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Electrochemical enzymatic biosensor with long-term stability by using hybrid mesoporous membrane

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Acetylcholinesterase immobilized sensor unit was successfully prepared by encapsulating the enzyme within hybrid mesoporous silica membranes (F127-MST). Through a novel combination with tetracyanoquinodimethane, both acetylcholine and organophosphorus pesticides were successfully detected with high sensitivity. Furthermore, we manufactured the working prototype of an enzyme sensor with this sensor unit for detecting dichlorvos, aldicarb and parathion. The detection limit in this working prototype was either equaling or surpassing compared with others at this moment. Also, we have an advantage on increased stability of enzyme against outer environment by encapsulating enzymes into silica nanospace. Consequently, acetylcholinesterase immobilized in F127-MST is a considerably practical sensor with high sensitivity, reusability, and storage stability.

Introduction

Development of acetylcholine sensor is of great interest[5–10] since acetylcholine is one of the most important neurotransmitters playing a vital role in the central and peripheral nervous system[1, 2] and is believed to affect our memory, sleep, concentration abilities, etc. An abnormally short supply of acetylcholine is associated with Alzheimer's disease[3, 4], which ranks fourth in the causes of death among adults.

Besides simply detecting concentration of acetylchloline, the major application is rather detecting organophosphorous compounds, which are considered to be the most toxic and used as pesticides, insecticides and chemical war agents. The high toxicity of organophosphorous neurotoxics and their large use in modern agriculture practices has increased public concerns, health risks and the consequent contamination of water, food sources[3].

Most commonly used techniques to detect organophosphorous are methods such as gas chromatography, liquid chromatography and thin film chromatography couple with different detectors and different types of spectroscopy. However, these techniques are time consuming and expensive. Also, qualified and experienced staffs are demanded.

Among several approaches to develop sensing ability, biological approaches using colorimetric techniques has recently been paid more attention. A number of biosensors based on the inhibition of acetylcholinesterase (AChE) have been studied for the determination of organophosphorous pesticides in the environment[11–17]. Especially amperometric ones, continue to be a topic of interest as they are rapid, simple and inexpensive methods.

Such amperometric detection of acetylthiocholine with tetracyanoquinodimethane (TCNQ) is previously described[18]:

$$acetylthiocholine + H_2O \xrightarrow{acetylcholinesterase} thiocholine + CH_3COOH \quad \text{---------(1)}$$

$$2thiocholine(red) + TCNQ(ox) \rightarrow 2thiocholine(ox) + TCNQ(red) + 2H^+ \quad \text{---------(2)}$$

$$TCNQ(red) \xrightarrow{electrode} TCNQ(ox) + 2e^- \quad \text{--------- (3)}$$

Since organophosphorus and carbamate pesticides have an inhibitory effect on AChE, they induce a decrease in the intensity of current generated by the transformations shown in Eqs. (1), (2), and (3).

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Here, enzyme immobilization method becomes one of the most important factors for enzyme based biosensors. The enzyme immobilized by conventional methods such as cross-linking and entrapping in polymeric gels cannot always exhibit satisfactorily high response or enough reproducibility owing to the loss of enzyme activity during the immobilization process[19, 20]. No method has been proposed that is generally applicable for all enzymes.

On the other hand, when enzymes are immobilized in the pores of an inorganic membrane, they are known to very useful reactors for organic synthesis. Based on this idea, the synthesis of nanoporous silica in anodic alumina pores has recently been tried elsewhere[21–26], and the resultant composite membranes have shown high potential as artificial membrane supports, owing to a large number of interconnected pores.

In our previous study, we have succeeded in assembling a highly durable membrane capable of high-density accumulation of enzymes by encapsulating them in mesoporous silica synthesized in the pores of an alumina membrane[27-32]. We have found that it is a suitable host for enzyme immobilization and useful for the fabrication of electrochemical biosensors, and also that it can be applied to various types of sensors by just selecting appropriate enzymes.

