Variation of the current signal slopes as a function of the rotation rate at a Pt/PPyox/ChO biosensor upon addition of acetylcholine 1 mM and AChE 10 U L^{-1}. Supporting electrolyte: phosphate buffer pH 7.8, I 0.1 M. Temperature: 25 °C.

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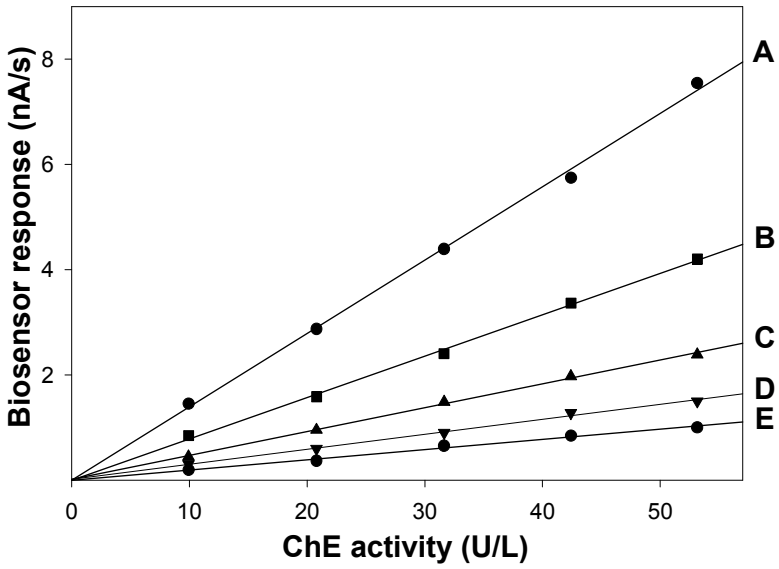

Figure S3 Calibration curves for various ChE/substrate couples: AChE/acetylcholine (A); BChE/butyrylcholine (B); BChE/s-butyrylthiocholine (C); BChE/benzoylcholine (D); BChE/acetylcholine (E). Substrate concentration: 1 mM. Supporting electrolyte: phosphate buffer pH 7.8, I 0.1 M. Temperature: 25 °C. Electrode rotation rate: 200 rpm.