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Optimization of Quartz Crystal Microbalance-Precipitation Sensor Measuring Acetylcholinesterase Activity

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Abstract The optimization of a batch-type quartz crystal microbalance (QCM)-precipitation sensor measuring acetylcholinesterase (AChE) activity was conducted. To covalently bind AChE onto the gold electrode of a QCM surface, glutaraldehyde cross-linking to a cystamine self-assembled monolayer was tried at different cystamine concentrations. At the optimum conditions of the QCM-precipitation sensor, 0.1 M potassium phosphate buffer (pH 8.0), containing 0.01% Tween 80, was used as the reaction buffer, with the enzyme amount of 5 units for immobilization and the substrate concentration of 50 mg/ml. The current biosensor might find a future applicability to the sum parameter detection on organophosphorus and carbamate pesticides.

Key words: Optimization, QCM-precipitation sensor, measurement, AChE activity

Organophosphorus and carbamate pesticides, the majority of which have reportedly been known as cholinesterase (ChE) inhibitors, belong to large families of insecticides having the composition of 55 and 11%, respectively. In spite of their low environmental persistence and high effectiveness, some exhibit potential dose-related acute and chronic toxicities in human beings, resulted from the accumulation of acetylcholine at cholinergic receptor sites due to ChE inhibition [7]. This can lead to various adverse clinical effects, particularly for infants and children [8, 12]. Therefore, sensitive, rapid, and reliable analysis for these agricultural chemicals has been strongly required for the protection of the environment and human health [1].

For a complete surveillance of organophosphates and carbamates in food matrices, however, quantitative analytical methods as well as screening ones should be developed simultaneously. The former methods that comprise GC, HPLC, and HPLC coupled with mass selective detector [14, 20] usually require tedious extraction and cleanup procedures before instrumental analysis and thus are not appropriate for routine analysis handling a large number of samples. The latter methods can be successfully applied to confirming the presence of these compounds in some positive samples, reducing the demand for quantitative analysis.

As rapid screening tools for the above pesticides, ChE inhibition tests and ChE-based biosensors, in particular, are useful. The principle of these tools is to determine the presence of these chemicals as the sum parameter of ChE inhibition with a high sensitivity [5, 26, 27]. Various spectrophotometric assays [6, 22] based on kinetic and end point methods, and ChE-based biosensors [5, 16, 24, 28] making use of immobilized ChE onto various transducers, have been used for this aim. Compared with the former tests, however, the sensitivity of ChE-based biosensors could be increased conspicuously because of the adaptability to new sensitive transduction principles for measuring sensor response [1, 15]. Moreover, they are useful for on-site monitoring and are easily arrayed for a real-time multi-sample analysis in a very short time [11, 15].

The quartz crystal microbalance (QCM) technique, which uses a mass-sensitive detector based on an oscillating piezoelectric quartz crystal resonating at a fundamental frequency, has been applied to biosensing, exploiting antibody or enzyme as the biological component [4, 13, 19]. It has been reported that the sensitivity of a QCM-based enzyme sensor can be amplified significantly by increasing the mass deposition via the precipitation of enzymatic reaction products [1, 3, 23]. Alfonta *et al.* [2] have measured acetylcholine with 3,3',5,5'-tetramethylbenzidine whose oxidation product is a signal-amplifying precipitate produced by a serial reaction of AChE, choline oxidase, and horseradish peroxidase. Karousos *et al.* [9] have applied this technique to the detection of organophosphorus and carbamate pesticides and reported on a QCM sensor detecting AChE inhibition

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by measuring the precipitation degree of an enzymatic reaction product, 4,4'-diimino-3,3'-diaminobiphenyl that is derived from 3,3'-diaminobenzidine substrate, over the QCM electrode.

We have tried to develop a QCM-precipitation sensor that exploits the precipitation of an AChE reaction product, 3-hydroxyindole dimer, over the surface of a QCM electrode and has a potential for screening the presence of organophosphorus and carbamate pesticides in food matrices. As an initial step, the operating conditions for the sensor were optimized with respect to cystamine concentration during enzyme immobilization, enzyme amount, and the concentrations of substrate and surfactant.

