Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 60 (2003) 53–61
Talanta
www.elsevier.com/locate/talanta

Real-time immunoassay of ferritin using surface plasmon resonance biosensor

Xiaoqiang Cui, Fan Yang, Yufang Sha, Xiurong Yang *

State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, People's Republic of China

Received 22 August 2002; received in revised form 10 January 2003; accepted 10 January 2003

Abstract

A surface plasmon resonance (SPR) biosensor was used for the first time to determine the concentration of ferritin in both HBS-EP buffer and serum. The monoclonal antibody was immobilized on the carboxymethyl dextran-modified gold surface by an amine coupling method. The interaction of antibody with antigen was monitored in real-time. The signal was enhanced by sandwich amplification strategy to improve the sensitivity and specificity of the immunoassay, especially in serum. The linear range of the assay in serum is over 30–200 ng ml^{-1} with the detection limit of 28 ng ml^{-1}. The sensitivity, specificity, and reproducibility of the assay are satisfactory. The analyte and enhancement antibody-binding surface could be regenerated by pH 2.0 glycine–HCl buffer and the same antibody-immobilized surface could be used for more than 50 cycles of ferritin binding and regeneration.

Keywords: Surface plasmon resonance; Immunoassay; Ferritin; Sandwich enhancement

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. The ferritin-stored iron is considered to play the role of resisting the cyclical reduction/oxidation reactions that result in the amplification of oxidative damage [2]. The increase of iron levels can promote the growth of a wide variety of tumors and infectious microorganisms. Therefore, it is very important to evaluate the iron stores in serum. Among the several methods, ferritin measurement in serum is considered to be the most reliable method for the evaluation of iron stores [3,4]. A sensitive, convenient, and inexpensive assay of ferritin would be useful for clinical consequences of ferropenic anemia [5], several tumor diseases (as a non-specific marker) [6], and the practice of autologous blood transfusion [7].

Conventional methods for measuring serum ferritin are radioimmunoassay (RIA) [8] and other non-isotopic immunoassays including enzyme-linked immunoassay [9,10], microparticle enzymoimmunoassay, and chemiluminescence assay

* Corresponding author. Fax: +86-431-5689711.
E-mail address: xryang@ns.ciac.jl.cn (X. Yang).

0039-9140/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0039-9140(03)00043-2

[11]. However, these methods are dangerous with respect to the isotope exposure (RIA) or inconvenient because of the need to label with enzyme or fluorescein, which may be complicated and time-consuming. Recently, a turbidimetric immunoassay [5] and quartz crystal microbalance immunosensor assay [6] were also used for the measurement of ferritin.

Optical biosensor methods, based on BIAcore's surface plasmon resonance (SPR) technology, have in recent years proven to be useful tools for the study of biospecific interaction including kinetic information, binding site, and concentration analysis [12,13]. This technique has also been used to detect the activity of antibody in serum [14]. To improve the sensitivity of this technology, a sandwich enhancement strategy has been used to detect IgE [12], pregnancy hormone human chorionic gonadotropin [15], and human complement factor 4 [16]. This report describes the development of an assay for real-time measurement of ferritin in buffer and serum. It gives a new method for immunoassay of ferritin in serum by using SPR, a biospecific interaction analysis technique. The results show that this real-time measurement method for the determination of ferritin is fast, label-free, and can be used more than 50 times with one surface.

2. Materials and methods

2.1. Instrument

BIAcore 1000 (Pharmacia Biosensor AB, Uppsala, Sweden) consists of a processing unit with liquid handling and optical system, a PC running BIAcore 1000 Control Software (version 2.1) for instrument operation and data handling. The processing unit contains the SPR detector and an integrated μ-fluidic cartridge (IFC) which, together with an autosampler, controls the delivery of sample into a transport buffer passing continuously over the sensor chip. BIAevaluation Software (version 3.0) was used for overlay immunoassay sensorgrams of different concentrations of ferritin.

2.2. Reagents

Sensor chip CM5 (research grade, a carboxymethyl dextran hydrogel coupled to a gold-coated glass surface), HBS-EP buffer (pH 7.4, consisting of 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 150 mM sodium chloride, 3 mM EDTA, and 0.005% (v v^{-1}) polysorbate 20), *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide (EDC), and ethanolamine hydrochloride were obtained from Pharmacia Biosensor AB (Uppsala, Sweden).