In this article, we have demonstrated how appreciable to various sensors our system is. AChE was immobilized within hybrid mesoporous membranes having 3-D concentric spherically layered structure with 13 nm pore diameter (F127-MST)[27]. The resulting AChE immobilized membrane (AChE–(F127-MST)) was applied for an amperometric biosensor. Also, AChE-(F127-MST) was developed as a sensor unit for our working prototype of an enzyme sensor. Then, sensitivity, response time, and stability of this sensor were particularly investigated for detecting organophosphorus pesticides.

Experimental

Preparation of F127-MST and AChE-(F127-MST): F127-MST was prepared through the previously reported synthetic procedure[28]. Typically, F127 (1.0 g) was dissolved in a mixture of ethanol (15 g), HCl (0.10 g of 37 wt% aqueous solution), and water (2.0 g). This mixture was stirred for 1 h at 60° C in a flask with a reflux condenser. TEOS (2.13 g) was then added to the mixture and further stirred at 60° C for 17 h, and then the resulting mixture was used as a precursor solution. An anodic alumina membrane was set in an ordinary membrane filtration apparatus, and the precursor solution (4 ml) was dripped onto the alumina membrane. Rapid vacuuming (20 L/min) was applied by using a diaphragm vacuum pump (Model UN820, KNF Neuberger, Inc.), so that the precursor solution penetrated into columnar alumina pores. After the complete penetration into alumina pores, it was dried under aspiration for 10 min and then calcined at 500° C for 6 h in the air to obtain the final product, F127-MST. BET surface area was found to be 19 m^2/g. Regarding to BJH average pore size, presence of the additive caused an increase in the pore size to 13 nm[27].

In the next step, AChE-(F127-MST) was prepared by placing F127-MST in 10.0 ml of the solution containing AChE (E.C. 3.1.1.7, from electric eel) (0-30 mg) in water. The membrane was then kept standing in that solution at 4° C for 12 h to establish an adsorption equilibrium. The amount of AChE adsorbed into the pores of F127-MST was determined by measuring absorbance of the supernatant at 280 nm, where characteristic absorption band of AChE was available. In addition, the amount of adsorption was also estimated by the TG decline on TG-DTA (Thermo plus TG8120 thermal analyzer, Rigaku Co., Ltd) measurements at a heating rate of 10 °C min^{-1}.

Electrochemical analyses:

A schematic diagram is shown in Fig. S1. Electrochemical cell VC-3 comprising a three-electrode setup (Bioanalytical Systems, Inc., West Lafayette, USA) was used with the potentiostat (Hokuto Denko Corporation, Kanagawa, Japan).The working electrode was constructed as follows: AChE–(F127-MST) (previously cut into 4 mm × 4 mm squares and washed in distilled water three times before use) was placed on the nylon mesh (pore diameter = ca. 1 μm, and thickness = ca. 75 μm). Then, it was placed directly on a glassy carbon working electrodes (i.d.: 3 mm) via rubber O-rings. A platinum auxiliary electrode, and an Ag/AgCl (+199 mV vs. NHE) reference electrode were also in use. The electrochemical detection system was based on amperometric measurements. The cell was filled with an electrolyte solution (15 ml) containing 0.5 mM Tetracyanoquinodimethane (TCNQ) in a phosphate buffer (pH 7.4). The electrodes were dipped in this electrochemical cell with stirring. After incubation for 10 min, a potential of 0.2 V vs. Ag/AgCl was applied. Measurements of the samples were performed after the background current had settled (100 s). That is, 50, 100, 200 μl (total 18, 32, 72 μM) of acetylthiocholine in a phosphate buffer (pH 7.4) was injected into the cell by a micropipette. All measurements were performed at 25±1◦C.

Results and discussion

Development of AChE adsorbed F127-MST (AChE-(F127-MST)) sensor.