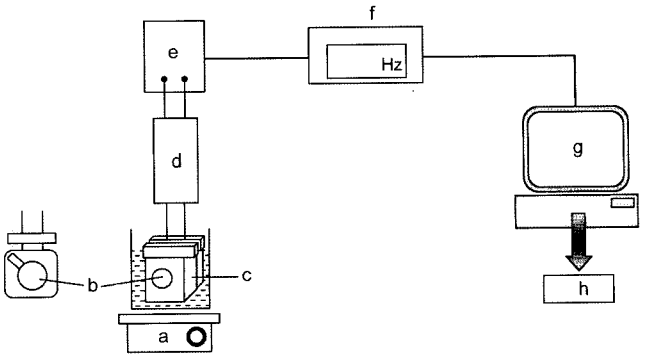

Fig. 1. Schematic diagram of the apparatus for the QCM-precipitation sensor with a batch-type dip holder.
a, Magnetic stirrer; b, Gold surface of QCM; c, Dip holder; d, Connector; e, Oscillator circuit; f, Quartz crystal analyzer; g, PC; h, Operating software.

MATERIALS AND METHODS

Reagents and Materials

AChE from *Electrophorus electricus* (an electric eel), which was used as the sensing element, was purchased from Sigma (MO, U.S.A.), and a histological substrate for the sensing element, 3-indolyl acetate, was the product of Aldrich (WI, U.S.A.). Most of the reagents for enzyme immobilization over the QCM surface such as cystamine, glutaraldehyde, and glycine were obtained from Sigma. The other reagents for buffer preparation, system optimization such as Triton X-100 and Tween 80, substrate dissolution like DMF, and Acetylcholine Enzyme Kit for measuring immobilization efficiency were purchased from Sigma, and double distilled water was used throughout this research. A 9 MHz AT-cut piezoelectric quartz wafer (QA 9RP-50, Seiko EG and G, Japan), attached with two gold electrodes of 5 mm diameter, was used as the transducer with a reproducibility of ±0.1 Hz in frequency response.

AChE Immobilization

Enzyme immobilization was carried out according to the method of Pariente *et al.* [17] with a slight modifications. The QCM was soaked in 1.2 M NaOH for 5 min, washed with distilled water, and immersed in 1.2 M HCl for 5 min. After washing with distilled water, it was treated with 20 μl of conc. HCl for 1 min with special care taken to keep the acid from touching the electrode leads, followed by washing again with distilled water and drying in a convection oven for 20 min. The pretreated QCM was separately incubated with different concentrations (1, 5, and 10%) of cystamine solution for 1 h, followed by washing with distilled water and dipping into 10% glutaraldehyde solution in sequence. After washing with 0.1 M potassium phosphate buffer (pH 8.0), the activated QCM surface was reacted with 40 μl of AChE dissolved in the same buffer solution for 2 h at 4°C. Afterward, the remaining active carboxaldehyde groups were blocked by treating with 0.1 M glycine solution for 30 min at room temperature. Finally, the enzyme-modified QCM was rinsed with the same buffer solution, followed by drying at room temperature. The sensor chip thus prepared was stored at 4°C until use.

System Setup

The construction of a batch-type sensor system for this study was done as follows (Fig. 1). The piezoelectric quartz crystal described above was mounted inside of a dip holder with a plug and was then connected to an oscillator module (QCA 917-11, Seiko EG and G) that was connected to a quartz crystal analyzer (QCA 917, Seiko EG and G). An analog frequency signal from the quartz crystal analyzer was converted to a digital one in an IBM-compatible PC via a GPIB interface. System operation was done with WinEchem software (version 1.12) from Seiko EG and G. To a small beaker filled with 19.5 ml of 0.1 M potassium phosphate buffer (pH 8.0) was inserted the dip holder having a QCM sensor chip immobilized with AChE, followed by the measurement of resonant frequency of the sensor chip until a steady-state baseline was obtained (F_1). Then, 500 μl of the substrate solution dissolved in DMF was injected into the reaction cell, with simultaneous stirring for 3 min, to induce complete substrate dissolution in the aqueous buffer solution. The steady-state resonant frequency (F_2) was read again to calculate the frequency shift ($\Delta F=F_1-F_2$).

Enzyme Binding Efficiency During Immobilization

The binding efficiency of AChE during immobilization was measured with an Acetylcholine Enzyme Kit as follows [25]. As AChE catalyzes the hydrolysis of cholinesters of various short-chain organic acids including acetylcholine, the enzymatic reaction in the presence of an acid-base indicator such as *m*-nitrophenol causes a pH decrease due to the acetic acid liberated, which in turn causes a color loss of the indicator. In this case, the percent absorbance values of the sensor chips prepared by the above two

protocols against the soluble enzyme were expressed as the binding efficiency.

