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Development of an immunosensor for human ferritin, a nonspecific tumor marker, based on a quartz crystal microbalance

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Abstract

A new human ferritin immunosensor was developed using anti-human ferritin antibodies (Abs) immobilized on the gold disc of a quartz crystal microbalance (QCM). Two kinds of self-assembled monolayers (SAMs) prepared by cystamine-glutaraldehyde and cystamine method were applied to immobilize anti-ferritin monoclonal antibodies (MoAbs) and polyclonal antibodies (PoAbs) on the quartz, respectively. The reusabilities of quartz crystal adopting the SAMs were found to be better than those of the other immobilization methods used. The 10 cycles of measurements could be performed on the gold surface of the same crystal regenerated with a solution of glycine·HC1. This sensor system could be continuously performed for 15 days, the relative frequency shifts (the frequency shifts measured are relative to the response at the first day) were all found to be above 95%. A linear relationship existed between the frequency shifts (Hz) and the log values of human ferritin concentrations in the range from 0.1 to 100 ng/ml in buffer and mouse serum. This human ferritin immunosensor had some advantages: high sensitivity, high specificity, low sample requirement, high reusability, no label and no pretreatment etc.

Keywords: Human ferritin; Immunosensor; Quartz crystal microbalance (QCM); Self-assembled monolayer (SAM); Monoclonal antibodies (MoAbs); Polyclonal antibodies (PoAbs)

1. Introduction

Ferritins are a class of storage proteins widely distributed in vertebrates, invertebrates, plants, fungi, and bacteria [1]. The major function of ferritin is to store and detoxify intracellular iron [2]. Ferritin is known to have two isomers, H-chain and L-chain ferritin. The H-chain of ferritin manifests ferroxidase activity [3]. It implies that ferritin-stored iron might resist cyclical reduction/oxidation reactions, which tend to propagate and amplify oxidative damage [2]. Elevated levels of available iron can also promote growth of tumors [4] and infectious microorganisms [5]. At extremely elevated iron levels, ferritin can be disproportionately increased [6]. However, the increased level of ferritin is known as a nonspecific marker of inflammatory processes and neoplasms. It was demonstrated to be elevated in the sera of patients of a wide variety of tumors, e.g. human breast cancer [7], renal cell carcinoma [8,9], hepatocellular carcinoma [10,11], larynx cancer and malignant neoplasms of maxilla [12], etc. or in significant diseases, like HIV infection, Still's disease, leukocytosis, reactive hemophagocytic syndrome [13,14], etc. Normal value of ferritin concentration of a healthy

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adult serum is less than 200 ng/ml for men and 90 ng/ml for women.

Conventional methods for the determination of ferritin 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. However, these methods must rely on the detection of labeled molecules to produce signal, they are complicated and time-consuming [15].

Immunosensors which combine the inherent specificity of antigen (Ag)–antibody (Ab) reaction with high sensitivity of various physical transducers have currently gained attention in clinical diagnosis [16]. 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 [17]:

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

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 [18]:

$$\Delta F = -f_0^{3/2} \left(\frac{\rho_1 \eta_1}{\pi \rho_q \mu_q} \right)^{1/2}$$

where f_0 is the resonance frequency of the crystal, ρ_1 and η_1 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 Abs on gold surface of the 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 [19]. For example, if the amino and sulfur-containing molecules adsorbed on gold surface and were then activated by glutaraldehyde, the aldehyde groups of glutaraldehyde could bind to the amino groups of protein to form Schiff bases [20]. Thus, the protein (e.g. enzymes, Abs) could be immobilized on the gold surface.

In this paper, immobilization methods of Abs on gold surface, reusabilities of quartz coated with Abs, operating stability of this immunosensor based on a quartz crystal microbalance (QCM) were studied. Furthermore, ferritin concentrations in buffer and mouse serum were determined by this QCM sensor.

