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Quantitative measurement of binding kinetics in sandwich assay using a fluorescence detection fiber-optic biosensor

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ABSTRACT

Fiber-optic biosensors have been studied intensively because they are very useful and important tools for monitoring biomolecular interactions. Here we describe a fluorescence detection fiber-optic biosensor (FD–FOB) using a sandwich assay to detect antibody–antigen interaction. In addition, the quantitative measurement of binding kinetics, including the association and dissociation rate constants for immunoglobulin G (IgG)/anti-mouse IgG, is achieved, indicating $0.38 \times 10^6\ M^{-1}\ s^{-1}$ for k_a and $3.15 \times 10^{-3}\ s^{-1}$ for k_d. These constants are calculated from the fluorescence signals detected on fiber surface only where the excited evanescent wave can be generated. Thus, a confined fluorescence-detecting region is achieved to specifically determine the binding kinetics at the vicinity of the interface between sensing materials and uncladded fiber surface. With this FD–FOB, the mathematical deduction and experimental verification of the binding kinetics in a sandwich immunoassay provide a theoretical basis for measuring rate constants and equilibrium dissociation constants. A further measurement to study the interaction between human heart-type fatty acid-binding protein and its antibody gave the calculated kinetic constants k_a, k_d, and K_D as $8.48 \times 10^5\ M^{-1}\ s^{-1}$, $1.7 \times 10^{-3}\ s^{-1}$, and 2.0 nM, respectively. Our study is the first attempt to establish a theoretical basis for the florescence-sensitive immunoassay using a sandwich format. Moreover, we demonstrate that the FD–FOB as a high-throughput biosensor can provide an alternative to the chip-based biosensors to study real-time biomolecular interaction.

Immunosensor is an important type of biosensor by means of selective affinity between antibodies and antigens or other related compounds [1]. This interaction allows developing quantitative and qualitative assays for target molecules. Since the development of the biosensor, enzyme-linked immunosorbent assay (ELISA)[1] has been widely used as a sensing technology to specifically target diverse antigens [2,3]. The ELISA technique is based on a sandwich format using two distinct monoclonal antibodies specifically binding to their common target. For clinical diagnosis and therapeutic applications, the utilities of biomarker-specific antibodies have been demonstrated for their intrinsic affinity and stability properties, although the conventional ELISA is less able to measure real-time kinetics due to the relatively long access time for data acquisition [4].

In this study, we report a kinetic study using a fluorescence detection fiber-optic biosensor (FD–FOB) with a sandwich system like ELISA. This system can be used to measure binding kinetics of biomolecules localized near the uncladded fiber surface where evanescent wave excitation of fluorescence is elicited by total internal reflection (TIR). The FD–FOB belongs to the attenuated total reflection (ATR) method whose fluorescence is excited by the evanescent wave in the vicinity of the interface between medium and fiber surface [5,6], where the penetration depth of the evanescent wave is an order of the incident laser beam wavelength. Thus, the fluorescence of a fluorophore-labeled antibody is excited by the localized evanescent wave only near the interaction region of fiber (Fig. 1). Therefore, the FD–FOB can serve as an important device to be able to perform real-time detection with high specificity. It allows us not only to measure the binding kinetics, such as the association and dissociation rate constants, but also to identify specific biomolecules in biomedical applications [7–10].

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[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; FD–FOB, fluorescence detection fiber-optic biosensor; TIR, total internal reflection; ATR, attenuated total reflection; IgG, immunoglobulin G; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PMMA, poly(methylmethacrylate); PMT, photomultiplier tube; LIA, lock-in amplifier; anti-IgG, anti-mouse IgG; $NaBH_4$, sodium borohydride; H-FABP, heart-type fatty acid-binding protein; BSA, bovine serum albumin; SPR, surface plasmon resonance.