Human spleen ferritin and its monoclonal antibody were obtained from Fitzgerald Industries International, Inc. (USA). Anti-human ferritin (delipidized whole antiserum) was obtained from Sigma (USA). Fetal bovine serum was obtained from Sino-American Biotechnology Company (Beijing, China).

All other reagents and solvents were of analytical reagent grade. All solutions were prepared with ultrapure water and filtered (0.22 μm) and thoroughly degassed prior to use.

2.3. Monoclonal antibody immobilization

Immobilization of monoclonal antibody was performed in the instrument. CM5 chip was inserted into the BIAcore and initialized by flowing HBS-EP buffer. A constant flow of HBS-EP of 5 μl min^{-1} and 25 °C temperature was chosen for the immobilization procedure.

Prior to immobilization, optimal conditions for antibody diffusion into the hydrophilic carboxylated dextran matrix were investigated. Antibody was dissolved in acetic buffers with different pH values or ionic strengths. Antibody immobilization was performed according to the general procedure described elsewhere [12].

2.4. Immunoassay

To reduce protein non-specific adsorption, fetal bovine serum (total protein concentration: 40.8 mg ml^{-1}) was diluted with the same volume of HBS-EP buffer. This 1/2 diluted serum was used to prepare different concentrations of ferritin in serum for measurements. Ferritin was diluted

with HBS-EP or fetal bovine serum and flowed over the sensor surface by injecting a 20 μl volume. Twenty microliters of ferritin polyclonal antibody (0.5 mg ml^{-1}) was subsequently injected to enhance the response signal. After each measurement, the surface was regenerated by injection of 5 μl glycine–HCl buffer of pH 2.0. The primary and enhanced response (ΔRU) was measured as the change in SPR signal, in arbitrary response units, of HBS-EP flow baseline before and after the injection of the sample and enhanced antibody.

The response value was read at 30 s before the injection started and 30 s after the injection ended. Three replicates of each concentration were analyzed on the same sensor chip surface. The limit of detection (LOD) of the assay was defined as the lowest concentration that can be determined to be statistically different (three standard deviations) from the average response of ferritin-free samples.

3. Results and discussion

3.1. Monoclonal antibody immobilization

The response resulting from the biospecific interaction between antigen and immobilized antibody will depend, to some extent, on the amount of antibody immobilized on the sensor chip surface. For concentration measurement using SPR technology, the antibody should be immobilized as densely as possible to obtain the highest response value. It is found for proteins that electrostatic attraction of the protein molecules to the activated dextran layer considerably enhances the coupling yields. Negative charges of carboxylated dextran matrix on sensor surface are used to concentrate antibody into the matrix for the actual coupling [17]. Parameters such as protein solution pH value, ionic strength, and protein concentration are the key factors for efficient electrostatic adsorption [18]. Therefore, the immobilization can be controlled by these parameters [19]. Before the immobilization procedure, the parameters mentioned above were optimized using real-time BIA technique in this study.

First, the influence of ionic strength on protein electrostatic adsorption was studied (Fig. 1). In this experiment, the pH and protein concentration were kept constant. Various ionic strengths were obtained by adding sodium chloride into acetic buffer. The results (Fig. 1) show a decrease in electrostatic adsorption as ionic strength increases. This is because there is a competition between the positively charged protein and other positively charged species in the protein solution (e.g., sodium ions in the buffer) for the matrix carboxyl groups [19]. For the best immobilization, the ionic strength of the protein solution should be kept as low as possible. In most cases, 10 mM buffer concentration has been found to be suitable.

The influence of antibody solution pH on the amount of electrostatic adsorption is shown in Fig. 2. The electrostatic force depends on the type of the charge and the charge density of both protein and matrix. Antibody protein has a net positive charge at pH values below the isoelectric point of antibody (p*I* 7.5). As shown in Fig. 2, the amount of protein adsorption increased with decreasing pH value from 5.8 to 4.25, however, it decreased from 4.25 to 3.3. When the pH value decreases, the net positive charge of protein molecule increases, at the same time, the negative charge density of carboxymethyl dextran decreases, as the degree of ionization becomes smaller. From Fig. 2, it can be seen that at pH 4.25, changes of the matrix charge will be the dominating influence factor for the adsorption. The amount of protein adsorbed is reasonably large during pH range 6.5–4.5. Taking into account that amine covalent coupling requires uncharged amino groups and is therefore favored by high pH, pH 4.8 buffer solution was chosen for the following immobilization in this work.