RESULTS AND DISCUSSION

The operating principle of the QCM-precipitation sensor of this study is based on the measurement of the enzymatic activity of AChE, bound on one side of the QCM and simultaneously exposed to the substrate solution, whose enzymatic reaction product is precipitated over the QCM surface because of solubility decrease after dimerization [1]. Then, the degree of reaction can be determined by measuring the frequency decrease caused by a mass deposit over the QCM surface. In this work, the QCM-precipitation sensor was optimized with respect to reaction conditions as follows to maximize the sensitivity and stability of the sensor before applying to an inhibition study with organophosphorus and carbamate pesticides.

AChE Binding and Sensor Response According to Cystamine Concentrations During Immobilization

The effects of cystamine concentration for self-assembled monolayer formation on enzyme binding efficiency and sensor response were determined. As described in Table 1, the absorbance value of the sensor chip treated with 5% cystamine solution was slightly higher than that of the sensor chip treated with 1 or 10% cystamine solution. The sensor chip undergoing 5% cystamine treatment also showed the highest sensor response of 548.0±1.6 Hz after the substrate addition, with a coefficient of variability (C.V., SD/Mean×100, %) of 0.29%. The above C.V. value was low enough to show a good repeatability of measurement, considering the normally accepted 5% level for a reasonable analytical method including biosensor technique [10].

Sensor Responses According to Immobilization Steps

To elucidate the effect of enzyme immobilization on the responses of the QCM-precipitation sensor, the time-dependent responses of the sensor chip at each step of immobilization were traced at 5% cystamine concentration

Table 1. AChE binding and sensor response according to cystamine concentration during immobilization.

Cystamine concentration	Absorbance (ΔA_{420})[a]	Binding efficiency (%)[b]	Frequency shift (Hz)
1	0.0398±0.0002[c]	69.7	267.0±2.7[c]
5	0.0423±0.0003[c]	74.1	548.0±1.6[c]
10	0.0387±0.0012[c]	67.8	196.0±2.9[c]

[a]The absorbance value measured by an Acetylcholine Enzyme Kit assay for the soluble enzyme was 0.0571.

[b]Percent absorbance value against that obtained with the soluble enzyme.

[c]Mean±SD (n=2).

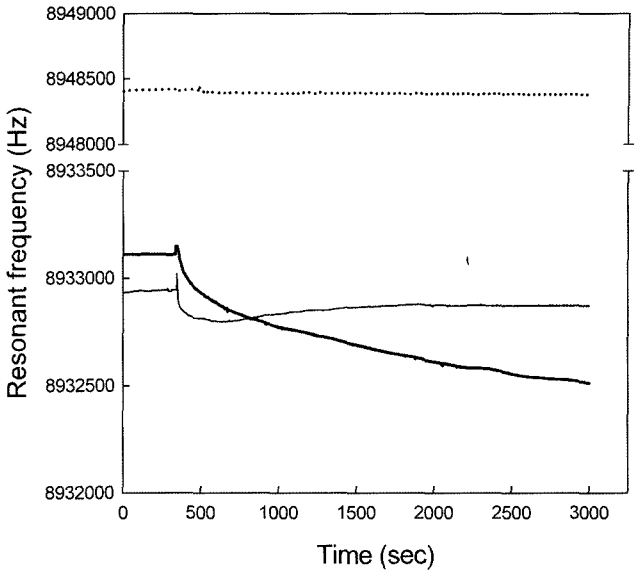

Fig. 2. Resonant frequency profiles of the QCM-precipitation sensor according to immobilization steps.
....., bare sensor chip; ——, only treated with cystamine and glutaraldehyde; ——, fully treated with cystamine, glutaraldehyde, and the enzyme.

(Fig. 2). Compared with the frequency shift of 30 Hz found for bare QCM, the sensor chip only treated with cystamine and glutaraldehyde showed a slightly increased frequency shift of 70 Hz. The above frequency shifts that were possibly caused by nonselective binding of the substrate [21] were not so meaningful considering that the frequency shift obtained with the sensor chip fully treated with cystamine, glutaraldehyde, and the enzyme was bigger than those values by 8.6–20.0-fold and thus could be corrected to measure the actual response. This finding clearly explained that the response of the sensor chip prepared by the complete immobilization was caused by AChE bound to the gold electrode of the QCM [19].

