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An optical biosensor for dichlovos using stacked sol–gel films containing acetylcholinesterase and a lipophilic chromoionophore

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Abstract

An optical biosensor consisting of a chromoionophore (ETH5294) (CM) doped sol–gel film interfaced with another sol–gel film immobilized with acetylcholinesterase (AChE) was employed to detect the insecticide dichlorvos. The main advantage of this optical biosensor is the use of a sol–gel layer with immobilized CM that possesses lipophilic property. The highly lipophilic nature of the CM and its compatibility with the sol–gel matrix has prevented leaching, which is frequently a problem in optical sensor construction based on pH indicator dyes. The immobilization of the indicator and enzyme was simple and need no chemical modification. The CM layer is pH sensitive and detects the pH changes of the acetylcholine chloride (AChCl) substrate when hydrolyzed by AChE layer deposited above. In the absence of the AChE layer, the pH response of the CM layer is linear from pH 6 to 8 ($R^2 = 0.98$, $n = 3$) and it showed no leaching of the lipophilic chromoionophore. When the AChE layer is deposited on top, the optical biosensor responds to AChCl with a linear dynamic range of 40–90 mM AChCl ($R^2 = 0.984$, $n = 6$). The response time of the biosensor is 12 min. Based on the optimum incubation time of 15 min, a linear calibration curve of dichlorvos against the percentage inhibition of AChE was obtained from 0.5 to 7 mg/L of dichlorvos (17–85% inhibition, $R^2 = 0.991$, $n = 9$). The detection limit for dichlorvos was 0.5 mg/L. The results of the analysis of 1.7–6.0 mg/L of dichlorvos using this optical biosensor agreed well with a gas chromatography–mass spectrometry detection method.

Keywords: Organophosphate pesticides; Toxicity; Stacked membranes; Disposable biosensor

1. Introduction

There are many advantages of using sol–gel as a membrane material for optical biosensors. The most important property of sol–gel is its optical clarity but other properties such as its ability to entrap large amount of enzymes, thermal and chemical stability, simplicity of preparation and flexibility in controlling pore size and geometry also are desirable features [1]. In the construction of optical sensing membrane, very frequently, a dye material that can change colour in response to interaction between an enzyme and substrate (or an analyte) are often immobilized in the sol–gel. The cholinesterases such as acetylcholinesterase and acetylcholine acylhydrolase are enzymes that can be inhibited by organophosphate and carbamate pesticides [2], and they have been proven useful for developing biosensors [3].

Many studies have reported the use of sol–gel and cholinesterase enzymes as optical biosensors for the determination of organophosphate and carbamate pesticides [1,3–5]. For optical biosensor based on sol–gel, the immobilization of a pH indicator dye is often an essential part of the biosensor fabrication process. A number of pH indicators has been incorporated into sol–gel film and in most cases, covalent binding is required to prevent leaching and loss of the pH indicator from the film. Some examples are covalent immobilized of thymol blue on aminopropyl glass [3] and chlorophenol red on porous sol–gel glass [4].

Attempt to prevent leaching of pH indicator from a sol–gel film through covalent attachment of the indicator required complicated chemical modification that involved many synthetic steps [3,5]. The leaching problem is mainly attributed to the highly water soluble nature of these pH indicator dyes. One way to prevent leaching without undergoing lengthy covalent attachment procedure is to employ pH indicator that is less soluble in water. Such a class of pH indicators has been synthesized and is commonly used in the fabrication of ion-selective optodes

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[6]. These lipophilic pH indicators, normally known as chromoionophores had been successfully used in the construction of ion-selective optodes and optical biosensors, and they were entrapped in plasticized poly(vinyl chloride) membranes [7,8] and also plasticizer-free acrylate membranes [9,10].

Chromoionophores are lipophilized pH indicators and can be classified depending on whether they are neutral and charged [11]. The neutral chromoionophore is positively charged when in its protonated form but neutral when it is unprotonated [6]. Ion-selective optodes employing chromoionophores were developed by Morf et al. and the response of such optodes is based on reversible mass transfer of the analyte ions or electrically neutral molecules from sample solution to the organic phase of the membrane [12,13]. The chromoionophore forms a complex with H^+ but not other metal cations, thus it demonstrates good selectivity to H^+ [14].

