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Studies on Development of an Immunosensor for Human Heart-type Fatty Acid Binding Protein Based on Surface Plasmon Resonance

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Abstract

A direct human heart-type fatty acid binding protein (HFABP) immunosensor was developed using anti- HFABP 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-protein A method was applied to immobilize the MAbs. 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 HFABP concentrations in the range from 0.1 to 300 ng/mL in buffer and artificial human serum. When used for 15 days, the angle shifts were all >95% of those on the response at the first day. A 1 M NaOH solution was used for clearing nonspecific binding in artifical human serum. The SPR sensor offers advantages of simplicity of immobilization, high sensitivity, high specificity, low sample requirement, high reusability, no label and no pretreatment etc.

Keywords: human heart-type fatty acid binding protein (HFABP), immunosensor, surface plasmon resonance (SPR), self-assembled monolayer (SAM)

1. Introduction

Myocardial damage ultimately results in several cardiospecific protein release from injured cardiomyocytes. Such proteins like creatine kinase-MB (CK-MB) and cardiac troponins have been reported to correlate well with the severity of myocardial damage (Cummins et al., 1987), and therefore, been widely used as diagnostic markers. Studies also demonstrated that these markers have been helpful in identifying risk conditions and determining the prognosis of cardiac events. However, their plasma levels increase slowly after reperfusion and need several hours to reach maximum level, which means that they are not fit for the early stage evaluation of myocardial damage and prediction of cardiac events. They also return very slowly to their baselines, and

will miss recurrent myocardial infarction or cardiac complication in the early stage after operation. An accurate, simple, and rapid noninvasive measurement is then needed.

Recently, heart-type fatty acid binding protein (HFABP), a small hydrophilic protein lying in the cytoplasm of both cardiac and skeletal muscle, function in uptake and transport of long-chain fatty acid, has entered the clinicians' insight for the early diagnostic value in acute coronary syndrome (ACS). Its release pattern is markedly different from routine markers due to its small size and physical properties, with a very early peak and a rapid decline thereafter. Preliminary studies suggested that HFABP is superior to CK-MB or cardiac troponins in the early detection of ischemic myocardial damage and is more specific and sensitive than myoglobin and CK-MB, and its total amount of leakage reflects the extent of the extent of the damage. However, few studies in the literature have prospectively investigated the diagnostic and prognostic values of HFABP in cardiac surgery with cardiopulmonary bypass (CPB) (Liu et al., 2005).

Recently, the methods for the characterization and quantification of HFABP are ELISA, Western blotting, as well as flow cytometric and microscopic analysis (Gutmann et al., 2005). The major quantitative detection method, ELISA, 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 which must rely on the detection of labeled molecules are complicated and time-consuming.

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 (κ) by a dispersion relation:

$$\kappa_{sp} = \omega c^{-1} [\varepsilon_1 \varepsilon_2 / (\varepsilon_1 + \varepsilon_{2)}]^{1/2}$$

where c is the speed of light in vacuum, ω is 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:

$$\omega c^{-1}\varepsilon_0^{1/2}\sin\Theta_R = \omega c^{-1}\,[\varepsilon_1\varepsilon_2 / (\varepsilon_1+ \varepsilon_{2)}]^{1/2}$$

where ε_0 is the dielectric constant of glass prism, Θ is 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-HFABP monoclonal antibodies (MAbs) on the gold surface of sensor chip is also discussed. When the protein molecules, such as IgG, are immobilized on the sensor surface, the activity is usually less than that in aqueous phase. The main reason is due to the random orientation and stereo hindrance of protein molecules on the solid surface. Recently, 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, cystamine, are adsorbed on the gold surface, the amino groups of cystamine may easily bind to carboxyl groups in the IgG molecule by the interaction presumably electrostatic, hydrogen bonding etc., not bind to the amino groups of the Ag binding sites in the IgG molecule. However, cystamine-Ab binding can not supply a good orientation to binding of Ag. For the construction of a well-defined antibody surface, protein A is used as a binding material. Protein A, a cell wall component of *Staphylococcus aureus*, binds with the Fc portion of the Ab (Harlow, et al, 1999), so the paratopes of Ab molecules are located on the surface of the protein A coating layer. Therefore, protein A-mediated Abs immobilization leads to highly efficient immunoreactions and enhances detection system performance (Lee, et al., 2004). The immobilization method using cystamine combined with protein A to bind the Abs was discussed in this study.