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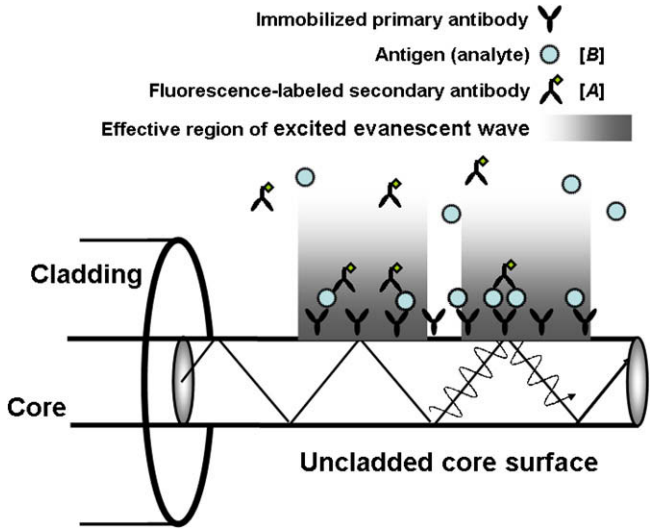

Fig. 1. Illustration of sandwich assay using the FD–FOB under TIR. Gradient grayness indicates the exponentially decayed electric field of the evanescent wave. As indicated in Eq. (1) below, fluorescence-labeled secondary antibody represents *A* molecule and antigen represents *B* molecule.

To take advantage of the specific features of antibodies, the developed FD–FOB uses a sandwich assay in which the primary antibody is immobilized on the core surface of the uncladded fiber. On the addition of antigen and a secondary fluorescence-labeled antibody, the ternary sandwich fluorescence-labeled antibody/antigen/antibody is formed near the fiber surface and, thus, sheltered in the electric field of the evanescent wave passed through the fiber core. In contrast, the unbound binary complex fluorescence-labeled antibody/antigen beyond the sensing volume of the evanescent wave will not contribute to the fluorescence signal, allowing the real-time monitoring of localized biomolecular interaction and measurement of binding kinetics near the fiber surface. In this study, the theory of binding kinetic analysis using a sandwich assay with the FD–FOB to measure the association and dissociation rate constants between the fluorescence-labeled antibody and antigen is derived and verified experimentally. Our result provides the theoretical basis for studying binding kinetics of antibody–antigen interaction via the FD–FOB and demonstrates the availability and applicability of this simple setup in kinetic analyses.

Materials and methods

Reagents and chemicals

All solvents and chemicals used in this study were of analytical grade or chemically pure grade in quality. Ethyl acetate, ethyldiamine, *n*-butyllithium, glutaraldehyde, sodium borohydride, mouse immunoglobulin G (IgG), and goat anti-mouse IgG were purchased from Sigma–Aldrich (St. Louis, MO, USA). A phosphate-buffered saline (PBS, pH 7.4) solution containing 10 mM phosphate and 145 mM NaCl was prepared and used. In addition, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG was purchased from Chemicon (Temecula, CA, USA).

Equipment

The instrumental setup of the FD–FOB was described previously by our group [6,11]. Briefly, a laser beam of a 488-nm solid-state laser was guided into optical fiber by using an objective lens (20×, NA = 0.5) to match the numerical aperture of the fiber (NA = 0.47). A poly(methylmethacrylate) (PMMA) multimode plastic fiber of 1000 μm in diameter (refractive index = 1.492, SK-40, Mitsubishi, Japan) was adopted. The reaction chamber of 1.5 ml in volume was modified from a plastic cuvette for loading solutions. The fluorescence signal was generated by exciting FITC fluorophore on FITC-conjugated antibody, filtered with a 520-nm interference filter to reduce background noise, and detected by a photomultiplier tube (PMT). The PMT was positioned perpendicular to the side of uncladded fiber core to provide high-fluorescence collection efficiency. A lock-in amplifier (LIA) was incorporated into the FD–FOB to further enhance the signal/noise ratio of the fluorescence signal.

