ASSAY OF ENZYMES OF CLINICAL AND BIOLOGICAL SIGNIFICANCE BY AN INTERFERENCE FREE CHOLINE BIOSENSOR

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INTRODUCTION

The fundamental role covered by various enzymes in clinical and biological fields justifies the increasing interest in developing fast, accurate and sensitive assay methodologies.

Phospholipase D (PLD) is a widespread enzyme which has multiple effects and significance on cellular functions[1]. The main methods[2] for PLD assay are limited by a poor sensitivity and specificity (spectrophotometric or titrimetric ones) or by the use of expensive and potentially health hazardous reagents (radioassay procedures).

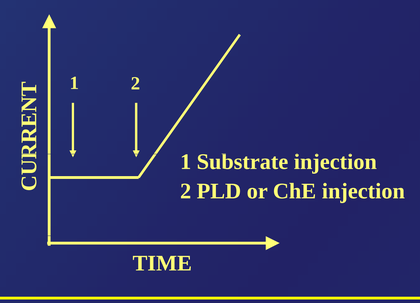
Measurement of serum cholinesterase (ChE) is important to assess liver function or excessive exposure to organophosphorus pesticides[3]. The reference procedure for such a measurement is the Ellman colorimetric method[4] which suffers from haemoglobin and glutathione interference.

Choline (Ch) is an important amine which can be selectively analysed detecting H_2O_2 enzymatically produced by choline oxidase (ChO). Based on this approach, several methods have been developed to detect Ch or species involving Ch release such as ChE, acetylcholinesterase (AChE), PLD and Ch containing phospholipids. Amperometric sensors based on immobilised ChO play a surely innovative role in this field. ChO immobilization is not an easy task. Covalent immobilization on nylon net[5], often coupled to multimembranes assembly[6] to preserve the electrode from interference and fouling, is complex and time consuming, moreover causing a significant slowing down of the sensor response.

In the present work a simple and sensitive enzyme assay is proposed based on ChO immobilization by co-crosslinking[7,8] on a platinum electrode previously modified by an overoxidized polypyrrole (PPy) film. Such a method, easier to realize, assures high enzyme stability, fast response time as well as anti-interferent and anti-fouling properties. The ChO biosensor has been employed to assay PLD and ChE in real matrices. In order to optimize the sensor response towards the enzyme to be assayed, the influence of experimental variables such as pH of buffer solution, rotation rate of the electrode and substrate concentration has been studied.

ENZYMES ASSAY BASED ON ChO BIOSENSOR

- The assay method developed is performed by immersing the sensor, assembled on a rotating disk electrode, in an electrochemical cell where a substrate of the enzyme to be assayed is added .
- Then an aliquot of enzyme standard solution is injected which hydrolyzes the substrate thus producing free choline. Choline oxidase immobilized at the electrode surface, catalyzes the oxidation of choline 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 within a certain time. From the slope of such current signal, enzyme activity in the cell solution can be computed after having opportunely calibrated the sensor.



$$\text{Substrate} \xrightarrow{\text{PLD or ChE}} \text{Ch}$$

$$\text{Ch} + 2O_2 + H_2O \xrightarrow{\text{ChO}} \text{Betaine} + 2H_2O_2$$

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \text{ (at the electrode)}$$

ChO BIOSENSOR

Realization and characterization of Pt/PPy/ChO biosensor

- A PPy film was electrosynthetized at a constant potential of +0.7 V versus SCE, until a deposition charge of typically 300 mC/cm² was achieved. The Pt/PPy modified electrode was overoxidized at +0.7 V versus SCE in phosphate buffer (pH 7.0) until a steady-state background current was obtained (at least 7 h).
- Choline biosensors were prepared in accordance with the following procedure[7]: 300 µl of a phosphate buffer (I 0.1 M, pH 6.5) solution containing 16 mg of BSA and 1 mg of ChO were carefully mixed with 30 µl of a 2.5% glutaraldehyde solution; 3 µl of the resulting solution were pipetted onto the Pt/PPy working electrode surface and carefully spread out to cover the electrode surface completely. The electrode was then air-dried at room temperature for few minutes.