We report methods for immobilizing anti- HFABP MAbs on gold surface, the reusabilities of coated chip, and the operating stability of the SPR immunosensor. Furthermore, we measured HFABP concentrations in buffer and artificial human serum using this sensor and compared this SPR method with ELISA in mouse serum samples.

2. Experimental

2.1 Reagents

HFABP from human heart tissue (> 98% purity was tested by 10-20% gradient SDS-PAGE) was purchased from Biodesign International, A Division of Meridian Life Science, Inc., Saco, ME, U.S.A. Anti-HFABP MAb (5B5) (IgG1) from mouse ascites (> 90% purity was tested by electrophoresis) was purchased from Biodesign International, A Division of Meridian Life Science, Inc., Saco, ME, U.S.A. Protein A from *Staphylococcus aureus* Cowan strain cell wall, cystamine dihydrochloride was purchased from Sigma-Aldrich Chem. Co., St. Louis, U.S.A. Bovine serum albumin (BSA) was obtained from Chemicon International, Inc., Temecula, CA. U.S.A. 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 the previous study (Chou, et al., 2004). Chromium and gold were successively deposited on a glass slide to give layers of approximately 5 nm 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, R.O.C.). Subsequently, a SAM was immobilized onto the gold surface. In this study, the immobilization of MAbs onto the SAM produced in the final sensor. Then the self-assembled SPR apparatus in the Kretschmann configuration (Kretschmann and Raether, 1968) 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, λ= 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 λ= 632.8nm). The slide was sealed to the prism using optical adhesive (n=1.524, Norland optical adhesive 65, Norland Products Inc., New Brunswick, N.J.). 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-CP240, Matsushita Communication Industrial Co., Ltd., Yokohama, Japan) connected with a computer containing the image treatment software (SpyGlass 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 sec (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 μL) was added into the gold surface. After 10 min, the chip was washed, dried and an angle shift value was read. The 3 μL 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 chip was treated with an Ab solution (10^6 μg/L) for 1 hr. Subsequently, the chip was washed with phosphate buffered saline (PBS) (5 mM phosphate buffer, 0.15 M NaCl, pH 7.0), distilled water, and dried. After drying, an angle shift value was read. All procedures were performed at room temperature under atmospheric pressure (Chou, et al., 2004).

2.4.2 Protein A method

Five microliter of a protein A solution (1 mg protein A in 1 ml of PBS, 50 mM, 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 immersed in distilled water for 30 min. Subsequently, 5 μl of a 10^6 μg/L Ab solution was spread over the surface. After drying, the crystal was washed with PBS, distilled water, and dried. Finally, an angle shift value was read. All procedures were performed at room temperature under atmospheric pressure (Chou, et al., 2004).

2.4.3 Cystamine-glutaraldehyde method

The chip was treated with a 10 mM cystamine solution (10 mM cystamine, 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0) for 1 hr, washed with distilled water and dried. The crystal was dipped into a 10% (v/v) aqueous glutaraldehyde solution for 30 min and washed twice with distilled water. After drying, the crystal was immersed with a 10^6 μg/L Ab solution for 1 hr, washed with PBS, distilled water, and dried. The crystal was blocked with a 0.1 M glycine-PBS solution (0.1 M glycine, 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0) for 30 min, and then washed with PBS, distilled water, and dried. Finally, an angle shift value was read. All procedures were performed at room temperature under atmospheric pressure (Chou, et al., 2004).