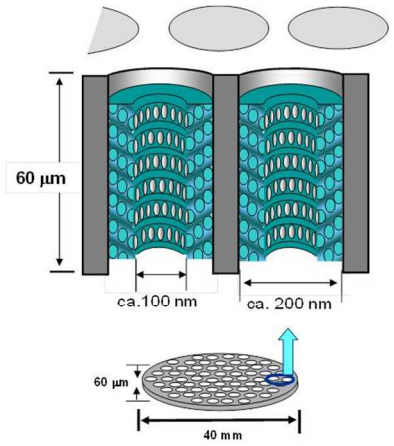

Figure 1. Schematic illustrations of the anodic alumina membrane and the assembly of silica-surfactant nanochannels (channel diameter = 13 nm) formed inside the columnar alumina pore (pore diameter = 200 nm).

In our previous study, we have successfully prepared silica nanotubes with nanochannel-wall in the columnar pores of a commercial anodic alumina membrane, F127-MST[27]. As shown in Fig. 1, the columnar pores have about 100 and 200 nm of inner and outer diameter, respectively. Nanochannels on the wall have 3D-hexagonal structure with the diameter of 13 nm.

In this study, a particular enzyme, an acetylcholinesterase, AChE, from Electrophorus electricus, consisting of monomeric subunit with a molecular weight of 237,170 (size: ca. 10 x 15 x 20 nm; PDB (1C2O), EC; 3.1.17) was chosen to make a hybrid to F127-MST in order to investigate application as an artificial membrane. AChE was adsorbed onto the composite membrane by immersing F127-MST in an aqueous solution containing the AChE. The amount of AChE adsorbed on the membrane was evaluated by changing original AChE concentration, as shown in Fig. 2a. Above 2 mg/ml of AChE, the amount of adsorbed AChE seemed saturated and was around 0.38 mg per 100 mg of F127-MST. Then, TG-DTA measurements were done for AChE-(F127-MST). As seen in Fig. 2b, the weight loss increased with an increase in the amount of AChE adsorbed, which was consistent with previously reported data[27,28]. Therefore, the composite membrane was confirmed to effectively immobilize AChE.

Electrochemical sensing ability of AChE-(F127-MST):

Electrochemical experiments were conducted with AChE-(F127-MST) based electrode as described in Fig. 3. The details of electrochemical set-up are described in experimental section. Amperometric measurements for sensing acetylthiocholine were performed in phosphate buffer (pH 7.4) solution containing 0.5 mM TCNQ as a mediator. Fig. 4 shows the amperometric responses of

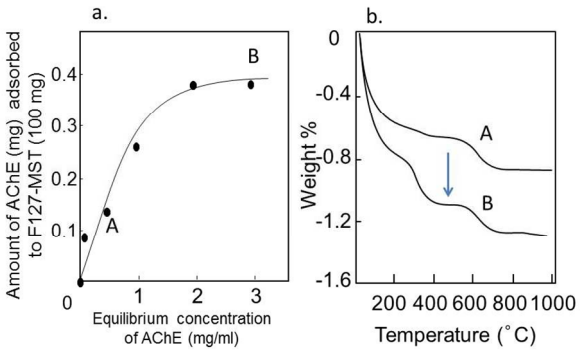

Figure 2. a) Spectrophotometrically measured adsorption of AChE onto F127-MST with respect to the equilibrium concentrations of AChE. b) TG-DTA curves for AChE-(F127-MST), where the amounts of AChE adsorbed to F127-MST (100 mg) for A and B are 0.14 and 0.38 mg, respectively.

AChE–(F127-MST) and the native AChE, which is not immobilized and dissolved in the electrolyte solution, after the injections of different concentrations of acetylthiocholine, ranging from 18 μM to 72 μM. Here,

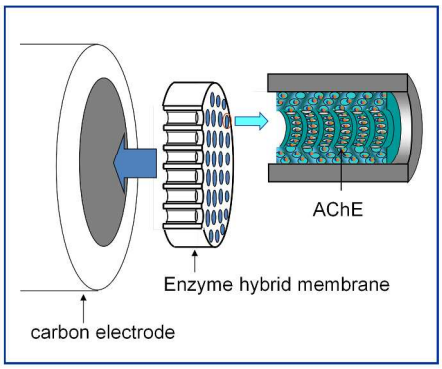

Figure 3. Schematic diagram of a working electrode in this study.

steady-state current was observed for AChE–(F127-MST), while steady-state current was not obtained and kept decreasing for the native, even though the instantaneous current response by the native was much higher than by AChE–(F127-MST). Also, Fig. 4a inset shows a corresponding output current against the different concentration, showing a linear relationship. The lowest detection we tried was 18μM, corresponding to 5 ppm.