2. Experimental

2.1. Reagents

Ferritin from human liver was purchased from Calbiochem-Novabiochem International, La Jolla, CA, U.S.A. Anti-human ferritin MoAbs and PoAbs were produced previously in our laboratory [21]. Protein A, cystamine dihydrochloride and concanavalin A (ConA) was purchased from Sigma, 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 (10 MHz, 8 mm × 8 mm × 0.18 mm, diameter of the gold electrode was 5 mm, Tai Tien Electric Co., LTD, Taiwan, R.O.C.) was employed throughout the work. Chromium and gold were successively deposited to give layers of 20 nm and 2000 nm thick, respectively. Chromium was deposited to obtain good adhesion between gold and crystal [22].

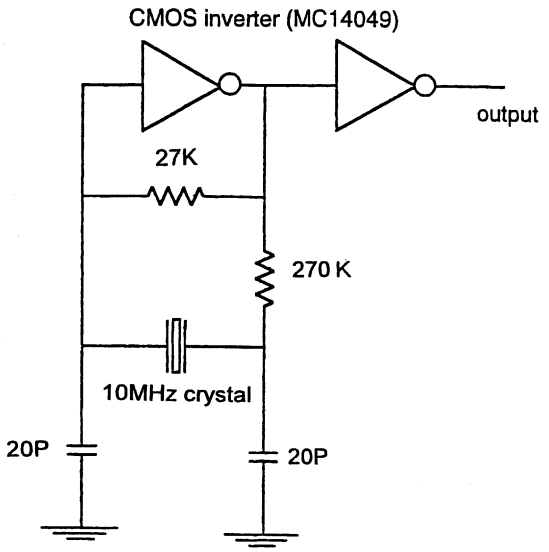

Fig. 1. Block diagram of oscillator circuit.

2.3. Apparatus

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

2.4. Measurement of resonant frequency by the QCM

The crystals were cleaned by immersing in 1.2 M NaOH for 10 min, 1.2 M HCl for 5 min and one drop of concentrated HCl was added for 30 s [23]. 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 marked on the gold surface in the repeated measurements. All the frequencies were measured at room temperature under atmospheric pressure.

2.5. Immobilization of Abs on gold disc

2.5.1. Adsorption method

The crystal was immersed with a 1 mg/ml Ab solution for 1 h, washed with phosphate buffered saline (PBS) (5 mM Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl, pH 7.0) and dried. Finally, a stable frequency value was reached and read [23]. All procedures were performed at room temperature under atmospheric pressure.

2.5.2. Protein A method

Five microliter of a protein A solution (1 mg protein A in 1 ml of PBS, 0.1 M, pH 7.0 and 1 ml of acetate buffer, 0.1 M, pH 5.5) was added to the electrodes on one side of the crystal. After drying, the crystal was immersed in distilled water for 30 min. Subsequently, 5 μl of a 1 mg/ml Ab solution was spread over the electrode surface. After drying, the crystal was washed with PBS and distilled water, and dried [24,25]. 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 cystamine solution (10 mM cystamine in 50 mM PBS, pH 7.0) for 1 h, 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 Ab solution for 1 h and washed with PBS, distilled water, and dried. The crystal was blocked with a 0.1 M glycine-PBS solution (0.1 M glycine in 50 mM PBS, pH 7.0) for 30 min [26], and then 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. Fig. 2 shows that the chemical steps of IgM immobilization by cystamine-glutaraldehyde method [27].

2.5.4. Cystamine method

The crystal was immersed with a cystamine solution (10 mM cystamine in 50 mM PBS, pH 7.0) for

