行政院國家科學委員會專題研究計畫　成果報告

多功能微生物毒素計測用免疫反應型生物感測器之開發研究(2/2)

計畫類別：個別型計畫
計畫編號：NSC91-2320-B-041-002-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：嘉南藥理科技大學食品衛生系

計畫主持人：周淑芬

計畫參與人員：陳建源、邱慶豐

報告類型：完整報告

處理方式：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中　華　民　國 92 年 10 月 30 日

行政院國家科學委員會補助專題研究計畫　V 成果報告
□期中進度報告

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執行單位：嘉南藥理科技大學食品衛生系

中　華　民　國　92　年　10　月 22　日

中文摘要

本研究利用自行生產之三種微生物毒素（包括：霍亂毒素、腸產毒性大腸桿菌熱不安定型毒素以及黃麴毒素B1）之單株及多株抗體，以兩種自製式單層膜有效固定於石英晶體微量天平之黃金表面，成功建立一新型多種微生物毒素免疫感測器。

本研究所採用兩種自製式單層膜(Cystamine-glutaraldehyde 及 Cystamine 法)之固定化方法均較其他方法包括：吸附法、Protein A 法、Concanavalin A 法為佳。晶片可以glycine · HCl 溶液（0.1M）再生，可連續使用十次而不會有活性降低之問題。此系統之操作穩定性達十四天以上。可檢測毒素濃度範圍均在0.1至 120 ng/ml 內。

本研究成果係有效利用壓電晶體微量天平技術配合抗原或抗體構築而成之多種微生物毒素免疫反應型生物感測器，具備有高特異性、高敏感性、不需樣品前處理及標識、可重複使用及所需樣品量少等多項優點，極具應用價值。

關鍵詞：微生物毒素、霍亂毒素、腸產毒性大腸桿菌熱不安定型毒素、黃麴毒素B1、單株抗體、多株抗體、自製式單層膜、壓電晶體微量天平、免疫感測器

Abstract

In this study, a new multi-microbial toxins immunosensor was developed using monoclonal and polyclonal anti-cholera toxin, anti-aflatoxin B1 and anti-*Escherichia coli* labile enterotoxin (LT) antibodies immobilized on the gold disc of a quartz crystal microbalance (QCM) system. Two kinds of self-assembled monolayers (SAMs) prepared by Cystamine-glutaraldehyde and Cystamine method were applied to immobilize monoclonal and polyclonal antibodies on the gold surface of the quartz, respectively. The reusabilities of quartz crystal adopting the SAMs were found to be better than those of the other immobilization methods used containing adsorption, Protein A, Concanavalin A methods. The 10 cycles of measurements could be performed on the gold surface of the same crystal regenerated with a solution of glycine · HCl. This sensor system could be continuously performed for 14 days, the relative frequency shifts were all found to be above 95% (relative to the response at the first day). A linear relationship existed between the frequency shifts (Hz) and the log values of three toxins concentrations in the range about from 0.1 to 120 ng/ml in buffer and mouse serum. This QCM immunosensor could efficiently detect three kinds of microbial toxins in the buffer solution and mouse serum and had some advantages: high sensitivity, low sample amount, high reusability, no label and no pretreatment etc.

Keywords: microbial toxins, immunosensor, monoclonal antibodies, polyclonal antibodies, cholera toxin (CT), aflatoxin B1 (AFB1), *Escherichia coli* labile enterotoxin (LT), quartz crystal microbalance (QCM), self-assembled monolayer (SAM)

Introduction

In this study, three kinds of microbial toxins were selected: (1) Fungal toxins: aflatoxin B1 (AFB1) produced from *Aspergillus flavus*. (2)Bacterial toxins: cholera toxin (CT) produced from *Vibrio cholerae* and labile enterotoxins (LT) produced from *Escherichia coli*. Mycotoxins are low weight metabolites produced by filamentous fungi growing naturally on food and feed commodities. Some mycotoxins, particularly aflatoxins, have been shown to have carcinogenic, mutagenic, teraogenic or oestrogenic effect [(1)(2)]. Among aflatoxins, AFB1 is the most toxic and carcinogenic member of a group of toxic haptenic compounds that are produced as secondary metabolites by toxigenic strains of the fungal species *Aspergillus flavus*, *A. parasiticus* and *A. nomius* [(3)(4)]. Due to the ubiquitous nature of these fungal species and the constant possibility of their growth and aflatoxins production in various foods and feeds, there has been a concerted effort to develop sensitive aflatoxins assay. In cholera, the ability to produce CT by *Vibrio cholerae* is critical for pathogenesis. Enterotoxigenic *Escherichia coli* (ETEC) is another example of an important agent of diarrheal diease in humans and some demestic animals. ETEC has been defined as *E. coli* that produces heat-labile (LT) and/or heat-stable (ST) enterotoxins[(5)].

Conventional methods for the determination of microbial toxins are radioimmunoassay (RIA) and nonisotopic immunoassays. Although RIA has advantages of high sensitivity and low cost in large amount of detection, the isotope used is dangerous and difficult for treatment of waste from the detection procedure and storage of reagents. In the majority of nonisotopic immunoassay, an enzyme or fluorescein is used to replace the radioactive label. Compared to RIA, nonisotopic immunoassay is less dangerous, but there are disadvantages of low sensitivity and high cost in the assay procedure [(5)(6)]. These detection methods must be labeled to produce signal, so they are time-consuming.

Immunosensors, which combines the inherent specificity of antigen-antibody reaction with high sensitivity of various physical transducers, have currently gained attention in clinical diagnosis[(7)] . This study focused on the microbalances based on piezoelectric crystals, where a decrease of the resonant frequency is correlated to the mass accumulated on its surface. The potential of piezoelectric devices for chemical sensor applications was realized by Sauerbrey who derived the following equation describing the frequency-to-mass relationship in air phase [(8)] :

$$\Delta F=-2.3\times 10^{6}F^{2}\Delta M/A$$

where ΔF is the measured frequency shift (Hz), F is the resonance frequency of the crystal (MHz), A is the area coated and ΔM is the change of the mass deposited. On the other hand, the relationship between the oscillation frequency change of a quartz resonator in contact with a liquid and accumulated mass was realized by Kanazawa who derived the following equation [(9)] :

$$\Delta F=-f_0^{3/2}(\rho_l \eta_l/\pi \rho_q \mu_q)^{1/2}$$

where f_0 is the resonance frequency of the crystal, ρ_l and η_l are the absolute density and viscosity of the solution, μ_q and ρ_q are the shear stiffness and density of quartz crystal, respectively.

In this study, immobilization of antibodies on the gold surface of quartz was discussed. The use of self-assembled monolayers (SAMs) in various fields of research is rapidly growing. In particular, many biomedical fields apply SAMs as an interface-layer between a metal surface and a solution. The pioneers in the assembly of sulfur-containing molecules, noticed that dialkane sulfides form highly ordered monolayers on metal surfaces. Van der Waals forces between methylene groups orient and stabilize the monolayer. The structure of a SAM depends on the morphology of the metal. Au (Ⅲ) is mostly applied for the formation of monolayers, because it is reasonably inert [(10)] . For example, if the amino and sulfur-containing molecules adsorbed on the gold surface and were then activated by glutaraldehyde, the aldehyde groups of glutaraldehyde could bind to the amino groups of the protein to form Schiff bases [(11)]. Thus the protein (e.g. enzymes, antibodies) could be immobilized on the gold surface.

In this paper, immobilization methods of antibodies on gold surface, reusabilities of coated quartz, operating stability of the sensor were studied. And the concentrations of three toxins in the buffer and mouse serum were determined by an immunosensor using a quartz crystal microbalance (QCM).