2.4.4 Cystamine method

The chip was treated with a 10 mM cystamine solution (10 mM cystamine, 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0) for 1 hr, washed with distilled water and

dried. The chip was then dipped into a 10^6 μg/L Ab solution for 1 hr, washed with PBS, distilled water, and dried. The chip was blocked with a 0.1 M glycine-PBS solution for 30 min, washed with PBS, distilled water, and dried. Finally, an angle shift value was read. All procedures were performed at room temperature under atmospheric pressure (Chou, et al., 2004).

2.4.5 Cystamine-Protein A method

The chip was treated with a 10 mM cystamine solution (10 mM cystamine, 50 mM phosphate buffer, 0.15 M NaCl, pH 7.0) for 1 hr, washed with distilled water and dried. The chip was blocked with a 0.1 M glycine-PBS solution for 30 min, washed with PBS, distilled water, and dried. Then 5 μL 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 for 30 min, washed with distilled water and dried. The chip was then blocked with a 0.1 M glycine-PBS solution for 30 min, washed with PBS, distilled water, and dried. Subsequently, 5 μL of a 1 mg/mL Ab solution was spread over the gold surface. After drying, the chip was washed with PBS and distilled water, dried and an angle shift value was read. All procedures were performed at room temperature under atmospheric pressure.

3. Results and Discussion

3.1 Reusabilities of sensor coated with anti-human HFABP MAbs

In this experiment, anti-HFABP MAbs (5B5) was immobilized on the gold surface of the chip using adsorption, protein A, cystamine, cystamine-glutaradehyde and cystamine-protein A method. Three μl of 10 ng/mL HFABP 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. 1 shows that the reusability of chip adopting cystamine-protein A method was better than those of the other above-mentioned methods. The chip could continuously operate 10 cycles for 10 ng/mL of HFABP 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 monolayer 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. In this study, if the amino and sulfur-containing molecules, cystamine, adsorbed on the gold surface, the amino groups of cystamine could easily bind to the carboxyl groups in the IgG molecule by the interaction presumably electrostatic, hydrogen bonding etc., not bind to the amino groups of the Ag binding sites in the IgG molecule. The Abs on the sensor surface were random, partly multi-layers and could not supply a good orientation to binding of Ag when using adsorption, protein A, cystamine, cystamine-glutaraldehyde method. For the construction of a well-defined

antibody surface, protein A is used as a binding material. Protein A (M.W. 42,000) has strongly specific binding with the Fc portion of the Ab (Harlow, et al, 1999), so the paratopes of Ab molecules are located on the surface of the protein A coating layer. Therefore, protein A-mediated Abs immobilization leads to highly efficient immunoreactions and enhances detection system performance (Lee, et al., 2004). The experimental results surely demonstrate the cystamine-protein A method was more suitable to immobilize the IgG molecule and the reusability of the chip was high. Fig.2. shows the chemical steps of IgG1 immobilization by cystamine-protein A method. Fig.3 shows a series of SPR curves obtained by successive immobilization treatment of cystamine, protein A and anti- HFABP MAbs.

3.2 Calibration curves of HFABP in buffer and artificial human serum

HFABP in the buffer and artificial human serum was detected in the range 0.1ng/mL to 1 μg/mL using MAbs. A linear relationship existed between the angle shifts (millidegrees) and the log values of HFABP in the range 0.1 to 300 ng/mL in artificial human serum shown in Fig.4 (the samples containing high HFABP 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 artificial 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.

3.3 Precision

Precision data for the determination of HFABP (0.1-300 ng/mL) in buffer and artificial human serum by this SPR immunosensor are shown in Table 1. Three replicates per specimen were measured.

