Sol–gel cholinesterase biosensor for organophosphorus pesticide fluorimetric analysis

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Abstract

Sol–gel crystals derived from tetramethyloxy silicate (TMOS) doped with cholinesterase using microencapsulation have been prepared. Organophosphorus pesticides (ORPs) are determined by fluorimetric detection based on enzymatic inhibition of immobilized cholinesterase; the percentage inhibition is correlated to pesticide concentration. The working substrate concentration (indoxyl-acetate) for ORP determination, $1.63 \times 10^{-4}$ M, is optimized by selecting a linear range in a plot of substrate concentration against signal. Precision as a relative standard deviation (RSD) is 10.2% ($n=7$) for the working substrate concentration. Fenitrothion, azinphos-ethyl, methidation, naled and mecarbam are analysed. Calibration plots have been established by measuring in a cuvette and through an optical fibre modified at its end with the immobilized cholinesterase gel. The biosensor performance is evaluated by measuring the percentage inhibition covering concentration ranges from $1.21-11.99$ µg ml$^{-1}$ (naled) up to $4.9-328.9$ µg ml$^{-1}$ (mecarbam). RSDs from 1.5 to 10.3% and detection limits from 0.12 (naled) to 57.6 µg ml$^{-1}$ (methidation) have been achieved. Mass production and good storage for one month of the immobilized enzyme render the gel disposable after a single use. ORP determination is fast (inhibition time of $5$ min) and the enzyme immobilization directly onto the end of the optical fibre enhances the sensitivity.

Keywords: Cholinesterase; Organophosphorus pesticides; Sol–gel methods; Optical fibres; Fluorimetry

1. Introduction

Chemical sensors are defined as miniaturized transducers that selectively and reversibly respond to chemical compounds or ions, yielding an electrical signal which depends on an analyte concentration [1]. One only has a true sensor if concentration changes in both directions can be detected. These conditions are not generally satisfied by devices such as indicator tubes, test strips and single-shot tests [2]. However, these systems are often discussed in the context of biosensor capabilities and future directions.

Biosensors can be regarded as a subgroup of chemical sensors in which a biologically based mechanism is used for detection of the analyte, and they have a potentially large application field, covering the areas of clinical diagnostics, process control, food, military and environmental monitoring. In the area of environmental monitoring there is a need for reducing expense in situ and continuous monitoring technologies. Analytes of interest include compounds such as pesticides, polychlorinated biphenyls, phenols and polycyclic aromatic hydrocarbons [3].

Enzymes are widely used as biological recognition elements of biosensors [4–8]; inhibitors of enzyme activity have been detected by their effect on the biosensor signal established in presence of the substrate. Thus, organophosphorus pesticides (ORPs) can be detected using the enzymatic inhibition of acetylcholinesterase (AChE) or cholinesterase (ChE). The immobilization of the enzymes dictates the reliability and performance of a biosensor. The maintenance of the catalytic activity of the enzyme and the mechanism of signal transduction are important considerations in the choice of immobilization method.

Chemical immobilization procedures of the enzymes involve the formation of a covalent bond between groups of an enzyme and a water-insoluble support or between two or more enzyme molecules [9]. Covalent attachment of proteins to a matrix requires that specific functionalities are present on the biomolecule, fixes the orientation of the exposed protein and does not preserve the integrity and directional homogeneity of the protein surface microstructure. Furthermore, these methods are tedious and time consuming. An ORP biosensor based on enzymatic inhibition of ChE or AChE by covalent immobilization on pre-activated membranes [10,11] or co-crosslinking [6,12,13] with potentiometric [6,10], amperometric [12,13] or optic detection [11] has been demonstrated.

Recent research has demonstrated that silicate glasses obtained by the sol–gel method can provide a host matrix for...
use in optical sensors [8,14–18]. The sol–gel material is chemically inert, thermally stable and transparent, enabling spectroscopic monitoring of the entrapped sample. Micro-encapsulation in the pores of the sol–gel matrix is independent of the functionalities on the protein, and does not significantly decrease the affinity and activity of biomolecules, because the enzyme is not really bound to the matrix. For enzymes, the absence of covalent interactions between the protein and the surrounding medium is of utmost importance for maintaining stability and reactivity. Silica biogels contain a sufficient amount of trapped interstitial water to provide essentially the same local aqueous microenvironment as in biological media, thereby contributing to the retention of the characteristics of the liquid state. The porous material permits permeation of the analyte into the sol–gel structure where the biological recognition reaction can occur [8]. The major disadvantage is that it requires a careful control of pore size to facilitate the accessibility of the analyte without leaching of the biological recognition element.

