Introduction

Electrochemical transducer systems based on acetylcholinesterase (AChE) are particularly promising for pesticide detections due to more advantages being offered compared to chromatography1-3 or electrophoresis methods.4,5 AChE is a well-known key enzyme in many important areas, such as neurobiology, toxicology and pharmacology, since AChE catalyzes the hydrolysis of acetylthiocholine to thiocholine.6 It is also known that different types of pollutants, especially various types of pesticides, like organophosphorus, carbamates and organochlorine, inhibit the enzymatic reactions of AChE.6-8 Their derivatives are harmful compounds found in insecticides, pesticides and chemical-warfare agents.2,9,10 Taking advantage of the inhibition behavior of pesticide, a pesticide sensor could be developed by monitoring the enzymatic activity during the reaction of acetylthiocholine/thiocholine.8,9,11-13

Meanwhile, colorimetric and spectrophotometric are the standard methods used to detect thiocholine.14-16 However, those methods require some chemicals to generate color14,15 or fluorescent products,16 which is less favorable compared to an easy and economic detection system based on the electrochemical oxidation of thiocholine at solid electrodes.9,17-25 Accordingly, direct detections of thiocholine using different types of solid electrodes were developed, including at gold17-19 and platinum-based20 electrodes. The use of carbon-based electrodes was also reported, including graphite paste,21 screen-printed carbon electrodes,22,23 and carbon nanotubes combined with other solid electrode.9 However, generally the use of some mediators, such as 7,7,8,8-tetracyanoquinonedimethane,21,22 cobalt(II) phthalocyanine,23 and prussian blue,24,25 to improve the kinetic reaction was also documented.

On the other hand, boron-doped diamond (BDD) electrodes are established as superior electrodes among other solid electrodes due to their wide potential windows, low background currents and excellent stability regarding physical and chemical properties.26 Moreover, the biocompatibility is also satisfactory.27 The applications of BDD electrodes as detectors and sensors for some important materials have been reported.26-29 However, it is very rare that enzymatic systems have been developed using BDD electrodes, since it is difficult to perform chemical modifications, such as enzyme immobilization, at a BDD
surface. In this work, the electrochemical detection of chlorpyrifos (CPF), as a model of organophosphorus pesticides, was developed based on thiocholine oxidation at bare BDD electrodes. AChE was used to generate thiocholine through the enzymatic hydrolysis of acetylthiocholine. Acetylcholine chloride (AT-Cl) was found to produce a more selective oxidation peak potential of thiocholine than acetylthiocholine iodide (AT-I). Furthermore, the extraordinary property of a collection of magnetic beads was utilized to immobilize AChE at a BDD surface. Then, the inhibition effect by CPF in AChE activity was used as a signal.

Meanwhile, the immobilization of AChE on magnetic beads has already been documented based on the adsorption, nickel-histidine affinity, and covalent coupling using glutaraldehyde. In this work, streptavidin-biotin interaction was used to perform self-assembly AChE immobilization on the magnetic beads. A selective and sensitive detection of CPF injected in tap water was successfully demonstrated. High stability of the current responses and a very low limit of detection (LOD) can be achieved. The results suggested that the combination of using magnetic beads with the superior properties of bare BDD electrodes is suitable for organophosphorous pesticide detection, especially CPF.

Experimental

Reagents and chemicals

Acetylcholinesterase (AChE, 844 U/mg from electric eel) was purchased from Sigma-Aldrich. The activity was confirmed by Pierce. AT-I, AT-Cl, Tris–HCl, bovine serum albumin (BSA), sulfo-NHS-biotin, and other chemicals were supplied by Wako, Japan.