The native AChE can be used only one time due to its low stability, while AChE–(F127-MST) is possibly reusable several times because it retains high stability. In order to confirm reusability of AChE–(F127-MST), current responses against 36 μM of acetylthiocholine were measured repeatedly every after washing AChE-(F127-MST) by distilled water for three times. Here, the

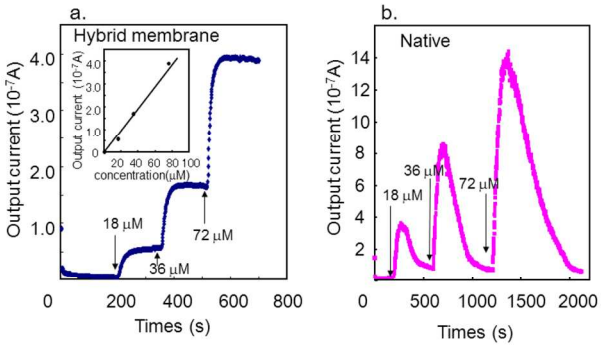

Figure 4. Typical responses of (a) AChE–(F127-MST) (0.014 mg/ 1.8 mg) and (b) the native AChE (0.015 mg) to the injections of acetylthiocholine solutions (18, 36 and 72 μM) at a constant potential of Eapp = 0.2 V. The electrolytes comprise 0.5 mM TCNQ in 90 μl of a phosphate buffer (pH 7.4).

electrolyte solution was exchanged to a fresh electrolyte solution after each measurement. As a result, even after 11 repetitive uses, AChE–(F127-MST) was able to reproduce over 95% of the original current response, as shown in Fig. 5a. The long-term storage stability of the AChE–(F127-MST) was also investigated by evaluating its response after 30 and 60 days of storage at 4 °C in a phosphate buffer (pH 7.4). As can be seen in Fig. 5b, the AChE–(F127-MST) electrode exhibited good stability. In fact, over 90% of its initial current response was retained after being stored even for 60 days. Further, electrochemical response of separately-synthesized samples in the same way have only a margin of error less than 5 % one way or

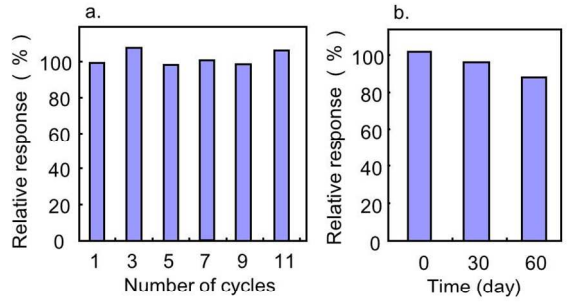

Figure 5. a)Relative current response of AChE–(F127-MST) (0.014 mg/1.8 mg) under the repeated use, and b) under the long-term storage stability (30 and 60 days). The current responses were measured by injections of a acetylthiocholine solutions (36 μM) at a constant potential of Eapp = 0.2 V. The electrolytes comprise 0.5 mM TCNQ in 90 μl of a phosphate buffer (pH 7.4).

the other (Fig. S2). We can provide a stable materials. Therefore, successful encapsulation of AChE into the pores of F127-MST brings up enough stability to be used for a practical application.

