

## Responses of Chloramphenicol Immunosensor to Analyte Types

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**Abstract** A well-holder type piezoelectric chloramphenicol (CAP) immunosensor which was prepared by binding an anti-CAP antibody to the chemisorbed monolayers of various thiol or sulfide compounds over the gold electrode surface of quartz crystals through a carboxyl-amine coupling procedure, using the activation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl and N-hydroxysulfosuccinimide, was determined for its responses to CAP, CAP succinate, and water-soluble CAP. The reaction phase used in the well holder was 0.01 M phosphate buffer (pH 7.4), and the solvent for analyte dissolution varied according to the solubility of the individual analyte. The analyte detection which was indicated by a steady-state frequency shift was finished within 10 min, except for CAP dissolved in methanol. The responses of CAP succinate and water-soluble CAP in the reaction phase were very stable, while a minute fluctuation was found with CAP.

**Key words:** Responses, chloramphenicol immunosensor, analyte types

With increasing possibility of contamination with antibiotics in the human food chain in spite of their benefit, a worldwide concern has been focused on the safety aspect of food under production and marketing [5, 8, 13, 16]. Chloramphenicol (CAP), which inhibits protein synthesis, has a broad spectrum against Gram-positive and negative bacteria, and rickettsia. However, owing to its residual persistence in the human food chain and innate toxicity, it has been banned in the European Union, Switzerland, U.S.A. and other countries, and a very harsh threshold value has been set [1, 9, 10]. Because of the low solubility of CAP in an aqueous solution [4], some CAP derivatives with enhanced solubility, such as CAP succinate and water-soluble CAP, are manufactured, resulting in the possible contamination of real samples with various CAP compounds.

A piezoelectric immunosensor, which measures the frequency shift caused by the mass change owing to antibody-antigen binding and uses a piezoelectric quartz crystal as the transducer, is economical in setting up the device and simple in the measurement procedure. Moreover, the minimal sample pre-treatment is possible due to its inertness to interferences by various coloring substances which might be present in the analytical samples [2, 6, 12]. Hence, the piezoelectric immunosensor seems to be an efficient tool to screen CAP compounds and might be used for constructing an efficient detection system together with the quantitative analytical methods such as HPLC/PDA, GC/MS selected ion monitoring (SIM), and LC/MS analysis in the sector of food safety [11, 14].

As the CAP compounds, including CAP, CAP succinate and water-soluble CAP, can be translocated to food via feed and injection to livestock, the responses of a piezoelectric CAP immunosensor to individual CAP compounds should precisely be studied for better understanding of CAP immunosensing. For this purpose, this study aims at the determination of response characteristics of a direct-binding CAP immunosensor prepared by a carboxyl-amine coupling procedure to various CAP compounds.

## MATERIALS AND METHODS

### Antibody and Reagents

An anti-CAP antiserum (preservative-free lyophilized powder) which was developed from rabbit, using CAP conjugated to bovine serum albumin as the immunogen, was purchased from Sigma (MO, U.S.A.). The analytes used were CAP (chloromycetin, D-threo-2,2-dichloro-N-[ $\beta$ -hydroxy- $\alpha$ -hydroxymethyl]-4-nitrophenylacetamide), CAP succinate, and water-soluble CAP having hydropropyl group, and were the products of Sigma. The thiol or sulfide compounds which were used for self-assembled monolayers (SAMs) formation on the gold electrode were homofunctional reagents such as 3,3-thiodipropionic acid (TDPA), 3,3-dithiodipropionic acid (DTDPA), and thiodiglycolic acid

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(TDGA), and heterofunctional reagents like thioctic acid (TCA), thiosalicylic acid (TSA), and 3-mercaptopropionic acid (MPA), and they were purchased from Sigma and Aldrich (WI, U.S.A.). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) were obtained from Sigma and Pierce (IL, U.S.A.). The other reagents were of analytical grade, and a doubly distilled water was used throughout this research.