In this work, we have entrapped a chromoionophore, which is a lipophilized Nile Blue dye (ETH5294) in sol–gel films and applied this as an optical biosensor after the immobilization of the enzyme AChE. Preliminary studies carried out on the ETH5294 doped sol–gel film have shown encouraging results [15]. In this paper, we report details for the construction of an optical biosensor based on a stacked sol–gel films for the determination of dichlorvos inhibition, an organophosphate insecticide. The chromoionophore (CM) doped sol–gel film was also examined for its pH response and the optimum condition for the best performance of the optical biosensor for the determination of dichlorvos after the second layer of AChE doped film was deposited on top of the CM sol–gel film.

2. Experimental

2.1. Reagents

Acetylcholinesterase (E.C. 3.1.1.7) from electric eel (Type V I-S, 480 U/mg-solid, AChE) and acetylcholine chloride (AChCl) were purchased from Sigma Chemicals, dichlorvos was purchased from Riedel-de Haen, tetraethoxysilane (TEOS), a precusor of sol–gel and the chromoionophore (CM) Nile blue derivative (9-(diethyl-lamino)-5-(octadecanoylimino)-5H-benzo[*a*]phenoxa-zine (ETH 5294) were purchased from Fluka. All other chemicals were of analytical grade and used as received without further purification. All solutions were prepared with double distilled or deionized water.

2.2. Instrumentation

Absorbance was measured using a double beam Varian (Model Cary 100) UV–vis spectrophotometer. A Dell personal computer was used for on-line data acquisition. The instrumental parameters were controlled by Cary-Win Software (Varian) and the data were collected and processed by this software. The immobilized AChE and CM sol–gel films coated on a cellulose acetate support was put in a cuvette (1.5 mL) for monitoring the absorbance changes. A Shimadzu gas-chromatograph with mass spectrometry detector was used to determine the dichlorvos for comparison study.

2.3. Enzyme and chromoionophore immobilization

The sol–gel was prepared as described before with minor modification [15–17] where the stirring time was extended to 4 h. The acid-catalyzed sol solution was prepared by mixing 4.5 mL of tetraethoxysilane (TEOS), 1.4 mL of double distilled and deionized water and 0.10 mL of 0.1 M HCl in a glass vial. The mixture was then stirred until a clear solution was formed and left overnight at room temperature before used. A solution of 5 mg/mL ETH5259 was prepared by dissolving the chromoionophore in absolute ethanol as a solvent. For the preparation of the chromoionophore sol–gel film, 3 μL of chromoionophore solution, 3 μL of sol solution and 10 μL of Tris–HCl buffer 0.1 M (pH 8) were mixed together. The mixture was then spin coated on a cellulose acetate film (1 cm × 3 cm) and left for 3 h at room temperature (30 °C) where the film formed. For the immobilization of enzyme AChE, 20 μL 100 U/mL enzyme solution in 0.1 M Tris–HCl buffer (pH 8) was added to 5 μL of sol solution before spin coated with speed of 340 rpm for 3 s on top of the sol–gel film that had been immobilized with chromoionophore earlier. The final biosensor film was stored in a sealed container and kept in a refrigerator (4 °C) until used.

2.4. Absorbance measurement of the optical biosensor

The immobilized chromoionophore gives maximum absorbance peaks at 543 and 660 nm. At 543 nm, deprotonation of the chromionophore occurs while at 660 nm protonation takes place. The changes of the absorbance attributed to the deprotonation–protonation processes at various pH values may be expressed as follows:

$$P = \left\{ \frac{A_{15} - A_0}{A_{15}} \right\} \times 100\%$$

where P is the percentage of absorbance changes (%), A_{15} the absorbance value recorded at 660 nm after 15 min and A_0 is the absorbance value recorded at 660 nm at 0 min.

The degree of deprotonation (α) of the CM sol–gel film may be calculated based on absorbance data [7,9], i.e.

$$\alpha = \frac{A - A_0}{A_1 - A_0}$$

A_1 and A_0 are the limiting absorbance values for $\alpha = 1$ (chromoionophore completely deprotonated) and $\alpha = 0$ (totally protonated), respectively.