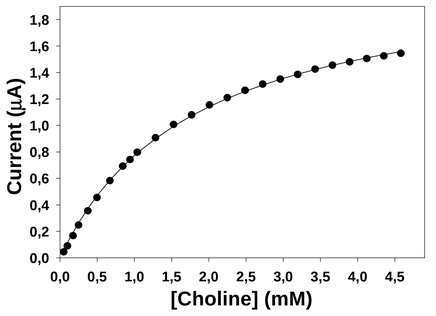


Figure 1 – Calibration curve realized in phosphate buffer at pH 6.5

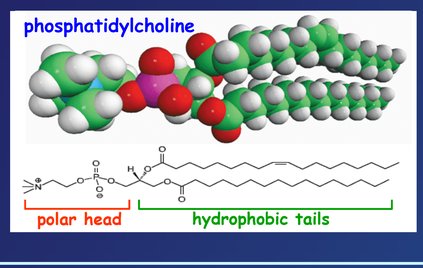
- **K'_M :** 2.18 ± 0.06 mM
- **Sensitivity:** 0.29 µA/(mM*mm²)
- **Linear range:** up to 0.5 mM
- **Response time:** 3 – 4 seconds
- **Long term stability:** the biosensor can be employed for more than 40 days without appreciable variation in sensitivity
- **Limit of detection:** 1µM (S/N =3)
- **Notable anti-interferent and anti-fouling properties**

PLD ASSAY

Experimental conditions:

- **buffer**: borate pH 8.0, I 50 mM
- **electrode rotation rate**: 200 r.p.m.
- **Ca^{2+} concentration**: 0.2 mM

  (PLD is a calcium dependent enzyme)
- **substrate concentration**: phosphatidylcholine 5 mM
- **surfactant**: Triton X-100 0.75% (V:V) (necessary to allow substrate dissolution in aqueous media with subsequent vescicles formation)



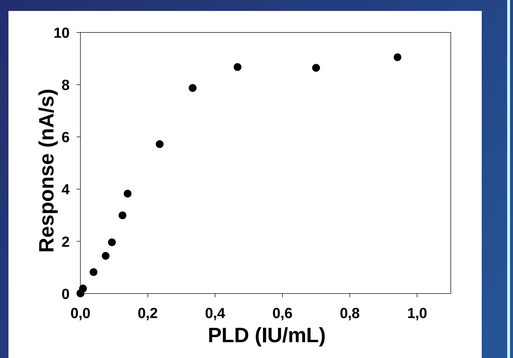


Figure 2 – Calibration curve realized in borate buffer at pH 8.0

- Sensitivity: 24 (nA/s)·(mL/IU)
- Linear range: up to 0.33 IU/ml
- Lowest detectable activity: 8.3×10^{-5} IU/ml

ChE ASSAY

There are two major types of ChE: true or acetylcholinesterase **(AChE)** and pseudo, or serum or butyrylcholinesterase **(BChE)**. They can be distinguished by their relative abilities to hydrolyse different choline esters.
AChE cleaves acetylcholine most actively and is much less potent in hydrolysing other alkyl esters.
BChE exhibits much less substrate specificity as it is able to hydrolyse acetylcholine (almost as well as AChE) as well as many other alkyl choline esters.

Experimental conditions:

- buffer: phosphate pH 8.0, I 0.1 M
- electrode rotation rate: 200 r.p.m
- substrate concentration at maximum activity:

- AChE: acetylcholine 1.50 mM
- BChE: acetylcholine 2.50 mM, butyrylcholine 1.75 mM, s-butyrylthiocholine 1.75 mM