2. Experimental

2.1 Reagents

Ferritin from human liver was purchased from Calbiochem-Novabiochem International, La Jolla, CA, U.S.A. Polyclonal and monoclonal anti-AFB1, anti-CT and anti-LT antibodies were produced previously in our laboratory. Protein A, cystamine dihydrochloride and concanavalin A (Con A) was purchased from Sigma Chem. Co. St. Louis, U.S.A. Bovine serum albumin was obtained from Chemicon International, Inc. Temecula, CA. U.S.A. All other chemicals used were of analytical grade.

2.2 Materials

AT-cut quartz crystal oscillator (10MHz, 8mm×8mm×0.18mm, diameter of the gold electrode was 5mm, Tai Tien Electric Co., LTD, Taiwan, R.O.C.) was employed throughout the work. Chromium and gold were successively deposited to give layer of 20nm and 2000nm thick, respectively. Chromium was deposited to obtain good adhesion between gold and crystal [(12)].

2.3 Apparatus

Fig.1 shows a quartz crystal oscillation circuit self-made for amplifying signal of resonance frequency of the oscillator. The frequency of oscillator was determined using a computer controlled frequency counter (Universal counter, Hewlett Packard 53131A, Boise, ID, U. S.A.). Fig.2 shows the measurement set-up used for all experiments. The crystal installed in the PVC cell (not a flow cell) was connected to the oscillation circuit. The diameter of this PVC cell well was 5mm.

2.4 Measurement of resonant frequency by the QCM

The crystals were cleaned by immersing in 1.2N NaOH for 10min, 1.2N HCl for

5min and one drop of concentrated HCl was added for 30sec [13]. After each step, the crystal was thoroughly washed with distilled water. The crystals were dried at room temperature and initial frequencies were read. Only one side of the crystal was exposed to the solutions (3 μl) added into the cell well and a stable frequency value was reached and read. The 3 μl drop was placed exactly onto the same place of the electrode in the repeated measurements. All the frequencies were measured at room temperature under atmospheric pressure.

2.5 Immobilization of antibodies on gold disc

2.5.1 Adsorption method

The crystal was immersed with a 1mg/ml solution of the antibody for 1hr, and then the crystal was washed and dried. Subsequently, the crystal was blocked with 0.1M glycine- phosphate buffered saline (PBS) solution for 30 min. The crystal was then washed with PBS, distilled water, and dried. Finally, a stable frequency value was reached and read [13]. All procedures were performed at room temperature under atmospheric pressure.

2.5.2 Protein A method

5 μl of a protein A solution (2 mg/ml protein A in PBS, pH 7.0) was added to the electrodes on one side of the crystal. After drying, the crystal was immersed in distilled water for 30mins. Subsequently, 5 μl of a 1 mg/ml antibody solution was spread over the electrode surface. After drying, the crystal was washed with PBS and distilled water, and dried. The crystal was then blocked with 0.1 M glycine-PBS solution for 30min, washed with PBS, distilled water, and dried [14,15]. Finally, a stable frequency value was reached and read. All procedures were performed at room temperature under atmospheric pressure.

2.5.3 Cystamine-glutaraldehyde method

The crystal was immersed with a solution of 10 mM cystamine in PBS (pH 7.0) for 1hr, washed with distilled water and dried. The crystal was dipped into 10%(v/v) aqueous glutaraldehyde solution for 30 min and washed twice with distilled water. After drying, the crystal was immersed with a 1 mg/ml antibody solution for 1hr and washed with PBS, distilled water, and dried. The crystal was blocked with a 0.1 M glycine-PBS solution for 30min, and then washed with PBS, distilled water, and dried [16]. Finally, a stable frequency value was reached and read. All procedures were performed at room temperature under atmospheric pressure. Fig.3 shows that the chemical steps of IgM immobilization by cystamine-glutaraldehyde method.

2.5.4 Cystamine method

The crystal was immersed with a solution of 10 mM cystamine in PBS (pH7.0) for 1hr, washed with distilled water and dried. The crystal was then dipped into a 1 mg/ml antibody solution for 1hr, washed with PBS, distilled water, and dried. The crystal was blocked with a 0.1 M glycine-PBS solution for 30 min, washed with PBS, distilled water, and dried. Finally, a stable frequency value was reached and read. All procedures were performed at room temperature under atmospheric pressure.

2.5.5 Con A method

The crystal was dipped into a Con A solution (85 nM Con A in PBS containing 100 μM Ca^{2+} and Mn^{2+}, pH 6.8) for 1 min, washed with distilled water and dried.

Subsequently, the crystal was immersed with a 1 mg/ml antibody solution for 1hr, washed with PBS and dried. The crystal was then blocked with a 0.1M glycine-PBS solution for 30 min, washed with PBS and distilled water, and dried [17]. Finally, a stable frequency value was reached and read. All procedures were performed at room temperature under atmospheric pressure.

3. Results and Discussion

3.1 Reusabilities of quartz crystals coated with anti-toxins antibodies using different immobilization methods

3.1.1 Immobilization of monoclonal anti-AFB1 antibodies (IgM)

Four immobilization methods including adsorption, protein A, Con A and cystamine-glutaraldehyde methods were used to immobilize antibodies. In this experiment, monoclonal anti-AFB1 antibody was immobilized on the gold surface of the crystal using the above-mentioned four methods. 3 μl of 10 ng/ml AFB1 in PBS (pH 7.0) was applied to the gold surface in the PVC cell well for detection. The regeneration solution used was 0.1 M glycine-HCl buffer (pH 2.1) after each measurement [13]. Fig.4 shows that the reusability of quartz crystal adopting cystamine-glutaraldehyde method was better than those of the other above-mentioned methods. The quartz could continuously operate 10 times for the measurement of 10 ng/ml ferritin and the relative frequency shifts obtained were all above 95% (relative to the response at the first time). Generally, the formation of the stable monolayers is based on the strong adsorption of disulfides (R-S-S-R), sulfides (R-S-R), and thiol (R-SH) on a metal (particularly gold) surface. In this method, disulfide bond of cystamine (2, 2'-dithiobisethanamine, $C_4H_{12}N_2S_2$) is broken and strongly adsorbed on the gold surface. The isotype of monoclonal anti-AFB1 antibodies had been classified as IgM previously (data not shown). In this study, if the amino and sulfur-containing molecules adsorbed on the gold surface were activated by glutaraldehyde, the aldehyde groups of glutaraldehyde could bind to the amino groups of the IgM molecule to form Schiff bases [11]. Thus the IgM could be easily immobilized on the gold surface. Fig.3 shows the chemical steps involved in IgM immobilization by cystamine-glutaraldehyde method. Thus cystamine-glutaraldehyde method was found to be suitable for immobilization of IgM.

3.1.2 Immobilization of polyclonal antibodies, monoclonal anti-CT and anti-LT antibodies

Fig.5 shows the reusabilities of quartz crystals coated with polyclonal antibodies, monoclonal anti-CT and anti-LT antibodies (IgG) using five immobilization methods including adsorption, protein A, Con A, cystamine-glutaraldehyde and cystamine method. The results demonstrated that the cystamine method developed in our laboratory was better than other methods. If the cystamine-glutaraldehyde method was used, the aldehyde groups of glutaraldehyde could easily bind to the amino groups of Fab portion in the IgG molecule (i.e. antigen binding site), and the binding of antigens to antibodies might be partly hindered (the major isotype of polyclonal antibodies in mice ascites were IgG; the isotypes of monoclonal anti-CT and anti-LT antibodies had been classified as IgG2a and IgG1, respectively, in previous study (data not shown)). Thus the cystamine-glutaraldehyde method was unsuitable to immobilize the polyclonal antibodies, monoclonal anti-CT and anti-LT antibodies. In cystamine method, the amino groups of

cystamine could bind to the carboxyl groups of Fc portion in the IgG molecule and not bind to the amino groups of the antigen binding sites. It may be similar to the binding between IgG and Fc receptor [18]. The true reasons are not completely clear thus far, but the experiment results demonstrated the cystamine method was suitable to immobilize the polyclonal antibodies.