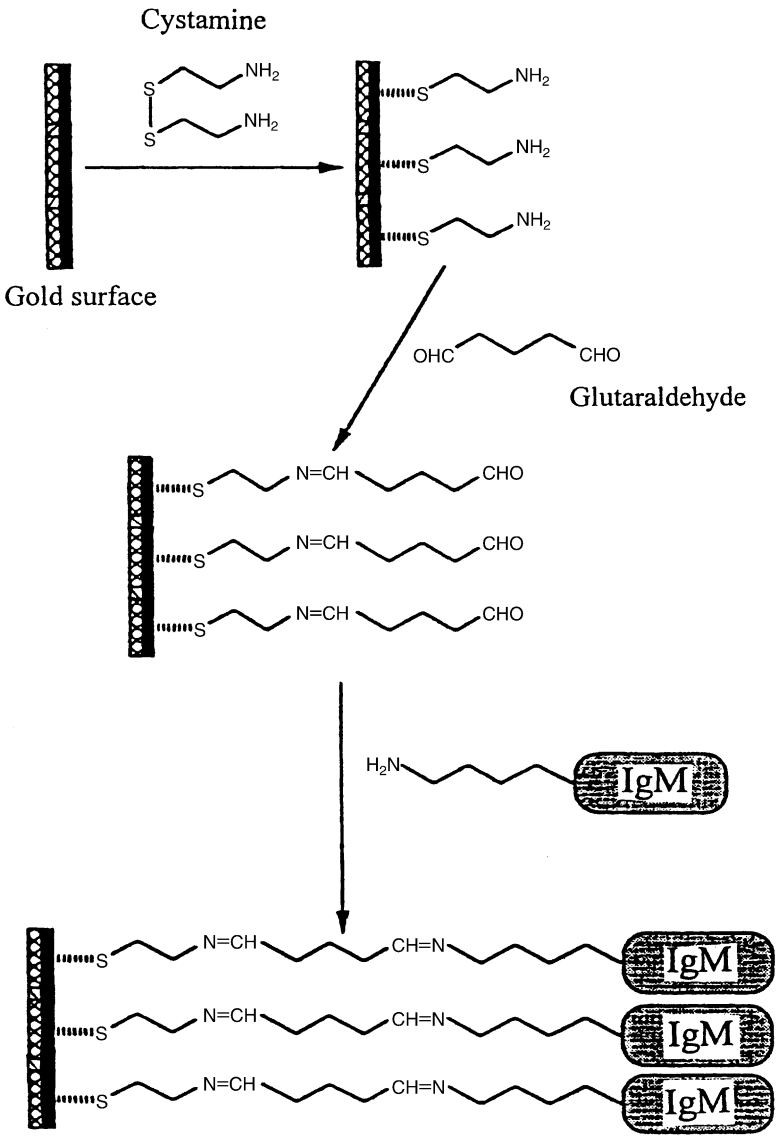

Fig. 2. The chemical steps of IgM immobilization by cystamine-glutaraldehyde method.

1 h, washed with distilled water and dried. The crystal was then dipped into a 1 mg/ml Ab solution for 1 h, 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. ConA method

The crystal was dipped into a ConA solution (85 nM ConA in 50 mM 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 Ab solution for 1 h, washed with PBS and dried. The crystal was then blocked with a 120 nM dextran solution for 30 min, washed with

PBS, distilled water, and dried [28]. 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-ferritin Abs using different immobilization methods

3.1.1. Immobilization of MoAbs

Four immobilization methods including adsorption, protein A, ConA and cystamine-glutaraldehyde methods were used to immobilize Abs. In this experiment, the MoAbs 1AD11F9 was immobilized on the gold surface of the crystal using the four methods. An amount of 3 μl of 10 ng/ml ferritin in PBS 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 [23]. Fig. 3 shows that the reusability of quartz crystal adopting cystamine-glutaraldehyde method was better than those of the other indicated methods. The quartz could continuously operate 10 cycles for 10 ng/ml of ferritin and the relative frequency shifts (the frequency shifts measured are relative to the response at the first time) were all above 95%. 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 MoAbs had been classified as IgM previously [21]. 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 [20]. Thus, the IgM could be easily immobilized on the gold surface. Fig. 2 shows the chemical steps involved in IgM immobilization by cystamine-glutaraldehyde method. Therefore, cystamine-glutaraldehyde method was found to be suitable for immobilization of IgM.

