

Development of an immunosensor for human ferritin, a nonspecific tumor marker, based on surface plasmon resonance

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Abstract

A direct human ferritin immunosensor was developed using anti-human ferritin monoclonal antibodies (MAbs) immobilized on the gold surface of a self-assembled surface plasmon resonance (SPR) apparatus. A kind of self-assembled monolayer (SAM) prepared by cystamine–glutaraldehyde method was applied to immobilize the MAbs. The reusability of the sensor chip adopting the SAM was found to be better than the other immobilization methods including adsorption, protein A, concanavalin A method. Ten cycles of measurements could be performed on the same chip regenerated with a 0.1 M HCl solution. A linear relationship existed between the angle shifts (millidegrees) and the log values of ferritin concentrations in the range from 0.2 to 200 ng/ml in buffer and human serum. When used for 15 days, the angle shifts were all >95% of those on the response at the first day. A 10 M NaOH solution was used for clearing nonspecific binding in human serum. Correlation coefficient was 0.991 between this SPR method and chemiluminescent immunoassay for determination of ferritin in clinical human serum samples. The SPR sensor offers advantages of simplicity of immobilization, high sensitivity, high specificity, low sample requirement, high reusability, no label and no pretreatment etc.

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1. Introduction

Ferritins are a class of iron storage proteins widely distributed in vertebrates, invertebrates, plants, fungi, and bacteria (Sun and Chasteen, 1992). The major function of ferritin is to store and detoxify intracellular iron (Levi et al., 1988). Ferritin is known to have two isomers, H-chain and L-chain ferritin. The H chain of ferritin manifests ferroxidase activity (Balla et al., 1992). It implies that ferritin-stored iron might resist cyclical reduction/oxidation reactions, which tend to propagate and amplify oxidative damage (Levi et al., 1988). Elevated levels of available iron can also promote growth of tumors (Stevens et al., 1994) and infectious microorganisms (including HIV, hepatitis virus, *Mycobacterium avium*, *Candida*, etc.) (Weinberg, 1978). At extremely elevated iron levels, ferritin can be disproportionately increased (Sullivan and Sullivan, 1996). 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 (Moroz et al., 1997), renal cell carcinoma (Ozen et al., 1995; Kirkali et al., 1995), hepatocellular carcinoma (Tatsuta et al., 1986; Ola et al., 1995), larynx cancer and malignant neoplasms of maxilla (Gierek et al., 1995) etc., or in significant diseases, like HIV infection, Still's disease, leukocytosis, reactive hemophagocytic syndrome (Olive' et al., 1996; Lee and Means, 1995), etc. Normal value of ferritin concentration of a healthy adult serum is less than 200 ng/ml for men and 90 ng/ml for women.

The 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, it requires special disposal and has stability problems (Biotechnology Information Service Report, 1993). The nonisotopic immunoassay, based on enzymes or fluorescein, has high sensitivity and is fully automated on many platforms (Morgan et al., 1996). However, these methods must rely on the detection of

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labeled molecules and are complicated and time-consuming (Sauerbrey, 1959).

Immunosensors, which combine the inherent specificity of antigen–antibody (Ag–Ab) reaction with high sensitivity of various physical transducers, have currently gained attention in clinical diagnosis (Kanazawa and Gordon, 1985). This study focused on a self-assembled surface plasmon resonance (SPR) apparatus. The concept of surface plasmons (SP) is coming from the plasmon approach of Maxwell's theory: the free electrons of a metal are treated as an electron liquid of high density (plasmon), and density fluctuations happening on the surface of such a liquid are called plasmons, SP (Raether, 1988). According to the Maxwell's theory, SP can propagate along a metallic surface and have a spectrum of eigen frequencies ω related to the wavevector (K) by a dispersion relation:

$$K_{\text{sp}} = \omega c^{-1} \left[\frac{\varepsilon_1 \varepsilon_2}{\varepsilon_1 + \varepsilon_2} \right]^{1/2}$$

where c is the speed of light in vacuum, ω frequency, ε_1 and ε_2 are the dielectric constant of the metal and of the medium in contact with it, respectively. Practically, SPR is realized in the so-called Kretschmann configuration (Kretschmann and Raether, 1968), where thin metal layer is deposited on a glass substrate, and plasmons are induced by p -polarized light undergoing total internal reflection (TIR) on the glass surface. More precisely, they are excited by an evanescent wave associated with TIR and penetrating through the metal thickness up to the metal/air interface. Exact matching of photons and plasmons happens for the resonance condition:

$$\omega c^{-1} \varepsilon_0^{1/2} \sin \Theta_{\text{R}} = \omega c^{-1} \left[\frac{\varepsilon_1 \varepsilon_2}{\varepsilon_1 + \varepsilon_2} \right]^{1/2}$$

where ε_0 is the dielectric constant of glass prism, Θ the internal angle of incidence of the light beam, and Θ_{R} is the resonance angle. Θ_{R} is a sensitive function of the dielectric constants of the two contacting media. SPR occurs at a given angle of incidence of the light at which thus the reflected light disappears. The resonance angle depends on the optical properties of the medium outside the metal film, which can be used, e.g. to detect Ag–Ab binding reactions on the metal surface.

In this study, immobilization of anti-ferritin monoclonal antibodies (MAbs) on the gold surface of sensor chip is also 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 (Wink et al., 1997). For example, if the amino

and sulfur-containing molecules are adsorbed on the gold surface and then activated by glutaraldehyde, the aldehyde groups of glutaraldehyde can bind to the amino groups of the protein to form Schiff bases (Chibata, 1978). Thus, the protein (e.g. enzymes, Abs) can be immobilized on the gold surface.

In this paper, we report methods for immobilizing anti-ferritin MAbs on gold surface, the reusabilities of coated chip, and the operating stability of the SPR immunosensor. Furthermore, we measured ferritin concentrations in buffer and human serum using this sensor and compared this SPR method with chemiluminescent immunoassay in clinical human serum samples.

2. Experimental

2.1. Reagents

Ferritin from human liver was purchased from Calbiochem-Novabiochem International, La Jolla, CA, USA. Anti-ferritin MAbs were produced previously in our laboratory (Chou and Chen, 2001). Protein A, cystamine dihydrochloride and concanavalin A (ConA) was purchased from Sigma, St. Louis, USA. Bovine serum albumin was obtained from Chemicon International Inc., Temecula, CA, USA. All other chemicals used were of analytical grade.

2.2. Principle and apparatus of the sensor

The basic design of the sensing chip is shown diagrammatically in Fig. 1a. Chromium and gold were successively deposited on a glass slide to give layers of approximately 5 and 50 nm thick, respectively. Chromium was deposited to obtain good adhesion between gold and glass slide (Yokoyama et al., 1995). (The metallic films on glass slide were produced from the Institute of Applied Mechanics of National Taiwan University, Taipei, Taiwan, ROC.) Subsequently, a SAM was immobilized onto the gold surface. In this study, the immobilization of MAbs onto the SAM produced the final sensor. A self-assembled SPR apparatus in the Kretschmann configuration (Kretschmann and Raether, 1968) as shown in Fig. 1b was used for monitoring the binding of specific Ags to the sensor surface. In the SPR apparatus, a beam of light from a 5 mW He-Ne laser (random polarization, $\lambda = 632.8$ nm) passed through a cube polarizer, which control the light intensity and produce p -polarized light at the sample. A plane/convex glass lens was used to compensate and ensure a parallel light beam into a right triangular prism. The laser beam passed through the prism and was incident on the back surface of a gold film deposited onto a glass slide. The prism and glass slide was made of BK7 glass ($n = 1.517$ at $\lambda = 632.8$ nm). The slide was sealed to the prism using optical adhesive ($n = 1.524$, Norland optical adhesive 65, Norland Products Inc., New Brunswick,

