



Development of a quantitative relationship between inhibition percentage and both incubation time and inhibitor concentration for inhibition biosensors—theoretical and practical considerations

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Abstract

Theoretical and practical insights into the design and development of immobilised enzyme inhibition biosensors are reported. A general mathematical expression relating the percent of enzyme inhibition (i.e. the analytical signal) to both the inhibitor concentration and the incubation time is presented. The relevant physical, chemical and biochemical parameters required by the model are developed and discussed in terms of the inhibition of acetylcholinesterase by the organophosphorous pesticide, paraoxon. A second enzyme, choline oxidase and an amperometric transducer are used to facilitate the determination acetylcholinesterase inhibitor. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Biosensors based on the inhibition of acetylcholinesterase (AChE) have been used for the determination of organophosphorous pesticides in the environment (Kumaran and Morita, 1995; Trojanowicz and Hitchman, 1996; Barceló and Hennion 1997; Jeanty and Marty, 1998; Mulchandani et al., 1999). These methods generally require a quantitative measurement of AChE activity or inhibition percentage as the basis of the analytical method. Numerous inhibition biosensing devices based on AChE have been reported. Both single enzyme systems (AChE) (Shi and Stein, 1996) and dual enzyme systems (AChE coupled with choline oxidase) (Fennouh et al., 1997) have been reported. These biological components can be coupled with optical or electrochemical transducers to form a biosensing device (Durand and Nicaud, 1984; Tran-Minh, 1985, 1993; Kumaran and Tran-Minh, 1992; Sklada, 1992; Wollenberger et al., 1992; Cremisini et al., 1995; Evtugyn et al., 1998, 1999).

Despite the large interest in inhibition biosensors, there appears to be confusion in the literature over what type of responses or quantitative relationships are expected from immobilised enzyme inhibition biosensors. A variety of linear, non-linear, logarithmic and other relationships between inhibition percentage and either inhibition concentration and/or incubation time have been reported (Kumaran and Tran-Minh, 1992; Sklada, 1992; Palleschi et al., 1992; Bernabei et al., 1993; Cremisini et al., 1995; Pandey and Weetall, 1995; Shi and Stein, 1996; Trojanowicz and Hitchman, 1996; Hart et al., 1997; Fennouh et al., 1997; Evtugyn et al., 1999). This confusion arises because there are no clear-cut 'rules' for the physical, chemical and biological aspects of biosensor design and fabrication—different responses/relationships appear to be obtained and/or expected from different biosensor configurations. Depending on the exact configuration of the biosensor device and the exact experimental conditions under which the devices are used, a variety of responses may be obtained.

Generally, the inhibition reaction during the incubation step of the biosensing process can be described as:



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where E represents the enzyme, I represents the inhibitor and EI refers to the inhibited enzyme complex.

Recently, we developed a theoretical model for inhibitor diffusion controlled enzyme inhibition of immobilised enzyme biosensors (Zhang et al., 2000a). The model was derived from Fick's Law of diffusion using three boundary conditions:

$$[I(x, 0)] = [I]_B \quad (2)$$

$$\lim_{x \rightarrow \infty} [I(x, t)] = [I]_B \quad (3)$$

$$[I(0, t)] = 0, \quad (t > 0) \quad (4)$$

where x is the distance from the interface of the immobilisation zone.

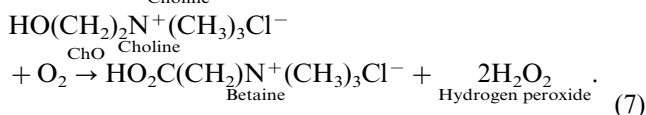
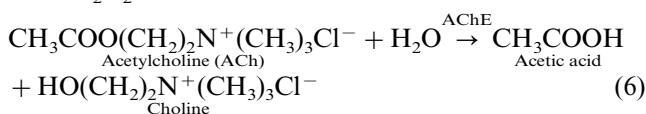
The mathematical expression relating inhibition percentage to inhibitor concentration and incubation time can be written as (Zhang et al., 2000a):

$$I\% = \frac{\{EI\}}{\{E_0\}} \times 100 = Kt^{1/2}[I]_B \times 100 \quad (5)$$

where $\{EI\}$ is the total amount of the inhibited enzyme complex formed at the incubation time t with an inhibitor concentration of $[I]_B$ using an initial enzyme loading of E_0 . At a given temperature, K is a constant and is dependent on the surface area, the diffusion coefficient of inhibitor and the initial enzyme loading.

