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Determination of affinities and antigenic epitopes of bovine cardiac troponin I (cTnI) with monoclonal antibodies by surface plasmon resonance biosensor

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Abstract

A surface plasmon resonance (SPR) biosensor based on wavelength modulation was used for real-time detection of the interaction of three monoclonal antibodies and antigens of bovine cardiac troponin I (cTnI). In order to recognize antigenic epitopes of bovine cTnI, two experimental modes were applied. In the first experimental mode, three monoclonal antibodies were divided into three groups and three experiments were performed on biosensor surfaces prepared with protein A. In the second experimental mode, antigen was immobilized on the biosensor surface prepared by the amine-coupling method and three monoclonal antibodies were detected in turn. The results obtained by the two modes are consistent. In addition, the affinities of the monoclonal antibodies for the antigen were also determined by the association rate and the disassociation rate in real-time. These results validate the biosensor technology and illustrate how biosensors based on wavelength modulation can be used to study the interaction of monoclonal antibodies and antigens in real time.

Keywords: SPR; Monoclonal antibody; Bovine cTnI; Antigenic epitopes; Affinity

For many years surface plasmon resonance (SPR)[1] biosensors have been extensively studied [1–6]. Various configurations of SPR biosensors have been developed and successfully applied for the simple, rapid, and nonlabeled assay of all kinds of biologic analytes. SPR has also shown promise in the real-time determination of concentration, kinetic constant, and binding specificity for individual biomolecular interaction steps. Many interactions between the biomolecule, such as antibody-receptor interaction [7], peptide-protein interaction [8], DNA-protein interaction [9], RNA-RNA interaction [10], and DNA-DNA interaction [11], have been analyzed.

The antibody plays an important role in the body's immune system and is a powerful diagnostic and research tool. Thereby, rapid and accurate determination of the activity and analysis of binding properties of antibodies is crucial in the estimation of their performance in various applications. For this purpose, the SPR biosensor is an effective tool [12]. The biosensor can be applied not only to determine the affinity and kinetics of single monoclonal antibodies in a short time, but also to determine simultaneously the affinity and kinetics of multimonoclonal antibodies and to recognize antigen epitopes rapidly. This is very useful for understanding the characteristics of monoclonal antibodies and for selecting monoclonal antibodies with the best applicable potential. Compared with enzyme-linked immunosorbent assay (ELISA) and fluorescence enzyme

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[1] *Abbreviations used*: SPR, surface plasmon resonance; cTnI, cardiac troponin I; AMI, acute myocardiac infarction; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MPA, 3-mercaptopropionic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; SAM, self-assembled monolayer.

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immunoassay [13,14], it possesses several inherent advantages. First, the interacting components do not need to be labeled, which allows the interaction between unmodified reactants to be monitored in real time. This avoids the possibility that the labeling interferes with the kinetics/affinity of the interaction. Second, the interaction of antibody and antigen can be studied in a relatively short period using automated programs. Third, the amount of reagent consumed is relatively small. This is of particular relevance where the components of the interaction of interest are difficult to produce.

Acute myocardiac infarction (AMI) is a familiar acute angiocardiopathy causing serious health problems. Diagnosis of AMI has received lots of attention in clinics for many years. Cardiac troponin I (cTnI) will release in the bloodstream when cardiac muscle is damaged or dead and the concentration of cTnI in serum will be elevated for a long time. Therefore, the content of cTnI in serum has a close relation with this disease. Compared with other biochemical markers, cTnI has a wider diagnostic window of time with an early and prolonged appearance and excellent sensitivity and specificity. Today, cTnI is gradually becoming the "gold standard" [15–17] for diagnosis of AMI, replacing other biochemical markers, and more and more specialists are focusing their attention on the determination of cTnI. To our knowledge, recognizing antigenic epitopes of cTnI with monoclonal antibodies has been not reported up to now by SPR biosensors.