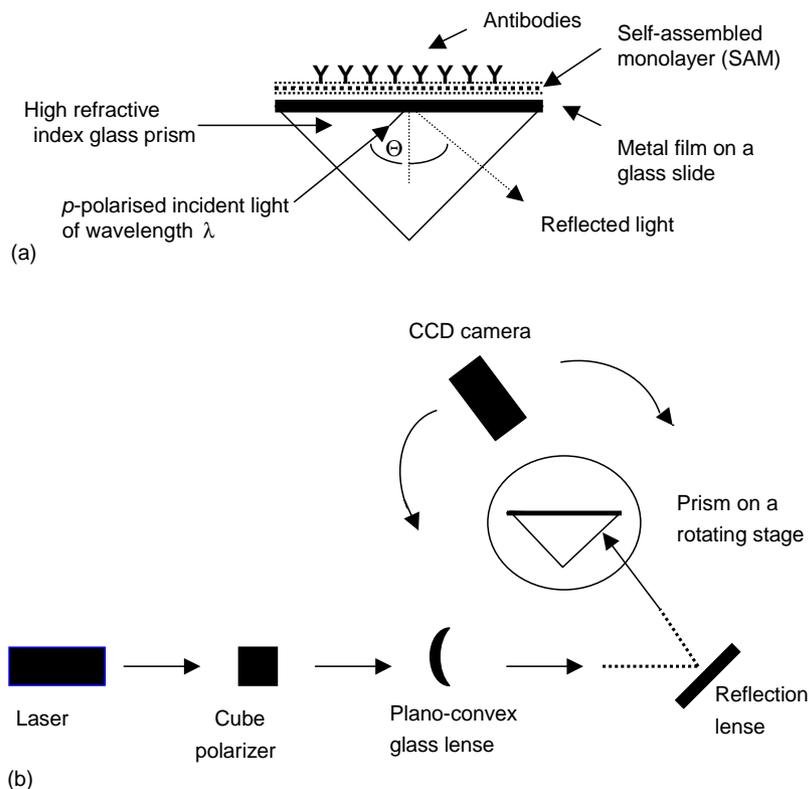


Fig. 1. (a) The basic design of the sensing chip. (b) A self-assembled SPR apparatus in the Kretschmann configuration.

NJ). The prism was mounted on a rotating stage. All optical components were obtained from Melles Griot, Irvine, California. The CCD camera (Panasonic Color CCTV Camera, Model WV-CP230, Matsushita Communication Industrial Co., Ltd., Yokohama, Japan) connected with a computer containing the image treatment software (Spy-Glass Transform 3.4) was used for detecting the reflected light. The resonance condition was determined by recording the reflected light intensity as a function of the incident angle. The SPR signal was presented as an angle shift in millidegrees.

The entire apparatus was mounted on an optical bench in a darkroom and routinely checked for alignment before any measurements were taken.

2.3. Measurement of angle shifts by the SPR apparatus

The gold surface of the chip was treated with 1.2 M NaOH for 10 min, 1.2 M HCl for 5 min and one drop of concentrated HCl for 30 s (Storri et al., 1998). After each step, the chip was thoroughly washed with distilled water. The chip was dried at room temperature and initial SPR angle was read. The sample solution (3 μ l) was added into the gold surface. After 10 min, the chip was washed, dried and an angle shift value was read. The 3 μ l drop was placed exactly onto the same place of the chip marked on the gold surface in the repeated measurements. All procedures were measured at room temperature under atmospheric pressure.

2.4. Immobilization of antibodies on gold

2.4.1. Adsorption method

The gold surface of the chip was treated with a 1 mg/ml solution of the antibody for 1 h, and the chip was then washed with phosphate buffered saline (PBS) (5 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 0.15 M NaCl, pH 7.0) (Storri et al., 1998). After drying, an angle shift value was read. All procedures were performed at room temperature under atmospheric pressure.

2.4.2. 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 gold surface of the chip. After drying, the chip was washed in distilled water for 30 min. Subsequently, 5 μ l of a 1 mg/ml antibody solution was spread over the gold surface. After drying, the chip was washed with PBS and distilled water, dried (Guibault et al., 1992; Nakanishi et al., 1996) and an angle shift value was read. All procedures were performed at room temperature under atmospheric pressure.

2.4.3. Cystamine–glutaraldehyde method

The gold surface of the chip was treated with a cystamine solution (10 mM cystamine in 50 mM PBS, pH 7.0) for 1 h, washed with distilled water and PBS, and dried. Subsequently, the chip was dipped into 10% (v/v) aqueous glutaraldehyde solution for 30 min and washed twice with