Eq. (5) is a quantitative relationship between inhibition percentage and both the inhibitor concentration and incubation time. This equation demonstrates that the inhibition percentage is directly proportional to the inhibitor concentration and square root of incubation time. Importantly, it depends only on those parameters that affect the incubation process and is independent of how the enzyme activity is actually determined.

The relationship between the enzyme activity and the measured analytical signal is normally determined using an appropriate transducer. One way to do this is to use an amperometric choline sensor to determine the amount of choline produced from the enzymatic hydrolysis of acetylcholine (see Eqs. (6) and (7)). Choline oxidase (ChO) can be co-immobilised with AChE and will turnover the choline produced in Eq. (6) to produce H_2O_2 .



The H_2O_2 produced can be detected amperometrically and the current signal (i) generated is directly proportional to the amount of active AChE $\{E\}$. Since $\{EI\} = \{E_0\} - \{E\}$ and i_0 and i_1 are directly proportional to $\{E_0\}$ and $\{E\}$, respectively, Eq. (5) can there-

fore be written as:

$$\begin{aligned} I\% &= \frac{\{EI\}}{\{E_0\}} \times 100 = \frac{\{E_0\} - \{E\}}{\{E_0\}} \times 100 = \frac{i_0 - i_1}{i_0} \times 100 \\ &= Kt^{1/2}[I]_B \times 100 \end{aligned} \quad (8)$$

Eq. (8) describes the quantitative relationship between the amperometric current signal and both inhibitor concentration and incubation time. In this equation, $I\%$ can be replaced by $RI\%$ (see Eq. (10)) when a successive incubation procedure is used.

It is important to note that Eq. (8) is only valid under certain conditions, namely when the reaction described by Eq. (6) is carried out under enzyme kinetic control and the reaction described by Eq. (7) is carried out under choline diffusion control.

The aim of this paper is to show how inhibition biosensor fabrication can be standardised by imposing certain physical, chemical and biochemical restrictions on biosensor design to obtain the relationships expected from the above model. The determination of the organophosphorous pesticide, paraoxon, was used as an example, as this is well known to inhibit AChE. AChE and ChO were selected for the fabrication of the sensing probe. The detection was based on the measurement of AChE activity and it was performed via an amperometric measurement of the hydrogen peroxide produced (Fig. 1). The effect of the experimental parameters on the validity of the model was investigated.

2. Experimental

2.1. Reagents and materials

All reagents were of analytical grade and used as received unless otherwise stated. All solutions were prepared with Milli-Q water. AChE (500 U/mg), choline oxidase (16 U/mg), acetylcholine chloride (ACh), choline chloride and diethyl *p*-nitrophenyl phosphate (90%, paraoxon) was obtained from Sigma. Polyurethane polyethylene oxide (PU-PEO) water dispersion (40% w/w) was a gift from IPRI, University of Wollongong, Australia. Platinum wires (99.99%) with a diameter of 50 μm were obtained from Goodfellow, UK. Dialysis membranes (MWCO: 12000–14000 Da) were commercially supplied by Medicell International Ltd.

2.2. Instrumentation

All amperometric experiments were carried out using a BAS LC-4C Amperometric Detector (Bioanalytical Systems, USA). All electrochemical data were recorded using Maclab/4e Analog/Digital interfaced with a