Chemical modification of PMMA optical fiber

The plastic fiber was first uncladded by immersing a segment of fiber into ethyl acetate for 1 min, followed by washing with distilled water and drying with nitrogen. The uncladded fiber surface was then modified by reacting with the monoanion of α,ω-diaminoalkanes to yield amine-terminated PMMA surface according to the literature [12]. Briefly, under nitrogen and cyclohexane, *N*-lithioethylenediamine was synthesized by reacting 6 mM ethyldiamine with 1 mM *n*-butyllithium. The uncladded PMMA fiber surface was then incubated with this *N*-lithioethylenediamine solution for 15 min and quenched with distilled water to generate the terminal amino groups on the uncladded PMMA fiber surface. Next, the modified surface was further activated with a homobifunctional cross-linker glutaraldehyde to establish a terminal aldehyde group that enables future covalent linkage with proteins, as reported previously [13]. Briefly, the amine-terminated surface of PMMA fiber was modified by reacting with 2.5% (v/v) glutaraldehyde (100 mM phosphate buffer, pH 7.0) at room temperature for 1 h, washed with distilled water, and nitrogen dried. The resulting aldehyde surface of uncladded PMMA fiber was now ready for coupling with the exposed lysine residues and N terminus of proteins (antibodies) to form stable covalent bonds.

Sandwich assay of IgG

The primary antibody, goat anti-mouse IgG (anti-IgG), was first immobilized onto the modified fiber surface through covalent bonding with surface aldehydes. The remaining unreacted aldehyde was quenched with sodium borohydride ($NaBH_4$), and the resulting antibody-conjugated fiber was placed inside the reaction chamber for the following analysis. Different concentrations of antigen (0.1, 0.5, 1, 5, and 10 μg/ml mouse IgG) were respectively injected into the reaction chamber and reacted with fiber surface for 1 h to form anti-IgG/IgG complex. After washing three times with PBS, an equal amount of the secondary antibody, FITC–anti-IgG, was added into the reaction chamber to produce the sandwich ternary complex anti-IgG/IgG/FITC–anti-IgG, which is able to emit fluorescence signal on excitation by the evanescent wave near the uncladded fiber surface. Because the penetration depth of the fiber-mediated evanescent wave in solution was approximately 200 nm from the uncladded fiber surface, only FITC-conjugated antibody bound with antigen in conjunction with fiber-immobilized primary antibody could be excited, but not for the unbound FITC–anti-IgG. Accordingly, the time response of IgG/FITC–anti-IgG interaction was measured by recording the fluorescence signals, leading to the deduction of k_a and k_d simultaneously.

Sandwich assay of heart-type fatty acid-binding protein

In the heart-type fatty acid-binding protein (H-FABP)/anti-H-FABP study, the primary antibody was covalently immobilized on the reaction area of the unclad optical fibers. First, modified optical

fibers were immersed in mouse anti-human H-FABP monoclonal antibody solution (concentration of 10 μg/ml) at 4 °C overnight. To avoid nonspecific binding, blocking treatment was carried out using bovine serum albumin (BSA) solution at room temperature for 1 h. Then the target molecules, human H-FABP, of different concentrations as 5, 1, 0.5, and 0.1 μg/ml were injected into the reaction chamber and interacted with immobilized anti-human H-FABP for 1 h at room temperature. Next, noninteraction molecules were washed out by using PBS solution, and the reaction chamber was set up in the optical system. Finally, 10 μg/ml FITC-conjugated anti-human H-FABP monoclonal antibody was injected into the reaction chamber and detected fluorescence signal simultaneously. Therefore, the sandwich format anti-H-FABP/human H-FABP/FITC–anti-H-FABP was constructed by this method and fluorescence signals were collected by PMT.

Results and discussion

In the current study, we intended to establish that, with suitable modification of fiber surface and theoretical calculation, the FD–FOB can serve as a high-throughput biosensor for real-time study of protein-binding kinetics. It was anticipated that this simple setup could provide an alternative device for chip-based biosensors to study biomolecular interaction in applications such as clinical diagnosis and drug discovery [14].