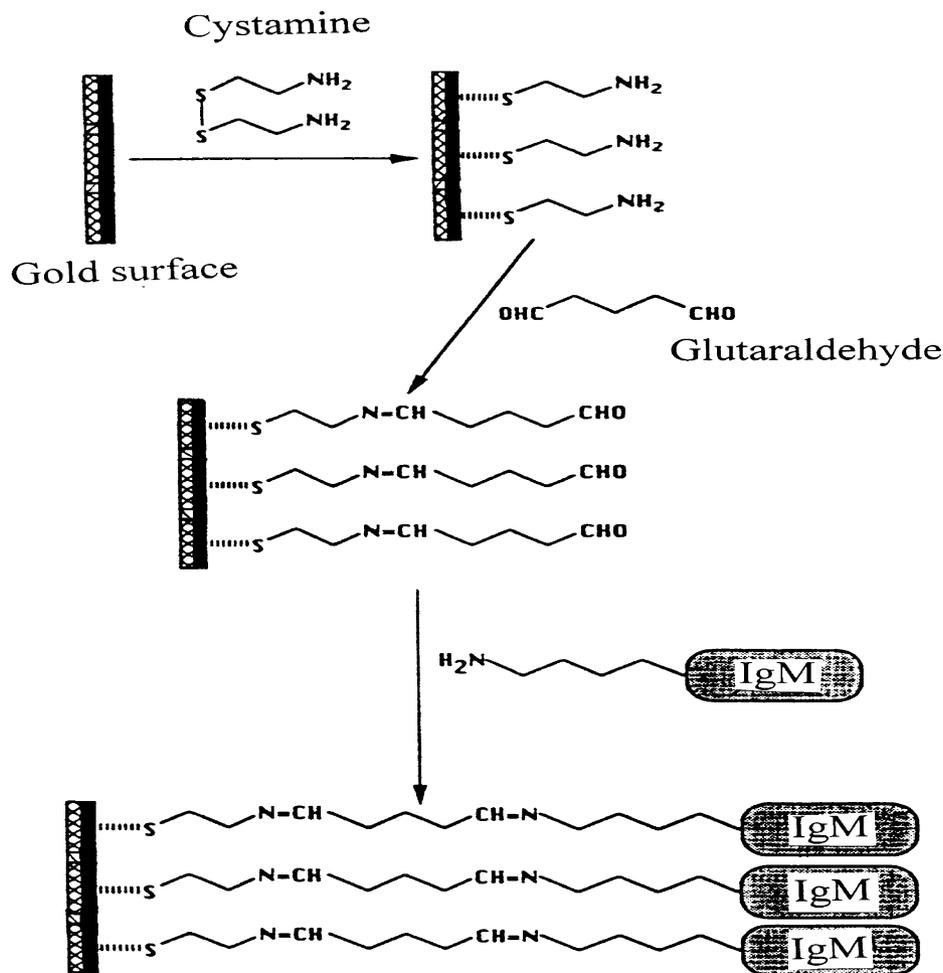


Fig. 2. The chemical steps of IgM immobilization by cystamine–glutaraldehyde method.

distilled water. After drying, the chip was immersed with a 1 mg/ml antibody solution for 1 h and washed with PBS, distilled water, and dried. The chip was blocked with a 0.1 M glycine–PBS solution (0.1 M glycine in 50 mM PBS, pH 7.0) for 30 min (Le et al., 1995), washed with PBS and distilled water, and dried. Finally, an angle shift value was obtained. All procedures were performed at room temperature under atmospheric pressure. Fig. 2 shows that the chemical steps of IgM immobilization by cystamine–glutaraldehyde method (Pariente et al., 1996).

2.4.4. ConA method

The gold surface of the chip was treated with a ConA solution (85 nM ConA in 50 mM PBS containing $100 \mu\text{M}$ Ca^{2+} and Mn^{2+} , pH 6.8) for 1 min, washed with PBS and distilled water, and dried. Subsequently, the chip was immersed with a 1 mg/ml antibody solution for 1 h, washed with PBS and dried. The chip was then blocked with a 120 nM dextran solution for 30 min, washed with PBS and distilled water, and dried (Mathewson and Finley, 1992). Finally, an angle shift value was obtained. All procedures were performed at room temperature under atmospheric pressure.

2.5. Chemiluminescent immunoassay

Ferritin in clinical human serum samples were analyzed using chemiluminescent immunoassay method in Department of Radiation Oncology of Tri-Service General Hospital, Taipei, Taiwan, ROC. The system of the chemiluminescent immunoassay kit used is that the Ags labeled-luminol competitive with ferritin in human serum samples bind to the immobilized specific Abs, then peroxidase and peroxide are added. Absorbance of the reaction product (*o*-methyl-acridone) at 430 nm is measured by an automated luminometer (ASC 180, CIBA-Corning).