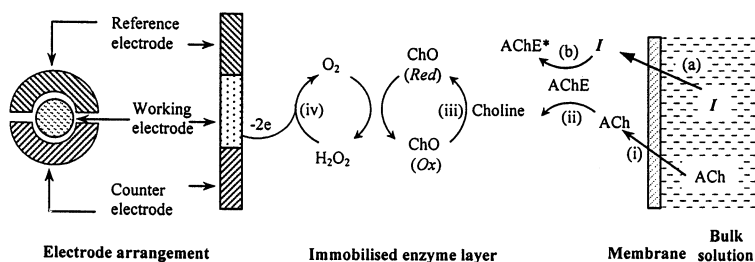


Fig. 1. Schematic of the detection mechanism. The inhibition process involves: (a) diffusion of the inhibitor across a membrane to the reaction zone; (b) inhibition of AChE by the inhibitor to produce the inhibited enzyme complex AChE*. The enzyme activity detection process involves: (i) diffusion of ACh to the reaction zone; (ii) AChE catalysed hydrolysis of ACh; (iii) ChO catalysed oxidation of choline in the presence of O_2 to produce H_2O_2 ; (iv) electrochemical oxidation of H_2O_2 . I represents the inhibitor. Ox and Red refer to the oxidised and the reduced forms of ChO, respectively.

Power Macintosh computer with Chart 3.5 software from AD Instruments, Australia. A single channel peristaltic pump (Pharmacia LKB Pump P-1) and a 6-valve injector were used in the flow injection analysis (FIA) measurements. An 89 μ l sample loop was used unless otherwise indicated.

2.3. Construction of a freestanding biosensing probe

All electrochemical experiments were conducted using a special homemade, freestanding biosensing probe. A platinum microdisc array ($50 \times 50 \mu$ m diameter disks) was employed as the working electrode. An Ag/AgCl reference electrode and a platinum auxiliary electrode were also employed. All three electrodes were integrated onto a planar surface with a diameter of 5.0 mm (Fig. 1). A very thin PU-PEO immobilisation layer ($< 10 \mu$ m) containing appropriate amounts of AChE and ChO, was cast on the tip of the sensing probe. A dialysis membrane was then placed on the top of the probe to form the self-contained electrochemical cell (freestanding biosensing probe). The probe can be conveniently used in either batch or flow injection analysis mode.

2.4. Incubation procedures

In the case of batch measurement, a simple preincubation method was used where the biosensing probe was exposed to the incubation solution containing paraoxon for a given time before the resulting enzyme activity change was determined.

For flow injection analysis, the incubation was carried out using an on-line incubation method. A given volume of the incubation solution (paraoxon) was injected in between the substrate (ACh) sample injections. The incubation time was taken as the contact time of the inhibitor solution with the sensing probe surface, which can be calculated based on the injection volume and the flow rate employed. The injection volumes were

varied from 89–445 μ l when the effect of incubation time experiments were conducted.

2.5. Inhibition percentage calculation

In this work, the inhibition percentage was calculated based on Eq. (9) (Tran-Minh, 1985; Sklada, 1992),

$$I(\%) = 100 \frac{E_0 - E_1}{E_0} = 100 \frac{i_0 - i_1}{i_0} \quad (9)$$

where $I(\%)$ represent the inhibition percentage, E is the enzyme activity and i is the steady state current in batch measurements and the peak current in FIA measurements. The subscripts (0) and (1) correspond to measurements performed before and after incubation, respectively.

For successive incubation measurements, the relative inhibition percentage, $RI\%$ was calculated according to Eq. (10):

$$RI(\%) = 100 \frac{E_n - E_{n-1}}{E_n} = 100 \frac{i_n - i_{n-1}}{i_n} \quad (10)$$

where the subscripts represent the number of successive incubation periods.

3. Results and discussion

A set of boundary conditions were used (see Eqs. (2)–(4)) to develop the theoretical model represented by Eq. (5). Special consideration must be given to the sensor configuration before the requirements of these boundary conditions can be met. Once the inhibition percentage is related to the analytical signal generated from the amperometric transducer (see Eq. (8)), more restrictions are introduced. These restrictions include (i) the enzyme activity must be determined when the reaction described by Eq. (6) is under enzyme kinetic control; and (ii) the reaction described by Eq. (7) must be carried out under diffusion control. In other words, the concentration of the substrate, ACh, must be suffi-

ciently high to ensure the former is limited by enzyme kinetics and the amount of ChO immobilised must be high enough to ensure the latter is limited by choline diffusion. To satisfy these requirements, sensor configuration and experimental parameters, such as the type of working electrode, the arrangement of the electrochemical cell, the type of immobilisation matrix, the thickness of the immobilisation layer, the amount of enzyme loading for AChE and ChO and other chemical and electrochemical conditions, must be optimised before the expected quantitative relationship can be obtained. The optimisation strategy to satisfy the proposed model is discussed in the following sections.