Inhibitor effect on sensing ability on AChE-(F127-MST):

Next, the inhibitor effect of organophosphorous pesticides on AChE-(F127-MST) was evaluated by monitoring the current responses in the presence of methamidophos. Fig. 6a shows typical amperometric responses in the presence of different concentrations of methamidophos, ranging from 0 to 1860 ppb, within the electrolyte solution. Fig. 6b summarizes the degree of inhabitation against concentration of methamidophos. Here, the concentrations of methamidophos denote final concentration of methamidophos in the electrolyte solution. As a result, values of steady-state current decreased with an increase in the amount of methamidophos. Since the inhibition of AChE is known to directly related to the

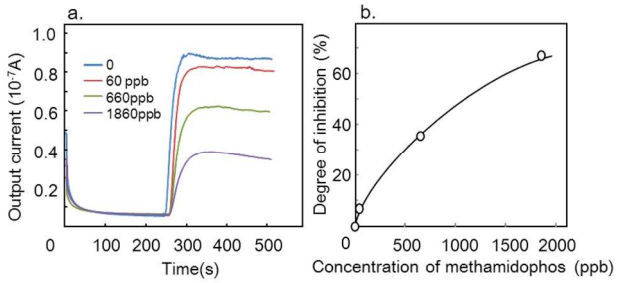

Figure 6. Current responses of AChE-(F127-MST) in the presence of different concentrations (0, 60, 660, and 1860 ppb) of inhibitor, methamidophos. and b) degree of inhibition against concentration of methamidophos. Measurements were done in 0.5 mM TCNQ / phosphate buffer (pH 7.4) by injecting acetylthiocholine solutions (36 μM).

amount of organophosphorous pesticides[10, 11], the result shows that we can effectively detect low concentration of methamidophos like 60 ppb by getting about 8 % inhibition.

Development of the working prototype of an enzyme sensor for the ready measurement:

Since AChE-(F127-MST) sensor unit was successfully made and its stability and sensitivity were well evaluated, development of an actual enzyme sensor with this unit was considered as a next step. We manufactured screen-printed carbon electrodes (SPCEs) containing TCNQ as seen in Fig. 7 in the same way as reference[32]. External views of the sensor unit (7 x 8 x 5 cm) composed with SPCE are also shown in Fig. 8. AChE–(F127-MST) cut into ϕ 4.5 mm by excimer laser irradiation was simply placed to the working carbon electrode without further fixing. A sampling unit was fixed to SPCE, and then 90 μL of 0.1 M phosphate buffer (pH 7.4) was poured into the unit. By using this sensor, amperometric measurements were performed with a PalmSens handheld potentiostat (Palm Instruments BV,

Netherlands) at room temperature (25 °C), by applying a constant potential (150mV) to the working electrode. After the transient current had settled (150 s), 10 μL aliquots of known concentrations of AChE were injected into the sampling unit by micropipette. Fig. 9 shows a

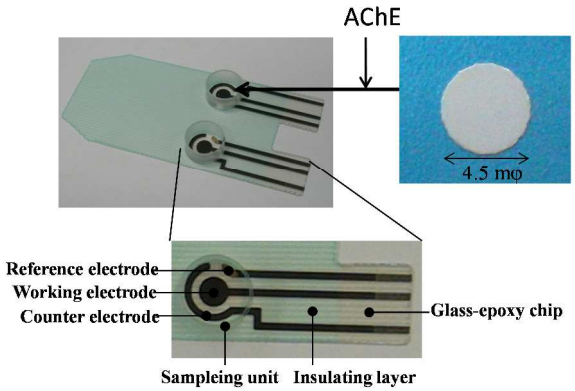

Figure 7. The manufactured screen-printed carbon electrode containing TCNQ on a glass-epoxy substrate.

corresponding output current against the different concentration at each right (A)-and-left (B) electrodes, showing a linear relationship and having little a margin of error of right-and-left. Also, this system can keep to a bare minimum external factor like temperature effect or pH taking difference between right-and-left electrodes. Therefore, this electrode permits the stable measurement.

Sensing ability of our working prototype of an enzyme sensor: By using our working prototype of an enzyme sensor, measurements were performed in the following.