An increase of protein concentration should lead to an increasing electrostatic adsorption. An antibody concentration of 0.1 mg ml^{-1} approached the maximum adsorption (data not shown).

In this study, the amine coupling method was used to immobilize the monoclonal antibody on the CM5 sensor chip surface. The sensorgram for immobilization of monoclonal antibody is shown in Fig. 3. After the stabilization of the baseline for about 10 min with flowing HBS-EP buffer, the carboxymethylated dextran on the sensor surface was activated by injection of NHS–EDC mixture

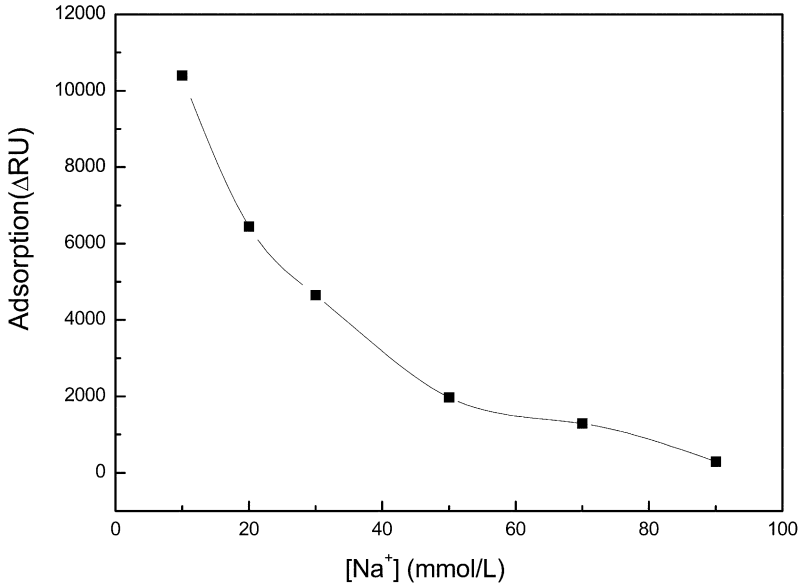

Fig. 1. Response of electrostatically adsorbed anti-ferritin mAb (0.1 mg ml^{-1} in 10 mM acetic acid buffer, pH 4.8) as a function of sodium ion concentrations.

(1:1) for 7 min. The protein (monoclonal antibody) was dissolved in 10 mM acetic buffer, pH 4.8, to a concentration of 0.1 mg ml^{-1} and the solution was injected over the activated sensor chip surface for 7 min. Remaining active esters were then blocked with a 7-min pulse of 1.0 M ethanolamine, pH 8.5. And then, 1-min pulse of 10 mM glycine–HCl buffer (pH 2.0) was injected to desorb noncova-

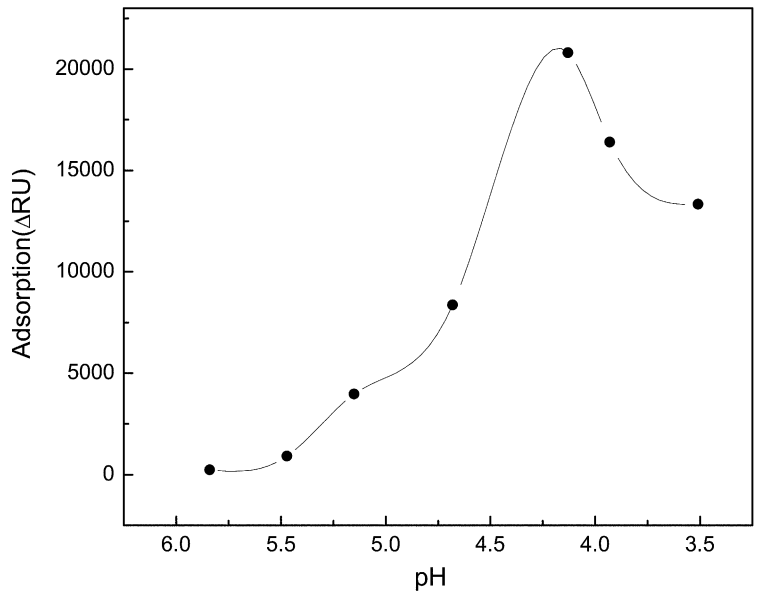

Fig. 2. Response of electrostatically adsorbed anti-ferritin mAb (0.1 mg ml^{-1} in acetic buffer containing 10 mM sodium ions) as a function of protein solution pH.