In this paper, we applied a SPR biosensor instrument installed in our laboratory for simultaneous multi-wavelength design [11] to detect affinities and antigenic epitopes of bovine cTnI with monoclonal antibodies. The SPR biosensor based on wavelength modulations has also been applied for the determination of albumin [18] and B factor [19] in our laboratory. The traditional SPR biosensor is an instrument based on the measurement of changes in the resonance angle induced by SPR. The surface plasmons are excited by a convergent beam of monochromatic light. It makes use of a coupling prism coated with a thin gold film and performs the sensing by varying the incidence angle with a goniometer [20]. The best angular accuracy of the goniometer is about 0.001°, which corresponds to a shift in optical wavelength of 0.6 nm [21,22], and its sensitivity is limited [23]. In contrast, the wavelength modulation SPR biosensor is based on the measurement of changes in the resonance wavelength induced by SPR. SPR spectra are shown in terms of reflected light intensity versus wavelength of the incident light. The intensity of the reflected light is the minimum at the resonant wavelength. A change in concentration or kind of analyte in the flow cell will lead to changes in the refractive index of the layer of the sensing membrane. Changes in the sensing of the membrane cause a shift of resonant wavelength. There are few reports about the sensitivity of wavelength modulation SPR [24,25]. A surface-plasmon-based optical sensor using acousto-opticals was developed by Jory et al. in 1995 [26] and the SPR minimum position was measured to within a precision of 0.0005 nm. Compared with other traditional SPR biosensors, it has many obvious characteristics: first, the instrument is cheaper and its sensitivity is high [24–26] based on wavelength modulation. Secondly, there are no moving parts in the biosensor. Thirdly, the biosensor measures reflected light intensities at all wavelengths in the range of 400–800 nm simultaneously. That is, it can monitor reaction in real time. Fourthly, wavelength modulation SPR sensors hold more potential for miniaturization as well as coupling the reflected light beam into an optical fiber and transmitting it for analysis to a remote site [27].

In this assay, the first aim was to recognize antigenic epitopes of bovine cTnI with three monoclonal antibodies, and two experimental modes were applied. The second aim was to determine the affinity of three monoclonal antibodies for antigens of bovine cTnI. The results illustrate that the SPR biosensor assay is an accurate and precise method for characterizing the interaction between antibody and antigen.

Materials and methods

Equipment and chemicals

A KQ118 ultrasonic clean up device was purchased from Kunshan Ultrasonic Cleaning Instruments Co. A halogen tungsten lamp and collimating device were purchased from Changchun Fifth Optics Precision Instrument. 3-Mercaptopropionic acid (MPA) was purchased from Sigma. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Shanghai Lizhu Dongfeng Biotechnology Co. Protein A and bovine serum albumin (BSA) were purchased from Shanghai Biology Product Research Institute. All the monoclonal antibodies and antigen of the bovine cTnI were produced in the laboratory [28]. Tetanus antibody was obtained from Changchun Biology Product Research Institute.

A 0.01 mol/L phosphate-buffered saline solution (PBS, pH 7.4) was prepared by dissolving 0.2 g KCl, 8.0 g NaCl, 0.24 g KH_2PO_4 and 1.44 g Na_2HPO_4, in 1000 ml ultrapure water. A 0.3 mol/L citrate buffer (pH, 2.7) was prepared by dissolving 21 g $C_6H_8O_7 \cdot H_2O$ and 11 g Na_2HPO_4 in 350 ml ultrapure water.

Instrument

The schematic diagram of the SPR optical biosensor based on wavelength modulation is shown in Fig. 1. The light source is a halogen tungsten lamp in conjunction with a constant voltage transformer. The light from this

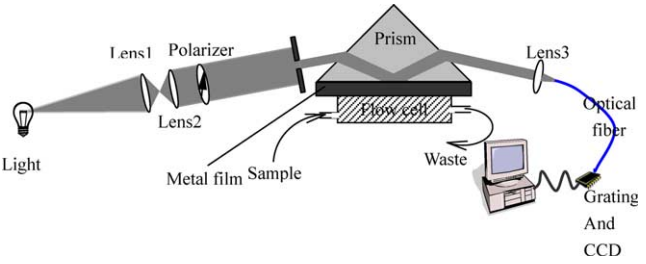

Fig. 1. Schematic diagram of the SPR optical biosensor based on wavelength modulation.

source passes through a polarizer and becomes TM polarized light. In order to make the light parallel, two lenses are employed. The parallel polychromatic light beam passes through an optical prism with a thin gold film and excites surface plasmon at the interface between the gold film and the analytes. The output light from the prism is guided into the optical fiber and then to the Fullwave spectrophotometer (Ocean Optics, Inc., USA). The spectrophotometer was inserted into the computer. Therefore, the apparatus is compact and small. The detector is a 1024 element charge-coupled device (CCD) linear-array detector and the wavelength range to be measured simultaneously is 400–800 nm. The gold surface of the biosensor was cleaned by exposure to ethanol for 5 min, followed by drying with nitrogen.

The incident angle is fixed at a suitable value to ensure that the surface plasmon resonance phenomenon occurs. The reflected light intensity is the minimum at the resonant wavelength. A smaller change in refractive index or layer thickness at the sensor surface would cause a clear shift of the resonant wavelength in SPR reflected spectra. A 90-μl volume flow cell ($18 \times 5 \times 1$ mm) was used for the reaction.