3.2 Calibration curves of three toxins in buffer and in mouse serum

The three toxins in the buffer and mouse serum was detected in the range 0.1 ng/ml to 1 μg/ml using monoclonal antibodies and polyclonal antibodies. A linear relationship existed between the frequency shifts (Hz) and the log values of AFB1 and CT in the range 0.1 to 120 ng/ml in mouse serum shown in Fig.6 and 7 (The samples containing high ferritin could be diluted into this range). In PBS, the similar results were obtained (data not shown). The reaction time to reach equilibrium in buffer was 10 min, but in mouse serum was 20 min (data not shown).

3.3 Operating stability of microbial toxins piezoimmunosensor

In the microbial toxins piezoimmunosensor with monoclonal antibodies immobilized, the 5 cycles of measurements *per* day were continuously performed for 14 days. Fig.8 shows that the operating stability of this piezoimmunosensor was more than 14 days and the relative frequency shifts were all above 95% (relative to the response at the first day). In case of microbial toxins piezoimmunosensor with polyclonal antibodies immobilized, the similar results were obtained (data not shown). Fig.9 shows the scheme of the multi-microbial toxins piezoimmunosensor.

4. Conclusion

In this study, the concentrations of three microbial toxins in buffer and mouse serum were determined using an immunosensor based on a QCM. Two kinds of SAMs prepared by cystamine-glutaraldehyde method and cystamine method were applied to immobilize monoclonal and polyclonal antibodies on the gold surface of the quartz, respectively. The 10 cycles of measurements could be performed on the gold surface of the same crystal regenerated with a glycine · HCl solution. A linear relationship existed between the frequency shifts (Hz) and the log values of toxins concentrations in the range from 0.5 to 120 ng/ml. The operating stability of this sensor was more than 14 days. The immunosensor will be applied for the detection of microbial toxins in foods and human serum samples and related clinical diagnosis in the near future.

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Figure legends:

Fig.1. Block diagram of oscillator circuit

Fig.2. Measurement set-up of a quartz crystal microbalance used

Fig.3. The chemical steps of IgM immobilization by cystamine-glutaraldehyde method

Fig.4.Comparison on the reusability of quartz crystals coated with monoclonal anti-AFB1 antibodies (IgM) using different immobilization methods applied in the AFB1 piezoimmunosensor system. Monoclonal anti-AFB1 antibodies were immobilized on the gold surface of quartz crystal using the indicated four kinds of immobilization methods. 10 ng/ml of AFB1 in PBS (pH 7.0) was applied to the gold surface for detection. The regeneration buffer was 0.1 M glycine-HCl buffer (pH 2.1). Relative frequency shift is the frequency shift relative to the response at the first time.

Fig.5. Comparison on the reusability of quartz crystals coated with polyconal antibodies (IgG) and using different immobilization methods applied in microbial toxins piezoimmunosensor system. Polyclonal antibodies were immobilized on the gold surface of quartz crystal using the indicated five kinds of immobilization methods. 10 ng/ml of AFB1, CT and LT in PBS (pH 7.0) were applied to the gold surface for detection. The regeneration buffer was 0.1 M glycine-HCl buffer (pH 2.1). Relative frequency shift is the frequency shift relative to the response at the first time.

Fig.6. Detection of AFB1 in mouse serum using monoclonal anti-AFB1 antibodies immobilized on the gold surface of quartz crystal. The immobilized antibodies used were monoclonal anti-AFB1 antibodies and anti-BSA (control). The immobilization method used was cystamine-glutaraldehyde method. The inset shows the linear relationship range between frequency shift (Hz) and the log values of AFB1 concentration (ng/ml). Each value is an average of three measurements. Bar is standard deviation (SD)

Fig.7. Detection of CT in mouse serum using monoclonal anti-CT antibodies immobilized on the gold surface of quartz crystal. The immobilized antibodies used were monoclonal anti-CT antibodies and anti-BSA (control). The immobilization method used was cystamine method. The inset shows the linear relationship range between frequency shift (Hz) and the log values of CT concentration (ng/ml). Each value is an average of three measurements. Bar is SD.

Fig.8. Operating stability of microbial toxins piezoimmunosensor using monoclonal antibodies immobilized. The immobilization method used was cystamine-glutaraldehyde method. The detections were performed continuously five times *per* day. Each assay was perfomed with 10 ng/ml AFB1. PBS was used as the blank. Each value is an average of five measurements. Bar is SD. Relative frequency shift is the frequency shift relative to the response at the first day.

Fig.9. The scheme of the multi- microbial toxins piezoimmunosensor.