3.1. Sensor configuration

The performance and the behaviour of the sensor device is strongly dependent on its configuration. An all-in-one enzyme based biosensing system was previously developed (Zhang et al., 2000b). In order to determine the inhibitor concentration, a freestanding biosensing probe (Fig. 1) was developed by modifying the all-in-one biosensing system. This probe has several features that improve upon existing systems. It is a self-contained sensing device—the entire electrochemical cell (including buffer and enzymes) resides within a well-defined hydrophilic, enzyme friendly, immobilisation matrix protected by a dialysis membrane. The geometrically fixed three-electrode configuration ensures the electrochemical characteristics of the sensing probe are fixed at the time of fabrication. This is crucial for obtaining reliable, reproducible responses in any dynamic electrochemical process. Another advantage of the probe is that it can be readily incorporated into stationary or flow systems for different applications.

3.1.1. Selection of the immobilisation matrix

The nature of the enzyme immobilisation matrix and the formation of the immobilisation layer greatly affect the enzyme activity and the analytical properties of the biosensing system. A water-based PU-PEO colloidal dispersion was selected as the immobilisation matrix in accordance with our previous work (Zhang et al., 2000b) and that of others (Hintsche et al., 1989; Shin et al., 1998). The selection was based on the criteria of physical and chemical stability, uniformity of enzyme distribution and water content within the matrix. The PU component serves to improve the adhesion and uniformity of the immobilisation layer (Hintsche et al., 1989; Shin et al., 1998), while the PEO, a well-known polyelectrolyte, was used to ensure homogenous distribution of enzyme and a high water content within the immobilisation layer. This latter point is crucial for the kinetics of the sensing process and for enzyme activity.

3.1.2. Selection of the membrane

The boundary condition shown in Eq. (4) (diffusion limited inhibition process) is an essential condition for the model. To ensure this condition is satisfied under a variety of experimental conditions, a membrane was introduced into the present configuration. A range of membranes was tested. The experimental results showed that fast response time and high sensitivity could be achieved only when hydrophilic membranes, such as dialysis membranes, were employed. When the pore size of the dialysis membrane was small (e.g. 1200 Da), sensitive detection could not be achieved, even with long incubation times. Another drawback of small pore size membranes was that a high concentration of ACh (i.e. > 20 mM) was required to satisfy the kinetic control condition for the acetylcholine hydrolysis reaction catalysed by AChE (Eq. (6)). It was found that a dialysis membrane with pore size of 12000–14000 Da was most suitable for this work. With this membrane, the diffusion limited incubation process can be readily achieved. Moreover it only required 1.0 mM of ACh (see later) to satisfy the kinetic control condition for the ACh hydrolysis reaction. A rapid response time (typically ≈ 40 s) was observed.

3.1.3. The microelectrode array working electrode

Biosensors employing amperometric transducers often suffer from high initial background currents and long equilibration times (the time required to reach the steady state). It is well known that microelectrodes have several advantages over conventional sized electrodes, e.g. high signal to noise ratio and short time to reach the steady state (Morf and Rooij, 1997). In order to improve the signal to noise ratio and to minimise equilibration time, a microelectrode array (see Fig. 2) was incorporated into the present configuration for the fabrication of the freestanding biosensing probe. The reason for using an array of microelectrodes is to maximise the analytical signal while still maintaining the inherent advantages of a single microelectrode. In this work, a series of microelectrode arrays ranging from a single 50 μm electrode up to 50 \times 50 μm electrode arrays were used. A linear relationship between the number of microelectrodes and the current response was observed (Fig. 3). This suggests that the characteristics of a single microelectrode were maintained by the arrays. Consequently a 50 \times 50 μm array was used for the majority of this work. When compared with a conventional macroelectrode, the results show that to detect sub-micromolar concentrations of substrate, the amperometric transducer required more than 3 h to reach a low enough background current to enable sampling of the current signal. The microelectrode array, on the other hand, took < 10 min to reach steady state.