Assay procedure

All the reagents were diluted with PBS and all the experiments were carried out at room temperature. In order to recognize antigenic epitopes of the bovine cTnI with three monoclonal antibodies, two experimental modes (A and B) were applied. In both modes, the biosensor surface was modified by self-assembled monolayers (SAMs). Fig. 2 shows the design of the mode A (a) and the corresponding sensorgram (b). In Model A, prior to immobilization of protein A, the biosensor surface was washed with PBS keeping the resonance wavelength almost constant, and then the surface was prepared by successive injections of protein A and BSA.

After immobilization of protein A on the biosensor surface, an experiment was performed with successive injections of PBS, MAb12 (or MAb5 or MAb11), antigen, tetanus antibody, and MAb5 (or MAb11 or MAb12). In Fig. 2a it is seen that three experiments were performed in the mode A and two antibodies were used in each experiment.

Fig. 3 shows the design of the experimental mode B (a) and the corresponding sensorgram (b). After the biosensor surface was prepared by the amine-coupling method, the experiment was performed by successive injections of PBS, antigen, MAb11, MAb5, and MAb12. It is seen in Fig. 3a that three monoclonal antibodies were used in the experiment.

In both modes, all procedures were performed at room temperature. The regeneration of the biosensor surface was done by the injection of citrate. The biosensor surface was washed with PBS buffer for 10 min between each solution injection. The concentrations of the all monoclonal antibodies are the same and are sufficient for association with antigen. The concentrations of all antigens are also the same and are sufficient for association with monoclonal antibodies.

Tetanus antibody binding to protein A

Tetanus antibody binding to protein A was performed in order to validate the reliability of the blocking with the tetanus antibody. After the biosensor surface was prepared with protein A, the tetanus antibody (1:20) was injected into the flow cell. The interaction of protein A and tetanus antibody was monitored in real time with the biosensor.

Determination of the antibody's affinities for antigen

Each monoclonal antibody was diluted with PBS to obtain different concentrations for determining the affinities of monoclonal antibodies for antigen. At room temperature, after immobilization of antigen on the biosensor surface prepared with the amine-coupling method, each monoclonal antibody at different concentrations was injected into the flow cell in turn, and the association rate and disassociation rate between antibody and antigen were determined, respectively.

Data processing

The interaction between antigen and each monoclonal antibody was monitored by observing changes in biosensor responses. A homogeneous 1:1 interaction between an immobilized antigen (M) and a monoclonal antibody (L) in solution to form a complex (ML) is described by

$$\mathrm{M} + \mathrm{L} \underset{k_\mathrm{d}}{\overset{k_\mathrm{a}}{\rightleftarrows}} \mathrm{ML} \tag{1}$$

where k_a and k_d are the association and disassociation rate constants, respectively. Assuming pseudo-first-order interaction kinetics, the association phase of the interaction kinetics measured as SPR signal can be expressed by [29]

$$\mathrm{d}R/\mathrm{d}t = k_\mathrm{a}[\mathrm{C}](R_\mathrm{max} - R) - k_\mathrm{d}R, \tag{2}$$