In this work, ChE is immobilized in a gel obtained by hydrolysis and condensation of tetramethyl orthosilicate (TMOS). The biosensor may be attached to the distal end of an optical fibre. ORP was determined by fluorimetric detection based on the inhibition of ChE. A non-fluorescent synthetic substrate (indoxyl-acetate) is hydrolysed by the enzyme to yield a highly fluorescent product (indoxyl). In the presence of the inhibitor, the rate of formation of this fluorescent product is lowered [19–21].

2. Experimental

2.1. Reagents

Cholinesterase was obtained from Merck (activity 50 U mg⁻¹; Ref. 2606). Stock standards were prepared in bidistilled water and stored at −40°C. Indoxyl-acetate was provided from Sigma (Ref. 13500). Substrate was prepared daily by dissolving 0.00725 g in 5 ml of dioxane.

The organophosphorus pesticides (ORPs) fenitrothion (96%, Ref. 45487) and azinphos-ethyl (98%, Ref. 35820) were obtained from Riedel de Hœn (Seelze, Germany); methidation (99%, Ref. Ch 51023), naled (98.6%, Ref. Ch 90426) and mecarbam (93.8%, Ref. Ch 10129) were from Dr S. Ehrenstorfer (Augsburg, Germany). Stock standards of 1000 µg ml⁻¹ concentration were prepared in methanol and stored at 4°C.

Tetramethyl orthosilicate (TMOS) was provided from Aldrich (grade 99%, Ref. 34, 143–6); dioxane, methanol and other chemicals used were from Merck. Buffer solution was prepared from phosphate (pH = 7.5) by dissolving 0.2721 g of H₃PO₄ and 1.8288 g of KH₂PO₄ in distilled water.

The disposable methacrylate cuvettes were obtained from Dispolab-Kartell, 4.5 ml PMMA, four clear sides, P.N. 1961.

2.2. Instruments

Direct measurements were accomplished in a Perkin-Elmer fluorescent spectrometer. An IBM PC was used for on-line data acquisition at an integration time of 10 s. The instrumental parameters were λₑₓ = 395 nm, λₑₘ = 470 nm and slit-width of the emission and excitation monochromators 5 nm. Data were collected and processed by FLDM software (Perkin-Elmer).

For measurements with the biosensor attached to an optical fibre a bifurcated high-grade fused silica fibre (Oriel, Stratford, CT, USA, Ref. 77533) with a high transmission range of 400–1500 nm was used to transfer the excitation and emission energies between the biosensor and the spectrometer. The ends are terminated with 11 nm ferrule adapters to couple our range of optical-fibre accessories.

2.3. Immobilization of the enzyme

The sol–gels were prepared in a similar manner to that described by Ellerby et al. [22]. The acid-catalysed silica sol was prepared by the sonication, in a crystal vial, for 20 min of TMOS (1.5 ml), deionized H₂O (320 µl) and 0.01 M HCl (30 µl). 60 µl of buffer pH = 7.5, 20 µl of enzyme 10 U ml⁻¹ and 26.7 µl of sol were put into a disposable methacrylate cuvette, and the sol–gel was formed after 2 min. Conservation and storage (after gelation) was carried out at 4°C, in water, and the gels were sealed with parafilm to prevent cracking, through rapid evaporation, during aging.

2.4. Construction of biosensor attached to optical fibre

Biosensor performance was studied using two different preparation protocols (Fig. 1).

For Biosensor I small plates of methacrylate (each of 1 cm² surface) were cut and 15 µl of buffer pH = 7.5, 5 µl of enzyme 10 U ml⁻¹ and 6.7 µl of sol were added to the glass slides. All films were then stored in ambient conditions. The plate is joined with a cyanacrylate adhesive to a black-film-covered plastic ring attached to an optical fibre. Adding the same quantities for Biosensor II, gel was prepared directly on the quartz window of the common end of the fibre bundle.

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**Fig. 1.** Two different preparations of biosensor attached to optical fibre: (a) Biosensor I, not directly attached; (b) Biosensor II, directly attached.
In this way, the optical path length was shortened and different.

For easy and comfortable handling, the fibre is held with a support.