Sensor Response According to Enzyme Amount for Immobilization

To determine the enzyme amount for immobilization, 1, 2, 3, 5, 7, and 10 units of AChE were separately immobilized onto the QCM surface, and the sensor chips thus prepared were measured for steady-state frequency shifts. As shown in Fig. 3, the sensor response increased sigmoidally according to the increment in enzyme amount until 5 units, but decreased conspicuously thereafter to the enzyme amount of 10 units. From this result, it was inferred that the adsorption by precipitation of the dimeric enzymatic reaction product over the QCM surface is slowed down by the closely packed enzyme molecules, which limits its access to active sites. In a similar way, the sensor response only increased to a critical AChE loading in a previous QCM analysis measuring the precipitation of oxidized benzidines

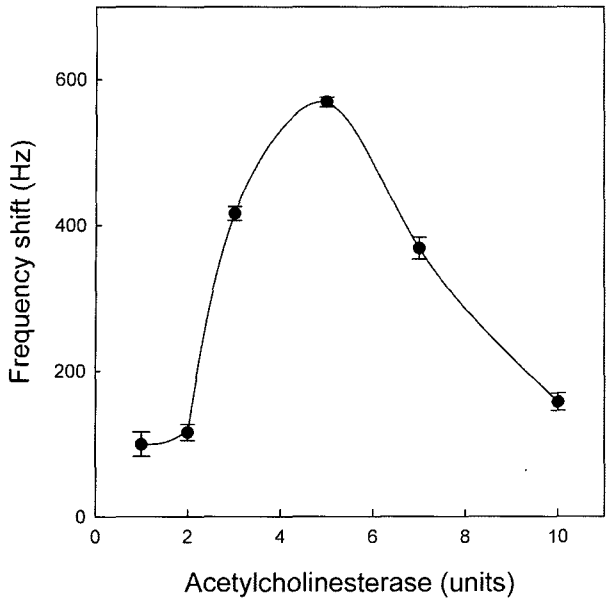

Fig. 3. Effect of enzyme amount for immobilization on the responses of the QCM-precipitation sensor.
Measurements were done in triplicate and the error bars were inserted.

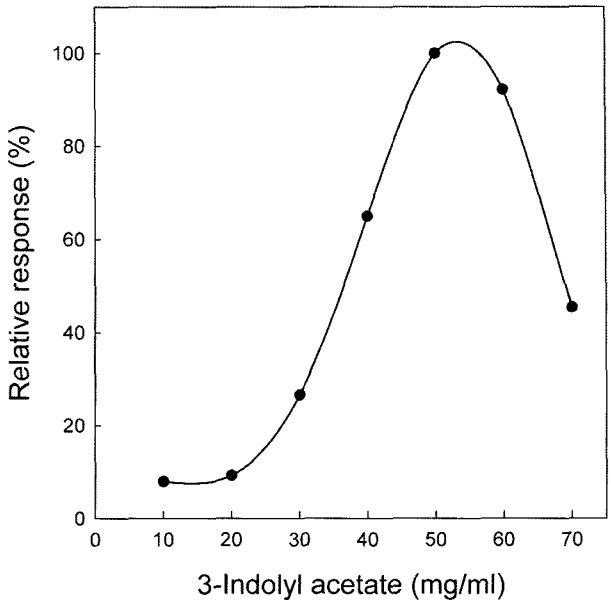

Fig. 4. Effect of substrate concentration on the responses of the QCM-precipitation sensor.
The 3-indolyl acetate concentrations tested were 10, 20, 30, 40, 50, 60, and 70 mg/ml, respectively, and the sensor response obtained with the injection of 50 mg/ml 3-indolyl acetate was arbitrarily taken as 100%.

[9]. Furthermore, it has been reported that it is not desirable to use an excessive esterase concentration for sensor preparation because of the possibility leading to a system tolerant to the presence of a pesticide in the following inhibition study, resulting in no attenuation in the sensor response [9]. Therefore, the optimized amount of AChE for the QCM-precipitation sensor was determined as 5 units. The same response profile as Fig. 3 was found when different amounts of BChE were immobilized onto the QCM surface (data not shown).

Sensor Response According to Substrate Concentration

The responses of the QCM-precipitation sensor at different substrate concentrations were measured at the enzyme amount of 5 units to optimize substrate concentration. The resonant frequency responses decreased drastically for approximately 10 min at all substrate concentrations after the initial spikes owing to magnetic stirring. After this time, the time-dependent decreases in resonant frequency diminished conspicuously, resulting in the establishment of steady-state frequencies. As shown in Fig. 4, the biggest sensor response was obtained at the substrate concentration of 50 mg/ml.