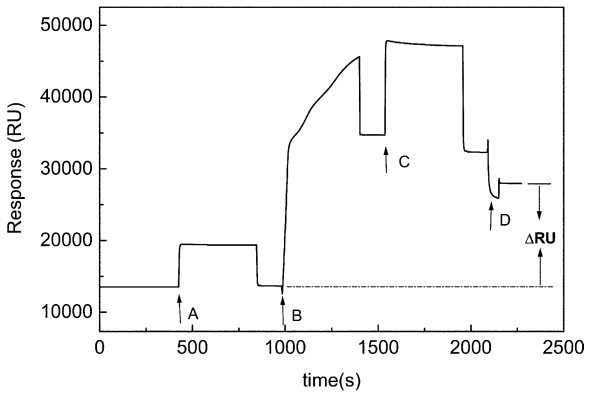

Fig. 3. Immobilization of anti-ferritin mAb on the sensor surface. HBS-EP was used as flow buffer at constant flow rate of 5 μl min^{-1}. The captions indicate injections of (a) 35 μl of 0.05 M NHS/0.2 M EDC, (b) 35 μl of 0.1 mg ml^{-1} anti-ferritin mAb (pH 4.8, acetic buffer), (c) 35 μl of 1 M ethanolamine (pH 8.5), and (d) 5 μl of 0.01 M glycine–HCl buffer (pH 2.0).

lently bound protein on the sensor chip surface. The actual immobilized amount of monoclonal antibody is about 12 180 RU after the complete washing. The surface concentration of the immobilized antibody on sensor chip surface is calculated to be 12.2 ng mm^{-2} according to the correlation of SPR response with surface protein concentration (1 kRU = 1 ng mm^{-2} [20]). Antibody immobilization is highly reproducible under the above optimal conditions with a relative standard deviation (RSD) of 2.5% ($n = 3$).

3.2. Immunoassay ferritin in HBS-EP buffer

After the immobilization, the analyte (ferritin protein) solution in HBS-EP buffer was injected over the surface, then the polyclonal antibody to ferritin was injected to enhance the signal for better sensitivity and specificity. The response due to antibody–antigen reaction was recorded in real-time as sensorgram by the BIAcore system. Fig. 4 shows the overlay sensorgrams of immunoassay for different concentrations of ferritin in HBS-EP buffer. As seen, the monitoring of SPR response as a function of time could be used for direct visual observation of binding and dissociation events during the procedure of reaction [13]. In phase a of Fig. 4, the increase signal shows the binding of ferritin to monoclonal antibody-immobilized surface. Then the signal was enhanced by injecting 0.5 mg ml^{-1} polyclonal antibody over the ferritin binding surface, as shown in phase b in Fig. 4. The surface was then regenerated by injecting pH 2.0 glycine–HCl solution (phase c in Fig. 4) to wash off the bound ferritin and antibody.

The amplifying antibody concentration is a major parameter that can influence the amplification effect of the sandwich strategy [21]. The amplification effect of the antibody (polyclonal antibody of ferritin) concentration was investigated in real-time. Different concentrations (0.1, 0.2, 0.5, 0.8 mg ml^{-1}) of rabbit anti-ferritin polyclonal antibody were separately injected over the sensor surface after each immune reaction of 100 ng ml^{-1} ferritin. The amplification factor for the four concentrations was 0.86, 1.34, 2.22, and 2.58, respectively. As seen, the enhancement times appeared to depend on the concentration of amplified antibody used. Considering both reducing the non-specific adsorption and the consumption of the reagents, we chose the concentration of 0.5 mg ml^{-1} for the amplification immunoassay.

