Potentiometric sensing of butyrylcholinesterase based on *in situ* generation and detection of substrates

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A novel reagentless biosensor has been developed in which the traditional ion selective electrode is used as a controlled-release system for *in situ* generation and detection of enzyme substrates.

Cholinesterase is one of the crucial enzymes responsible for the nervous system functioning. Some compounds such as natural and synthetic drugs and pesticides are known to be reversible and irreversible inhibitors of cholinesterases.[1] Accordingly, many researchers are seeking to design highly sensitive biosensing systems for inhibitor determination and drug screening.[2] Nevertheless, manual addition of the substrate to the sample solution is always required before analysis. In this work, we report a reagentless biosensor for butyrylcholinesterase (BuchE) based on an ion-selective electrode (ISE).

During the past decade, ion transfer across the interface of two immiscible solvents has attracted significant attention especially in view of its applicability in the field of ISEs.[3] It has been fully realized that ions can be transported through polymeric membranes of ISEs when an external voltage or a current is applied, and even under zero-current conditions.[4,5] Ion fluxes across ion-selective electrode membranes have been found analytically useful, and most current applications are based on the ion fluxes of primary ions in the direction of the inner solution. Such examples include polyion sensors,[6] pulstrodes,[7] switchtrodes,[8] ion-channel biosensors,[9] electrodes sensitive to total ion concentrations[10] and ISE indicators for complexometric titrations.[11]

Herein, we present a novel detection system that makes use of outward ion flux through an ISE membrane, *i.e.* flux in the direction of the sample solution, to provide a controlled-release substrate for measuring the enzyme. The constant release of substrate under zero-current conditions from the inner solution into the sample solution generates a measurable potential signal *in situ*. However, the resulting ion flux induced membrane potential may be perturbed as a result of enzyme catalysis and thereby the activity of the enzyme can be detected.

To prove this concept, we first constructed an ISE for butyrylcholine (Buch).[12] Based on the toroidal cavity of trimethyl *o*-β-cyclodextrin (β-CD), and its molecular recognition for three types of interactions, *i.e.* direct N–H···O and N–C–H···O hydrogen bondings and van der Waals forces, β-CD was used as ionophore for the present electrode.[13] The emf response of the polymeric membrane electrode with 10^{-3} M Buch as inner solution indicated a Nernstian slope of 57.4 ± 0.5 mV per decade in the range of 10^{-1}–10^{-6} M Buch. The selectivity of such a β-CD based ISE was characterized by using Bakker's method to evaluate the influence of the discriminated ions, for which the electrode was conditioned in 10^{-3} M NaCl.[14] The logarithmic Nikolskii coefficients for Buch (K^{pot}_{BuchJ}) over choline (Cho) and chloride are -2.54 (± 0.04) and -5.93 (± 0.05), respectively. In the case of the membranes without β-CD, the corresponding values were -1.90 (± 0.04) and -4.90 (± 0.05). The better response of the electrode using trimethyl *o*-β-cyclodextrin as ionophore may be caused by its enhanced lipophilicity due to the three alkyl groups at the 2, 3 and 6 positions.[15]

In previous research, direct evidence of ion flux across an ISE membrane has been tracked by atomic absorption spectroscopy[16] or by scanning electrochemical microscopy.[17] In our system, the ion fluxes diffusing from the inner solution to the sample solution were monitored by UV/Vis absorption spectroscopy at 210 nm.[18] It was found that a constant release of Buch could be available after conditioning the electrode overnight. Buch at the sample–membrane interface, induced by ion flux from the inner solution, is detectable by the ISE. This is the basis for further enzyme determination. The concentration of released Buch at the electrode membrane surface can be readily modulated by the Buch concentration in the inner solution. As shown in Fig. 1, the surface Buch activity can be increased with increase of its concentration in the inner filling solution. For the present work, 10^{-2} M Buch was used as inner solution for the detection of enzyme and pesticide. In this case, the activity of Buch released at the sample–membrane phase boundary, which can be used to evaluate the detection limit of the ISE for Buch,[4] was measured as 3.2×10^{-6} M in 0.02 M of pH 7.4 sodium phosphate buffer solution (PBS). With respect to higher concentrations of ion-exchangers, thinner membranes and higher contents of polar plasticizers, which could promote the ion flux from the inner filling solution into the sample solution and therefore cause higher substrate concentrations at the sample–membrane interface, the detection method can be further controlled. In addition, the ion flux of primary ions in the direction of the outer solution could be modulated by an external voltage or a current.[19]

BuchE, as a target enzyme for pesticide determination and drug screening, can catalyze the hydrolysis of Buch. The ISE membrane potential generated by the surface Buch may therefore be sensitive to BuchE. When the electrode is

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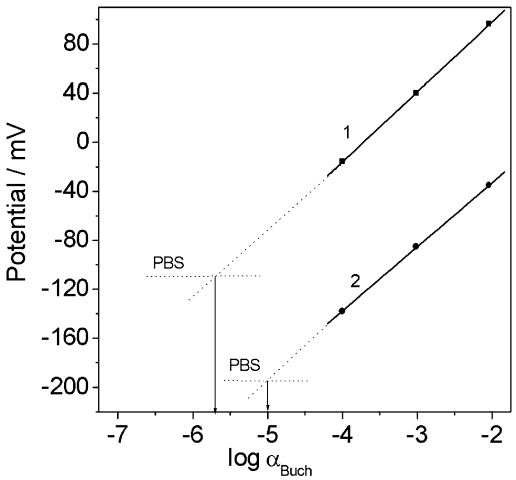

Fig. 1 Response of the ISE to Buch and 0.02 M PBS with different Buch concentrations in the inner filling solutions: (1) 10^{-4} M and (2) 0.1 M Buch.