The specific and efficient immobilization of primary antibody on fiber core surface is critical to the detection sensitivity of the FD–FOB [15]. To ensure the steady formation of a sandwiched antibody/antigen/antibody complex on fiber surface, the surface of PMMA plastic fiber was chosen and chemically modified to build an FD–FOB with covalently immobilized primary antibody, as described in Materials and Methods [16]. Accordingly, the immobilization of biomolecules onto fiber surface was significantly improved and standardized. Thus, the following binding of antigens and FITC-labeled secondary antibody to form a sandwiched complex can be monitored by fluorescence detection, enabling the fiber to probe the real-time binding kinetics in solution (Fig. 1).

To measure the binding kinetics using the FD–FOB, we derived a theoretical basis to calculate the resulting fluorescence signals. Conventionally, binding kinetics can be analyzed using the solid phase-immobilized antigen and labeled primary antibody in liquid phase or vice versa [4]. However, antigens prepared from a complex mixture may lower binding specificity due to high background interference. In contrast, a sandwich assay using two distinct antibodies recognizing different epitopes of the target antigen can largely diminish the chance of intervention from other similar molecules [17]. Furthermore, our FD–FOB adopted the sandwich assay using the fiber-immobilized primary antibody, free antigen, and fluorescence-labeled secondary antibody to form immobilized antibody/antigen/fluorescence-labeled antibody complex whose fluorescence can be excited only by the evanescent wave through fiber. This allowed us to study the binding kinetics of the antigen and secondary antibody using the recorded fluorescence on concentration and time changes. Therefore, the interaction between fluorescence-labeled secondary antibody (indicated as A) and target antigen (indicated as B) can be described in the equation

$$[A] + [B] \underset{K_d}{\overset{K_a}{\rightleftarrows}} [AB] \tag{1}$$

where k_a is the association rate constant describing the rate of molecular complex formation and k_d is the dissociation rate constant describing the stability of the complex (i.e., the fraction of complexes that decays per second). In addition, the equilibrium dissociation constant and association constant [18] are defined as

$$K_D = \frac{k_d}{k_a} \tag{2a}$$

$$K_A = \frac{k_a}{k_d} \tag{2b}$$

To simplify the derivation of rate constants determined by the FD–FOB using the sandwich format, the following premises are assumed. First, the first-order reaction kinetic scheme is applied for antigen/fluorescence-labeled antibody interaction at the second reaction that the concentration of fluorescence-labeled secondary antibody is assumed to be a constant over the entire course of reaction. Therefore, the reaction proceeds at a rate directly proportional to one of the reactants [4,18]. Second, the binding of target antigen onto the primary antibody immobilized on fiber surface has reached equilibrium and keeps constant during interaction of antigen and fluorescence-labeled secondary antibody. Third, the binding sites are limited and the amount of consumed binders is small during antigen/fluorescence-labeled antibody interaction to ignore the influence of diffusion [4]. Fourth, the dissociation and rebinding effect between primary antibody and antigen can be ignored due to the stagnant configuration of the reaction chamber [19,20]. According to the associated rate equation [21],

$$\frac{d[AB]}{dt} = K_a[A][B] - K_d[AB] \tag{3}$$

$[A]$ is the concentration of FITC–anti-IgG, and $[B]$ is the concentration of antigen, IgG, which is bound with the immobilized primary antibody on the uncladded surface of the optical fiber. $[AB]$ is the concentration of complex of $[A]$ and $[B]$ in the sandwich. In the measurement, $[B]$ is gradually reducing during the binding process between A and B. Therefore, the values of $[A]$ and $[B]$ at time t are equal to

$$[A] = [A]_0 - [AB] \tag{4}$$

$$[B] = [B]_0 - [AB]. \tag{5}$$

In this setup, $[A]_0$ is the maximum number of $[A]$ at $t = 0$ and $[A]_0 >> [AB]$. Similarly, $[B]_0$ is the maximum number of $[B]$ at $t = 0$, which is identical to the number of antigens bound onto primary antibody immobilized onto the uncladded fiber surface. Then Eq. (3) becomes