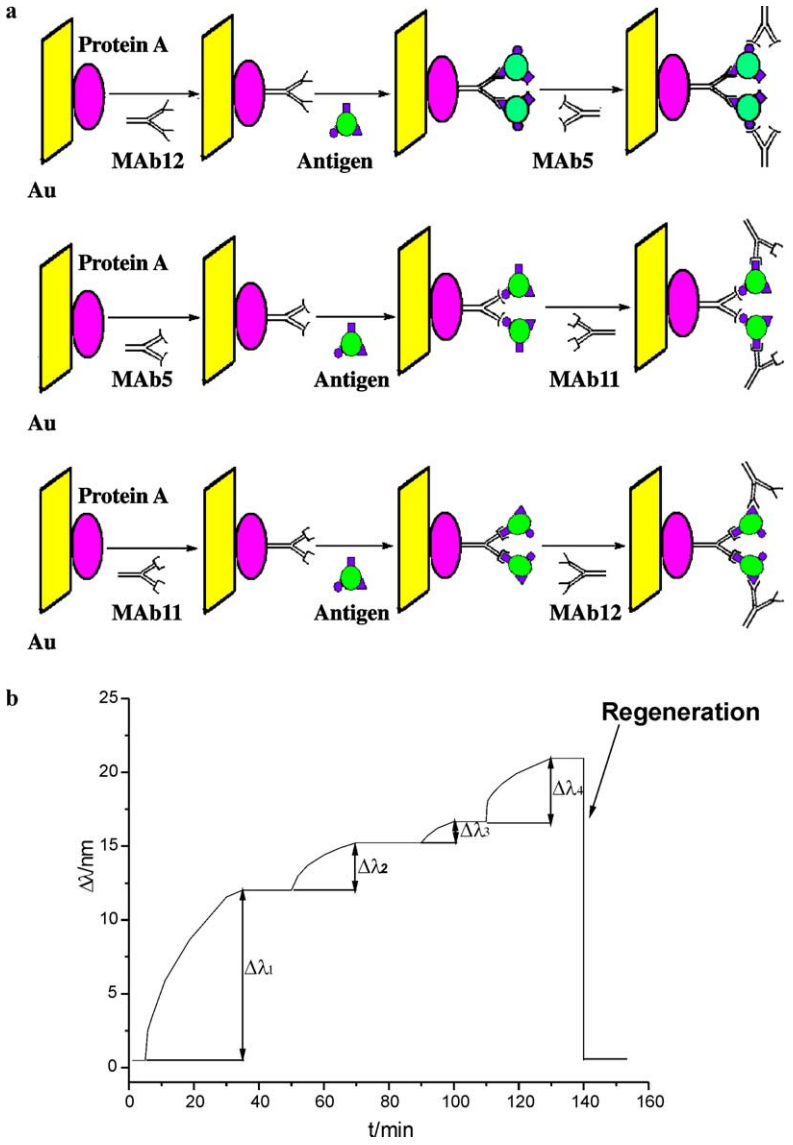

Fig. 2. Monoclonal antibody recognizing antigenic epitopes of the bovine cTnI in experiment mode A. (a) After immobilization of protein A on the biosensor surface the experimental design was composed successive injections of MAb12 (or MAb5 or MAb11) (50 μg/ml), antigen (26.4 μg/ml), MAb5 (or MAb11 or MAb12) antibody 2 (50 μg/ml). (b) The corresponding sensorgram was done by plotting the shift in resonance wavelength as a function of time.

where dR/dt is the rate of change of SPR signal, [C] is the concentration of the analyte, R_{max} is the maximum analyte-binding capacity in resonance unite ($\Delta\lambda$), R is the amount of bound monoclonal antibody measured as the biosensor response ($\Delta\lambda$) at time t. The integrated form of the Eq. (2) yields

$$R = \{k_a[C]R_{max}/(k_a[C] + k_d)\}\{1 - \exp(-([C]k_a + k_d)t\}. \quad (3)$$

Using Eq. (3), binding data can be fitted at different concentrations to determine k_{on}, where $k_{on} = [C]k_a + k_d$. A linear plot of k_{on} against monoclonal antibody concentrations [C] will give a straight line with a slope of k_a and a y intercept of k_d. The association constant, K_A, is then calculated from k_a/k_d.

Results and discussion

Preparation of the biosensor surface

Protein A is a type of polypeptide of the staphylococcus cell wall, and the molecular weight is about 40,000 Da. Protein A has four binding sites with antibodies. The polycomplex of protein A and antibody is formed by means of two sites in protein A. The association process of protein A and antibody has four significant characteristics: (1) Binding sites of protein A and antibody locate on the Fc fragment of the antibody; therefore, the association capacity of antibody for antigen cannot be changed. (2) Protein A will resume its character readily. (3) The affinity of protein A for