4. Conclusion

In this study, the concentrations of HFABP in the buffer and artificial human serum were efficiently determined using an immunosensor based on a self-assembled SPR. A SAMs prepared by cystamine-protein A 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 1 M NaOH solution was used for clearing nonspecific binding in artificial human serum. A linear relationship existed between the angle shifts (millidegrees) and the log values of interferon-γ concentrations in the range from 0.1 to 300 ng/mL. The operating stability of this sensor was more than 15 days. The direct SPR immunosensor measured HFABP 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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Figure legends:

Fig.1. Comparison on the reusability of chip coated with anti-HFABP MAbs using different immobilization methods applied in the SPR system. Anti-HFABP MAbs were immobilized on the gold surface of sensor chip using the indicated four kinds of immobilization methods. 10 ng/mL of HFABP 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.

Fig.2. The chemical steps of IgG1 immobilization by cystamine-protein A method

Fig.3. SPR scanning curve for each successive adlayer

The successive adlayer was obtained: the plain gold was immersed in cystamine solution for 1 hr, protein A for 30 min, and 5B5 (IgG1) solution for 1 hr. The SPR curves for each successive adlayer were then detected.

Fig.4. Detection of HFABP in artificial human serum using anti-HFABP MAbs (5B5) immobilized on the gold surface of the sensor chip. The immobilized antibodies used were 5B5 and anti-BSA (control). The immobilization method used was cystamine-protein A 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 1 M NaOH solution was used for clearing nonspecific binding in artificial human serum. The inset shows the linear relationship range between angle shift (millidegree) and the log values of HFABP concentration (ng/mL). The vertical bars designate the standard deviation (SD) for the mean of 3 measurements.