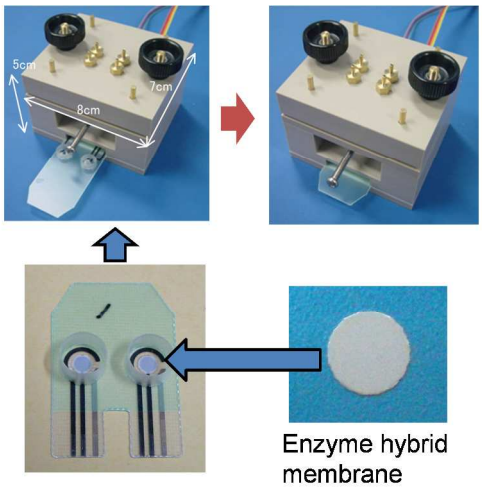

Figure 8. External views of the sensor unit developed in this study.

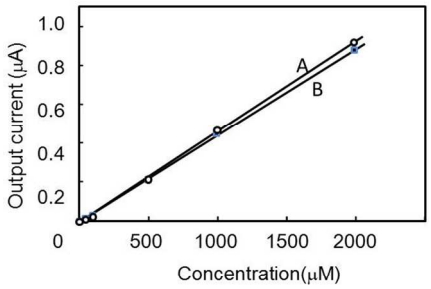

Figure 9. Output current against the different concentration of acetylthiocholine at each right (A)-and-left (B) electrodes.

First, 40 μL of 0.1 M phosphate buffer (pH 7.4) was poured into the left side (for reference) and right side (for detection) chambers. Next, 80 μL of distillated water and diluted solution of target organophosphorous pesticides were injected into the left and right sides of chambers, respectively. After an adequate incubation time, 40 μL of 500 μM concentrations of acetylthiocholine solutions were injected into both sides of chambers by micropipette. Finally, the sensor responses were measured until the

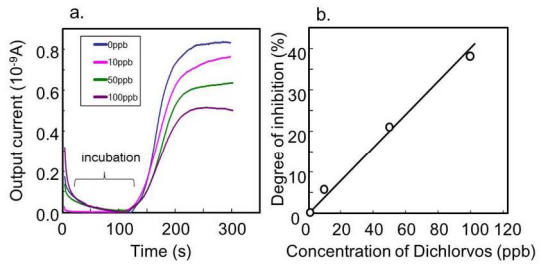

Figure 10. a) Current responses of AChE-(F127-MST) in the presence of different concentrations (0, 10, and 100 ppb) of inhibitor, i.e., dichlorvos, and b) degree of inhibition against concentration of dichlorvos. Measurements were done in phosphate buffer (pH 7.4) by injecting acetylthiocholine solutions.

resulting current reached the steady-state values. Then, the degree of inhibition, I (%), was calculated according to the following formula:

$$I(\%) = \frac{i_0 - i}{i_0} \times 100$$

Where i_0 and i are the response currents in the absence (left side electrode) and the presence (right side electrode) of target organophosphorous pesticides after the addition of acetylthiocholine solutions, respectively.

In case that dichlorvos was chosen, typical amperometric responses in the presence of different concentrations of dichlorvos, ranging from 0 to 100 ppb, are shown in Fig. 10a. Degree of inhibition and

Table 1. Comparisons of different methods prepare to biosensors for pesticides based on electrode and AChE