Electrode preparation

BDD thin films were deposited on Si(111) wafers in a microwave plasma chemical vapor deposition system (Cores Technology). Acetone was used as a carbon source, whereas trimethoxyborane was used as a boron source with a 1% B/C ratio. The details of the preparation are described elsewhere. The surface morphology and the crystalline structure of the films characterized using a scanning electron microscope (SEM) showed a polycrystalline diamond grain size of ~5 μm with ~13 μm thickness. Raman spectroscopy showed a peak at ~1300 cm⁻¹ attributable to the sp³ hybridization of diamond. The absence of a peak at ~1600 cm⁻¹ suggested a fine quality of the diamond films. Prior to use, the BDD film was cleaned by ultrasonication in 2-propanol for 10 min, followed by rinsing with high-purity water. In order to oxidize the surface of BDD, the as-deposited BDD was electrochemically oxidized in 0.1 M H₂SO₄ at +3.0 V (vs. Ag/AgCl) for 20 min. Characterization with XPS showed that the process increased the O/C ratio of BDD from 0.03 to 0.3.

Preparation of AChE-modified magnetic beads

Prior to use, the magnetic beads were cleaned based on a procedure proposed by Dynal Biotech. A volume of 15 μL of the bead solution was homogenized and equilibrated in a vial containing 150 μL of a 50 mM phosphate buffer solution (PBS). The process was repeated three times. Meanwhile, the AChE was modified with biotin according to a procedure proposed by Gao et al. Briefly, the AChE was reconstituted in PBS and desalted over a PD-10 column equilibrated in PBS. An aliquot of sulfo-NHS-biotin was added into a 2.5 mL solution of 50 mM PBS pH 7.4 containing 75 mU AChE, then conjugated for 60 min at room temperature. Finally, 50 μL of the conjugated AChE was added and mixed in a vial of washed magnetic beads, and allowed to undergo a conjugation process for 24 h at 4 °C to obtain the AChE-modified magnetic beads. Prior to use, the vial was normalized at room temperature, followed by a three-times washing process to remove any excess of enzyme, and allowed to exist in the form of a suspension in 150 μL PBS before being measured.

Electrochemical measurements

The electrochemical experiments were performed in a conventional three-electrode cell system with a BDD film used as a working electrode, an Ag/AgCl electrode as a reference electrode, and a platinum wire as a counter electrode. The volume of the cell was 5 mL. The BDD working electrode along with the conducting silicon substrate was pressed against the bottom of the cell using a Viton O-ring (3 mm diameter). Electrical contact was made through the backside of the silicon by contacting to a brass plate. A piece of magnet bar with a diameter of 3 mm was then placed under the brass plate at the same position as the working electrode. Electrochemical measurements were carried out in 50 mM PBS at ambient temperature using a galvanostat (ALS CHI BAS).

Enzymatic assay

The AChE-modified magnetic beads suspension from the vial was added into a cell that contained 1 mL of CPF in PBS. In order to dissolve CPF, a mixture of methanol and NaOH solution is required. After the optimum inhibition time, 3 mL of acetylthiocholine chloride in PBS was added, and final concentration was adjusted to 1 mM. Cyclic voltammetry was conducted after an optimum contact time between AChE and acetylthiocholine chloride. Schematic steps of the electrochemical measurements of the enzymatic assay are displayed in Fig. 1. The measurements were performed for various concentrations of CPF standard solutions, and repeated three times. The application of a real sample was performed using a Yokohama (Japan) tap water sample. Prior to analysis,
the sample was filtered using a filter paper (Whatmann-41, pore diameter of 20 – 25 μm). Then, a volume of 5 mL filtered tap water was injected by a CPF standard solution. A volume of 1 mL of the CPF-injected sample was then filled into the cell to be treated using the same procedure as the standard solution of CPF for electrochemical measurements.

Results and Discussion

Electrochemical characteristic of thiocholine at BDD electrodes

Figure 2 shows cyclic voltammograms (CVs) performed using BDD electrodes for 0.1 M PBS pH 7.4 in both the absence and the presence of 1 mM acetylthiocholine iodide and 1 mM acetylthiocholine chloride, and at 5 min after the addition of 25 μU AChE in the system of (a) acetylthiocholine iodide and (d) acetylthiocholine chloride. Inset of (d) shows its related voltammograms observed at glassy carbon electrode. Scan rate was 100 mV/s.