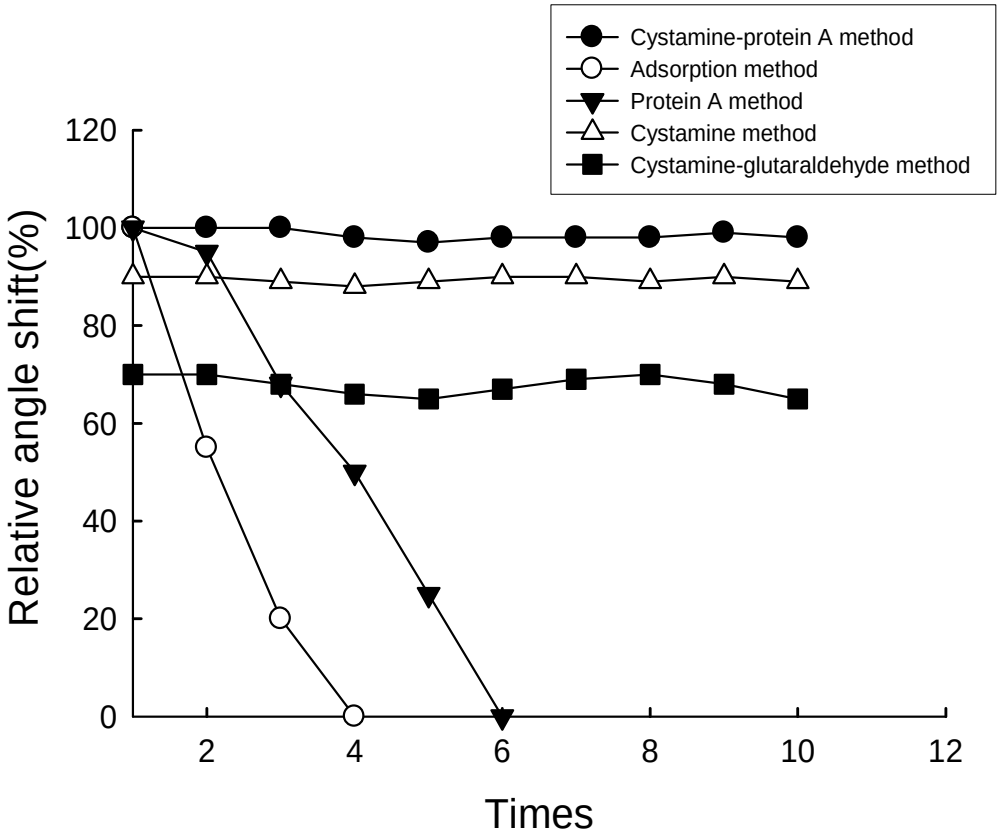
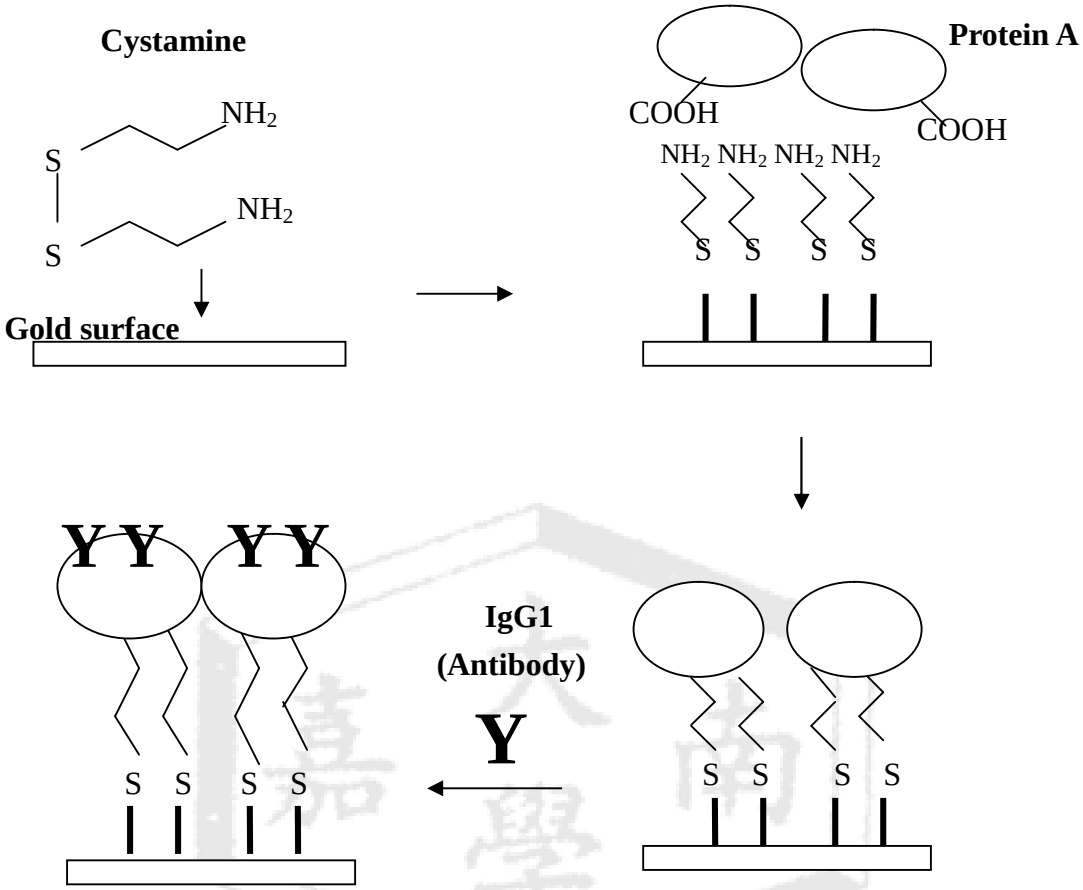