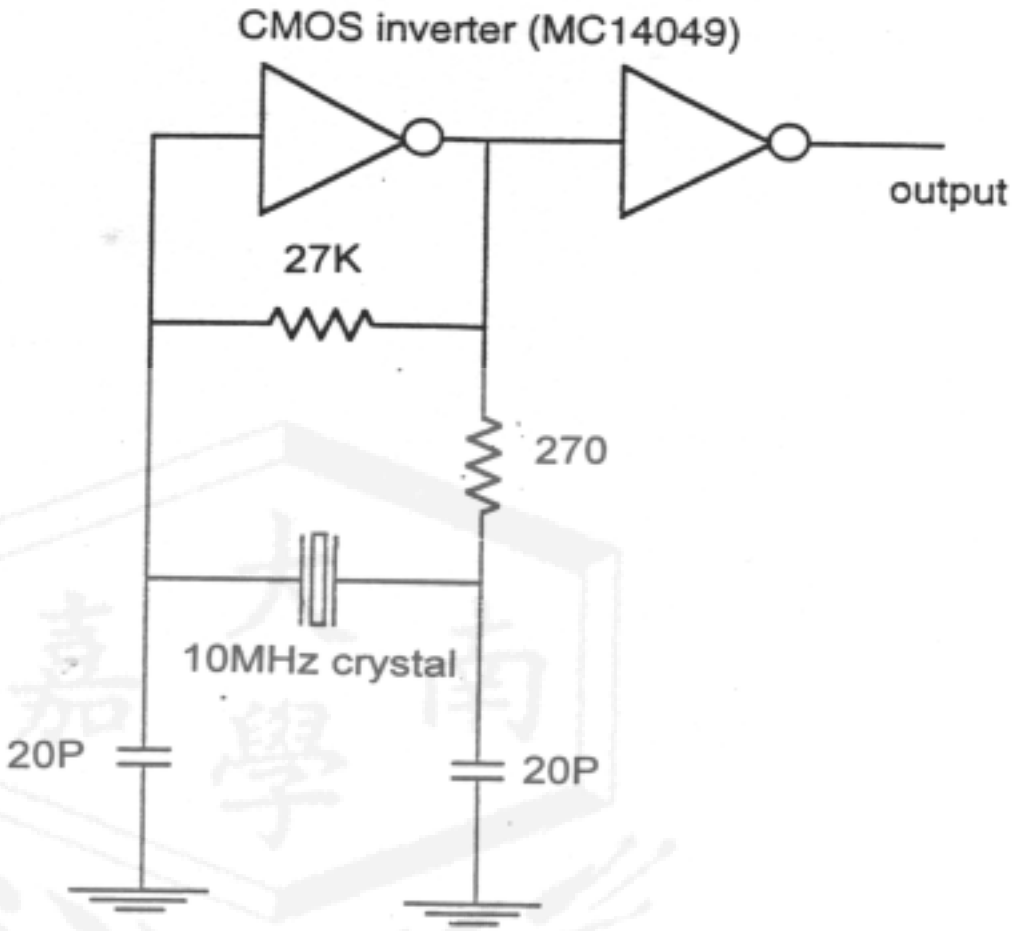


Fig.1. Block diagram of oscillator circuit

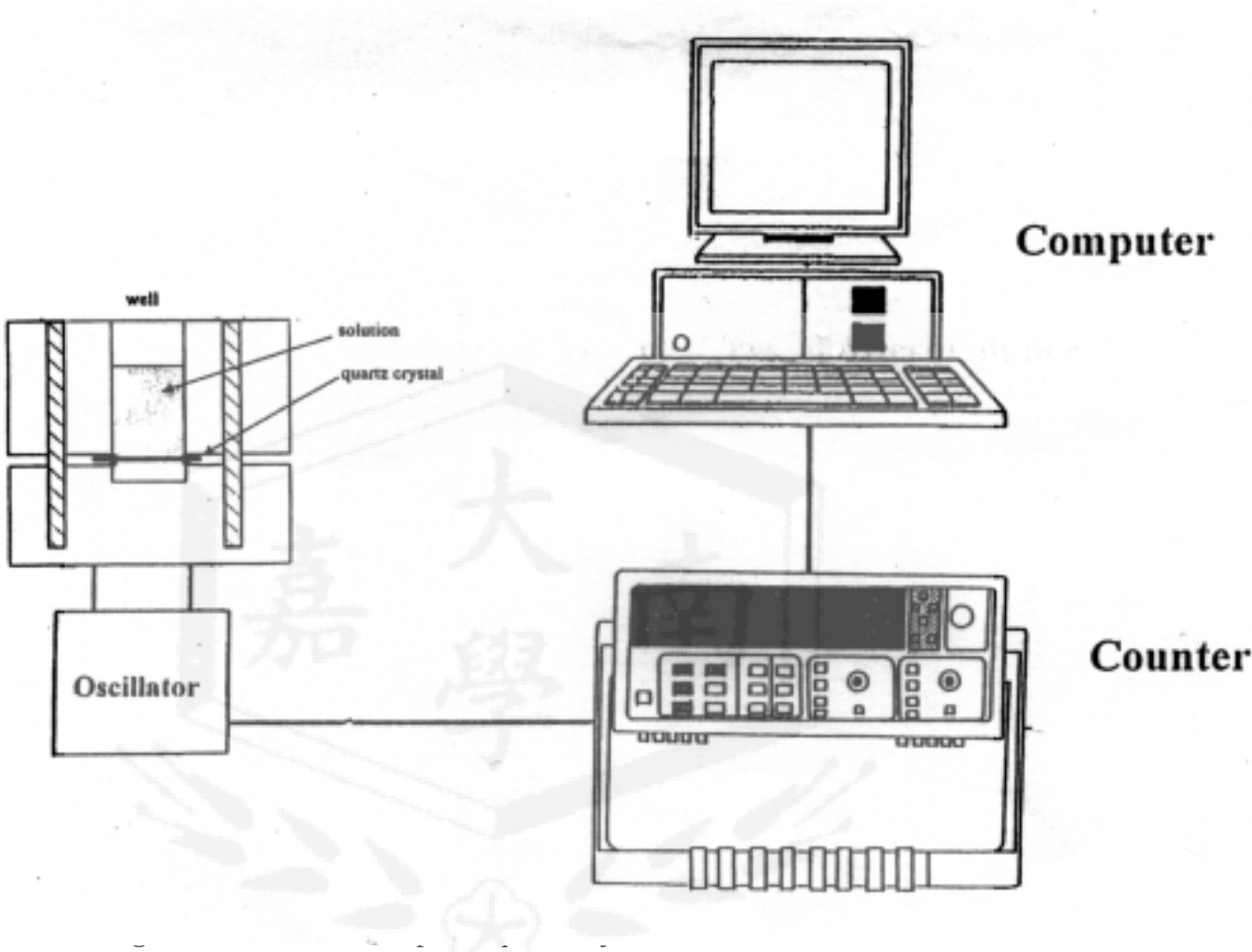


Fig.2. Measurement set-up of a quartz crystal microbalance used

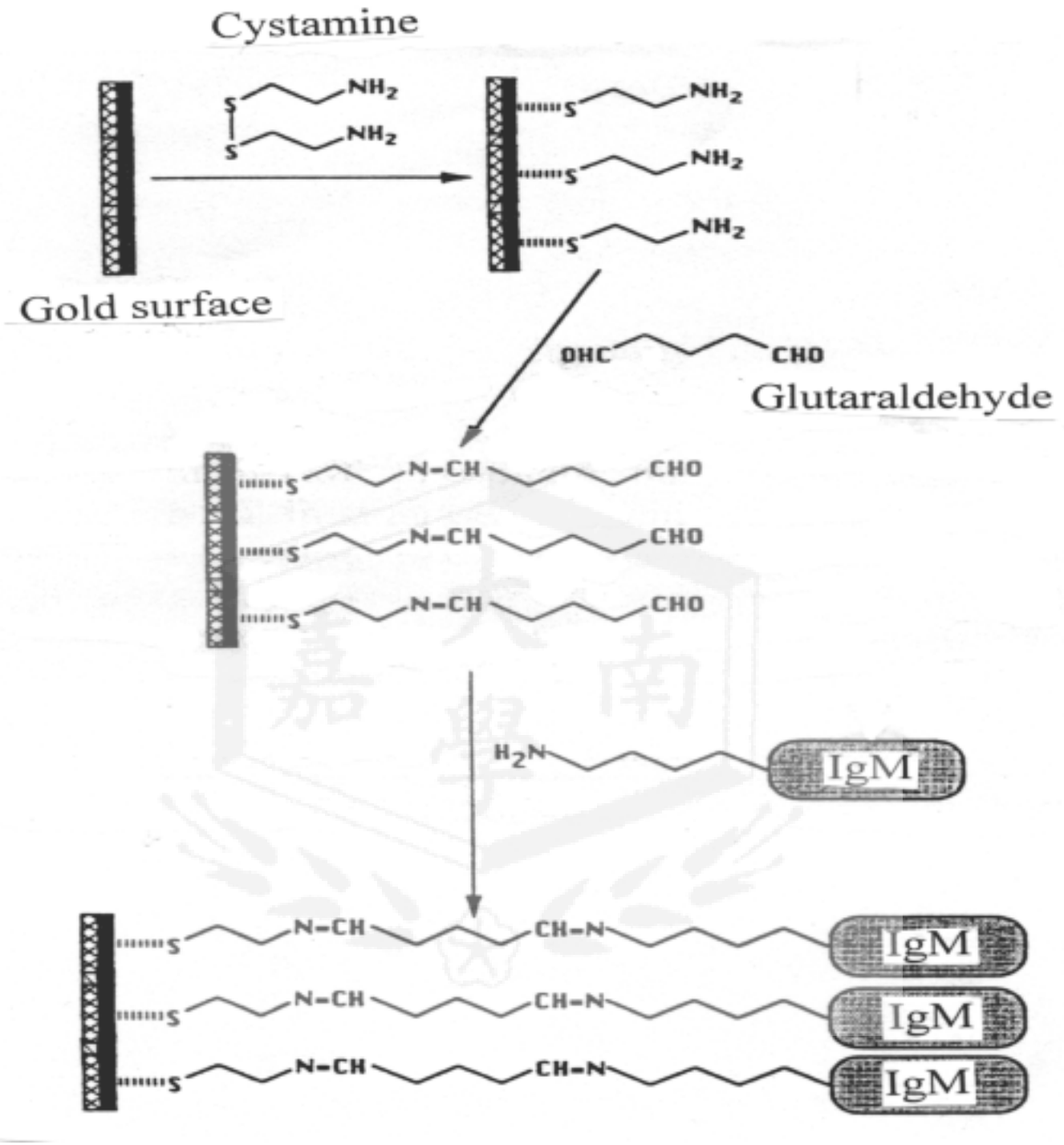


Fig.3. The chemical steps of IgM immobilization by cystamine-glutaraldehyde method