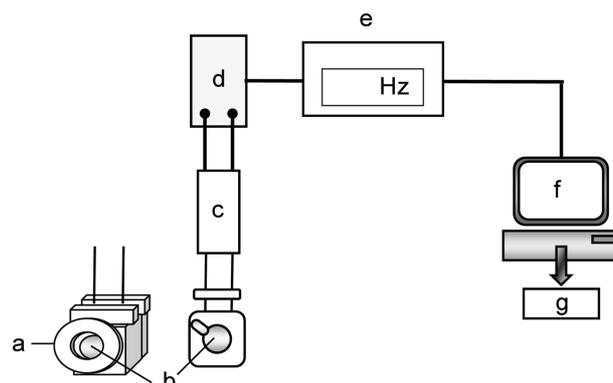
### Antibody Immobilization

The antibody immobilization over the gold electrode of a piezoelectric quartz crystal was carried out according to the procedure of Vaughan *et al.* [17]. The quartz crystal was pre-treated by soaking in 1.2 M NaOH for 5 min, followed by rinsing with distilled water, and then soaked in 1.2 M HCl for 5 min. After rinsing with distilled water, 20  $\mu$ l of concentrated HCl were spread on the gold electrode of the crystal for 1 min, with special care to keep the acid from touching the electrode leads. The crystal was then rinsed with ethanol and distilled water, and air-dried. The cleaned crystal was submerged into 10 mM thiol or sulfide solution prepared in absolute ethanol for 24 h, rinsed with absolute ethanol, and allowed to dry. To activate the SAM, 100  $\mu$ l each of 46 mM EDC and 46 mM NHS (both prepared in distilled water) were consecutively placed over the crystal surface with a gentle mixing. These reagents were left to react on the crystal surface for 1 h. After rinsing with distilled water and 0.01 M phosphate buffer (pH 7.4), the reaction buffer, 10 ml of the antibody solution (500–1,000  $\mu$ g rabbit serum proteins including sufficient antiserum to CAP/ml of the reaction buffer) was placed over the activated crystal surface. The antibody binding to the SAM was allowed for 60 min, followed by washing with the reaction buffer.

### Biosensor System and Experimental Procedure

A 9 MHz AT-cut piezoelectric quartz crystal (QA 9RP-50, Seiko EG and G, Japan) attached with two gold electrodes of 5 mm diameter had a reproducibility of  $\pm 0.1$  Hz in frequency response and was used in this study as the transducer of the quartz crystal microbalance (QCM) biosensor. It was mounted on a well holder with a plug, and then was connected to an oscillator module (QCA 917-11, Seiko EG and G) and a quartz crystal analyzer (QCA 917, Seiko EG and G). The analog frequency signals from the quartz crystal analyzer were converted to the digital ones in an IBM-compatible personal computer with a GPBI interface. The system operation was done with the WinEchem software (version 1.12) of Seiko EG and G (Fig. 1).

The experiments were done as follows [14]. Two hundred microliters of the reaction buffer were added into the reaction cell of the well holder, having a nominal capacity of 500  $\mu$ l, followed by the measurement of the resonant frequency of the quartz crystal until a steady-state baseline



**Fig. 1.** Schematic diagram of the apparatus for a CAP immunosensor system with a batch-type well holder.

(a) Well holder, (b) QCM/gold surface, (c) connector, (d) oscillator module, (e) quartz crystal analyzer with analog reading window, (f) PC, and (g) software.

was obtained ( $F_1$ ). Then, 200  $\mu$ l of a standard sample was injected into the reaction cell. The steady-state resonant frequency ( $F_2$ ) was read again to calculate the frequency shift ( $\Delta F = F_1 - F_2$ ).

## RESULTS AND DISCUSSION

As the solubility of CAP in an ordinary buffer solution is low [4], the responses of the CAP immunosensor for CAP were measured first at the reaction phase of 0.01 M phosphate buffer (pH 7.4), the reaction buffer, and dissolution phases of methanol and ethanol. The steady-state resonant frequency of the biosensor at the reaction buffer was  $8,878,065 \pm 4.5$  Hz, and this value was maintained stably. When the reaction buffer, methanol, and ethanol themselves were injected into the reaction cell of the biosensor system, the frequency shifts of  $19 \pm 1.5$ ,  $171 \pm 3.2$ , and  $107 \pm 12.5$  Hz, respectively, were attained. The possible reason for higher frequency shifts in the case of alcohol injections seemed to be due to the difference in solvent composition in the reaction cell between the reaction and dissolution phase. When  $10^{-1}$  M CAP dissolved in methanol

**Table 1.** Responses of the CAP immunosensor prepared by the MPA-mediated antibody immobilization to CAP according to the reaction and dissolution phase.