Fig.1 of Chou, S-F



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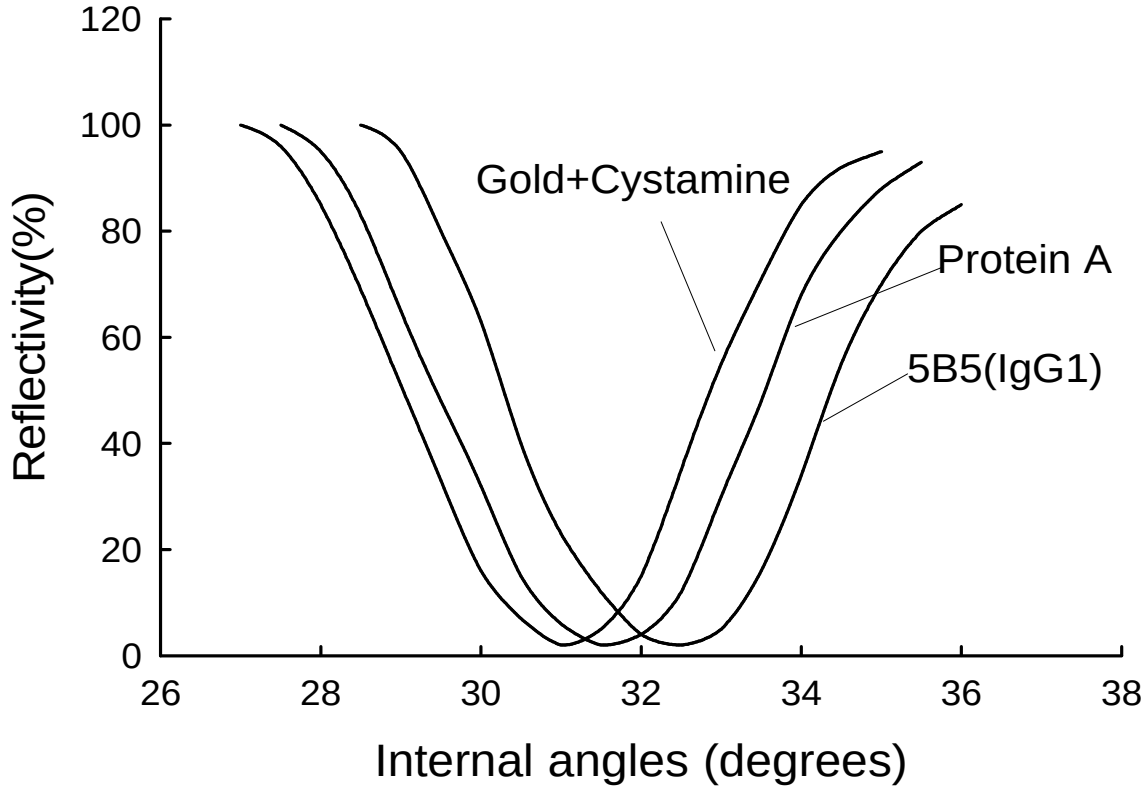

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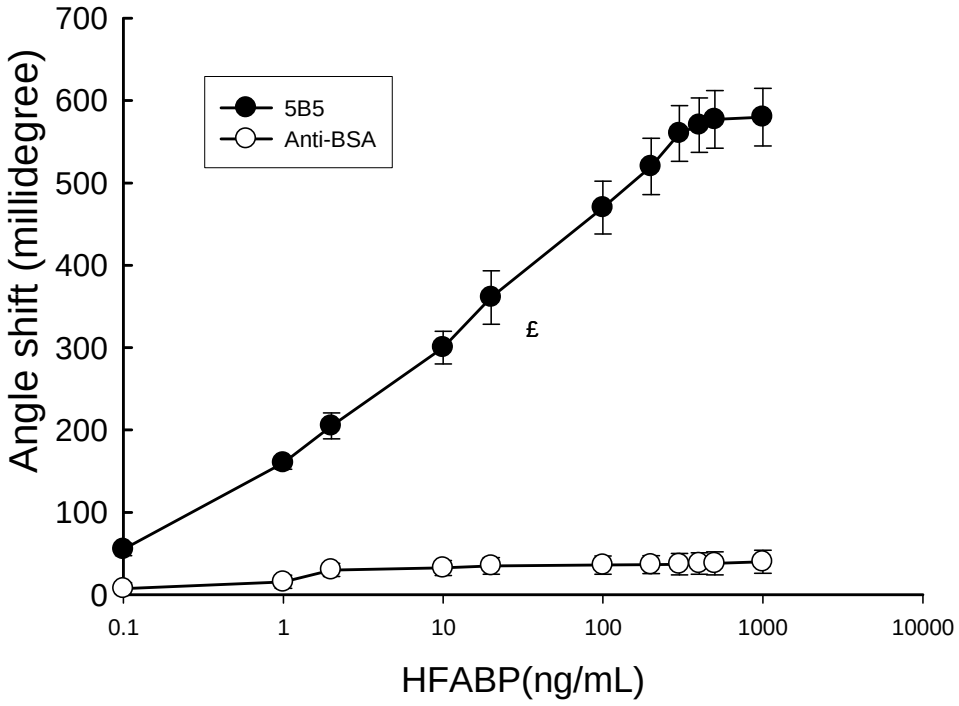