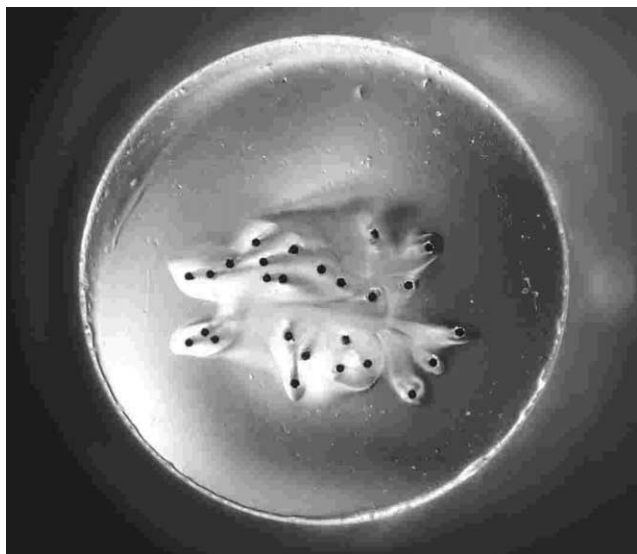


Fig. 2. Photograph of $26 \times 50 \mu\text{m}$ Pt microdisc array. External diameter = 3.3 mm.

3.2. Optimisation of chemical, electrochemical and biochemical conditions

3.2.1. Optimum pH

The pH was varied from 4.0 to 10 and a maximum response obtained between 6.5 and 7.5. It was found that the choline sensor lost its activity when the pH was < 6.0 and the hydrolysis of paraoxon occurred when pH was > 8.0 . These results agree with previous reports (Palleschi et al., 1992; Cremisini et al., 1995). Therefore, pH 7.0 buffer solution was selected for subsequent experiments.

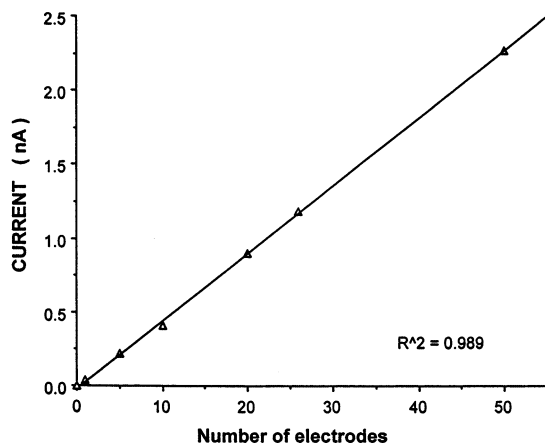


Fig. 3. Electrochemical behavior of different number of platinum microdisc array in batch measurement mode. 1.0 U of ChO and 0.02 mg of PU-PEO, 0.10 M NaCl and phosphate buffer (pH 8.0), +0.60 V versus Ag/AgCl. The current was obtained with 10 μM choline.

3.2.2. Optimum potential

The optimum potential for the detection of H_2O_2 was then obtained by varying the applied potential. A maximum current was generated when a potential of +0.60 V versus Ag/Ag/Cl was applied. This again agreed with previously reported results (Palleschi et al., 1992; Cremisini et al., 1995).

3.2.3. Optimum biochemical conditions

In order to obtain the expected quantitative relationship (see Eq. (8)), the biochemical conditions have to be optimised according to the conditions imposed by the assumptions of the model. The relationship between the inhibitor concentration and both the incubation time and the inhibition percentage are established during the incubation process. These relationships are largely determined by the transport properties of the membrane. The expected linear relationship (Eq. (5)) was achieved by employing a suitable membrane, which ensured the incubation process was under inhibitor diffusion control. The relationship given in Eq. (5) does not show the relationship between the measured analytical signal and percentage of inhibition, since this was independent of the type of transducer used.