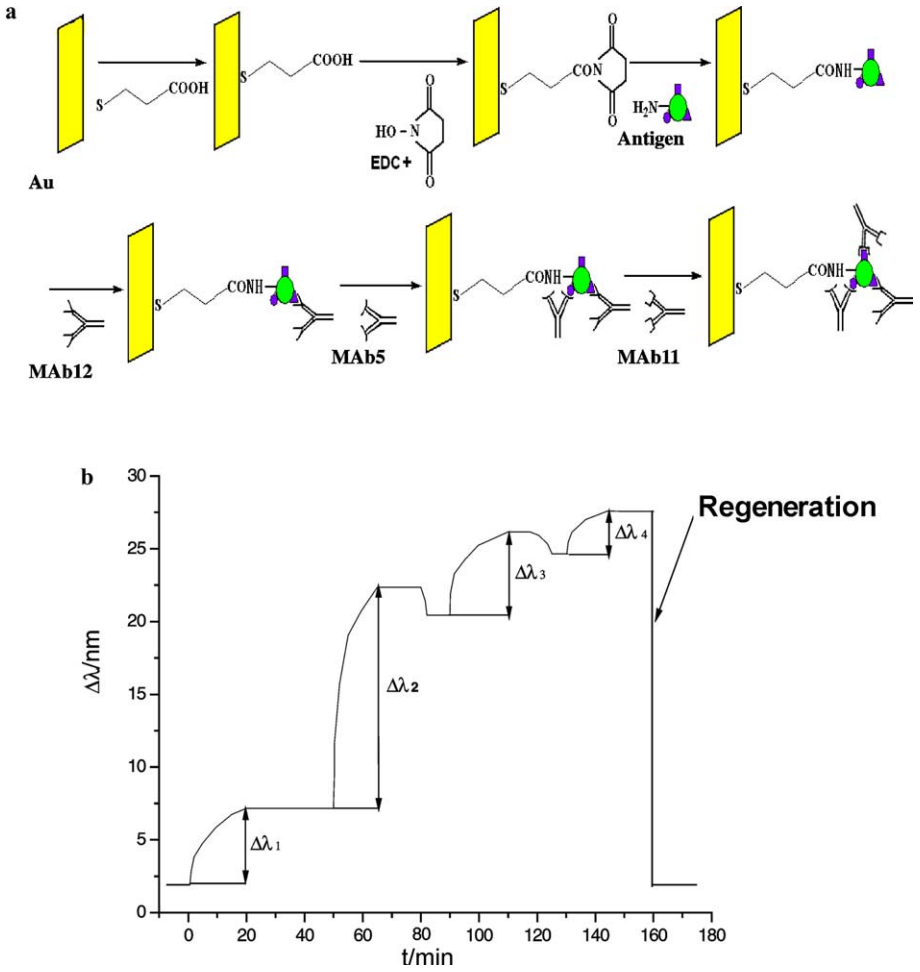

Fig. 3. Monoclonal antibody recognizing antigenic epitopes of the bovine cTnIin experimental mode B. (a) After the biosensor surface was prepared by the amine-coupling chemistry method, the experimental design was composed of successive injections of antigen (26.4 μg/ml), MAb11, MAb5, MAb12. (b) The corresponding sensorgram was done by plotting the shift in resonance wavelength as a function of time.

antibody is very high; however the association of protein A and antibody will be lost by an acidic solution. (4) The combined strength of protein A and Au is very strong. Thereby, protein A is especially amenable for making a membrane used to connect antibody and Au. In experimental mode A, the biosensor surface was modified with protein A.

Fig. 4a shows a sensorgram of the interaction of protein A and Au. It can be seen from Fig. 4a that the association of protein A and Au is very fast. The shift of the resonant wavelength reaches about 95% of its total shift within 10 min in a 0.1 g/L protein A solution. After 10 min, the resonant wavelength remarks almost constant. This means that the self-assembly is complete and the monolayer formed. The maximum shift of the resonant wavelength is 11.54 nm.

Because the 3-mercaptopropionic acid monolayer can stick firmly to the gold surface with its sulfide bonds, in experimental mode B, MPA was used to prepare the biosensor surface. A solution containing MPA was injected into the flow cell. The changes in resonant wavelength were measured in real time. The shift of the resonant wavelength reaches about 99% of its total shift within 5 min in the 10 mmol/L MPA solution. Further increasing the self-assembling time, the resonant wavelength remains almost constant. That means that the self-assembly is complete and the monolayer formed. Fig. 4b shows a sensorgram of the interaction of MPA and Au. The sulfide bond of MPA easily forms S–Au binding. Meanwhile the –COOH group reacts with *N*-hydroxysuccinimide. Therefore, MPA forms a good monolayer. The resonant wavelength shift for the formation of this monolayer is 11.56 nm. NHS reacted with MPA under catalysis of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The maximum resonant shift wavelength is 2.4 nm.

Detection of the blocking reliability of tetanus antibody

Fig. 5 shows the interaction response of the tetanus antibody and protein A and illustrates the reliability of