Reaction phase	Dissolution phase	CAP concentration (M)	Frequency shift (Hz)
PB <sup>a</sup>	PB	0	$19 \pm 1.5^b$
PB	Methanol	0	$171 \pm 3.2$
PB	Ethanol	0	$107 \pm 12.5$
PB	Methanol	$10^{-1}$	$689 \pm 2.4$
PB	Ethanol	$10^{-1}$	$487 \pm 12.8$

<sup>a</sup>0.01 M phosphate buffer (pH 7.4).

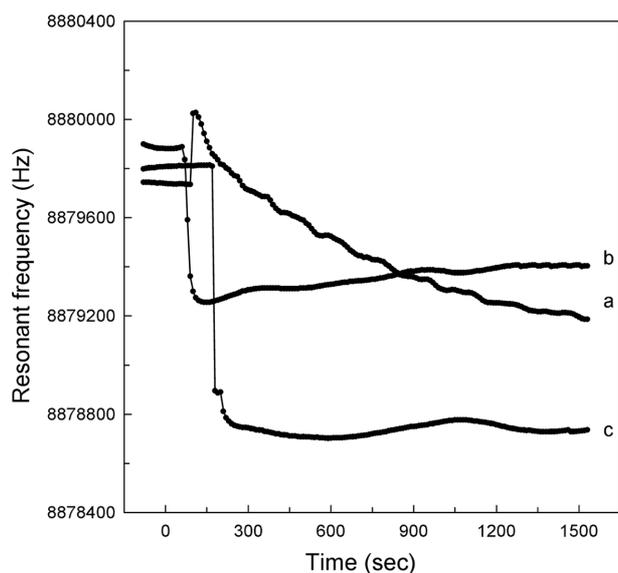
<sup>b</sup>Mean  $\pm$  SD (n=2).

or ethanol was separately injected into the reaction cell, the frequency shifts amounting to  $689 \pm 2.4$  and  $487 \pm 12.8$  Hz, respectively, were obtained (Table 1). As the biosensor response of CAP in methanol was relatively high, methanol was used as the dissolution phase of CAP in further experiments. When the analytes were CAP succinate and water-soluble CAP, the reaction buffer was dually used as the reaction and dissolution phase.