3. Results and discussion

3.1. Reusabilities of sensor coated with anti-human ferritin MAbs using different immobilization methods

Four immobilization methods including adsorption, protein A, ConA and cystamine–glutaraldehyde methods were used to immobilize the MAbs. In this experiment, anti-ferritin MAbs 1AD11F9 was immobilized on the gold

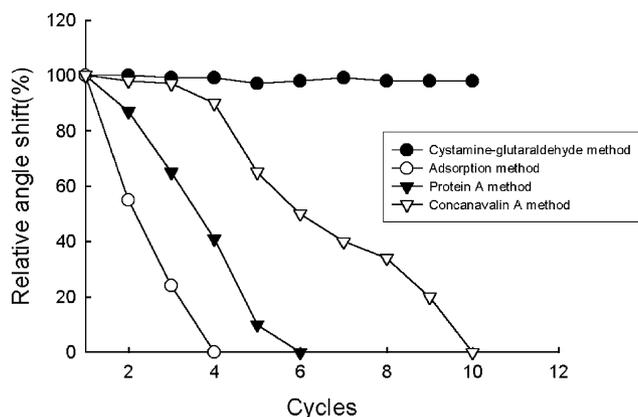


Fig. 3. Comparison on the reusability of chip coated with anti-human ferritin MAbs using different immobilization methods applied in the SPR system. Anti-human ferritin MAbs (1AD11F9) were immobilized on the gold surface of sensor chip using the indicated four kinds of immobilization methods. A 10 ng/ml of human ferritin in PBS (pH 7.0) was applied to the gold surface for detection. The regeneration buffer was 0.1 M HCl buffer (pH 2.1). Relative angle shift (%) means the angle shift measured is relative to the response at the first time.

surface of the chip using the above-mentioned four methods. Three μ l of 10 ng/ml ferritin in PBS was applied to the gold surface for detection. The regeneration solution used was 0.1 M HCl buffer (pH 2.1) after each measurement. Fig. 3 shows that the reusability of chip adopting cystamine–glutaraldehyde method was better than those of the other above-mentioned methods. The chip could continuously operate 10 cycles for 10 ng/ml of ferritin and the relative angle shifts (the angle shifts measured were 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 MAbs had been classified as IgM previously (Chou and Chen, 2001). In this study, if the amino and sulfur-containing molecules adsorbed on the gold surface were activated by glutaraldehyde, the aldehyde groups of glutaraldehyde molecule could bind to the amino groups of the IgM molecule to form Schiff bases (Chibata, 1978). The IgM could be easily immobilized on the gold surface. Thus, cystamine–glutaraldehyde method was found to be suitable for immobilization of IgM. Fig. 2 shows the chemical steps involved in IgM immobilization by cystamine–glutaraldehyde method. Fig. 4 shows a series of SPR curves obtained by successive immobilization treatment of cystamine, glutaraldehyde and anti-human ferritin MAbs.

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

Human ferritin in the buffer and human serum was detected in the range 0.1 ng/ml to 1 μ g/ml using MAbs

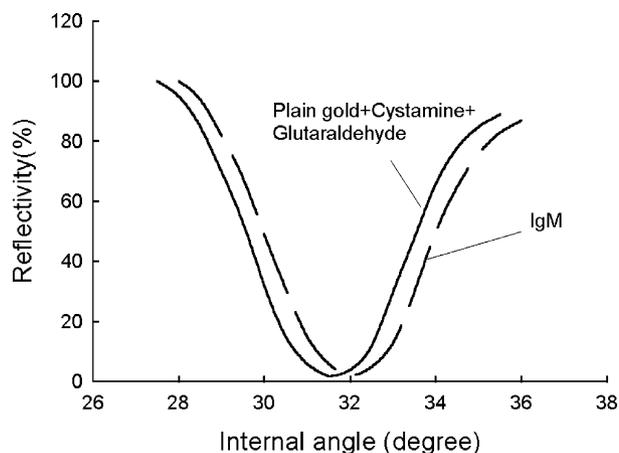


Fig. 4. SPR scanning curve for each successive adlayer. The successive adlayer was obtained: the plain gold was immersed in cystamine solution for 1 h, glutaraldehyde for 30 min, and IgM solution for 1 h. The SPR curves for each successive adlayer were then detected.

1AD11F9. A linear relationship existed between the angle shifts (millidegrees) and the log values of ferritin in the range 0.2–200 ng/ml in human serum shown in Fig. 5 (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 human serum was 20 min (data not shown). The regeneration buffer used was 0.1 M HCl buffer (pH 2.1) for clearing the Ags binding to the Abs. A 10 M NaOH solution was very suitable to clear nonspecific binding in human serum as shown in Fig. 6.