The relationship between the measured analytical signal and the percentage of inhibition is established during the detection process. This relationship is dependent on the type of transducer used and how the measurement conditions of analytical signal are carried out. For the transducer used in this study, the expected relationship between the inhibition percentage and current signal (Eq. (8)) can be obtained only when the current was measured under the following conditions:

1. ACh hydrolysis reaction catalysed by AChE (Eq. (6)) must be carried out under the kinetic controlled condition; and
2. Choline oxidation reaction catalysed by ChO (Eq. (7)) must be performed under diffusion controlled conditions.

To satisfy these conditions, parameters, such as AChE and ChO loading and substrate concentration must be optimised accordingly.

Experiments were performed to establish the relationship between AChE loading and inhibition percentage when ACh concentration and incubation time were varied. Fig. 4(a) shows the relative inhibition percentages ($RI\%$) obtained after the first and second 5 min of incubation in 100 nM paraoxon for an AChE loading of 0.0025 U. It was found that $RI\%$ observed after the second incubation was much greater than that observed after the first incubation. Furthermore, $RI\%$ observed after the first 5 min of incubation was found to be ACh concentration dependent (curve 1). This indicated that the ACh hydrolysis reaction was not fully controlled by the AChE kinetic and excess enzyme activity existed within the immobilisation layer. $RI\%$ obtained after the

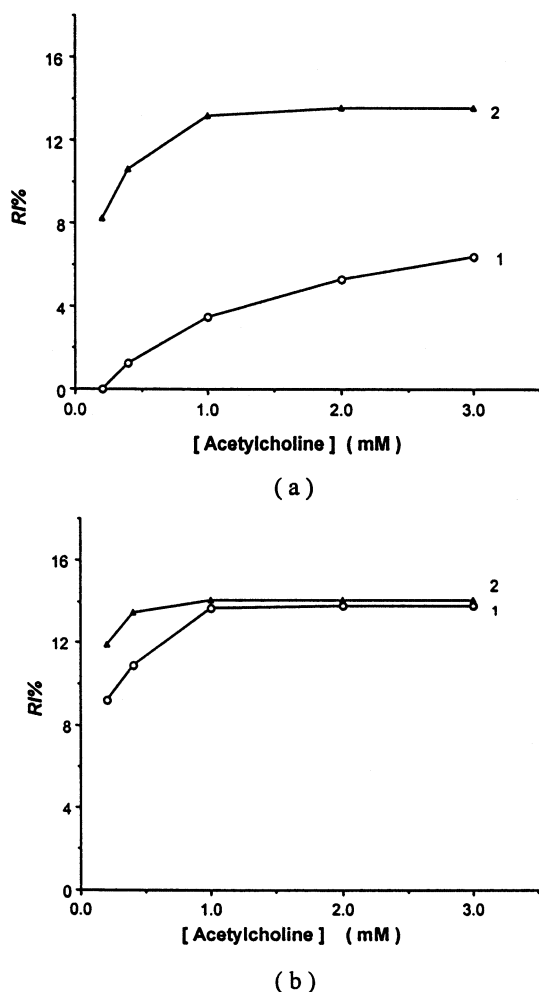


Fig. 4. Effect of ACh concentration and AChE amount on the detection of AChE activity: (a) 0.0025 U of AChE immobilized; (b) 0.00025 U of AChE immobilized. Curve 1 represents the first 5 min incubation in 100 nM paraoxon; curve 2 represents the second 5 min incubation in 100 nM paraoxon. $50 \times 50 \mu\text{m}$ microarray was used. Other conditions were as in Fig. 3.

second 5 min incubation was saturated (independent of ACh concentration) when the ACh concentration was >1.0 mM indicating a full enzyme kinetic control process (curve 2). When the enzyme loading was decreased 10-fold (to 0.00025 U), the incubation behaviour observed was quite different (Fig. 4b). For ACh concentration >1.0 mM, no significant difference was observed between RI% obtained after the first and second incubation periods. This suggests that with low enzyme loading, the first incubation was sufficient to eliminate all excess enzyme activity within the immobilisation layer. This is very important for the detection of inhibitor since the quantitative relationship between the enzyme activity (or $I\%$) and the concentration of inhibitor can only be obtained when the excess enzyme activity is completely eliminated (Tran-Minh, 1985, 1993; Evtugyn et al., 1998). Fig. 4(b) shows that en-

zyme kinetic control (saturated RI%) was achieved when the concentration of acetylcholine was >1 mM. Consequently, an AChE loading of 0.00025 U and acetylcholine concentration of 1.0 mM was selected for the subsequent experiments.