### Comparison of the Biosensor Responses to Different Analyte Types

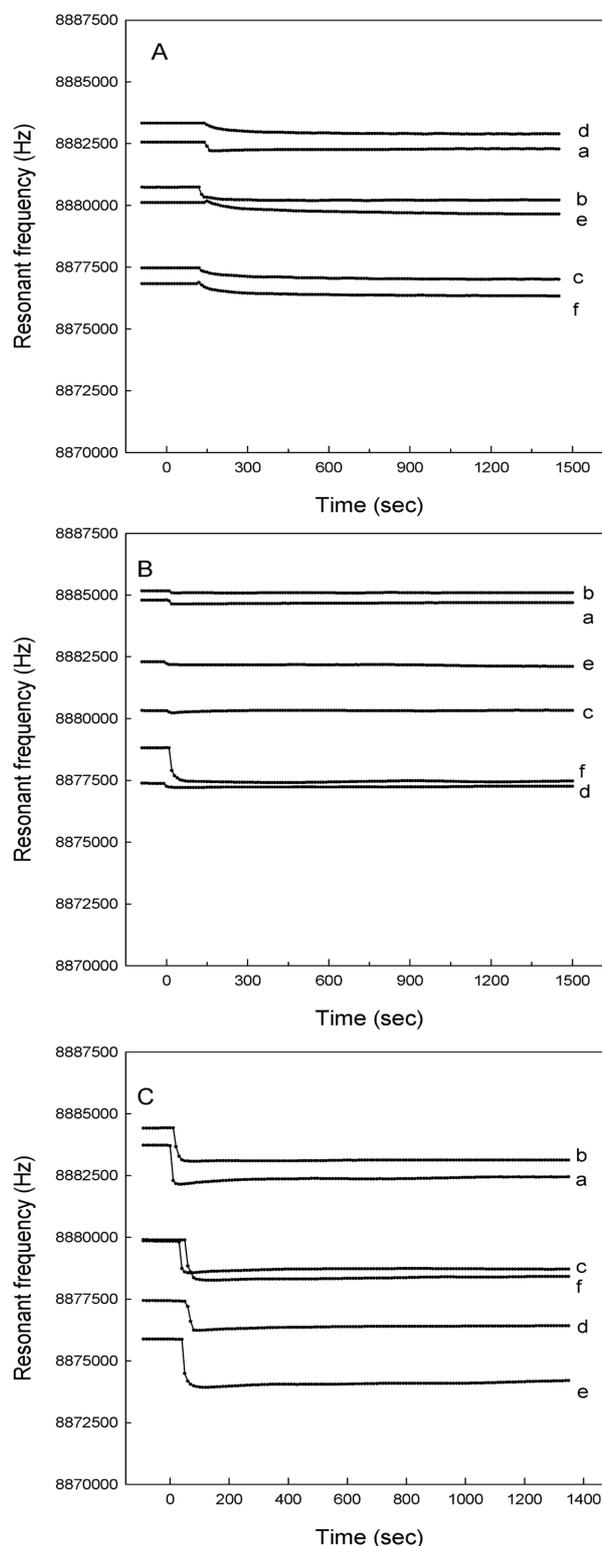
According to our previous works on the batch type QCM immunosensors, detecting *Salmonella* spp., and chloramphenicol [14, 15], the non-specific analyte adsorption onto the gold surface of the piezoelectric quartz crystal was negligible. Hence, the analyte measurements were performed without any correction with control run.

The time-dependent resonant frequency profiles of the CAP compounds in the present study were compared at  $5 \times 10^{-2}$  M concentration (Fig. 2). When CAP in methanol was used as the analyte, a short and abrupt increase in resonant frequency occurred, followed by a gradual decrease in resonant frequency until around 25 min. Also, a minute fluctuation in resonant frequency which possibly occurred by difference in solvent composition in the reaction cell was found. When CAP succinate was the analyte, the steady-state frequency shift was easily attained within 5 min, possibly due to homogeneity of the reaction mixture in the reaction cell. A similar trend was found in the case of water-soluble CAP, and the significant frequency shift