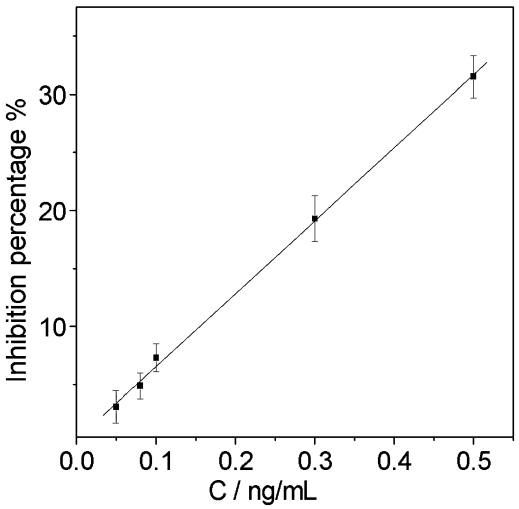

Fig. 3 Calibration curve of the ion selective electrode for detection of parathion.

immersed into the BuchE solution, the ion flux is disturbed due to the hydrolysis of Buch. The potential change, which serves as an indication of the enzyme activity, can be measured by the electrode. As can be seen from Fig. 2, with increasing enzyme concentration, the measured potential decreased due to the decrease of substrate concentration at the sample–membrane interface.[20] More importantly, detailed experimental results revealed that there was a linear dependence of the potential difference (ΔE) on the concentration of enzyme (see inset in Fig. 2). It should be noted that since the ion flux is controllable, the sensor sensitivity and dynamic range for BuchE may be further improved.

To test the application of the sensor, parathion, which is widely tested as a representative organophosphate pesticide, was determined.[21] As shown in Fig. 3, the inhibition of parathion on BuchE was proportional to its concentration in the range of 0.05–0.5 ng mL^{-1} (r = 0.995) with a detection limit of 0.03 ng mL^{-1} (3σ). The detection limit was one order of magnitude lower than those reported by other researchers.[22]

In conclusion, the present study has introduced a method for *in situ* generation and detection of enzyme substrates by ISE. This new technique allows the detection of both free and labeled cholinesterase in biosensors and enzyme immunoassays at ultralow concentrations. The practical advantages of the method include the ease of construction and reagentless design for biosensors. It is possible to localize the enzyme on the ISE membrane surface to develop a controllable and reagentless biosensor for pesticide.

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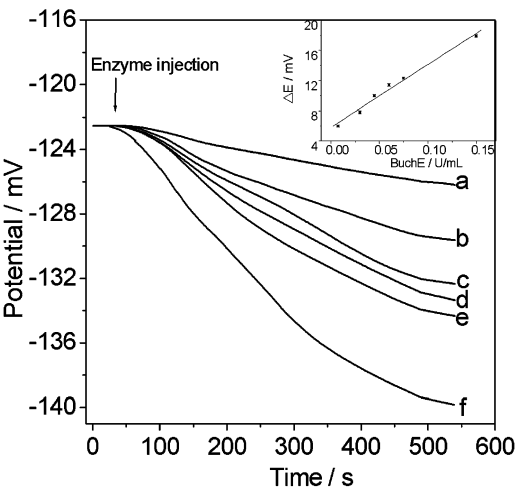

Fig. 2 Potentiometric response of the ion selective electrode in 0.02 M PBS upon addition of increasing concentrations of BuchE: (a) 0.0075, (b) 0.03, (c) 0.045, (d) 0.06, (e) 0.075 and (f) 0.15 U ml^{-1}. The inset is the calibration curve of the ISE for BuchE.

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12 The butyrylcholine selective membranes contained 1 wt% sodium tetrakis[3,5 bis(trifluoromethyl)phenyl]borate, 1 wt% 2,3,6-trimethyl *o*-β-cyclodextrin (β-CD), 65 wt% 2-nitrophenyl octyl ether and 33 wt% high molecular weight poly(vinyl chloride). Each electrode membrane had a thickness of *ca.* 200 μm and a diameter of 6 mm. All measurements of emf were performed at 25 ± 1 °C using a PXSJ-216 pH meter (Shanghai, China) with a saturated calomel electrode as reference electrode. The activities of butyrylcholine were based on the activity coefficient γ, as calculated from the modified Debye–Hückel equation: $\log\gamma = -0.511Z^2[\mu^{1/2}/(1 + 1.5\,\mu^{1/2}) - 0.2\,\mu]$, where μ is the ionic strength and Z is the valence. Before measurements, all the electrodes were conditioned in a solution identical to the inner filling solution overnight. For determination of enzyme and pesticide, 0.02 M pH 7.4 sodium phosphate buffer solution (PBS) and 10^{-2} M Buch were used as sample buffer and inner filling solution, respectively.
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20 The ISE potential was first measured in 0.02 M PBS to obtain a baseline. After addition of BuchE into the buffer solution, the potential change was recorded with time. The potential difference (ΔE) between the baseline and the potential measured at 8 min after enzyme addition was used for quantification of enzyme activity.
21 The inhibition of parathion was calculated as follows: inhibition (%) = $100\% \times (E_{\text{pesticide}} - E_{\text{enzyme}})/(E_{\text{basline}} - E_{\text{enzyme}})$, where E_{baseline} is the ISE potential measured in 0.02 M PBS, $E_{\text{pesticide}}$ and E_{enzyme} are the potentials measured at 8 min after adding 0.03 U mL^{-1} BuchE into 0.02 M PBS with and without parathion, respectively.
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