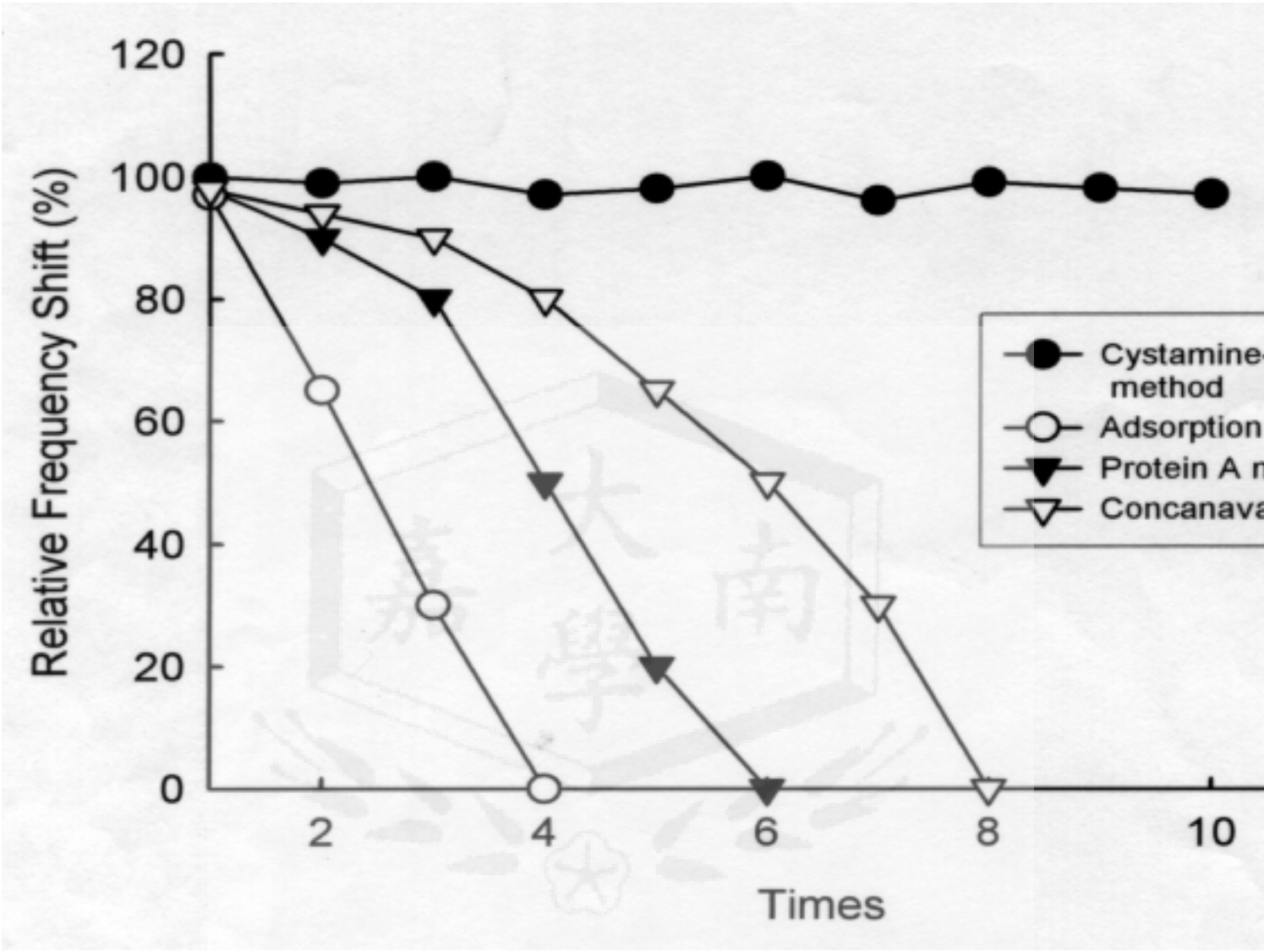


Fig.4. Comparison on the reusability of quartz crystals coated with monoclonal anti-AFB1 antibodies (IgM) using different immobilization methods applied in the AFB1 piezoimmunosensor system.