Fig. 5 shows the typical calibration curves obtained over a ferritin range 20–1000 ng ml^{-1} in HBS-EP buffer; the vertical bars represent the standard deviation for three replicates of each concentration on the same sensor chip surface. The change of the response units between the start and the end of injection were plotted as a function of ferritin concentration. The curves (a) and (b) represent the primary and amplified response, respectively. From the plots, it is evident that the SPR biosensor can be used for the determination of ferritin concentration. The inset in Fig. 5 shows the linear range of the calibration data. For the primary response (curve a), the linear range is over 20–200 ng ml^{-1} ferritin and the linear regression equation was (RU) $Y = 10.92 + 0.9939$[ferritin] ng ml^{-1} ($R = 0.9991$, $n = 6$). LOD was found to be 15 ng ml^{-1} ferritin. For the amplified response (curve b), the linear range is over 20–200 ng ml^{-1} ferritin and the linear regression equation was (RU) $Y = 77.12 + 1.712$[ferritin] ng ml^{-1} ($R = 0.9995$, $n = 6$). LOD was found to be 18 ng ml^{-1} ferritin.

For SPR biosensor assays, the response can be amplified based on the sandwich method by introducing the analyte-specific antibody for bet-

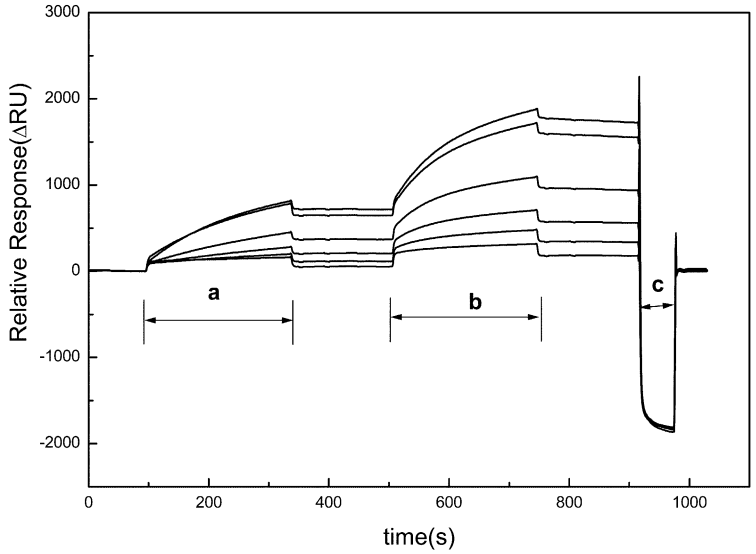

Fig. 4. Representative overlaid sensorgrams illustrating the real-time immunoassay of ferritin in HBS-EP buffer at different concentrations (40, 100, 200, 400, 800, and 1000 ng ml^{-1}, from bottom to top). Arrows indicate phases of (a) primary response of 20 μl ferritin, (b) 20 μl of 0.5 mg ml^{-1} enhanced antibody, and (c) 5 μl of pH 2.0 glycine–HCl buffer. Besides phases a, b, and c, HBS-EP buffer was continuously flowed over the surface.

ter sensitivity and specificity of the analyte concentration assay [13]. From the above results, we know that the sensitivity was improved from 0.9939 to 1.712 RU (ng $ml^{-1})^{-1}$ after sandwich

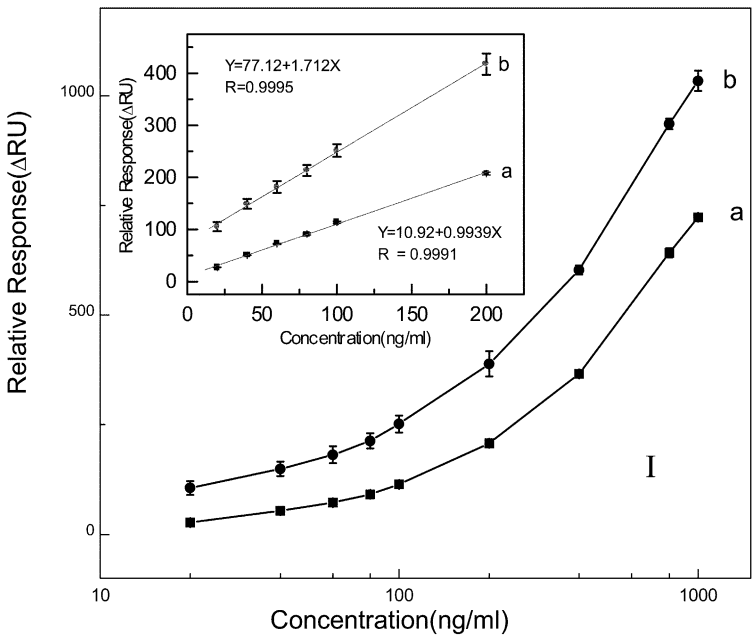

Fig. 5. Calibration graphs for ferritin in HBS-EP buffer. The inset presents the linear range of the assay. (a) The primary response and (b) the amplified response. Log scale in I and linear scale in inset.