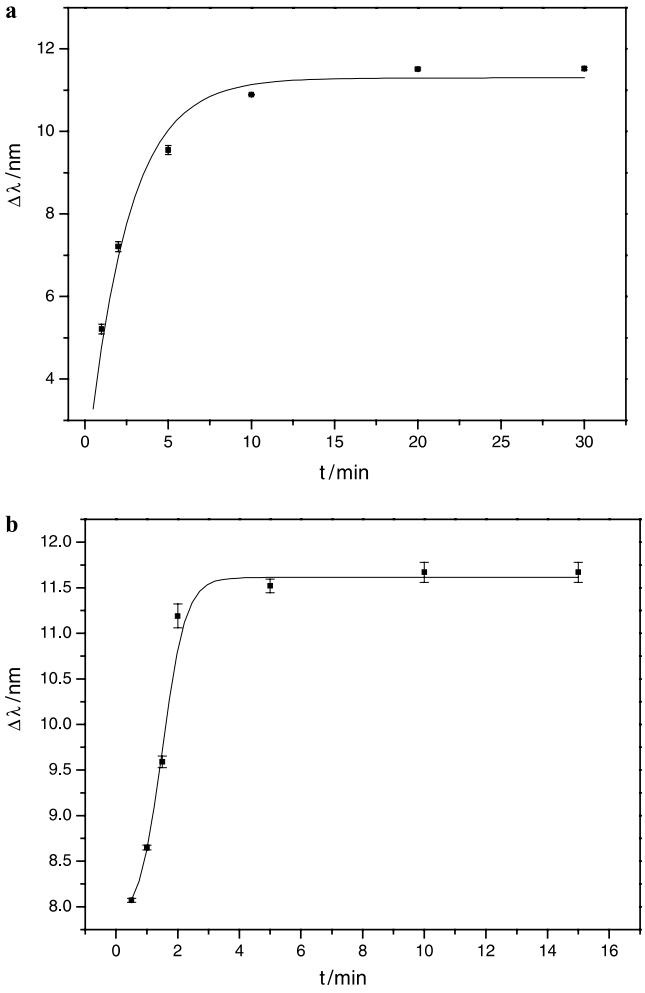

Fig. 4. Sensorgram for the interaction of protein A and MPA with Au. (a) Kinetic adsorption curve of protein A on the gold film. The error bars indicate the standard deviations. (b) Kinetic adsorption curve of 3-mercaptopropionic acid (MPA) on the gold film. The error bars indicate the standard deviations.

the blocking with the tetanus antibody. As expected, a biosensor surface prepared with protein A is exposed to tetanus antibodies and shows a strong response. The shift of the resonant wavelength is 8.18 nm within 1 min. After 30 min, the resonant wavelength remains almost constant. The maximum resonance wavelength shift is 16.34 nm. Almost no change in the resonant wavelength was observed the biosensor surface was washed with PBS. This suggests that the tetanus antibody bound tightly to protein A on the biosensor surface (Fig. 5, curve 1). But after the monoclonal antibody was immobilized on the biosensor surface prepared with protein A, tetanus antibody was injected into the flow cell to block a nonspecific on the protein A. The shift of resonance wavelength is only 1.43 nm (in Fig. 5, curve 2). It is obvious from these results that it is very reliable for blocking nonspecific binding sites on protein A with tetanus antibodies.

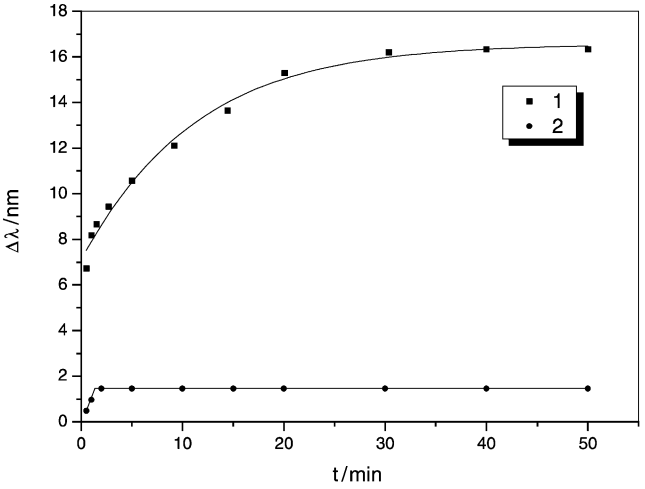

Fig. 5. Kinetic adsorption curve of tetanus antibody assembled on the protein A monolayer. (1) Without injection of monoclonal antibody. (2) With injection of monoclonal antibody.

Determination for recognizing antigenic epitopes of bovine cTnI

Based on the assay procedure, two different experimental modes were carried out to recognize antigenic epitopes at room temperature. In experimental mode A, three experiments were performed (Fig. 2a). Fig. 2b shows the sensorgram obtained by one of three experiments. The results obtained from experimental mode A are shown in Table 1. In the first experiment, for the same amount of MAB12 and MAB5 bound on antigens of bovine cTnI, the binding responses are 3.21 and 4.29 nm, respectively. This shows that binding sites of MAB12 and MAB5 on the antigen of bovine cTnI are different and the affinity of MAB12 and MAB5 for antigens of bovine cTnI is MAb5 > MAb12. In the subsequent two experiments, the corresponding responses of MAB5 and MAB11 bound on antigen of bovine cTnI are 4.30 and 14.35 nm, respectively. This shows that the binding sites of MAB5 and MAB11 on antigens of bovine cTnI are different and the affinity of MAB5 and MAB11 for antigens of bovine cTnI is MAb5 > MAb11. The corresponding responses of MAB11 and MAB12 bound on antigen of bovine cTnI are 13.42 and 2.86 nm, respectively. This shows that the binding sites of MAB11 and MAB12 on antigens of bovine cTnI are different and the affinity of MAB11 and MAB12 for antigen of bovine cTnI is MAb11 > MAb12.