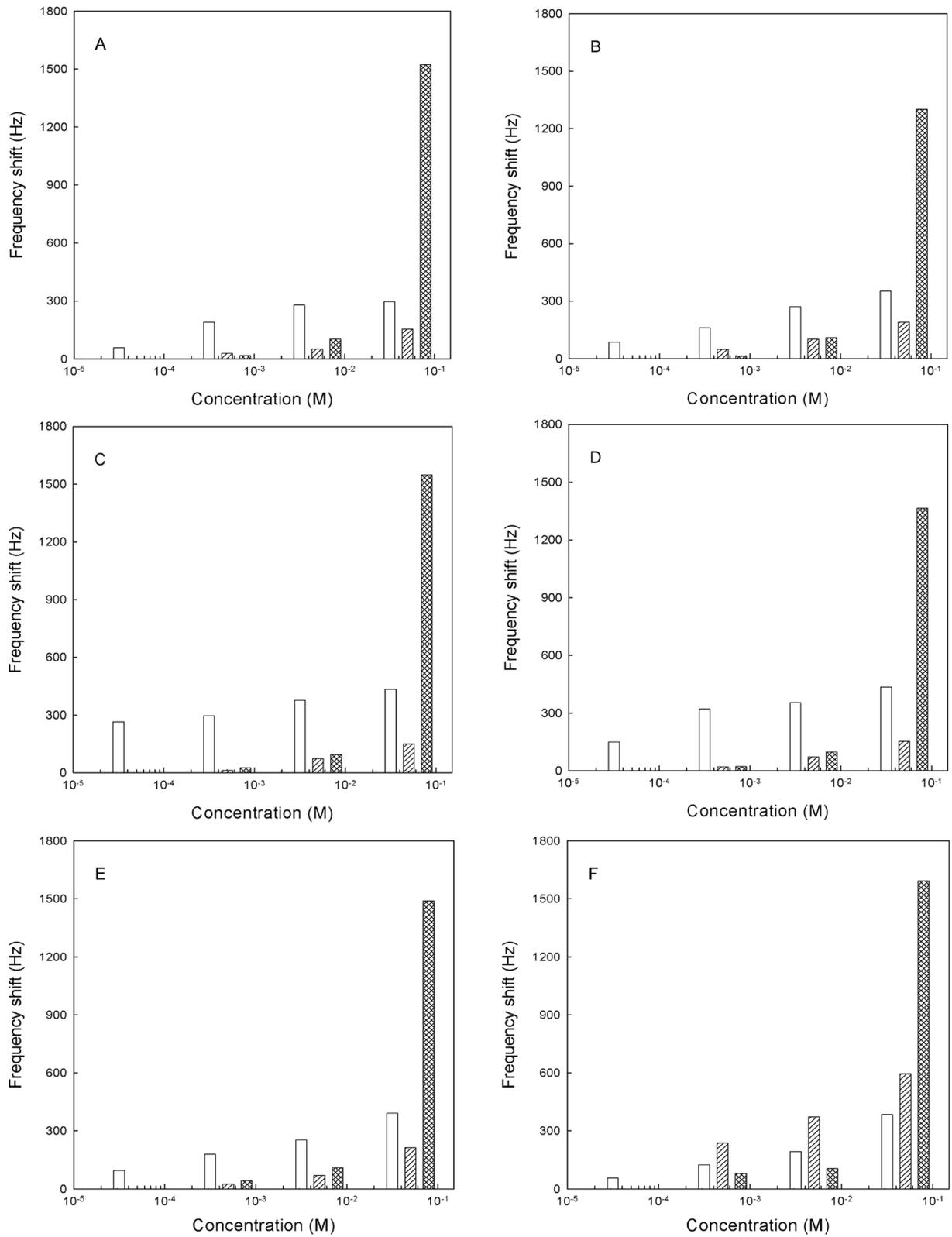


**Fig. 2.** Time-dependent resonant frequency profiles in the reaction buffer following the analyte injections.

The antibody was immobilized over the MPA monolayer, and the analyte concentration was  $5 \times 10^{-2}$  M. (a) CAP in methanol, (b) CAP succinate in the reaction buffer, and (c) water-soluble CAP in the reaction buffer.



**Fig. 3.** Time-dependent resonant frequency profiles of the CAP immunosensor to analyte types in  $5 \times 10^{-2}$  M in the presence of various thiol or sulfide compounds for antibody immobilization. Analyte types: (A) CAP in methanol, (B) CAP succinate in the reaction buffer, and (C) water-soluble CAP in the reaction buffer. Thiol or sulfide compounds: (a) TCA, (b) TSA, (c) TDGA, (d) TDPA, (e) DTDPA, and (f) MPA.



**Fig. 4.** Responses of the CAP immunosensor to analyte concentrations in the presence of various thiol or sulfide compounds for antibody immobilization.

Thiol or sulfide compounds: (A) TCA, (B) TSA, (C) TDGA, (D) TDPA, (E) DTDPA, and (F) MPA. Analyte types: (□) CAP in methanol, (▨) CAP succinate in the reaction buffer, and (⊠) water-soluble CAP in the reaction buffer.

around 1,100 Hz was obtained. The main reason for this drastic change in resonant frequency seemed to be its high molecular weight (i.e., 100 mg of CAP is equivalent to 1 g of water-soluble CAP having hydropropyl group), as the response of a QCM sensor is proportional to mass deposit [7].