amplification. However, the RSDs increased significantly from 0.1–2.2 (primary) to 1.3–10.7% (amplified) in 20–1000 ng ml^{-1} ferritin concentration range. Also, the LOD increased a little (from 15 to 18 ng ml^{-1}) after the amplification. We attributed these increases of RSD and LOD to the non-specific adsorption of amplified antibody to the sensor surface. This can be seen from the assay of ferritin-free sample (only HBS-EP as the sample). The primary and amplified response of ferritin-free sample is -7.9 ± 3.2 and 51.2 ± 10.8 RU, respectively. The non-specific adsorption effect on RSD and LOD will be discussed further in the following section.

3.3. Immunoassay in serum

Ferritin in fetal bovine serum was detected in the range 20–800 ng ml^{-1} under the same procedure as the previous section. Fig. 6 shows the real-time overlay sensorgrams of immunoassay in serum. In Fig. 6, a sudden increase and decrease of response at the beginning and end of injection can be observed in phase a, which is different from that in Fig. 4. This is due to the refractive index change caused by the high concentration of protein (total protein concentration: 20.4 mg ml^{-1}) and some hemachrome in the serum. Also, the primary response was significantly higher than that in HBS-EP buffer. For example, at 80 ng ml^{-1}, the primary response was 96 RU in HBS-EP buffer, however, it was 495 RU in serum. Although the serum used for preparing the sample has been diluted with HBS-EP buffer, which can reduce the non-specific adsorption of protein because of the high ionic strength and surfactant, the non-specific adsorption was still high (about 300 RU).

Fig. 7 shows the typical calibration curves obtained over a ferritin range 40–800 ng ml^{-1} in serum. The inset in Fig. 7 shows the linear range of the calibration data. For the primary response (curve a), the linear range was over 40–300 ng ml^{-1} ferritin and the linear regression equation

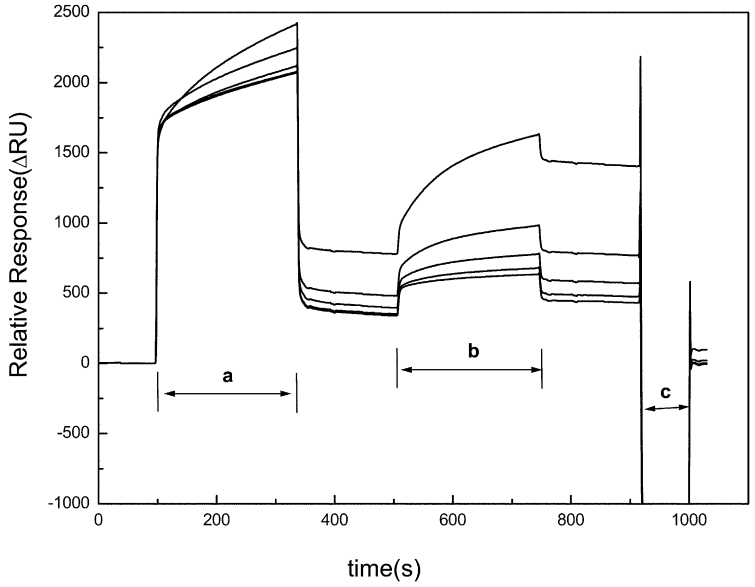

Fig. 6. Representative overlaid sensorgrams illustrating the real-time immunoassay of ferritin in serum at different concentrations (20, 40, 80, 200, and 800 ng ml^{-1}, from bottom to top). Arrows indicate phases of (a) injecting 20 μl of ferritin in serum, (b) 20 μl of 0.5 mg ml^{-1} enhanced antibody, and (c) 5 μl of pH 2.0 glycine–HCl buffer. Besides phases a, b, and c, HBS-EP buffer was continuously flowed over the surface.