![Figure 2](image-url)

Fig. 2  Cyclic voltammograms performed for 0.1 M PBS pH 7.4 in both the absence and presence of (a) 1 mM acetylthiocholine iodide and (b) 1 mM acetylthiocholine chloride, and at 5 min after the addition of 25 μU AChE in the system of (c) acetylthiocholine iodide and (d) acetylthiocholine chloride. Inset of (d) shows its related voltammograms observed at glassy carbon electrode. Scan rate was 100 mV/s.

The oxidation and reduction peaks in Fig. 2(a) could be attributed to the oxidation of iodide ions, which are presented in the system as counter ions of AT-I.

Considering that the current of the thiocholine oxidation peak was relatively small in comparison to those of iodide oxidation, and therefore was predicted to be discomposed in the presence of iodide ions, AT-Cl was then used for the next experiments. A comparison was also performed with the oxidation peak of thiocholine at glassy carbon electrodes, as shown in the inset of Fig. 2(d). Although a similar oxidation potential as well as current were observed at both the glassy carbon and BDD electrodes, a lower background current of the BDD electrode results in about an order higher signal-to-background ratio of the current, which generally leads to better limits of detection.26
is in agreement to the oxidation of the –S–H functional group at BDD electrodes, as reported by Terashima et al.\textsuperscript{34} Based on this report, it is predicted that the –S–H bond was oxidized to be the S–S functional group, as follows:

\[
2 \text{R–SH} \rightarrow \text{R–SS–R} \rightarrow 2 \text{H}^+ + 2e^– (1)
\]

The oxidation can continue to –S–O–S– at the higher potential. However, in this work we limited the measurements at +1.5 V and used the peak at +0.8 V for the following experiments.

The peak current at +0.8 V increases with the contact time between AT-I and ACHE, then reaches the maximum currents after 30 min (Figs. 3(a) and 3(b)). Therefore, a contact time of 30 min was fixed for the next experiments. Furthermore, using 1 mM AT-Cl as the substrate, the oxidation peak currents at +0.9 V were linear ($R^2 = 0.99$) in the ACHE activity range from 5 mU to 30 mU (Figs. 3(c) and 3(d)). The results suggested that this system is promising to be utilized to monitor the activity of ACHE.

The dependence of the peak current of thiocholine on the scan rate was also investigated from the scan rate of 5 to 400 mV/s. A linearity ($R^2 = 0.99$) to the square root of the scan rate was achieved, suggesting that the oxidation mechanism was under diffusion control.

Basically, the enzymatic assay was performed using an inhibition reaction of ACHE by organophosphorus compounds. Since organophosphorus compounds have very similar molecule structures with acetylthiocholine, they can irreversibly bind to the active site of ACHE through the phosphorylation adduct.\textsuperscript{36} In order to avoid any competition between acetylthiocholine and CPF to react with ACHE in this sensor, AT-Cl needs to be added after the inhibition reaction of CPF to ACHE is completed.

Furthermore, since this reaction involves enzymatic activity, it is important to control the pH. Accordingly, the oxidation current of thiocholine was investigated at different pH values in the presence of CPF. Solutions of 1 mM CPF with different pHs were conjugated to 25 mU ACHE for 10 min, then, an AT-Cl solution was added into the solution. The final concentration of AT-Cl was adjusted to 1 mM and voltammetry was performed after 30 min contact. Figure 4(a) shows plots of the peak currents at a potential of +0.8 V. The presence of CPF decreases the peak current of thiocholine oxidation, since CPF inhibits the ACHE activity. The maximum decrease occurred at pH 7.4, and remained the same when the pH was continually decreased to 7.0. Therefore, pH 7.4 was selected for the condition of the following experiments.