Based on the results and analysis noted above, we can conclude that binding sites of the MAb12, MAb5, and MAb11 on the antigens of bovine cTnI are different and the affinity of the three monoclonal antibodies for antigens of bovine cTnI is MAb11 > MAb5 > MAb12.

Fig. 6a shows the kinetic curves obtained by injection of secondary antibodies in the three experiments of mode A. As from Fig. 6a, the MAB11 binding on antigen was

Table 1
Change of resonance wavelength with the time in experimental mode A

Experiment	Injection solution (concentration)	t (min)	$\Delta\lambda$ (nm)
The first experiment	BSA	0–5	0.48
	MAb12 (50 μg/ml)	5–35	11.55
	PBS	35–45	0.00
	Antigen (26.4 μg/ml)	45–75	3.21
	PBS	75–85	0.00
	Tetanus antibody (1:20)	85–115	1.43
	MAb5 (50 μg/ml)	115–145	4.29
The second experiment	BSA	0–5	0.96
	MAb5 (50 μg/ml)	5–35	12.96
	PBS	35–45	−0.48
	Antigen (26.4 μg/ml)	45–75	4.3
	PBS	75–85	0.00
	Tetanus antibody	85–115	0.00
	MAb11 (50 μg/ml)	115–145	14.35
The third experiment	BSA	0–5	0.48
	MAb11 (50 μg/ml)	5–35	13.42
	PBS	35–45	−0.48
	Antigen (26.4 μg/ml)	45–75	6.21
	PBS	75–85	0.00
	Tetanus antibody (1:20)	85–115	0.97
	MAb12 (50 μg/ml)	115–145	2.86

relatively fast and relatively better compared to MAB5 and MAB12. The maximum shifts of the resonance wavelength are 14.35, 4.29, and 2.86 nm within 30 min, respectively. For comparison, Fig. 6b shows the binding response in the mode B. It is clear that MAB11 binding on antigen was relatively fast and relatively better compared to MAB5 and MAB12. Fig. 3b also shows the biosensor response to the interaction between three monoclonal antibodies and antigens in the experimental mode B. It can be seen from Fig. 6b and Fig. 3b, that their affinity is also MAb11 > MAb5 > MAb12 and their binding site on the antigens of bovine cTnI are different. This is consistent with results obtained by the experimental mode A. The observed discrepancies between two experimental modes may in part be due to differences in biosensor functionalizations and a slight influence of the first antibody for the interaction of the secondary antibody and antigen in experiment A.

We applied two different experimental modes to recognize antigenic epitopes of bovine cTnI with three monoclonal antibodies and the results obtained are consistent with those obtained by the ELIAS method and the assay is easily reproducible. Thus it can be concluded that this assay is an accurate method.

Kinetic analysis of antibody and antigen

Three monoclonal antibodies were used to determine the binding affinities of antigens of bovine cTnI in real time. These antibodies were examined previously for antigen affinities by ELISA. In the experiments, equilibrium responses were collected for the MAb11 at concentrations of 2.5, 3.33, and 10 μg/ml, the MAb5 at concentrations of 16.67, 25, and 50 μg/ml, and the MAb12 at concentrations of 25, 50, and 100 μg/ml (all on the same prepared biosensor surface). Fig. 7 shows the binding responses of MAB12 (differential concentration) injected over a biosensor surface. Association and dissociation phases of the measured sensorgrams were used to calculate kinetic and equilibrium constants [Eq. (3)]. An overview of the results is given in Table 2. The affinities among MAb11, MAb5, and MAb12 and the antigen of bovine cTnI are 1.956×10^6, 1.49×10^6 and 2.3×10^5, respectively. These results appear to agree well with the result obtained in experiments of the three monoclonal antibodies recognizing antigenic epitopes of the bovine cTnI and these results are also consistent with those obtained by ELISA.