2.5. Analytical procedure

Cholinesterase catalyses the hydrolysis of substrate, indoxyl-acetate, to indoxyl and acetic acid according to the reaction

\[ \text{Indoxyl - acetate} + \text{H}_2\text{O} \rightarrow \text{Indoxyl} + \text{acetic acid} \]

The indoxyl produced is fluorescent, its maximum wavelengths of excitation and emission being 395 and 470 nm, respectively [19].

For direct measurements 2 ml of deionized H₂O, ORP and substrate are added to a cuvette containing the gel with immobilized enzyme. The relative fluorescence intensity (RFI) 5 min after substrate addition is determined. For measurements with optical fibre the cuvettes are covered with black film and the RFI at 5 min is determined from RFI-time curves; the emission slit width used is 20 nm, because the RFI decreases due to transmittance losses.

3. Results and discussion

3.1. Choice of working conditions

The RFI is measured at a fixed time instead of the initial rate of enzymatic reaction because an agitation system would damage the gel. A measurement time of 5 min after substrate addition is sufficient to produce a noticeable analytical signal.

Separate experiments were conducted to study the activity of cholinesterase in different conditions, in aqueous solution, immobilized enzyme without pesticide and immobilized enzyme in the presence of the pesticide fenitrothion. Fig. 2 shows the fluorescent spectra obtained by using the same substrate concentration. It can be deduced from the maximum RFI obtained from these spectra that immobilized enzyme conserves 80% of activity in dissolution because immobilization by sol–gel methods does not significantly decrease the catalytic activity of the enzyme. A fluorescence intensity decrease is observed in the presence of the pesticide fenitrothion.

To obtain the gels, initial volumes of phosphate buffer, enzyme and sol were added in this order. The buffer is needed to avoid losses of the enzymatic activity by denaturalization during the gelification process (since the pH of the sol is acid), making the enzyme remain at an optimum pH [8].

It can be observed that the biosensor responses depended on the proportion of the initial volume (buffer + enzyme) / sol used in obtaining the gel. On increasing this proportion the enzyme remained in a medium similar to a biological one; it holds more activity and increases the obtained fluorescent intensity. On decreasing this proportion the denaturalization increases, the activity decreases and the obtained fluorescent intensity decreases. On the other hand, the decreased fluorescent intensity with the decreased (buffer + enzyme) / sol can be explained by the small size of the pores of the tridimensional network obtained by silanos condensation. The substrate molecule cannot reach the enzymes in the gel [6].

The quantity of enzyme in the gel for work conditions is 0.2 U, which approximately binds 50% of substrate indoxyl-acetate. If a smaller amount of the enzyme is taken, the fluorescence obtained is small and the ORP cannot be analysed. If a much higher quantity of enzyme is chosen, the amount of pesticide required to produce a significant shift in the fluorescence is high, increasing the detection limits of the ORP.

Fig. 3 presents a calibration plot of the sensor to indoxyl-acetate with these conditions, correlating RFI against substrate concentration in a semilogarithmic scale. Earlier studies have demonstrated the high repeatability of measurements using immobilized enzymes. Using the gels obtained from

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**Fig. 2.** Fluorescent spectra (for substrate concentration of $1.68 \times 10^{-4} \text{ M}$) 6 min after substrate addition, with enzyme in aqueous solution (a), immobilized enzyme without pesticide (b) and immobilized enzyme in the presence of the pesticide fenitrothion (c).

**Fig. 3.** Calibration plot for substrate, indoxyl-acetate. RFI measured at 5 min.
TMOS, the precision as relative standard deviation (RSD) is 10.2% \((n = 7)\) for a working concentration of indoxyl-acetate of \(1.63 \times 10^{-4} \text{ M}\), selected from the linear zone of the calibration curve.

### 3.2. ORP pesticide analysis

Calibration plots of the biosensor were made showing the extent of enzyme inactivation with varying concentrations of the different pesticides in the cuvette, when they were added directly to it. For each pesticide calibration, plots were obtained correlating the percentage inhibition 5 min after substrate addition with pesticide concentration on a semilogarithmic scale. The percentage inhibition is calculated as follows:

\[
\text{% INH} = \frac{\text{RFI}_0 - \text{RFI}}{\text{RFI}_0} \times 100
\]

where RFI_0 is the fluorescence without inhibitor, and RFI is the fluorescence with inhibitor [21].

Fig. 4(a)–(e) are calibration plots for fenitrothion, azinphos-ethyl, methidathion, naled and mecarbam, respectively. Depending on the inhibitory power of the compound, different limits of detection were observed. In Table 1 the pesticides analysed, RSD and detection limits are indicated. The obtained RSDs are less than or equal to 10.2% for \(n \geq 4\). Here, the enzyme was more sensitive to naled (detection limit: 0.36 \(\mu\text{g ml}^{-1}\)).