$$\frac{d[AB]}{dt} = K_a[A]_0[B]_0 - (K_a[A] + K_d)[AB] \tag{6}$$

Because $[A]_0$ and $[B]_0$ are independent of fluorescence signal, in this experiment $[A]_0$ and $[B]_0$ remain constant where C and C_0 represent the concentrations of $[A]_0$ and $[B]_0$, respectively. Then the rate equation of the fluorescence signal (R) becomes

$$\frac{dR}{dt} = K_a C C_0 - (K_a C + K_d)R \tag{7}$$

$$\frac{d}{dR}\left(\frac{dR}{dt}\right) = -(K_a C + K_d) \tag{8}$$

and the solution of Eq. (7) can be represented by

$$R(t) = \alpha e^{-(K_a C + K_d)t} + K \tag{9}$$

$$R(t) = R_{bg} + \left(R_{max} - R_{bg}\right)\left(1 - e^{-(K_a C + K_d)t}\right), \tag{10}$$

where $K = R_{max}$ when the fluorescence signal is saturated at $t \to \infty$ and $\alpha = R_{bg} - R_{max}$ where R_{bg} is the background fluorescence signal at $t = 0$. This result is identical to the output signal of protein–protein interaction analysis in the real-time detection of antigen/antibody complex of surface plasmon resonance (SPR) biosensor [22]. From Eq. (8), the linear relationship between $\frac{d}{dR}\left(\frac{dR}{dt}\right)$ and concentration of FITC-labeled secondary antibody $[A]$ is measured. The k_a zand k_d are obtained simultaneously by the slope and intersection of the linear response of Eq. (8) apparently.

To investigate the feasibility of this FOB, characteristic measurement of FITC-conjugated rabbit anti-mouse IgG (FITC–anti-IgG) interacting with mouse IgG at various concentrations were carried out according to the literature [23]. Goat anti-mouse IgG (anti-IgG) as primary antibody was first immobilized onto the uncladded fiber surface and placed in the reaction chamber. Subsequently, mouse IgG was added and incubated with fiber to form the anti-IgG/IgG binary complex in chamber. Finally, the FITC–anti-IgG was injected into the chamber to allow the formation of sandwiched anti-IgG/IgG/FITC–anti-IgG complex, and the binding affinity between IgG and FITC–anti-IgG was monitored by recording the fluorescence changes in real time. The time responses of fluorescence intensity from sandwiched anti-IgG/IgG/FITC–anti-IgG at diverse concentrations (0.1, 0.5, 1, 5, and 10 μg/ml) in the reaction chamber were measured and are shown in Fig. 2A. The detected intensity oscillation in Fig. 2A might be caused by free secondary antibody moving into the reaction region of the evanescent wave. A linear equation was obtained as $y = 383132x + 0.00315$ ($R^2 = 0.998$) in Fig. 2B. After calculation, the association rate constant $k_a = 0.38 \times 10^6\ M^{-1}\ s^{-1}$ and the dissociation rate constant $k_d = 3.15 \times 10^{-3}\ s^{-1}$ according to Eq. (8) above. Thus, the equilibrium association constant $K_A = 1.21 \times 10^8\ M^{-1}$ and the equilibrium dissociation constant $K_D = 8.3$ nM are obtained from the interaction of IgG and FITC–anti-IgG. These results are close (in order of magnitude) to the values reported by Ivnitski and coworkers, who obtained k_a, k_d, K_A, and K_D values of $1.3 \times 10^6\ M^{-1}\ s^{-1}$, $0.2 \times 10^{-3}\ s^{-1}$, $6.7 \times 10^9\ M^{-1}$, and 0.15 nM, respectively [23].