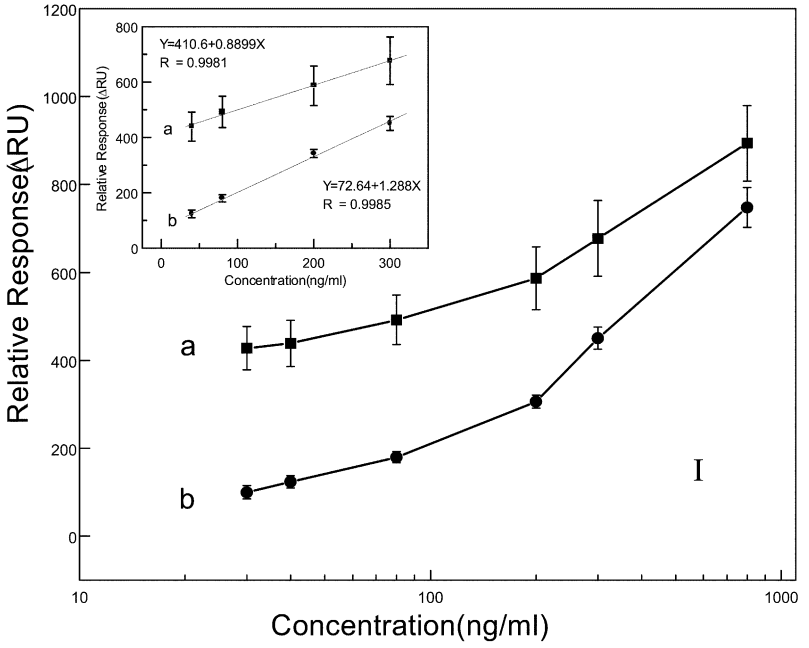

Fig. 7. Calibration graphs for ferritin in serum. The inset presents the linear range of the assay. (a) The primary response and (b) the amplified response. Log scale in I and linear scale in inset.

was (RU) $Y = 410.6 + 0.8899$[ferritin] ng ml^{-1} ($R = 0.9981$, $n = 4$). The LOD was found to be 37 ng ml^{-1} ferritin. For the amplified response (curve b), the linear range is over 30–300 ng ml^{-1} ferritin and the linear regression equation was (RU) $Y = 72.64 + 1.288$[ferritin] ng ml^{-1} ($R = 0.9985$, $n = 4$). LOD was found to be 28 ng ml^{-1} ferritin.

The calibration curve for detection in serum (Fig. 7) is different from that in HBS-EP buffer (Fig. 5). In Fig. 5, the primary curve (a) was lower than amplified curve (b). However, the situation was opposite in Fig. 7, the primary curve (a) was higher than amplified curve (b). As mentioned above, this is due to the high non-specific adsorption of protein during the primary immunoassay. Although the amplified response is lower than primary response, the sensitivity was improved from 0.8899 to 1.288 RU (ng ml^{-1})$^{-1}$. The linear range in serum was 30–300 ng ml^{-1} ferritin and LOD was 28 ng ml^{-1} ferritin after the amplification. The most attractive character of the amplified response in serum is that it can improve the specificity of the assay, because the amplified antibody can only interact with the target analyte. Therefore, the sandwich amplification served as a function of purification of the sample. Also, the high total protein concentration (20.4 mg ml^{-1}) in serum can block the surface to reduce greatly the non-specific adsorption of the analyte. After the amplification, the RSD of the assay in serum decreased from 11.8–12.7 (primary) to 4.5–5.7% (amplified) in 40–800 ng ml^{-1} ferritin concentration range.

The detection limit (28 ng ml^{-1}) in this assay was higher than particle-enhanced turbidimetric immunoassay [5] (3 ng ml^{-1}). As we know, the normal serum ferritin concentrations are within the range 15–300 ng ml^{-1} [3]. Therefore, this method can be used for direct and fast determination of ferritin in serum. The results show that secondary amplification for ferritin immunoassay in serum is very important because it can improve the sensitivity and specificity of the immunoassay.

3.4. Reproducibility of the immunoassay

The same immobilized surface could be used for a series of measurements in real-time SPR immunoassay. Chemical reagents such as acid, base, or salt with high ionic strength can be used to desorb antigens or antibodies bound on a sensor surface [14]. Glycine–HCl (0.1 M, pH 2.0) buffer was used to regenerate the surface in our experiments. The amplifying immunoassay of ferritin (80 ng ml^{-1}) in serum was carried out for 50 cycles on the same immobilized surface. The results show that the baseline drifted upward about 600 RU for 50 cycles. This was mainly due to the non-specific accumulation of protein in the serum in the matrix of sensor chip [21]. A decrease of the response signal of about 15.6% was observed over 50 cycles. The average loss of reactivity per regeneration is 0.31%. The results demonstrate that the immobilized antibody surface is remarkably stable for 50 cycles of binding per elution. After 50 cycles, the antibody-immobilized surface could still be used for more assays.