3.1.2. Immobilization of PoAbs

Fig. 4 shows the reusabilities of quartz crystals coated with the PoAbs using five immobilization methods including adsorption, protein A, ConA, cystamine-glutaraldehyde and cystamine method. The results demonstrated that the cystamine method developed in our laboratory was better than the other methods. If the cystamine-glutaraldehyde method was used, the aldehyde groups of glutaraldehyde could

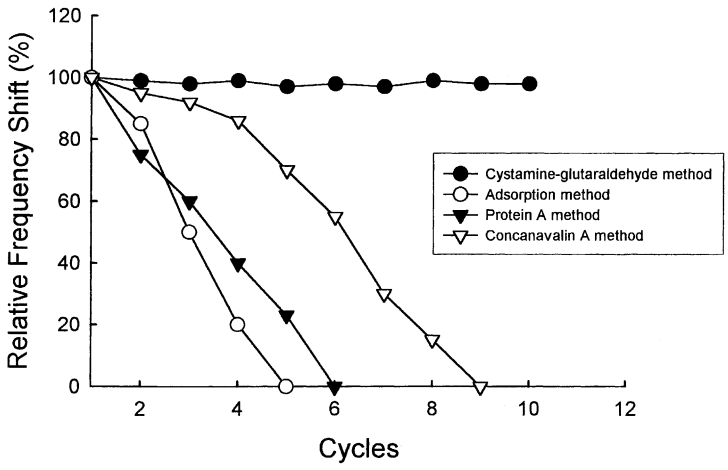

Fig. 3. Comparison on reusability of quartz crystals coated with anti-ferritin MoAbs using different immobilization methods applied in the ferritin piezoimmunosensor. Anti-ferritin MoAbs 1AD11F9 were immobilized on the crystal using the four indicated immobilization methods. 10 ng/ml of ferritin in PBS (pH 7.0) was applied to the gold disc for detection. The regeneration buffer was 0.1 M glycine-HCl buffer (pH 2.1). Relative frequency shift (%) means that the frequency shift measured is relative to the response at the first cycle.

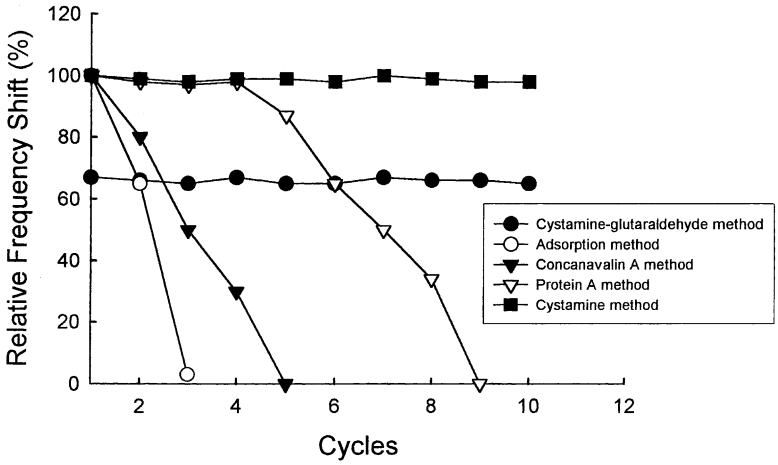

Fig. 4. Comparison on reusability of quartz crystals coated with anti-ferritin PoAbs using different immobilization methods applied in the ferritin piezoimmunosensor. Anti-ferritin PoAbs were immobilized on the crystal using the indicated five kinds of immobilization methods. 10 ng/ml of ferritin in 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). PBS was used as the blank. Relative frequency shift (%) means the frequency shift measured is relative to the response at the first cycle.

easily bind to the amino groups of Fab portion in the IgG molecule (i.e. antigen binding site), so the binding of Ags to Abs might be partly hindered (the major isotype of PoAbs in mice ascites was IgG). Therefore, the cystamine-glutaraldehyde method was unsuitable to immobilize the PoAbs. In cystamine method, the amino groups of cystamine could bind to the carboxyl groups in the IgG molecule by the interaction presumably electrostatic, hydrogen bonding etc., but not bind to the amino groups of the antigen binding sites