Using a chromoionophore sol–gel film without immobilized AChE, the pH response of the film was first evaluated in Tris–HCl solutions of pH 5–10. With the same sol–gel film, the pH response of the substrate AChCl at various concentrations (10–150 mM in deionized water) was also determined to establish the range of AChCl that can be used for inhibition study without pH interference from the substrate alone.

2.5. Substrate and inhibition measurements

After the AChE layer was deposited on the chromoionophore film, the response of the optical biosensor towards various buffer

capacities (Tris–HCl) and AChCl concentrations were studied. To study the inhibition effect of the optical biosensor by the insecticide dichlorvos, two biosensor films were used, one was to measure the response in the absence of inhibition and the other was to determine the response after inhibition. Based on these responses, the percentage of inhibition was determined from the equation below:

$$\text{percentage inhibition, } I(\%) = \left\{ \frac{P_0 - P_a}{P_0} \right\} \times 100$$

P_0 and P_a are the percentage absorbance changes due to no inhibition and inhibition at certain concentration of dichlorvos, respectively. For the inhibition evaluation, the incubation time of the biosensor in dichlorvos was optimized and a calibration plot of percentage of inhibition against dichlorvos at concentrations of 0–50 mg/L was constructed. All experiments involving enzyme were conducted at a fixed pH of 7.5 in Tris–HCl buffer.

2.6. Analysis of dichlorvos with gas chromatography and mass spectrometry detection

To compare the results of analysis of dichlorvos with other conventional techniques for dichlorvos determination, gas chromatography using mass spectrometry detection (GC-MSD) method was chosen. Water samples for analysis were first spiked with different concentrations of dichlorvos from 1.7 to 6.0 mg/L. Direct determination of these artificial samples was carried out with the optical biosensor. For GC-MSD analysis, the samples were first extracted with SOLUTE SPE column (International Sorbent Technology) by eluting through the SPE column with 2 mL × 2 mL of ethyl acetate. The final extract was then concentrated before GC-MSD analysis was carried out.

3. Results and discussion

3.1. Response of immobilized chromoionophore (CM) in sol–gel film towards pH changes

Fig. 1 shows the absorbance spectrum of an immobilized CM in sol–gel film exposed to Tris–HCl solution at pH 10. The chromoionophore that was originally protonated (blue colour) gradually transformed into the deprotonated form (red colour) [18]. Three distinct absorption maxima (λ_{max}) at 543, 610 and 660 nm were demonstrated in the spectrum [9] The isobestic point indicates the transformation of the protonated and deprotonated forms [19].

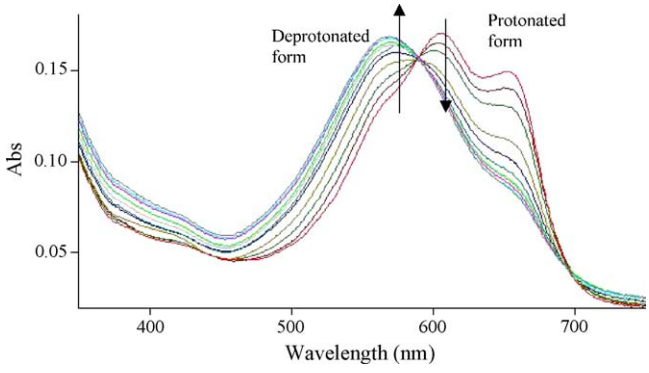

Fig. 1. The progressive changes of the spectra of CM sol–gel film at 1 min interval after exposure to Tris–HCl buffer at pH 10.

In a plasticized PVC and bulk optode films, the processes of deprotonation and protonation were known to involve extraction of protons into the PVC membrane by mass transfer [20,8], thus changing the degree of protonation of the chromoionophore when the pH is varied. Obviously, similar behaviour of the chromoionophore was observed in the sol–gel film even though sol–gel is a very different matrix from that of plasticized PVC optode film. The response to pH of the sol–gel film was approximately 10 min and is much slower than that of plasticized PVC films, which is less than 1 min (film thickness 1–5 μm). But for the pH response of the sol–gel film, thickness of film used in this work was approximately 200 μm.