4. Conclusions

Real-time SPR immunosensor has been used for the first time to determine the ferritin in both HBS-EP buffer and serum. The results show that the sandwich enhancement strategy is very important for improving the sensitivity and specificity of the immunoassay, especially in serum. The linear range of the enhanced immunoassay in serum was 30–300 ng ml^{-1} with the LOD of 28 ng ml^{-1} ferritin. This is in the normal range of serum ferritin concentration [3]. In this paper, we also investigated the antibody electrostatic adsorption, antibody immobilization, immune reaction, and regeneration by real-time SPR technique. The amplification immunoassay of ferritin in this work demonstrates that the real-time SPR biosensor can be a useful tool in clinical chemistry for the detection of protein components in serum.

Acknowledgements

This work was supported by the National Natural Science Foundation of China with the Grant No. 20075027 and the National Key Basic Research Development Project "Research on Human Major Disease Proteomics" with the Grant No. 2001CB5102.

References

[1] S. Sun, N.D. Chasteen, J. Biol. Chem. 267 (1992) 25160.
[2] S. Levi, A. Luzzago, G. Cesareni, A. Cozzi, F. Franceschinelli, A. Albertini, P. Arosio, J. Biol. Chem. 263 (1988) 18086.
[3] M. Worwood, Clin. Chim. Acta 259 (1997) 3.
[4] J.M. Simo, J. Joven, X. Clivile, T. Sans, Clin. Chem. 40 (1994) 625.
[5] F. Gomenz, J.M. Simo, J. Camps, X. Cliville, N. Bertran, N. Ferre, C. Bofill, J. Joven, Clin. Biochem. 33 (2000) 191.
[6] S.F. Chou, W.L. Hsu, J.M. Hwang, C.Y. Chen, Anal. Chim. Acta 453 (2002) 181.
[7] S.M. Kasper, H. Lazansky, C. Stark, M. Klimek, R. Laubinger, U. Borner, Transfusion 38 (1998) 764.
[8] J.W. Halliday, K.L. Gera, L.W. Powell, Clin. Chim. Acta 58 (1975) 207.
[9] V.F. Fairbanks, G.G. Klee, Biochemical aspects of hematology, in: Ca. Burtis, Er. Ashwood (Eds.), Tietz Textbook of Clinical Chemistry, 2nd ed., Saunders, Philadelphia, PA, 1994, pp. 1974–2072.
[10] S. Zhang, K. Jiao, H. Chen, M. Wang, Talanta 50 (1995) 95.
[11] J. Weeks, J.S. Woodhead, J. Clin. Immunoassay 7 (1984) 82.
[12] S. Lofas, M. Malmqvist, I. Ronnberg, E. Stenberg, B. Liedberg, I. Lundstrom, et al., Sens. Actuators B 5 (1991) 79.
[13] L.G. Fagerstam, A.F. Karlsson, R. Karlsson, B. Persson, I. Ronnberg, J. Chromatogr. 597 (1992) 397.
[14] R. Pei, X. Cui, X. Yang, E. Wang, Talanta 53 (2000) 481.
[15] A.H. Severs, R.B.M. Schasfoort, Biosens. Bioelectron. 8 (1993) 365.
[16] R. Pei, X. Yang, E. Wang, Anal. Chim. Acta 453 (2002) 173.
[17] S. Lofas, Pure Appl. Chem. 67 (1995) 829.
[18] S. Lofas, B. Johnsson, J. Chem. Soc., Chem. Commun. (1990) 1526.
[19] B. Johnsson, S. Lofas, G. Lindquist, Anal. Biochem. 198 (1991) 268.
[20] E. Stenberg, B. Persson, H. Roos, C. Urbaniczky, J. Colloid. Interf. Sci. 143 (1991) 513.
[21] R. Pei, X. Cui, X. Yang, E. Wang, Chin. J. Chem. 20 (2002) 441.