We then further applied such an approach to study the interaction between human H-FABP and its antibodies, a clinically important marker in myocardial infarction [24]. The linear response of $\frac{d}{dR}\left(\frac{dR}{dt}\right)$ versus target concentration (5, 1, 0.5, and 0.1 μg/ml) of human H-FABP was derived, and the correlation is 0.9714 (Fig. 3). Subsequently, the kinetic constants k_a, k_d, and K_D are calculated as $8.48 \times 10^5\ M^{-1}\ s^{-1}$, $1.7 \times 10^{-3}\ s^{-1}$, and 2.0 nM, respectively. Previously, only Orban and coworkers had employed a grating coupler sensor to obtain an average association rate constant of H-FABP $k_a = 4.2 \times 10^3\ M^{-1}\ s^{-1}$, a dissociation rate constant of $k_d = 1.3 \times 10^{-4}\ s^{-1}$, and an equilibrium constant of $K_D = 30$ nM [25]. It seems like our measurement indicates a tighter affinity between H-FABP and its antibody. By using a simple setup of the FD–FOB, our results provide an alternative technique to characterize the antibody–antigen interaction of this clinically important biomarker.

With the FD–FOB, experimental errors on kinetic measurement can arise from several factors, including laser intensity fluctuation and diffusion rate of FITC–anti-IgG [11]. Theoretically, laser intensity fluctuation during the measurement is insensitive to the uncertainty of k_a and k_d according to the equation $\frac{d}{dR}\left(\frac{dR}{dt}\right) = -(K_a C + K_d)$ by the ratio of fluorescence. Or, a reference laser beam can be added to reduce the effect of laser intensity fluctuation as well by measuring the ratio of the detected and reference intensities in real time. On the other hand, the diffusion rate of FITC–anti-IgG in the reaction chamber concerns kinetic measurement. Theoretically, the effect of diffusion of secondary antibody is largely contributing to the starting time of fluorescence signals in this setup. However, our experimental results using different concentrations of secondary antibody showed a similar starting time for each experiment (<1 s) (Fig. 2A). The fact that the volume concentration of secondary antibody in the reaction chamber is kept constant during the whole measurement for fluorescence signal detection ensures that the diffusion rate equation of secondary antibody can be ignored [11].

Although we could not exclude the errors from the variation of used optical fibers and experimental conditions, the high correlation coefficients indicate that the developed FD–FOB is capable of performing measurements with high reproducibility in protein–protein interaction. In addition, the current literature has described the range of k_a, k_d, and K_D of IgG/anti-IgG interactions as 1.3 to $55 \times 10^6\ M^{-1}\ s^{-1}$, 1.8 to $20 \times 10^{-4}\ s^{-1}$, and 0.11 to 1.4 nM, respectively [11,23,26,27]. Our measured results of IgG/anti-IgG interaction demonstrated the capability of the FD–FOB to study this biospecific interaction and the availability of the alternative technique. Moreover, in comparison with the cost-effective SPR on measuring the binding constant of biomolecules [22], the FD-FOB is comparable to SPR not only in sensitivity but also in time response experimentally.

Probable weaknesses of the FD–FOB could come from the batch variation of plastic fiber as optical biosensor, including the surface modification of fiber and the immobilization efficiency of primary antibody. In addition, the output intensity fluctuation of the used laser beam is critical to this method. All of these would cause deviations of the resulting constants. To solve the variation for each produced fiber, we have carefully kept the fiber modification in the chemical hood to reduce any unwanted side reactions. In addi-