The mechanism of ion transport in sol–gel film is unlikely to be similarly to that of plasticized PVC membrane. For example, the diffusion of the chromoionophore ETH5294 in a plasticized PVC film is influenced by the polymer:plasticizer ratio and also the type of plasticizer used. Higher polymer content reduces diffusion coefficient [21]. Long response time of more than 10 min for plasticizer-free acrylate films of about several microns has also been observed, presumably attributed to the slow transport of chromoionophore in the polymer film because of the absence of a plasticizer [9]. In the case of sol–gel film, the response time may be affected by the pore size and the water molecules behaviour in the sol–gel film, which affects the proton transport. The rate of transport of proton in sol–gel derived porous vitreous silica is known to depend on the pore size. Pore size of too large or small will lead to poor proton conduction and a 4 nm pore size provides fast proton conduction [22]. Thus, by optimizing the film thickness and the sol–gel pore size, faster response time can be achieved for the sol–gel matrix employed here.

The pH response demonstrated a linear range from pH 6 to 8 ($\alpha = 0.179$, pH 0.615, $R^2 = 0.9812$, $n = 3$) (Fig. 2), which is similar to that reported for plasticiser free acrylate films, which

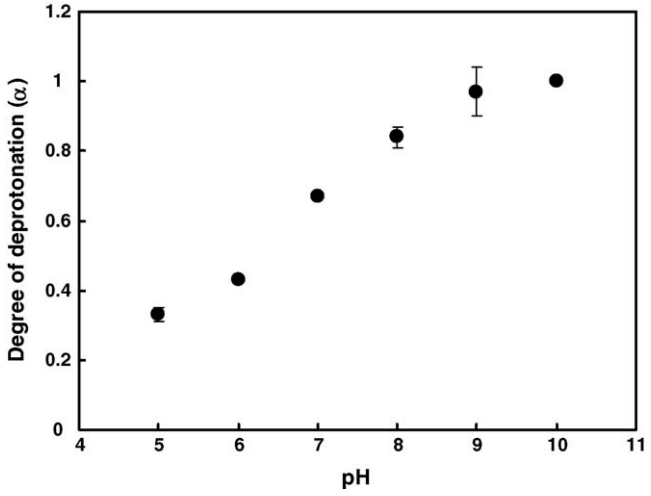

Fig. 2. The variation of the degree of deprotonation (α) of the chromoionophore immobilized in a sol–gel film with pH changes. (Each data point is an average of three replicates.)

showed linear pH range of 5.5–8.0 [9]. This range is lower than that observed for plasticized PVC film (pH 9–11) [20]. The linear response range of the CM sol–gel film falls within the pH working range of the enzyme AChE (pH 6.0–10.0) with optimum pH at 8.5 [23]. The immobilized CM in sol–gel film also demonstrated no leaching of the chromoionophore even after the film was exposed to Tris–HCl buffer for several hours. The absence of leaching even without covalent immobilization is attributed to the lipophilic nature of the CM and also the low water absorption behaviour of the sol–gel [24].

3.2. The response of the optical biosensor to acetylcholine (AChCl) substrate concentrations

The construction of the optical biosensor is accomplished by depositing a sol–gel film doped with AChE enzyme on top of the CM sol–gel layer. Film that doped simultaneously with CM and AChE was attempted but the AChE was inhibited by the CM. Therefore, a two-layer construction for the biosensor film was necessary. In the top layer of AChE sol–gel film, the enzyme catalyzes the hydrolysis of AChCl to choline and acetic acid [14]. Thus, AChCl was used as a substrate for the characterization of the enzyme optode [3]. The release of the acid resulted in a decrease of the pH, which could be detected optically by the change of colour of the CM sol–gel film at 660 nm [9,25].

However, the amount of pH change was dependent on the buffer capacity of the medium used [1]. Using 100 mM of AChCl, the effect of Tris–HCl buffer concentrations on the response of the biosensor was examined with 10–50 mM Tris–HCl. The observed percentage change in the absorbance at various concentrations is shown in Table 1. The Tris–HCl concentration of 10 mM demonstrates the largest absorbance change when compared with 30 or 50 mM buffers. This leads to a highest change in the pH as detected by the CM sol–gel layer. Therefore, 10 mM of Tris–HCl was selected as the medium of the enzymic reaction for subsequent investigation because it gave better sensitivity to the biosensor.