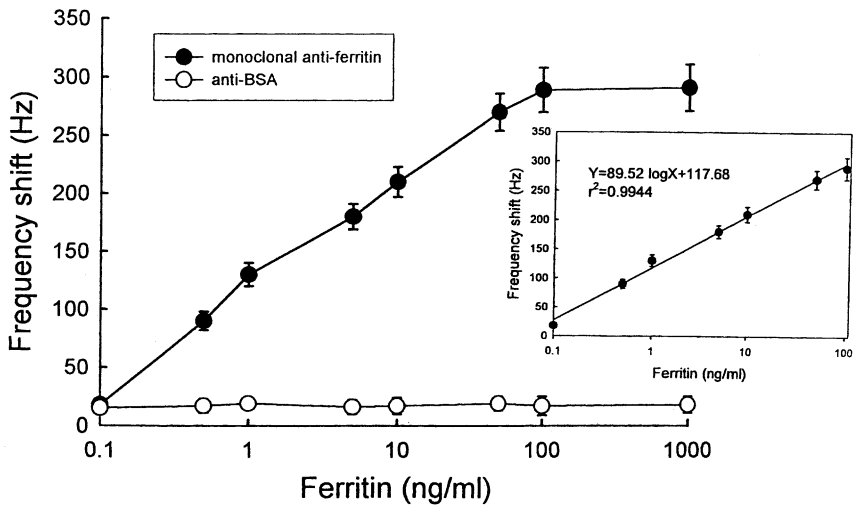

Fig. 5. Detection of human ferritin in mouse serum using anti-ferritin MoAbs immobilized by this QCM immunosensor. The immobilized Abs used were anti-ferritin MoAbs and anti-BSA (control). The immobilization method used was cystamine-glutaraldehyde method. PBS was used as the blank. The inset shows the linear relationship range between frequency shift (Hz) and the log values of human ferritin concentration (ng/ml). The vertical bars designate the standard deviation (S.D.) for the mean of three measurements.

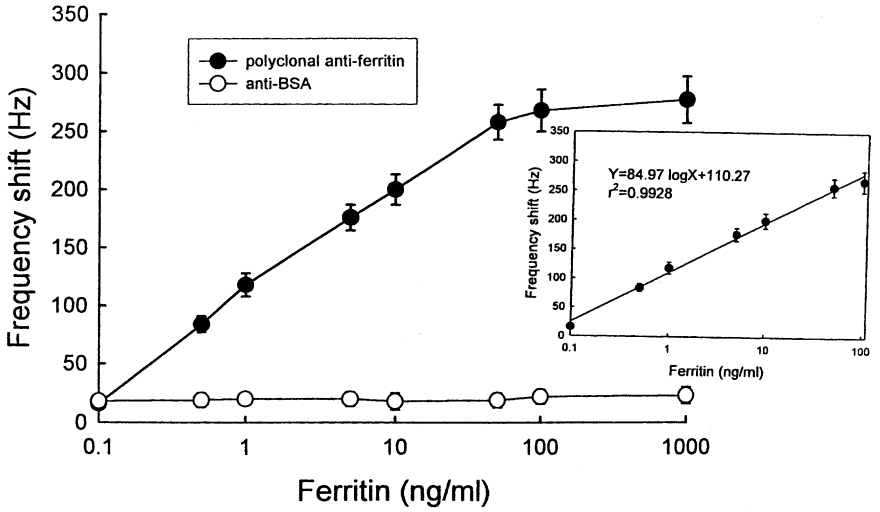

Fig. 6. Detection of human ferritin in mouse serum using anti-ferritin PoAbs immobilized by this QCM immunosensor. The immobilized Abs used were antiferritin PoAbs and anti-BSA (control). The immobilization method used was cystamine method. PBS was used as the blank. The inset shows the linear relationship range between frequency shift (Hz) and the log values of human ferritin concentration (ng/ml). The vertical bars designate the S.D. for the mean of three measurements.

in IgG. It may be similar to the binding between IgG and Fc receptor [29]. The low pH regeneration buffer could easily disrupt Ag–Ab, but not disrupt Ab–SAM. The experiment results demonstrated the cystamine method was suitable to immobilize the PoAbs.