immobilization	Enzyme	OP compound	Stability (storage /repeat)	Incubation time	Working range	detection limit	Ref.
dialysis membrane	AChE/ChOx	Paraoxon, carbofuran and aldicarb	---	30 min	3.0 x 10^{-7}, 9.1 x 10^{-8}, 3.0 x 10^{-7}M	1 x 10^{-9}M	33
CNT (carbon nanotube)	AChE/ChOx	Methyl prathion	---	10 min	Up to 200 μM	0.05 μM	34
Silica sol-gel	AChE	Oxydemeton methyl	21 days at −20 °C /60 times	20 min	2-200 ppb	2 ppb	35
PVA-SbQ polymer, Silica sol-gel and metal–chelate affinity	AChE	Paraoxon, dichlorvos and chlorpyrifos	11 weeks at 4 °C	20 min	10^{-8}-10^{-9}M	2.4 x 10^{-8}, 1.5 x 10^{-8}, 1.12 x 10^{-8} M	36
Silica sol-gel on Au NPs	AChE	Monocrotophos	---	10 min	0.001-1 μg/ml, 2-15 μg/ml	0.6 ng/ml (0.6 ppb)	37
Silica-monolith microreactor	AChE/ChOx	Malaoxon, eserine and methomyl	---	20 min	---	0.5, 0.2 and 1.0 μM	38
Au-Pt NPs	AChE/ChOx	Paraoxon, carbofuran and DFP	15 days at 25 °C	10 min	0.1-100 μM	0.2 μM at 25% inhibition level	39
AChE–Au–PPy/GCE	AChE	Methyl parathion	30 days at 4 °C	30 min	0.005 -0.12, 0.5-4.5 μg/mL	2 ng/mL (2 ppb)	40
Mesoporous silica membrane (F127-MST)	AChE	dichlorvos	60 days at 4 °C /10 times	2 min	Up to 100 ppb	14 ppb	This paper

concentration of dichlorvos gave a linear relation for the range 0-100 ppb with a correlation coefficient of 0.996 as shown in Fig. 10b. The biosensor has good analytical characteristics for dichlorvos, including linear range (up to 100 ppb), and low detection limit (14 ppb). Also, we can increase the selectivity of our sensor if we give more incubation time (Fig. S3). However, we set incubation time of our sensor in 2 minutes, becouse in-situ measurement using enzymatic biosensor need to high-speed. Additionally, we tested the current responses in the presence of aldicarb and parathion (Fig. S4). The lower detection limits causing 27 % inhibition for aldicarb and 1 % inhibition for paratheion were 1 ppb in this study. The analytical features achieved for pesticides determination and those reported previously in the literature, using biosensors, are presented in Table 1. It shows that the proposed biosensor has good storage stability longer than that of other biosensors[35, 39, 40]. The limit of detection was comparable to that of the sensor presented in [34, 35, 36] or was better than that of the sensor reported in [39, 40]. The response time was lot shorter than that of other biosensors[33-40]. But in comparison to biosensors using microreactor, Llopis et al.[41] have reported the sensor developed with enzyme-labeled magnetic beads and shown the limit of detection producing 5 % inhibition is 0.34 ppb for 90 seconds. Even though our sensing ability is lower than theirs, we have an advantage on increased stability of enzyme against outer environment, such as heat, organic solvent, and so on by encapsulating enzymes into silica nanospace[42-46]. Actually, our sensor is a considerably practical sensor with high reusability and storage stability. Also, it can be used in in-situ measurement. Therefore, we believe that our sensor is applicable to inhibition biosensors, i.e., the determination of organophosphorus pesticides.

Conclusion

In this study, we have presented the amperometric biosensor based on enzyme immobilization in F127-MST. AChE-(F127-MST) was tested for both sensing acetylcholine and organophosphorus pesticides. A novel combination of AChE-(F127-MST) and TCNQ allowed us to detect acetylcholine rapidly with high sensitivity.

Since AChE-(F127-MST) sensor unit was successfully made and its stability and sensitivity were well evaluated, development of an actual enzyme sensor, the working prototype with this unit was considered as a next step. The working prototype was tested within the range of 0-100 ppb of organophophorous pesticides. Resulting response time was approximately 5 min, showing its possible usage at practical level. The detection limits for target pesticides in this study are not especially good comparing to the previously reported sensor. For example, concentration of dichlorvos causing 6 % inhibition was 10 ppb. However, we have an advantage on increased stability of enzyme against outer environment, such as heat, organic solvent, and so on by encapsulating enzymes into silica nanospace[42-46]. Consequently, AChE-(F127-MST) is a considerably practical sensor with high sensitivity, reusability, and storage stability.

Acknowledgements

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