In the absence of the AChE sol–gel layer, the CM sol–gel layer alone did not give obvious response to the changes of AChCl from 0 to 150 mM (Fig. 3). But when AChE is immobilized, the biosensor based on this double sol–gel membrane gave a linear response to AChCl at concentrations of 40–80 mM with a sensitivity slope of 0.461 absorbance change per millimole AChCl ($R^2 = 0.983$, $n = 6$; Fig. 3). Above 80 mM, the enzyme reaction saturates and no further change of absorbance can be observed with increase in AChCl concentrations. Below 40 mM, a linear response ($R^2 = 0.966$, $n = 4$) was also obtained from 10

Table 1
The percentage changes of the optical biosensor response when exposed to 100 mM of AChCl at different concentrations of Tris–HCl buffer

Tris–HCl concentration (mM)	Absorbance change (%)	Change of pH
10	26.3	0.78
30	23.0	0.45
50	20.7	0.30

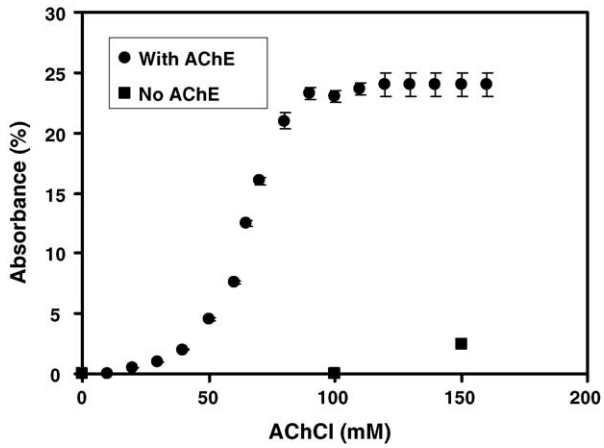

Fig. 3. The response of the optical biosensor towards changes of AChCl substrate concentrations. (Each data point is an average of three replicates.)

to 40 mM AChCl but the sensitivity was seven times lower than that observed at 40–80 mM AChCl. The difference in behaviour of the biosensor towards different concentrations of substrate can be explained by a slower diffusion of the substrate to the sol–gel especially at low substrate concentrations [26] and also possible slow reaction kinetics in the alkoxysilane derived matrices [27] when less substrate is available. Most of the reported linear range for AChCl that based on the enzyme AChE are smaller than the present study, i.e. typically from 5 to 20 mM [1,3,5,28]. Lower detection limit for AChCl was 10 mM where the percentage absorbance change was 0.85%. This is higher when compared with 0.5 mM reported by Doong and Tsai [1]. The upper limit of detection was at 100 mM, beyond which the optical biosensor did not show any further change in absorbance (constant at ~24%). The biosensor for AChCl exhibited average reproducibility with relative standard deviation of 15% ($n = 3$).

For inhibition study that involved separate steps of inhibitor incubation and substrate exposure, the substrate concentration at saturation [29] can be used to maximize the optical signal. In this work, the saturation concentration of 100 mM was used. The response of the optical biosensor with time when exposed to 100 mM of AChCl is demonstrated in Fig. 4. There is a rapid

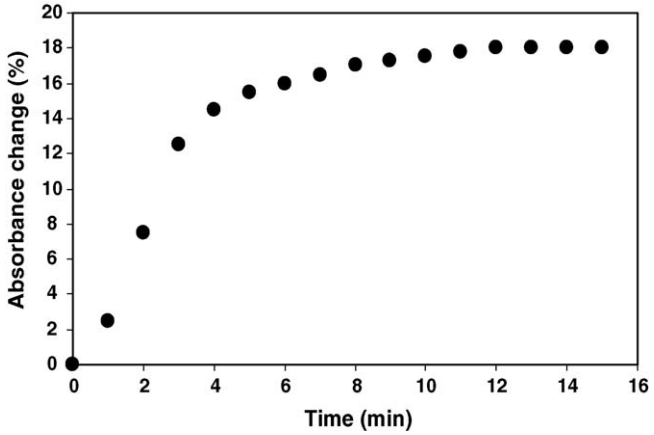

Fig. 4. The response time profiles of optical biosensors after the exposure to 100 mM AChCl substrate.