3.3. Precision

Precision data for the determination of ferritin (0.2–200 ng/ml) in buffer and human serum by this SPR immunosensor are shown in Table 1. Three replicates per specimen were measured.

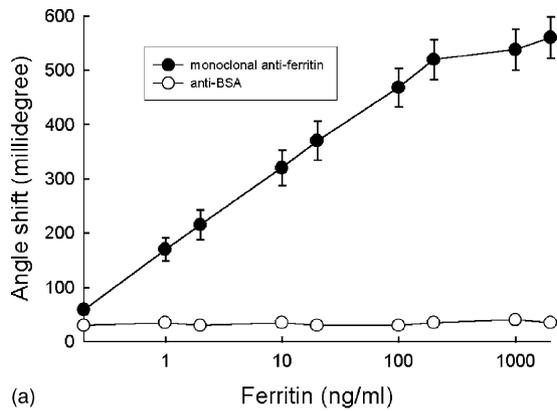
Table 1
Precision of the ferritin determination by SPR immunoassay

Ferritin concentration (ng/ml)	Angle shifts (millidegree)					
	In PBS ($n = 3$) ^a			In human serum ($n = 3$) ^a		
	Mean	S.D. ^b	CV ^c	Mean	S.D. ^b	CV ^c
0.2	50	5.00	10	59	8.03	13.6
1.0	152	8.12	5.3	170	21.2	12.5
2.0	200	10.1	5.0	215	27.5	12.8
10.0	310	15.2	4.9	320	32.0	10.0
20.0	350	16.8	4.8	370	36.2	9.78
100.0	453	20.2	4.5	468	35.0	7.48
200.0	506	25.9	5.1	519	36.7	7.07

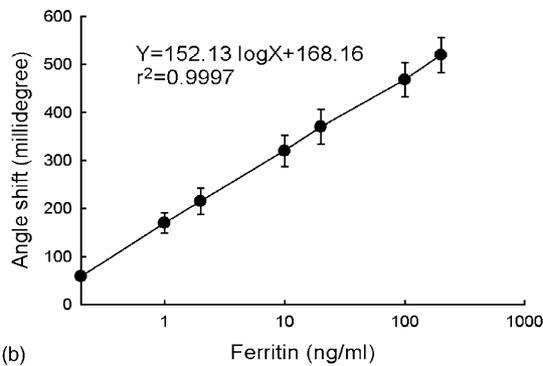
^a Three replicates per specimen were measured.

^b S.D. means standard deviation.

^c CV means coefficient of variation.



(a)



(b)

Fig. 5. Detection of human ferritin in human serum using anti-human ferritin MAbs immobilized on the gold surface of the sensor chip. The immobilized antibodies used were anti-human ferritin MAbs and anti-BSA (control). The immobilization method used was cystamine–glutaraldehyde method. PBS was used as the blank. The regeneration buffer was 0.1 M HCl buffer (pH 2.1) for clearing the Ags binding to the Abs. A 10 M NaOH solution was used for clearing nonspecific binding in human serum. The inset shows the linear relationship range between angle shift (millidegree) 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.

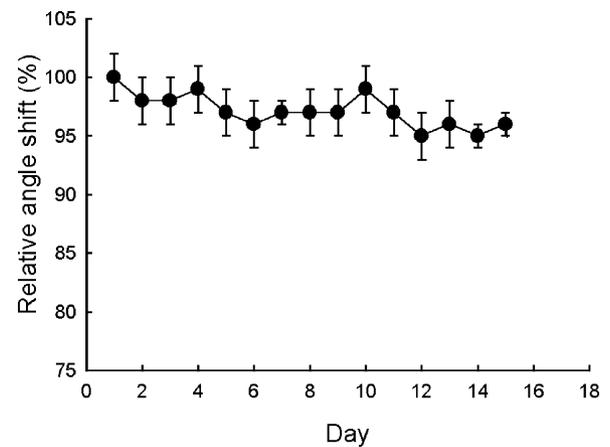


Fig. 7. Operating stability of human ferritin SPR immunosensor. The detections performed continuously were five times per day in human serum. Each assay was analyzed with ferritin 100 ng/ml. The vertical bars designate the standard deviation (S.D.) for the mean of five measurements.