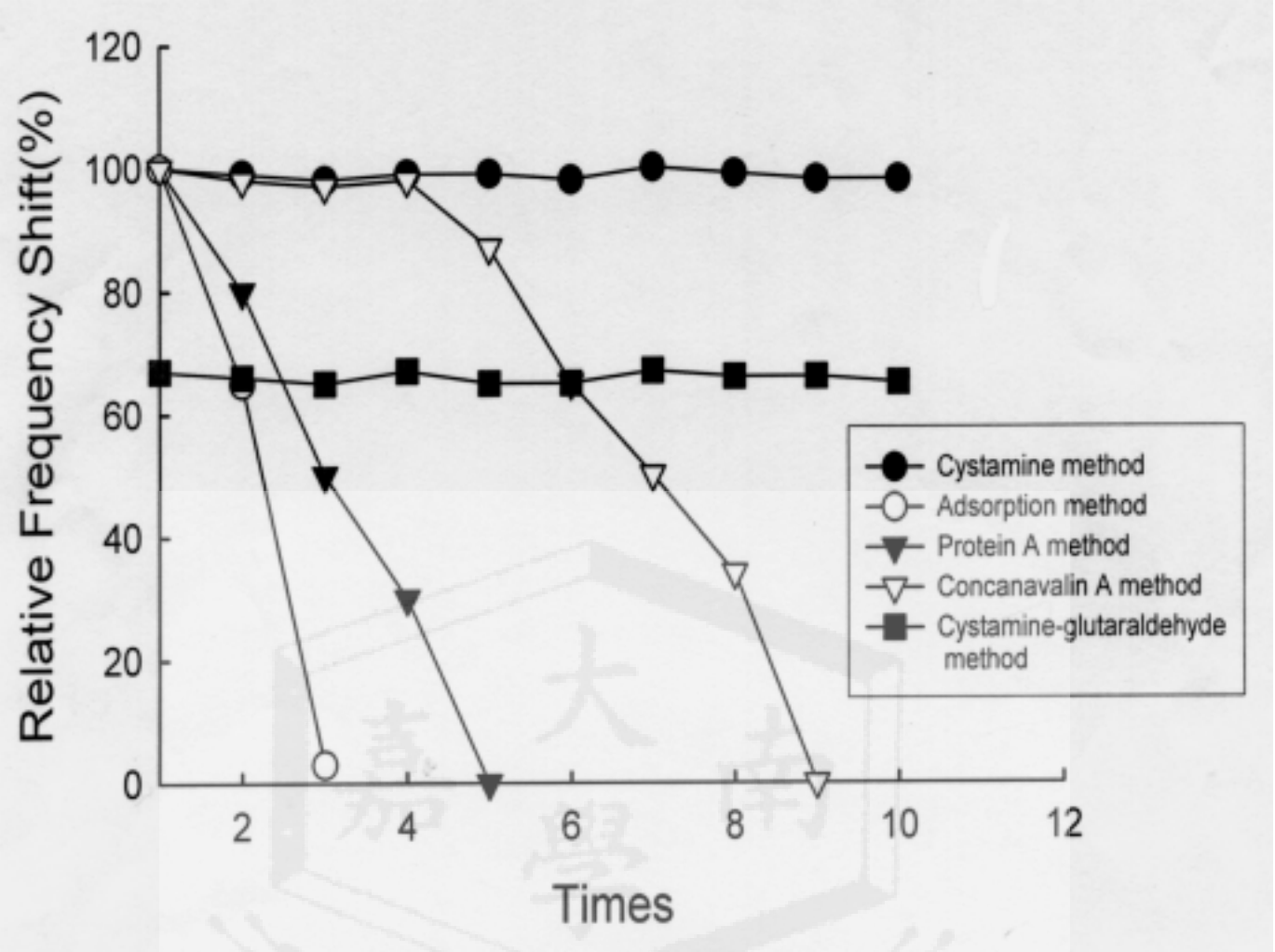
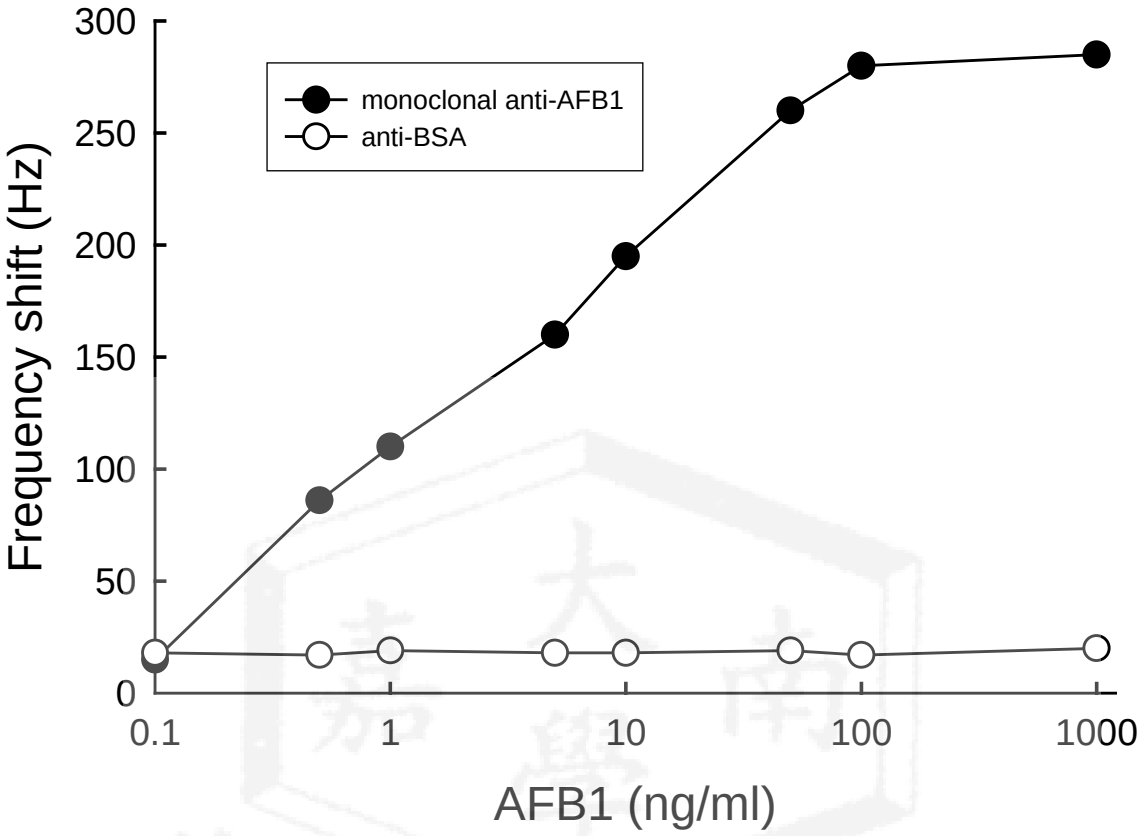


Fig.5. Comparison on the reusability of quartz crystals coated with polyconal antibodies (IgG) and using different immobilization methods applied in microbial toxins piezoimmunosensor system.


300
250
200
150
100
50
0
Frequency shift (Hz)
monoclonal anti-AFB1
anti-BSA
0.1
1
10
100
1000
AFB1 (ng/ml)

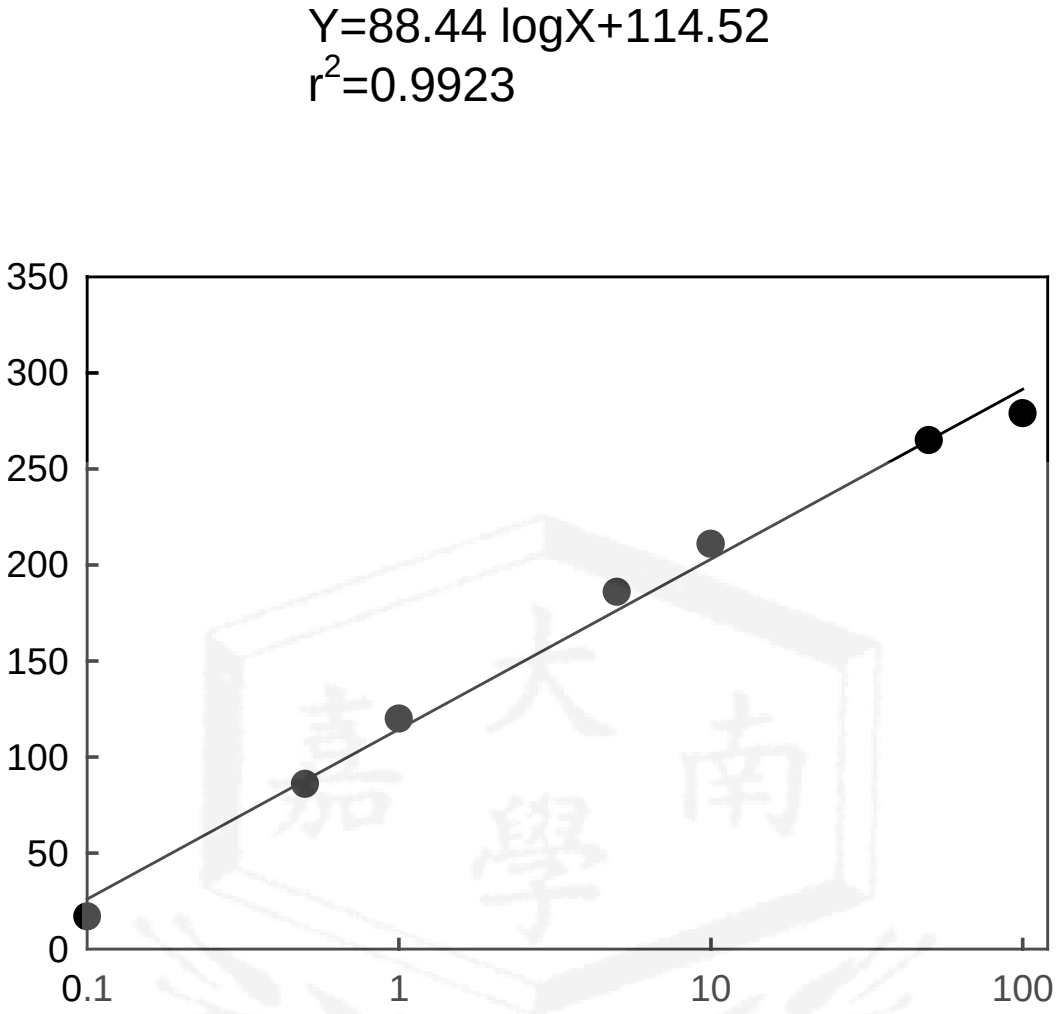
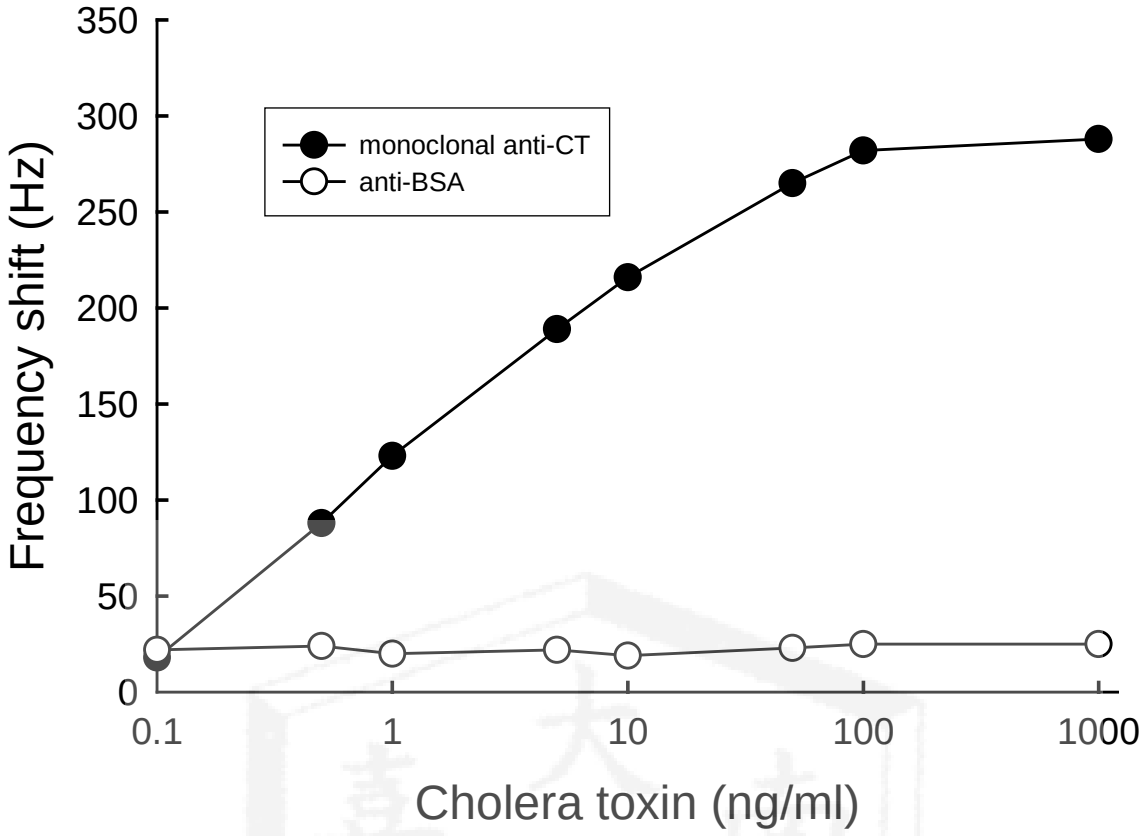