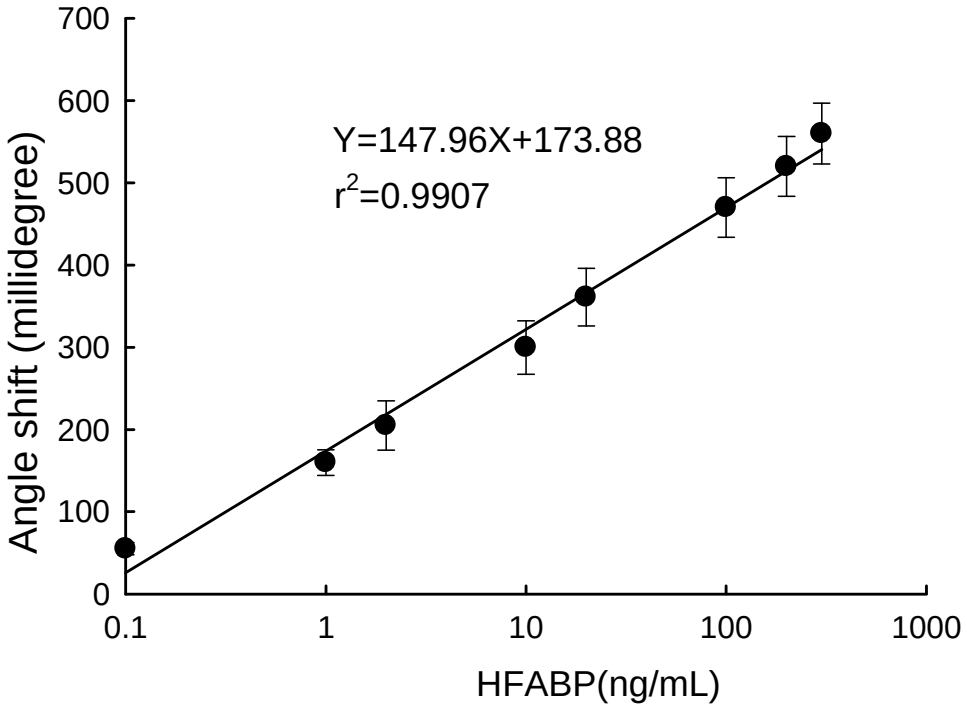

Fig.4 of Chou, S-F

Table 1. Precision of the HFABP determination by SPR immunoassay

HFABP Concentration, ng/mL	Angle shifts, millidegree					
	In PBS (n=3)[a]			In artificial human serum (n=3)[a]		
	Mean	SD[b]	CV[c]	Mean	SD[b]	CV[c]
0.1	54	5.6	10.4	55	7.5	13.6
1.0	152	9.5	6.25	160	15.6	9.75
2.0	207	11.1	5.36	205	30.1	14.7
10.0	315	16.2	5.14	300	32.5	10.8
20.0	350	18.3	5.08	361	35.0	9.70
100.0	360	22.3	4.85	470	36.0	7.66
200.0	516	27.4	5.31	520	36.5	7.02
300.0	546	30.2	5.53	560	37.0	6.61

[a] Three replicates per specimen were measured.

[b]SD means standard deviation.

[c]CV means coefficient of variation.