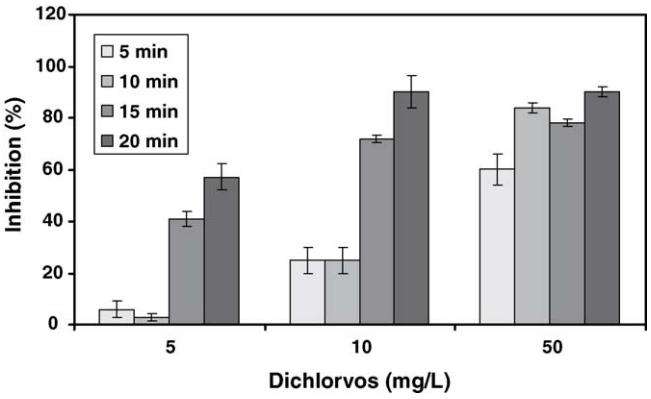

Fig. 5. The effect of duration of incubation on the AChE inhibition by different concentrations of dichlorvos as determined by the optical biosensor. (Each data point is an average of three replicates.)

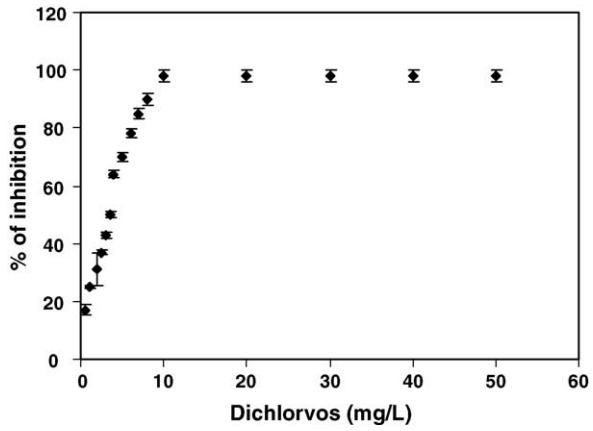

Fig. 6. The inhibition profile of the optical biosensor with various concentrations of dichlorvos. A linear response range was obtained from 0.5 to 7 mg/L dichlorvos. (Each data point is an average of three replicates.)

increase in the absorbance at the first 3 min and it then levels off. Based on this response profile, the response time of the optical biosensor for AChCl is approximately 12 min.

3.3. Determination of dichlorvos using the optical biosensor

For enzyme AChE, the incubation time of the immobilized enzyme in dichlorvos can influence the degree of inhibition. The influence is particularly obvious at lower concentrations of dichlorvos (<10 mg/L) where longer incubation time tends to give higher level of inhibition (Fig. 5). For a concentration 10 mg/L of dichlorvos, the inhibition achieved after an incubation time of 15 min was almost three times higher than that incubated for 5 or 10 min. Above 15 min, the percentage of inhibition appears to level off and this is particularly obvious for higher concentration such as 50 mg/L dichlorvos. Thus, for the purpose of determination of dichlorvos by AChE inhibition, the most suitable incubation time chosen was 15 min as this gave a larger inhibition signal, especially for lower concentrations of dichlorvos.

In order to demonstrate the usefulness of the AChE-based optical biosensor constructed from immobilized chromoionophore for the determination of dichlorvos, the optical biosensor was exposed to dichlorvos concentrations of 0.5–50 mg/L. The inhibition response of the biosensor towards dichlorvos is shown in Fig. 6. Clearly, almost maximum inhibition was attained above 10 mg/L of dichlorvos, i.e. approximately 100%. Below 10 mg/L, the inhibition pattern was linear from 0.5 to 7 mg/L dichlorvos with a sensitivity slope of 10.1% inhibition per unit change in dichlorvos concentration ($R^2 = 0.992$, $n = 9$). The relative standard deviation of the biosensor ($n = 3$) was <16% indicating a reasonably good reproducibility for most measurements. The percentage of inhibition, which was in the range of 25–98% for the dichlorvos concentrations of 1–10 mg/L was higher than those reported by Andreou and Clonis [5] where the range was between 10 and 85%. Using the optical biosensor reported here, dichlorvos concentration as low as 0.50 mg/L (17% inhibition) can be detected and this is approximately 30 times higher than the limit of detection by an AOAC method (Method 991.07, 1999) for dichlorvos [30]. However, the detection limit of 0.50 mg/L is within the limit permitted by the Malaysian Food Act for dichlorvos [31], i.e. <2 mg/L.