In this work, the AChE activity change was monitored according to the concentration change of choline, which was produced by the ACh hydrolysis reaction (Eq. (6)) under AChE kinetic controlled condition. The concentration of choline was determined indirectly by measuring the H_2O_2 oxidation current when the choline oxidation reaction, catalysed by ChO (Eq. (7)), was performed under diffusion controlled conditions (see Fig. 1). In order to obtain the diffusion limited current, the ChO loading must be sufficient to rapidly convert all the choline produced by the ACh hydrolysis reaction. When a ChO loading of 1.0 U was used, a linear current response was obtained up to the $400 \mu\text{M}$ of choline. With our sensing system, when AChE loading was fixed at 0.00025 U and ChO loading was varied from 0.5 to 5.0 U, a 50 nA saturated current response (diffusion limited current) was observed in the presence of 3 mM ACh. This result indicated that 1.0 U of ChO loading is sufficient to convert the choline generated by 0.00025 U of AChE from 3 mM of ACh. The saturated current response of 50 nA in this case represented $190 \mu\text{M}$ choline, which was definitely within the linear range of choline detection. Consequently, a ChO loading of 1.0 U was therefore used for all further experiments.

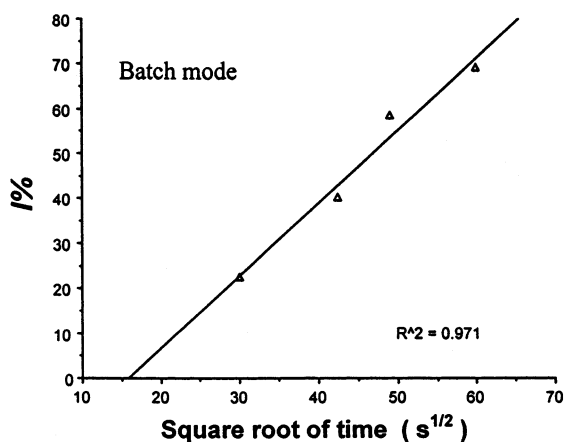
3.3. Validation of quantitative relationships

After the sensing system had been optimised to satisfy the conditions imposed by the proposed model, the expected quantitative relationships were examined. Fig. 5 shows the experimental results obtained from batch and flow measurement modes under the optimal conditions. In both cases, $I\%$ was calculated from the amperometric responses using Eq. (9). These results agreed with the proposed model (see Eq. (8)). A linear relationship between inhibition percentage and the square root of the incubation time was obtained, indicating the optimisation strategy used was appropriate.

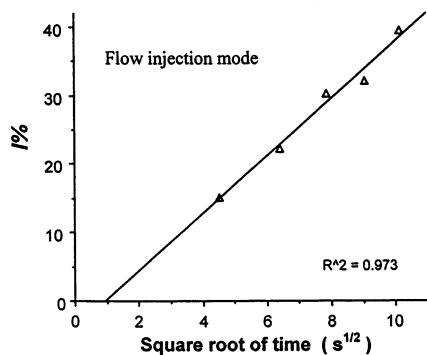
The relationship between the inhibition percentage ($I\%$) and the concentration of inhibitor ($[I]_B$) was also examined. Figs. 6 and 7 show the typical amperometric current profiles and the calibration curves (i.e. a plot of RI% versus paraoxon concentration) for measurements carried out in batch and flow analysis modes. In both cases, Eq. (10) was used to calculate RI% since the successive incubation procedure was employed. A simple linear relationship between RI% and $[I]_B$ was observed in both analysis modes. These results agreed with the proposed model (see Eq. (8)) and again confirmed the optimisation strategy used was successful.