3.4. Operating stability of human ferritin SPR immunosensor

In the ferritin immunosensor with MAbs immobilized, the five cycles of measurements *per* day were continuously performed for 15 days in buffer and human serum. Fig. 7 shows the operating stability of this immunosensor was more than 15 days in human serum, and the relative angle shifts were all above 95% of those measured on the first day, similar to the data obtained in buffer (this data not shown).

3.5. Correlation between this SPR method and chemiluminescent immunoassay

Ferritin concentrations in the clinical human serum samples were simultaneously detected by this SPR and

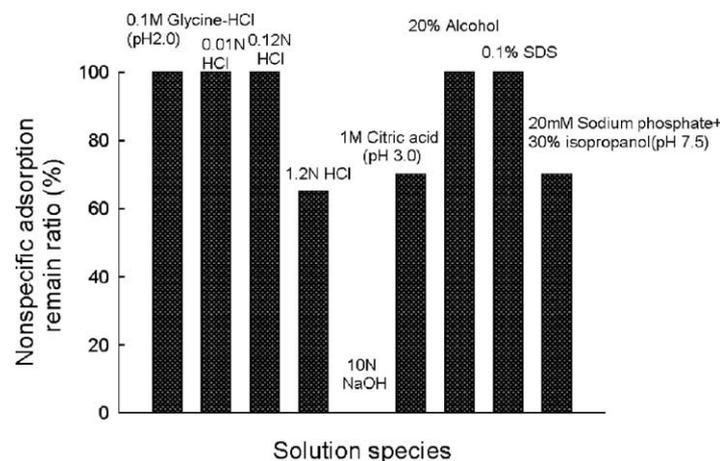


Fig. 6. Effects on clearing non-specific binding in human serum using different reagents. The immobilized antibodies used were anti-human ferritin MAbs. The immobilization method used was cystamine–glutaraldehyde method. The chip coated with Abs was dipped in human serum containing Ags for 30 min. The regeneration buffer was 0.1 M HCl buffer (pH 2.1) for clearing the Ags binding to the Abs. Treatment time of each solution for clearing non-specific binding was 30 min.

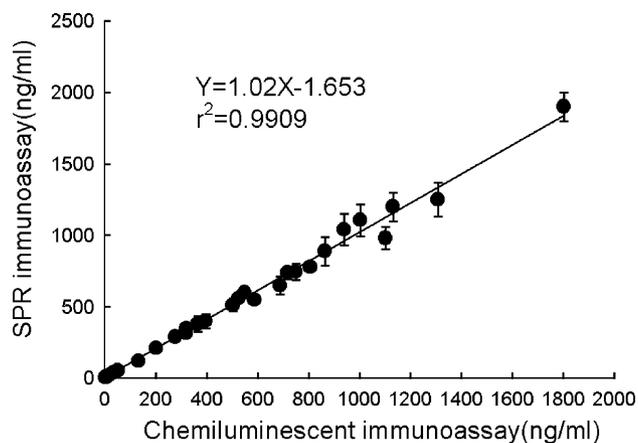


Fig. 8. Correlation between this SPR method and chemiluminescent immunoassay for determination of ferritin in clinical human serum samples. The vertical bars designate the standard deviation (S.D.) for the mean of three measurements.

chemiluminescent immunoassay. The results of chemiluminescent immunoassay were obtained from Department of Radiation Oncology of Tri-Service General Hospital, Taipei, Taiwan, ROC. Fig. 8 shows the correlation coefficient obtained was 0.991 between SPR and chemiluminescent immunoassay for determination of ferritin in 30 clinical human serum samples. Three replicates per specimen were measured.

4. Conclusion

In this study, the concentrations of human ferritin in the buffer and human serum were efficiently determined using an immunosensor based on a self-assembled SPR. A SAMs prepared by cystamine–glutaraldehyde method was applied to immobilize MAbs on the gold surface of the chip. The 10 cycles of measurements could be performed on the gold surface of the same chip regenerated with a 0.1 M HCl solution for clearing the Ags binding to the Abs. A 10 M NaOH solution was used for clearing nonspecific binding in human serum. A linear relationship existed between the angle shifts (millidegrees) and the log values of ferritin concentrations in the range from 0.2 to 200 ng/ml. The operating stability of this sensor was more than 15 days. The direct SPR immunosensor measured ferritin without the need for a labeled reagent and pretreatment of samples. The assay format of

the sensor was more rapid and simpler than conventional methods.

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