Effect of Surfactant Concentration on the Augmentation of Sensor Response

It has been reported that a surfactant molecule produces a stable interface by reducing the interfacial energy between the precipitating species from 3,3'-diaminobenzidine and the QCM surface [9]. Like the oxidized benzidine, 3-hydroxyindole, which is one main AChE-induced cleavage product from the substrate of this study, is highly hydrophobic although the 3-hydroxyl group gives the molecule hydrophilic character [1]. As such an amphiphilic character is also manifested by surfactant molecules, the use of the modified buffer solution containing nonionic surfactants such as Triton X-100 and Tween 80 was tried to make the precipitating 3-hydroxyindole dimer more surface-active (Fig. 5). In the case of Triton X-100 addition, the responses of the QCM-precipitation sensor were drastically augmented according to the increases in surfactant concentration in 0.1 M potassium phosphate buffer (pH 8.0), reaching to the maximum frequency shift of 2,250 Hz at 0.5% Triton X-100 addition. This value was bigger than that in the buffer solution by only 11.0-fold. Likewise, the addition of Tween 80 to the buffer solution also significantly increased the sensor response by 3.6-fold up to the addition of 0.01% Tween 80. In both cases, however, the sensor response decreased abruptly after the critical surfactant concentrations, as reported previously [9]. From the above result, it was clear that the surfactant addition up to a critical concentration produces a stable interface, possibly by reducing the interfacial energy between the precipitating species and the sensor surface [9]. Although the addition of Triton X-100 increased the sensor response more conspicuously compared with that in Tween 80 addition, the stability in resonant frequency response was better in the latter case. Therefore, the optimum surfactant concentration in the above buffer solution was determined as 0.01% Tween 80.

From the above experiments, 0.1 M potassium phosphate buffer (pH 8.0) comprising 0.01% Tween 80 as the reaction

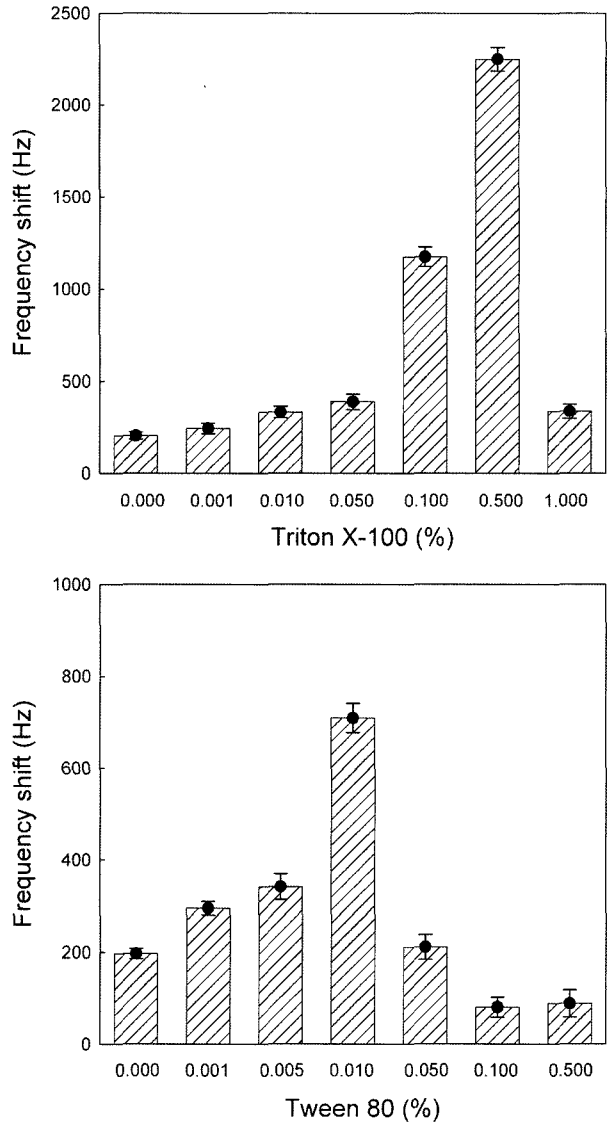

Fig. 5. Effects of surfactant concentration on the responses of the QCM-precipitation sensor.
Measurements were done in triplicate and the error bars were inserted.

buffer, 5 units of AChE for immobilization, and 50 mg/ml 3-indolyl acetate as the substrate solution were selected as the optimum operating conditions for the QCM-precipitation sensor of this study. The use of the sensor at the optimized conditions is expected to make it possible to detect the presence of organophosphorus and carbamate pesticides with a high sensitivity in the following inhibition test, compared with the routinely used spectrophotometric methods. Moreover, as the QCM-precipitation sensor of this study does not require additional reagents such as visualizing chromogens for sensor response, it might be used as a simple, rapid, and highly sensitive screening tool for the above pesticides, possibly present in various food samples, in real-time scale.

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