Conclusion

The surface plasmon resonance biosensor is an ideal tool for determining the interaction of monoclonal antibodies and antigens of bovine cTnI. The assay based on SPR biosensors is fast with high accuracy and very good reproducibility. Kinetic analyses can provide detailed insights into the mechanism of complex formation. Most importantly, recognizing antigenic epitopes is more accurate using biosensors, which is of great practical value for selecting the best antibody combination applied to ELISA. Such applications could be of particular interest for the development of immunology.

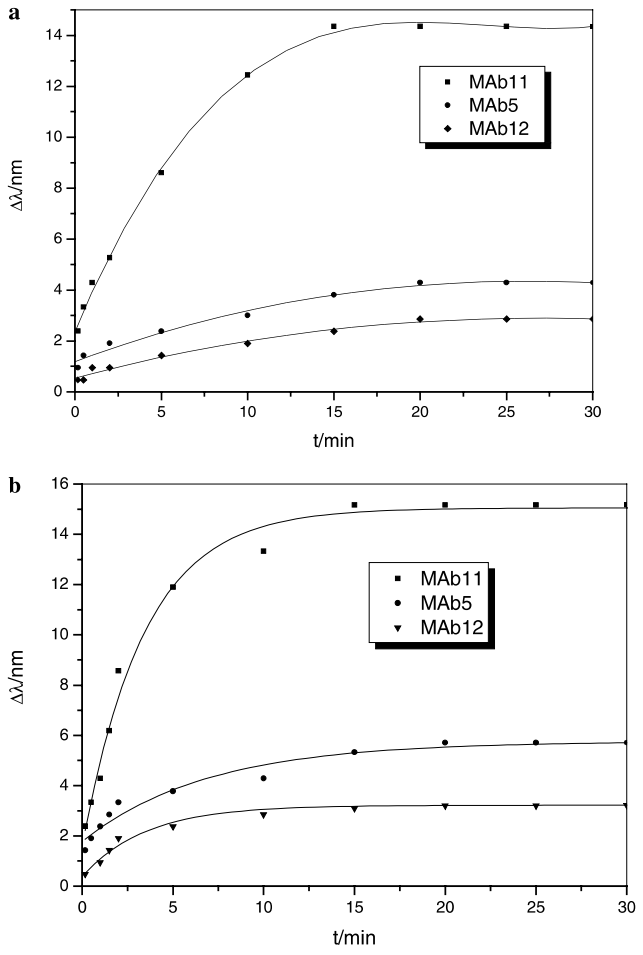

Fig. 6. Kinetic curves of the interaction between monoclonal antibody and antigen. (a) The kinetic curves of the interaction between the secondary antibody and the antigen of every group in experimental mode A. (b) The kinetic curves of the interaction among between MAb12, MAb5, MAb11 and antigen in experimental mode B.

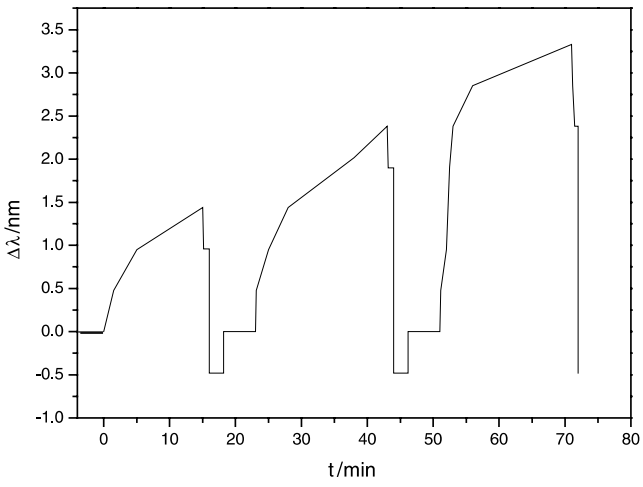

Fig. 7. Kinetic analysis of the interaction between MAB12 and antigen of bovine cTnI.

Table 2
Kinetic data of monoclonal antibodies to antigens of bovine cTnI

Monoclonal antibody	k_a ($mol^{-1} L s^{-1}$)	k_d (s^{-1})	k_a/k_d ($mol^{-1} L$)
MAb11	1.15×10^4	5.88×10^{-3}	1.96×10^6
MAb5	8.21×10^3	5.49×10^{-3}	1.49×10^6
MAb12	1.40×10^3	6.10×10^{-3}	2.30×10^5

The presented wavelength modulation SPR biosensor is a new generation of biosensor instruments which is under development to provide methods for real time, fast, and label-free analysis of crude samples in the field. But the biosensor presented also has some limitations for detection, which may be further improved by adopting a monochromator with a higher resolving power. At present, this aspect of the biosensor system is being researched in our laboratory.

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