Further, the influence of the inhibition time of ACHE by CPF was investigated. A solution series of 1 mM CPF was conjugated to 25 mU ACHE in 0.1 M PBS pH 7.4 for various inhibition times of from 0 to 25 min. Then, AT-Cl was added and cyclic voltammetry was conducted. Figure 4(b) shows that increasing the inhibition time by CPF resulted in a decrease of the oxidation peak current at +0.8 V. As the decrease reached its maximum after 15 min, the inhibition time of 15 min was fixed for the next experiments.

Modification of magnetic beads with ACHE

Modification of the magnetic beads was performed using a specific interaction between streptavidin and biotin. Streptavidin-modified magnetic beads supplied from Invitrogen AG (Basel, Switzerland) were used, whereas the ACHE was an in-house modified with biotin to provide the attachment of ACHE on the magnetic beads. Sulfo-NHS biotin was used to perform biotinylized ACHE. Then, biotinylized ACHE was
conjugated to the magnetic beads. The steps involved in the assembly of the structure of the enzymatic assay for the determination of CPF are shown in Fig. 1. Since streptavidin is a tetrameric protein in which each sub unit binds to one molecule of biotin, larger capacities of streptavidin than biotin allow the immobilization of biotinyl AChE on the surface of magnetic beads. An amount of 15 μL of the magnetic bead suspensions is expected to cover the active site for 75 mU of biotynilized AChE. However, problems related to unspecific sites need to be considered, including the unspecific sites of the magnetic beads that was not covered by AChE, and the surface of sulfo-NHS-biotinyl-streptavidin at the magnetic beads as the result of the reaction between sulfo-NHS-biotin with magnetic beads. In order to cover the unspecific sites, BSA was then added.

Prior to measurements, a method was examined for measuring 1 mM CPF using unmodified magnetic beads (without AChE) at 30 min after the addition of AT-Cl, as shown in Fig. 5(a). Another measurement of 1 mM using AChE-modified magnetic beads without the addition of AT-Cl in the solutions was also tested, as shown in Fig. 5(b). Two very small oxidation peaks at potentials of around +0.3 and +0.6 V are shown in the CVs of both tests. Theoretically, there is no thiocholine peak that will be generated at the system in the absence of AChE or AT-Cl. It was previously reported that Bovine Serum Albumin (BSA) are electroactive at hydrogen terminated BDD but not at oxidized terminated BDD electrodes. Since the oxidized terminated one was employed in this work, it can be presumed that the oxidation peaks that appeared at the CVs were related to the oxidation of biotin-streptavidin systems. Furthermore, these peaks appeared at different potentials with very small currents in comparison with the thiocholine peak. Therefore, these peaks can be ignored, and the system can be considered to be suitable for the detection of CPF.

Application of AChE-modified magnetic beads in chlorpyrifos sensors

The AChE-modified magnetic beads were then applied for electrochemical sensors of CPF. A comparison was made with the system with free AChE. Figure 6 shows typical CVs resulted from those systems with free AChE (Fig. 6(a)) and with immobilized AChE at magnetic beads (Fig. 6(b)) in the presence of various concentrations of CPF. The same number of enzyme activity, i.e. 25 mU, and the same concentration of AT-Cl, i.e.
1 mM, were employed. In the CVs of both systems, a well-defined peak was observed at around +0.7 V. This peak potential is slightly shifted compared to the thiocholine peak in Figs. 2 and 3 due to the change of the pH, since methanol and NaOH are required to dissolve CPF. The dependences of the inhibition percentage of AChE to the CPF concentrations are shown in Figs. 6(c) and 6(d). Good linearity in the CPF concentrations range from 10^{-9} to 10^{-5} M was shown at both systems ($R^2 > 0.9$). However, a slightly higher slope was observed for the system using the magnetic beads. Small error bars shown in these figures, suggested good stability of the current responses, which indicated the stability of the BDD surfaces. Furthermore, the sensor’s performance was also examined for 50 and 10% AChE inhibition (IC_{50} and IC_{10}, respectively).40 About one order lower IC_{50} and IC_{10} can be achieved using a system with immobilized AChE. The limits of detection (LODs) were then estimated using IC_{10}, which showed LOD values of 6.5 x 10^{-9} and 5.7 x 10^{-10} M for free and immobilized AChE, respectively. The results suggested that the immobilization of AChE at magnetic beads system provides better performance in the measurements of CPF. A summary of the analytical performance is displayed in Table 1. These LODs are also comparable to other reported detection methods based on thiocholine (Table 2), considering that the system applied by cyclic voltammetry instead of amperometry.17,18,22,25,39–43 The AChE-modified magnetic beads could be stored in 4°C for 30 days with less than a 5% change of the current response. However, the recovery of the magnetic beads (ex. using a microfluidic system) for repetitive measurements is an important aspect to be considered in the future.25