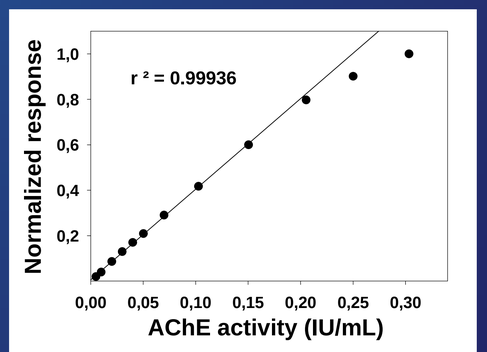


Figure 3 – Calibration curve for **acetylcholine**

AChE – acetylcholine

- sensitivity: 4.05 $(IU/mL)^{-1}$
- linear range: up to 0.20 IU/mL

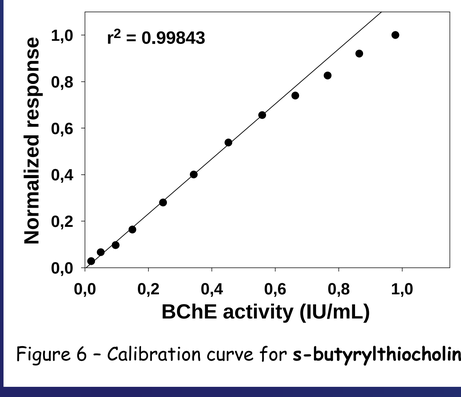


Figure 6 – Calibration curve for **s-butyrylthiocholine**

BChE – butyrylthiocholine

- sensitivity: 1.18 $(IU/mL)^{-1}$
- linear range: up to 0.60 IU/mL

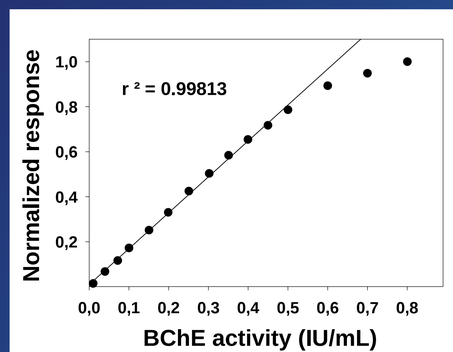


Figure 4 – Calibration curve for **acetylcholine**

BChE – acetylcholine

- sensitivity: 1.60 $(IU/mL)^{-1}$
- linear range: up to 0.50 IU/mL

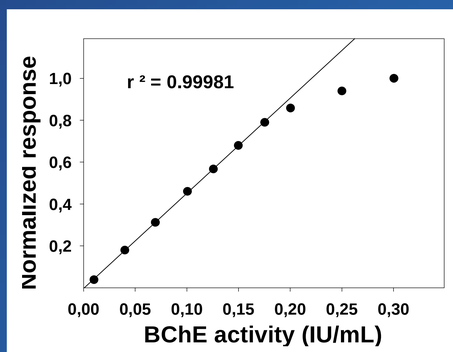


Figure 5 – Calibration curve for **butyrylcholine**

BChE – butyrylcholine

- sensitivity: 4.55 $(IU/mL)^{-1}$
- linear range: up to 0.18 IU/mL.

Application: BChE assay in sera

In order to perform BChE electrochemical assay in real serum samples by using the developed biosensor, s-butyrylthiocholine was employed, being the substrate reported in the spectrophotometric reference procedure[4]

Serum sample	BChE activity (IU/mL) by spectrophotometric assay	BChE activity (IU/mL) by electrochemical assay
1[a]	3,48 ± 0,09[c]	3,38 ± 0,04[d]
2[b]	4,22 ± 0,12[c]	4,15 ± 0,05[d]
3[b]	4,17 ± 0,11[c]	4,22 ± 0,05[d]
4[b]	3,58 ± 0,11[c]	3,61 ± 0,04[d]

[a] Control Serum from Sigma
[b] Serum Samples from Hospital
[c] Mean ± Standard Deviation (n = 3)
[d] Mean ± Standard Deviation (n = 3)

The precision and accuracy of the electrochemical method are comparable with those obtained by spectrophotometric techniques

CONCLUSIONS

An amperometric biosensor for the determination of PLD and ChE activity based on choline oxidase immobilized by co-crosslinkling on a platinum electrode previously modified by an overoxidised polypyrrole film has been developed. The influence of some experimental parameters (i. e. pH, rotation rate of the electrode, type and concentration of substrate) on the sensor behaviour has been studied. The developed method is characterized by high sensitivity, wide linear range, fast response time and appreciable long term stability. Allowing polypyrrole layer to remove interference from electroactive compounds, the present method revealed suitable to assay ChE and PLD in real matrices at activities value respectively down to 5×10^{-4} IU/ml and to 8×10^{-5} IU/ml.

References

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