The high detection limit of both AChCl and dichlorvos may be attributed to the poor diffusion in the sol–gel film. Jin and Brennan [26] suggested that the reduction of enzyme activity in sol–gel matrix is due to an increase in viscosity of entrapped solvents, which leads to slower diffusion of analytes. The apparent diffusivities of acetylcholine in a polymeric material is in the range of 10^{-8} to 10^{-9} cm^2/s, which is lower than the diffusivities of acetylcholine in an aqueous phase (10^{-5} to 10^{-6} cm^2/s) [32], and the calculated apparent Michaelis–Menten constant (k_m) for AChE immobilized in sol–gel was higher than those obtained in free AChE [1,33].

3.4. Comparison of determination of dichlorvos with optical biosensor and GC-MSD method

Fig. 7 depicts the correlation between the analysis of six spiked water samples by optical biosensor and GC-MSD

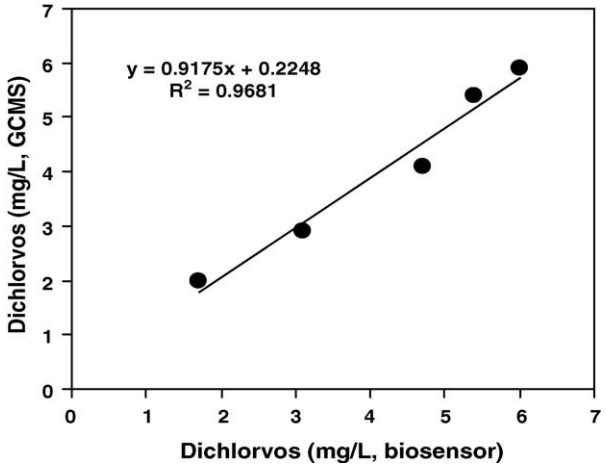

Fig. 7. A correlation of the concentrations of dichlorvos determined directly by the optical biosensor with that determined by GC-MS after solid-phase extraction procedure.

method. The amount of dichlorvos analyzed in all samples by the optical biosensor correlates well with that of GC-MSD. The correlation coefficient was close to 0.97 and the slope of the correlation is close to 1. This shows that the optical biosensor can be used to determine dichlorvos when other substances that can cause AChE inhibition are absent.

4. Conclusion

An optical biosensor consisting of stacked sol–gel films where the enzyme AChE layer was deposited on top of the chromoionophore layer has been successfully developed for the determination of the insecticide dichlorvos based on enzyme inhibition concept. The main advantage of this optical biosensor is the use of a sol–gel layer with immobilized chromoionophore that possesses lipophilic property. The highly lipophilic nature of the chromoionophore and its compatibility with the sol–gel matrix has prevented leaching of the optical sensing material, which is frequently a problem in optical sensor construction based on pH indicator dyes. The immobilization of the indicator and enzyme was simple and need no chemical modification compared with other reports where glutaraldehyde and bovine serum albumin were introduced for enzyme immobilization with complicated fabrication procedures and a long period of stabilization (up to 20 days) after the chemical modification [5,34–36].

The biosensor developed can be used for the screening of insecticides and the chromoionophore doped sol–gel film is expected to find many applications in optical biosensors that utilized pH transduction and this is currently been explored in our laboratory.