4. Conclusion

An optimisation strategy for immobilised enzyme inhibition biosensors was presented. The important considerations of inhibition biosensor design, configuration and optimisation have been addressed. The simple linear relationship between the inhibition percentage and the inhibitor concentration can be obtained only when the sensor configuration enables the inhibition reaction take place under inhibitor diffusion controlled conditions. Additionally, predicted relationships between the measured signal and inhibition percentage (see Eq. (8)) can only be achieved when the AChE catalysed choline production (from ACh) is carried out under kinetic controlled condition—i.e. the amount of choline produced depends only on AChE activity, not



(a)



(b)

Fig. 5. (a) Relationship between inhibition percentage and the incubation time in batch measurement mode. The inhibition of AChE was carried out in a pH 7.0 (0.1 M NaCl and 0.1 M phosphate) buffer solution containing 100 nM paraoxon. Some 0.00025 U of AChE and 1 U of ChO were immobilised. The enzyme activity was determined in a pH 7.0 (0.1 M NaCl and 0.1 M phosphate) buffer containing 1.0 mM ACh. Other conditions were as in Fig. 4. (b) Relationship between inhibition percentage and the incubation time in flow injection analysis mode. The concentration of paraoxon used for the inhibition reaction was 1.0 μM . The incubation time was changed by varying the injection volume of the incubation solution at a constant flow rate of 0.25 ml/min. Other conditions were as in Fig. 5(a).

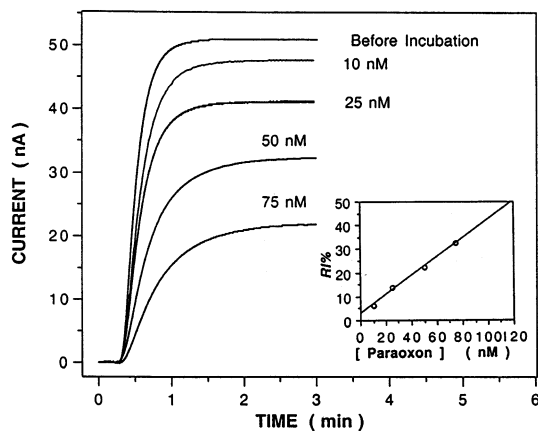


Fig. 6. Detection of paraoxon in batch measurement: 0.00025 U AChE immobilized. ACh (1 mM) was used to detect the residue AChE activity after the 30-min incubation of paraoxon (concentration as indicated). All other conditions were the same as in Fig. 5(a).

acetylcholine concentration. Finally, when ChO is used in conjunction with an amperometric transducer, the ChO loading must be sufficiently high to ensure that conversion of choline is controlled by choline diffusion and choline production must lie within the linear detection range of the amperometric transducer. In this work, using the particular probe design discussed, this was achieved by having an AChE loading of 0.00025 U, ACh concentration of 1.0 mM and ChO loading of 1.0 U. This allowed paraoxon determination down to 10 nM.

In summary, for inhibition biosensors, the quantitative relationships expected between inhibition percentage and either incubation time and/or inhibitor concentration will depend on a variety of physical, chemical and biochemical parameters associated with

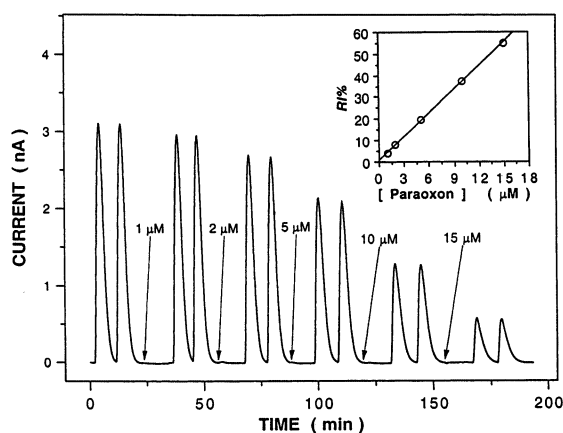


Fig. 7. Flow injection amperometric current responses resulting from ACh injections before and after exposure to paraoxon. Paraoxon (89 μl) was injected at the indicated concentrations. Other experimental conditions were as in Fig. 5(b). The relationship between relative inhibition percentage and inhibitor concentration is shown in the insert.

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