### Analysis of chlorpyrifos in tap water

In order to study the interference effect, Yokohama (Japan) tap water spiked with CPF was selected as a model sample. The tap water, according to Yokohama Waterworks Bureau, can be used as drinking water. The water is well controlled and its quality information is available to be accessed by public with a pH of 6.99 and contains in ppm of 0.04 Al, 49 Ca, 6.5 Cl–, 0.01 Fe, 49 Mg, and 1.17 NO3–. Negative contents of Cu, As, Zn, Cr, and Pb have been reported. While Ca^{2+}, Cl–, and NO3– ions are known not to be electroactive, Al, Fe, and Mg was found to have oxidation-reduction potentials far from +0.8 V (vs. Ag/AgCl).26 Therefore, the interference was expected to come from the inhibition of AChE by these compounds.

The CPF-injected tap water solutions of 10^{-7} and 10^{-6} M were measured using systems with free and immobilized AChE. While the free AChE systems showed that the interference of tap water increases the signals, the immobilized AChE systems showed a better recovery of the measurements. Apparently, the accumulation of AChE immobilized at magnetic beads at the surface of BDD limits the interference of some metals to AChE. It is well known that activity of AChE decreases with the presence of metals.6,8 The results suggested that the detection of

#### Fig. 6
Linear sweep voltammograms performed for 1 mM acetylthiocholine chloride in 0.1 M PBS pH 7.4 at 15 min after being added in various concentrations of CPF conjugated to (a) free AChE in the solutions and (b) immobilized AChE at 15 μL magnetic beads. The linear dependence inhibition % on the CPF concentrations are respectively shown in (c) and (d). Scan rate was 100 mVs.
Comparison with other reported chlorpyrifos biosensors based on AChE

<table>
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<tr>
<th>Electrode</th>
<th>Immobilization support</th>
<th>Linearity/M</th>
<th>LOD/M</th>
<th>Ref.</th>
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<td>Strip test</td>
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<tr>
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<td>PEDOT</td>
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</table>

CPF based on thiocholine oxidation using an AChE-modified BDD electrode using a magnetic platform is more selective and suitable at BDD electrodes. A summary of the detection of CPF injected in Yokohama Tap Water is displayed in Table 3.

Conclusions

The detection of CPF based on thiocholine oxidation using AChE immobilized at magnetic beads has been successfully performed at bare BDD electrodes. AT-Cl was found to produce a more selective oxidation peak potential of thiocholine +0.8 V (vs. Ag/AgCl) than acetylthiocholine iodide. The optimum contact time between AT-Cl and AChE was found to be 30 min, whereas the inhibition time of AChE by CPF was 15 min. The system with immobilized AChE at magnetic beads provided a better limit detection and less interference in a tap water matrix compared to that of free enzyme. It can be previewed that the developed pesticide detection system can be implemented easily in (micro) fluidic measurements for on-line monitoring as well and can be extended to several other analytes with interest for the environment, health and safety, and security fields.

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