Fig.6. Detection of AFB1 in mouse serum using monoclonal anti-AFB1 antibodies immobilized on the gold surface of quartz crystal.


Frequency shift (Hz)
350
300
250
200
150
100
50
0
monoclonal anti-CT
anti-BSA
0.1
1
10
100
1000
Cholera toxin (ng/ml)

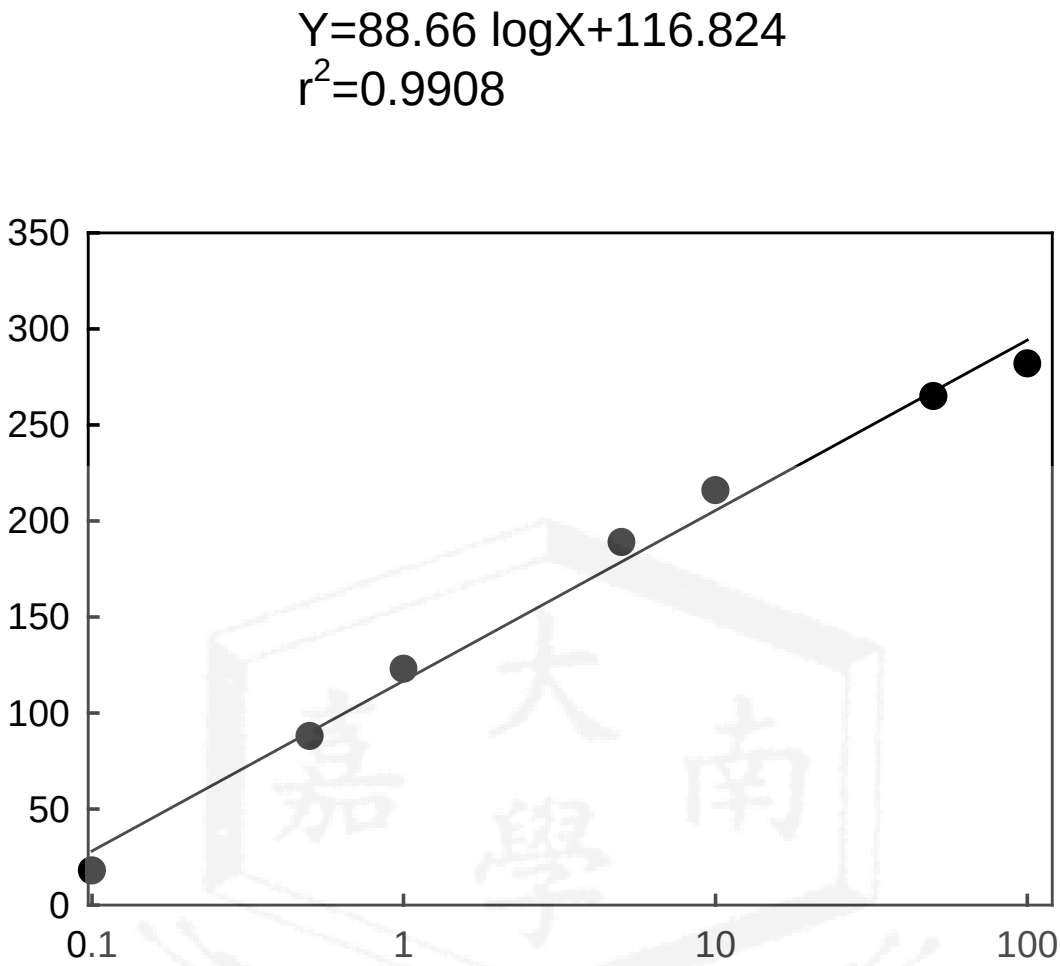


Fig.7. Detection of CT in mouse serum using monoclonal anti-CT antibodies immobilized on the gold surface of quartz crystal