### 3.3. Characterization of immobilized enzyme in gel in cuvette

The gel is prepared and stored easily. The activity of the immobilized enzyme is rapidly lost with continuous use. After seven uses the enzyme maintains 50% of its initial activity, and after one month of storage at 4°C conserves 60% of its initial activity. Fig. 5 shows the RFI-time curves for gel recently obtained and stored at 4°C for one month. These results show that a stable fluorescent signal is achieved after a month of storage when the gel is stored in water, in this

<table>
<thead>
<tr>
<th>Pesticides</th>
<th>Detection limit ((\mu\text{g ml}^{-1}))</th>
<th>RSD (%)</th>
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<tbody>
<tr>
<td>Fenitrothion</td>
<td>17.8</td>
<td>10.3</td>
</tr>
<tr>
<td>Azinphos-ethyl</td>
<td>17.9</td>
<td>7.2</td>
</tr>
<tr>
<td>Methidathion</td>
<td>57.6</td>
<td>7.9</td>
</tr>
<tr>
<td>Naled</td>
<td>0.36</td>
<td>7.2</td>
</tr>
<tr>
<td>Mecarbam</td>
<td>13.9</td>
<td>5.2</td>
</tr>
</tbody>
</table>

![RFI-time curves for gel recently obtained (O) and stored at 4°C for 1 month (▼). Substrate concentration = 1.63 \(\times 10^{-4} \text{ M}\).](image)
way avoiding the aging and drying that cause a subsequent increase in the diffusion time of analyte and products within the evolving sol-gel network.

The rapid loss in activity with continuous use need not be viewed as a disadvantage, because the gel can be mass produced, possesses good storage stability for one month and can be discarded after a single use.

3.4. Biosensor attached to optical fibre

Two different preparations, as indicated in Section 2, were used for the attachment of the biosensor to the optical fibre, obtaining different results in calibration plots of substrate. Biosensor I presents a very low sensitivity. The calibration plot for indoxyl-acetate with Biosensor II exhibits a linear correlation ($r = 0.997$). The RFI–time curves for different substrate concentrations are shown in Fig. 6(a). The precision as RSD is 10.3% ($n = 5$) for the working concentration of indoxyl-acetate, $2.42 \times 10^{-4}$ M.

The calibration plot of biosensor to substrate display an increase in signal and higher sensitivity when Biosensor II is used. This could be due to the optical path length with Biosensor I being greater than with Biosensor II, and through which the light passes at distinct levels with different refractive indices. Given that there is a large sensitivity and its construction is much easier, Biosensor II was chosen for ORP analysis.

Fenitrothion and naled have been analysed with Biosensor II, correlating the percentage inhibition at 5 min from the RFI–time curves against pesticide concentration. In Fig. 6(b) the RFI–time curves at different naled concentrations are represented. The RSD is calculated for $n = 3$, and the analytical characteristics are given in Table 2. The detection limits achieved with optical fibre were smaller than those for direct measurements in the cuvette; so the immobilization of enzyme directly onto the end of the optical fibre enhances the sensitivity. The enhancement of the sensitivity can be attributed to the low volumes of samples used in order to reach the gel attached to the optical fibre, obtaining larger changes in the RFI for lower concentrations of pesticide. Besides, the fluorescence with the optical fibre is measured in the gel, and the indoxyl local concentration is greater than in direct measurements in solution.

4. Conclusions

The simple enzyme immobilization procedure described here permits the construction of indoxyl-acetate biosensors. We report the biosensor attachment to optical fibre; once calibrated, it can be used as an analytical instrument to screen for ORPs on a routine basis. Two different preparation protocols have been used in attaching the biosensor to the optical fibre (I and II). Biosensor II has been chosen for ORP analysis since it shows an increase in the signal and a greater sensitivity than Biosensor I. Determination of ORPs with the indoxyl-acetate biosensor has been carried out with two detection methods: directly measurement in the cuvette and with optical fibre (Biosensor II). The enzyme immobilization directly onto the end of the optical fibre enhances the sensitivity, giving lower detection limits.

The most important advantage is the short time for analysis, as in only 30 min the enzyme is immobilized, the gel is attached to the optical fibre and an analytical signal is obtained. Other advantages are the achievement of satisfactory detection limits and the possible application in various analytical arrangements.
Acknowledgements

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References