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References

[1] R.A. Doong, H.C. Tsai, Anal. Chim. Acta 434 (2001) 239.
[2] D.W. Moss, A.R. Henderson, in: C.A. Burtis, E.R. Ashwood (Eds.), Tietz Textbook of Clinical Chemistry, second ed., W.B. Saunders, Philadelphia, 1994, p. 877.
[3] R.T. Andres, R. Narayanaswamy, Talanta 44 (1997) 1335.
[4] M.P. Xavier, B. Vallejo, M.D. Marazuela, M.C. Moreno-Bondi, F. Baldini, A. Falai, Biosens. Bioelectron. 14 (2000) 895.
[5] V.G. Andreou, Y.D. Clonis, Biosens. Bioelectron. 17 (2002) 61.
[6] E. Bakker, M. Lerchi, T. Rosatzin, B. Rusterholz, W. Simon, Anal. Chim. Acta 278 (1993) 211.
[7] B. Kovács, G. Nagy, R. Dombi, K. Tóth, Biosens. Bioelectron. 18 (2003) 111.
[8] S. Ursula, S. Wilhelm, E. Bakker, L. Markus, B. Philippe, Sens. Actuators B 11 (1993) 1.
[9] L.Y. Heng, H.F. Teh, H.C. Loh, A. Musa, Sensors 3 (2003) 83.
[10] S. Peper, I. Tsagkatakis, E. Bakker, Anal. Chim. Acta 442 (2001) 25.
[11] E. Bakker, W. Simon, Anal. Chem. 64 (1992) 1805.
[12] W.E. Morf, K. Seiler, B. Lehmann, Ch. Behringer, S.S.S. Tan, K. Hartman, P.R. Sørensen, W. Simon, in: E. Pungor (Ed.), Ion-Selective Electrodes, vol. 5, Akadémiai Kiadó, Budapest, 1989, p. 115.
[13] W.E. Morf, K. Seiler, P.R. Sørensen, W. Simon, in: E. Pungor (Ed.), Ion-Selective Electrodes, vol. 5, Akadémiai Kiadó, Budapest, 1989, p. 141.
[14] Y. Qin, E. Bakker, Talanta 58 (2002) 909.
[15] F. Wong, M. Ahmad, L.Y. Heng, Proceeding of ASIASENSE International Conference, IEEE, 2003, p. 225.
[16] A. Kumar, R. Malhotra, B.D. Malhotra, S.K. Grover, Anal. Chim. Acta 414 (2000) 43.
[17] U. Narang, P.N. Prasad, F.V. Bright, K. Ramanathan, N.D. Kumar, B.D. Malhotra, M.N. Kamalasanan, S. Candra, Anal. Chem. 66 (1994) 3139.
[18] M.M.F. Choi, K.O.P. Chung, X. Wu, Talanta 56 (2002) 1027.
[19] O. Satoshi, C.H. Peter, S. Kurt, S.S.T. Susie, E.M. Werner, S. Wilhelm, Anal. Chem. 63 (1991) 640.
[20] E. Bakker, S. Wilhelm, Anal. Chem. 64 (1992) 1805.
[21] R. Long, E. Bakker, Anal. Chim. Acta 511 (2004) 91.
[22] Y. Daiko, T. Kasuga, M. Nogami, Microporous Mesoporous Mater. 69 (3) (2004) 149.
[23] Enzyme Handbook, Springer-Verlag, Berlin, Heidelberg. Cholinesterase 3.1.1.8, 1991, pp. 1–6.
[24] J. Lin, Trends Anal. Chem. 19 (2000) 541–552.
[25] S. O'Neill, S. Conway, J. Twellmeyer, O. Egan, K. Nolan, D. Diamond, Anal. Chim. Acta 398 (1999) 1–11.
[26] W. Jin, J.D. Brennan, Anal. Chim. Acta 461 (2002) 1.
[27] C.Y. Shen, M.N. Kostiç, J. Am. Chem. Soc. 119 (1997) 1304.
[28] C. Cremisini, S. Di Sario, J. Mela, R. Pilloton, G. Palleschi, Anal. Chim. Acta 311 (1995) 273.
[29] R. Shi, K. Stein, Anal. Chim. Acta 324 (1996) 21.
[30] W. Horwitz, Official Methods of Analysis of AOAC International, 17th ed, SOSC International, Maryland, 2000, Chapter 10, p. 27.
[31] Malaysia Food Act, Food Act 1983: Food Regulation. Warta Kerajaan 29(20): Regulation 41 Pesticide Residue, 1985.
[32] M.L. Chang, S.C. Wu, C.Y. Chen, Environ. Sci. Technol. 31 (1997) 2307.
[33] G. Tunnicliff, Y. Tsukada, A. Barbeau, Can. J. Biochem. 54 (1976) 389.
[34] B.D. Gupta, D.K. Sharma, Optics Commun. 140 (1997) 32.
[35] B.D. Gupta, D.K. Sharma, Optics Commun. 154 (1998) 282.
[36] O.S. Wolfbies, I. Oehme, N. Papkovskaya, I. Klimant, Biosens. Bioelectron. 15 (2000) 69.