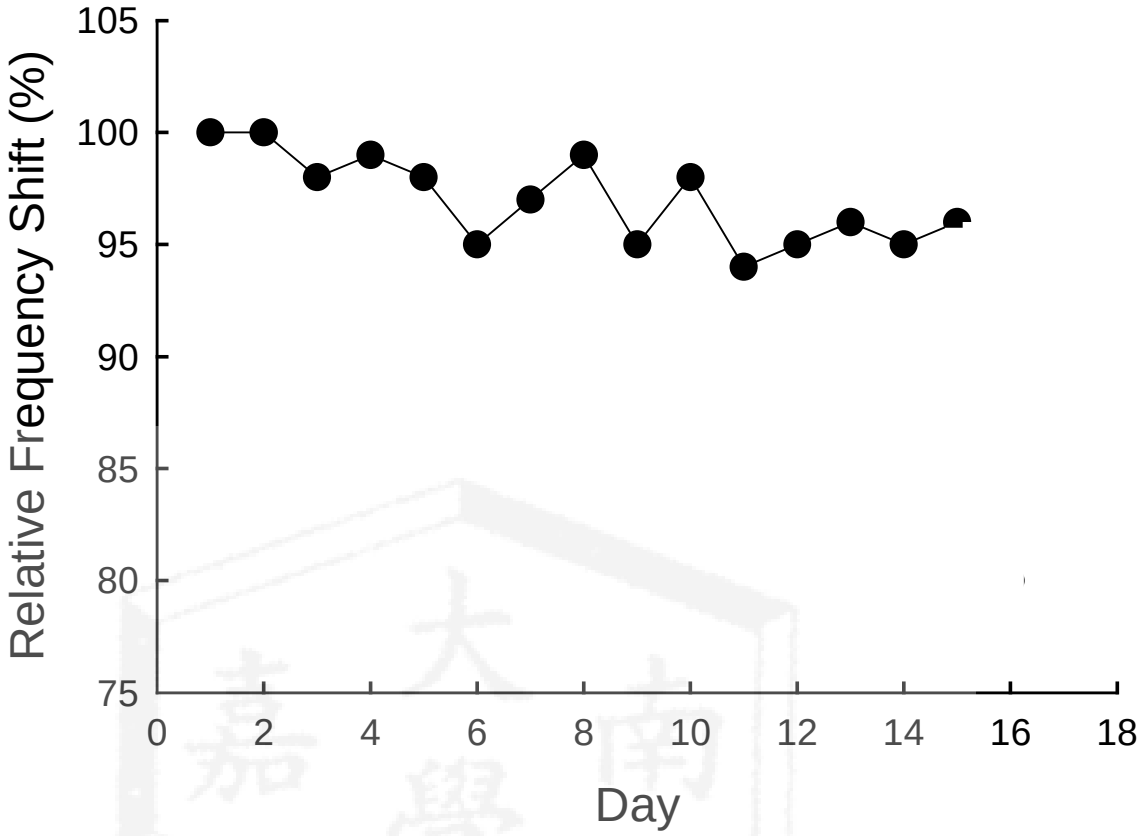


Fig.8. Operating stability of microbial toxins piezoimmunosensor using monoclonal antibodies immobilized.

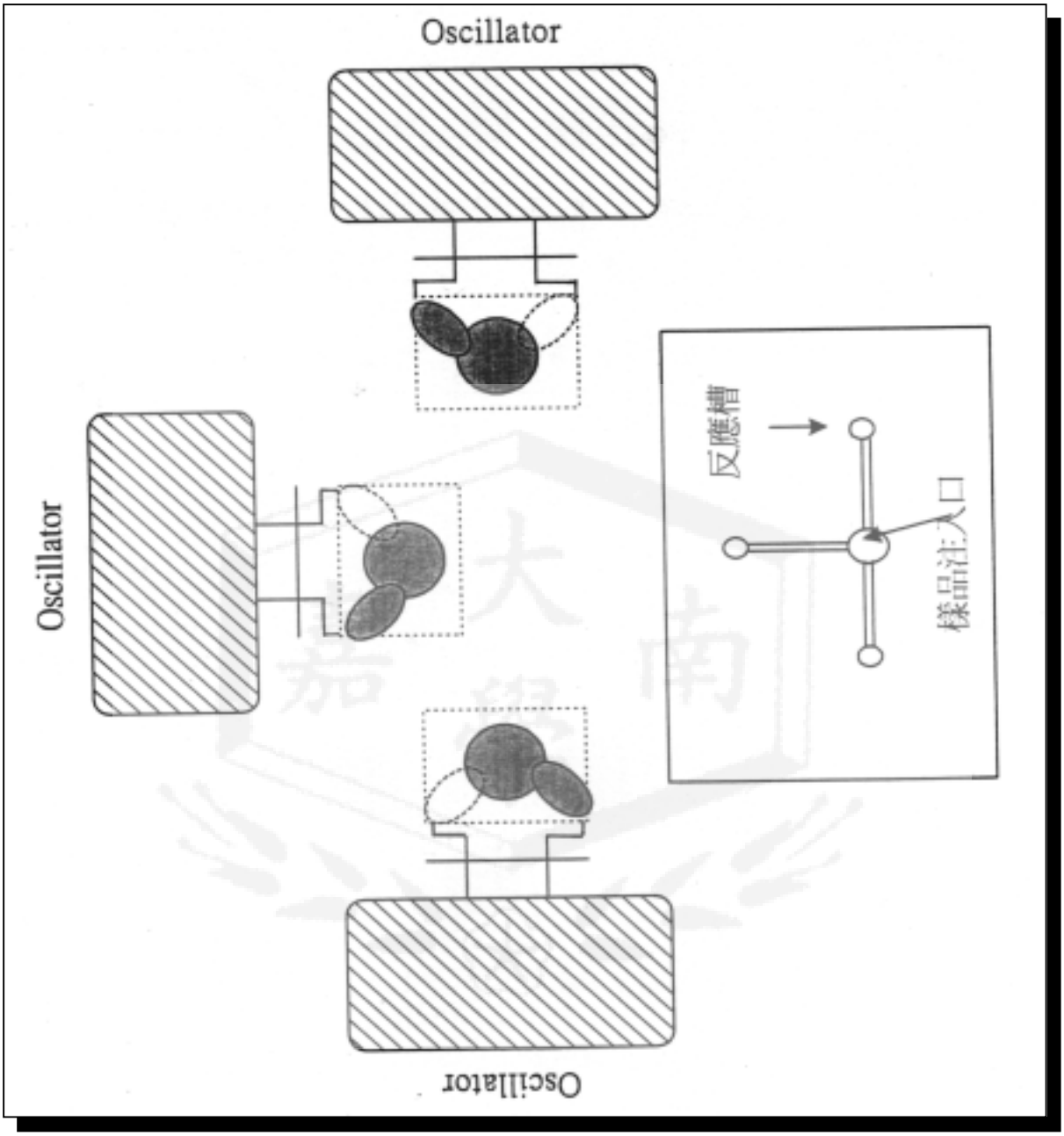


Fig.9. The scheme of the multi- microbial toxins piezoimmunosensor.

可供推廣之研發成果資料表

V 可申請專利　　V 可技術移轉　　日期：92 年 10 月 22 日

國科會補助計畫	計畫名稱：多功能微生物毒素計測用免疫反應型生物感測器之開發研究(2/2) 計畫主持人：　周淑芬 計畫編號：NSC 91-2320-B-041-002　學門領域：醫學工程
技術/創作名稱	微生物毒素免疫感測器
發明人/創作人	周淑芬
技術說明	中文：本研究利用自行生產之三種微生物毒素（包括：霍亂毒素、腸產毒性大腸桿菌熱不安定型毒素以及黃麴毒素）之單株及多株抗體，以兩種自製式單層膜有效固定於石英晶體微量天平之黃金表面，成功建立一新型多種微生物毒素免疫感測器。 英文：In this study, a new multi-microbial toxins immunosensor was developed using monoclonal and polyclonal anti-cholera toxin , anti-aflatoxin B1 and anti-*Escherichia coli* labile enterotoxin (LT) antibodies immobilized on the gold disc of a quartz crystal microbalance (QCM) system. Two kinds of self-assembled monolayers (SAMs) were applied to immobilize monoclonal and polyclonal antibodies on the gold surface of three quartz chips, respectively.
可利用之產業及可開發之產品	可廣泛利用於醫藥、農業、食品衛生等多方面之生物產業上之檢測
技術特點	以自行生產之多株及單株抗體有效固定於所建立之免疫感測系統上，具有高靈敏度、樣品不需前處理、抗體不需標示且感測晶片可重複使用等多項特點
推廣及運用的價值	具快速、直接、準確、可重複使用、樣品量少等特性來同時進行多種微生物毒素之檢測，極具實用價值。

※　1.每項研發成果請填寫一式二份，一份隨成果報告送繳本會，一份送　貴單位研發成果推廣單位（如技術移轉中心）。

※　**2.本項研發成果若尚未申請專利，請勿揭露可申請專利之主要內容。**

※　3.本表若不敷使用，請自行影印使用。