### Biosensor Responses to Analyte Types at Various Thiol or Sulfide Compounds

The time-dependent resonant frequency profiles as a function of analyte types were measured at various thiol or sulfide compounds for antibody immobilization (Fig. 3). All QCM sensor chips showed stable resonant frequencies of 8,854,000–8,891,000 Hz in the reaction buffer within 5 min. When each CAP compound was injected into the reaction cell, the resonant frequency started to decrease due to the binding between the antibody and analyte, until a new steady-state resonant frequency was established. In this case, the degrees of frequency shift were quite different, depending on the species of thiol or sulfide compounds used for antibody immobilization, and MPA was found to be the most effective, mainly due to the strongest cross-linker-antibody complex binding to the gold electrode of the QCM, which is attributable to the presence of free SH group with a straight carbon chain [3].

### Biosensor Responses to Analyte Concentrations at Various Thiol or Sulfide Compounds

Figure 4 depicts the frequency shifts obtained with the CAP compounds in various concentrations, when the chemisorption cross-linkers for antibody immobilization varied. The frequency shifts obtained with  $5 \times 10^{-5}$ – $5 \times 10^{-2}$  M concentrations of CAP were in the range of 229–562 Hz, and no drastic changes in frequency shift according to the analyte concentrations were found. This fact might result in a narrow dynamic range. When CAP succinate was used as the analyte, the frequency shifts of 13–595 Hz were attained. Compared with those obtained by the CAP injections, more significant changes in biosensor response were found according to the analyte concentrations, showing the best concentration-dependency in the case of MPA. When water-soluble CAP was added as the analyte, the frequency shifts of 12–1,593 Hz were obtained, with the bigger changes in frequency shift over  $5 \times 10^{-2}$  M. The coefficient of variabilities (SD/ Mean  $\times 100$ , %) of repetitive measurements were generally below 5%, indicating a good reproducibility of the CAP immunosensor of this study.

From the calibration curves obtained with the CAP compounds at various thiol or sulfide compounds for antibody immobilization, correlation coefficients ( $r$ ,  $r^2$ ) and calibration curve slopes (Hz/one log scale change) were determined (Table 2). When CAP in methanol was used as the analyte, the  $r$  values of 0.909–0.994 and the calibration curve slopes of 58.5–105.8 were obtained. The  $r$  values of 0.911–0.998 obtained with CAP succinate were as comparably

**Table 2.** Correlation coefficients and calibration curve slopes to analyte types in the presence of various thiol or sulfide compounds.

Thiol or sulfide compound	Correlation coefficient ( $r$ , $r^2$ )/calibration curve slope (Hz/one log scale change)		
	CAP in methanol	CAP succinate in PB <sup>a</sup>	Water-soluble CAP in PB
TCA	0.909/80.7	0.911/67.5	0.793/- <sup>b</sup>
TSA	0.994/90.6	0.980/70.5	0.806/-
TDGA	0.975/58.5	0.998/69.5	0.783/-
TDPA	0.913/88.9	0.984/74.5	0.792/-
DTDPA	0.979/96.3	0.925/94.5	0.649/-
MPA	0.930/105.8	0.989/170.2	0.668/-

<sup>a</sup>0.01 M phosphate buffer (pH 7.4).

<sup>b</sup>Not determined due to nonlinear responses.

good as those obtained with CAP and the calibration curve slope was the biggest in the case of MPA immobilization, resulting in the calibration curve of a reasonably good dynamic range. When water-soluble CAP was used as the analyte, first-order correlations were not obtainable due to the big responses at the higher analyte concentrations described above, and the small responses at the lower concentrations below  $5 \times 10^{-2}$  M and the  $r^2$  values of 0.649–0.806 were obtained.

The responses of the QCM immunosensor, which was immobilized with an anti-CAP antibody via a procedure of SAMs formation, to various CAP compounds were measured by using a batch-type well-holder system. The analytical results clearly showed that the biosensor responses were strongly influenced by analyte types and thiol or sulfide compounds for antibody immobilization. As the primary usage of this biosensor might be an initial screening of CAP compounds, it could be used as a simple and rapid detection method for CAP compounds possibly present in real foods, after enhancing its sensitivity through the introduction of a competitive assay format. During this process, stable and reproducible QCM chips would strongly be required and the chemisorption procedure described in the present study via MPA seems to meet this requirement.

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