3.2. Calibration curves of human ferritin in buffer and mouse serum

Ferritin in the buffer and mouse serum was detected in the range 0.1 ng/ml to 1 μg/ml using the MoAbs lAD11F9 and PoAbs. A linear relationship existed between the frequency shifts (Hz) and the log values of ferritin in the range 0.1 to 100 ng/ml in mouse serum shown in Figs. 5 and 6 (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. Precision

Precision data for the determination of ferritin (0.1–100 ng/ml) in mouse serum using the MoAbs

Table 1
Precision of the human ferritin determination in mouse serum using anti-ferritin MoAbs and PoAbs immobilized by QCM immunoassay

Concentration of ferritin (ng/ml)	Frequency shift (Hz)					
	MoAbs ($n = 3$)[a]			PoAbs ($n = 3$)[a]		
	Mean	S.D.	CV (%)	Mean	S.D.	CV (%)
0.1	18	3.67	20.4	16	2.92	18.2
0.5	90	7.34	8.16	84	8.97	10.7
1.0	130	7.41	5.70	118	9.79	8.30
5.0	180	8.97	4.98	176	10.6	6.02
10.0	210	11.0	5.23	200	13.2	6.61
50.0	270	13.5	5.00	258	14.4	5.58
100.0	289	14.4	4 97	269	14.8	5.50

[a] Three replicates per specimen were measured.

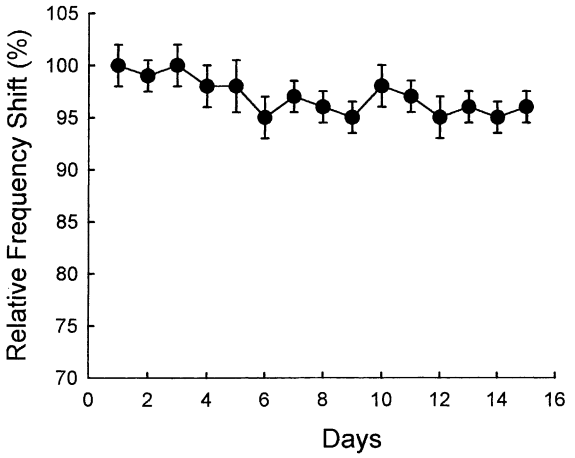

Fig. 7. Operating stability of the ferritin piezoimmunosensor using anti-ferritin MoAbs immobilized. The immobilization method used was cystamine-glutaraldehyde method. The detections were performed continuously five times per day for 10 ng/ml of ferritin. PBS was used as the blank. Relative frequency shift (%) means the frequency shift measured is relative to the response at the first day. The vertical bars designate the S.D. for the mean of five measurements.

1AD11F9 and PoAbs immobilized by this QCM immunosensor are shown in Table 1. Three replicates per specimen were measured.

3.4. Operating stability of human ferritin piezoimmunosensor

In the ferritin piezoimmuno sensor with the MoAbs 1AD11F9 immobilized, the five cycles of measurements per day were continuously performed for 15 days. Fig. 7 shows that the operating stability of this piezoimmunosensor was more than 15 days and the relative frequency shifts (the frequency shifts measured are relative to the response at the first day) were all above 95%. In case of ferritin piezoimmunosensor with the PoAbs immobilized, the similar results were obtained (data not shown).

4. Conclusion

In this study, human ferritin concentrations in buffer and mouse serum were efficiently determined using an immunosensor based on a QCM. Two kinds of SAMs prepared by cystamine-glutaraldehyde and cystamine method were applied to immobilize the anti-ferritin MoAbs and PoAbs on 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 ferritin concentrations in the range from 0.1 to 100 ng/ml. The operating stability of this sensor was more than 15 days. The immunosensor will be applied for the detection of ferritin in human serum samples and related clinical diagnosis in the near future.

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