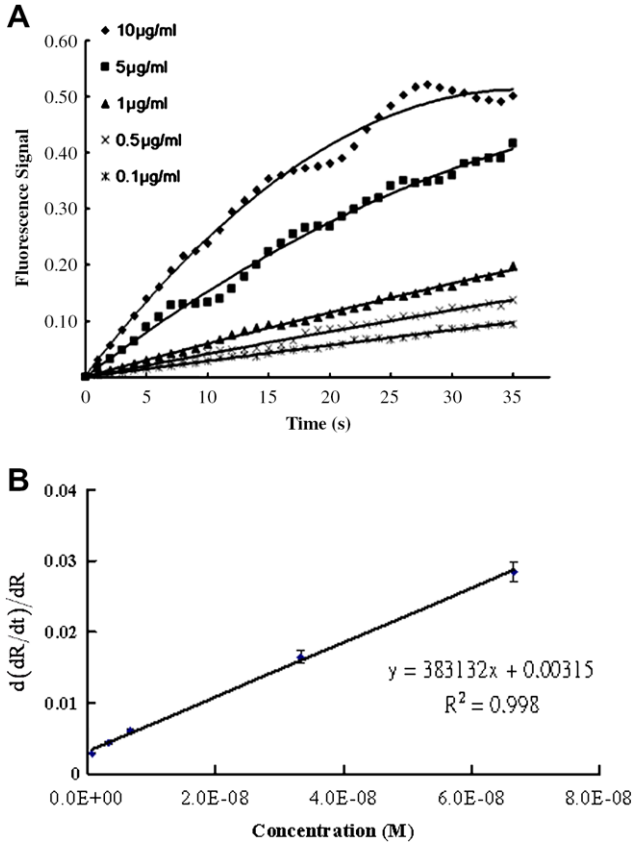

Fig. 2. (A) Fluorescence signals measured by the FD–FOB at different concentrations of FITC–anti-IgG on interacting with an equal amount of IgG. (B) Determination of binding kinetics for IgG/FITC–anti-IgG interaction.

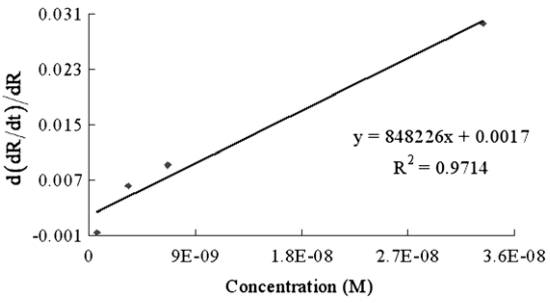

Fig. 3. Determination of binding kinetics for H-FABP/FITC–anti-H-FABP interaction.

tion, 20 fibers are produced each time to make sure that there are enough fibers for reproducible experiments. The high immobilization efficiency can be achieved by the prolonged incubation time of fiber and primary antibody. On the other hand, the ratio method can be used to reduce the effect of intensity fluctuation via normalization by using a reference laser beam in the setup. Moreover, the synchronized detection by using a lock-in amplifier increases the signal/noise ratio of detected signal significantly in this experiment. In addition, a tapered optical fiber can also be used to enhance the detection sensitivity [28]. Theoretically, the V number mismatch of optical fiber without being tapered will decrease the efficiency of fluorescence signal back to the fiber core. However, the fluorescence signal is detected beside the optical fiber in the FD–FOB of this setup, which is insensitive to the V number of optical fiber under this consideration. A high collective efficiency on fluorescence signal results from this setup experimentally.

Conclusions

Kinetic constant measurement of antibody–antigen interaction in real time is very important to characterizing such biospecific affinity and exploring the future feasibility of its clinical availability regarding sensitivity, efficiency, and accuracy. In this study, we have demonstrated that an FD–FOB is capable of measuring and calculating the kinetic constants using our proposed theoretical basis. Our results of IgG/anti-IgG kinetic constants match values reported previously. In addition, a further measurement of H-FABP/anti-H-FABP interaction provides kinetic constants in addition to those in previous studies, helping to increase the understanding of H-FABP interaction by the alternative technique. Moreover, this immunosensor setup can be applied to measure the real-time interaction of biomarker and antibody in a complex medium such as patient serum. In summary, we believe that the FD–FOB as a biosensor has several advantages. First, a multimode plastic fiber is able to provide a larger sensing area than is a single-mode fiber. Second, a uniformly distributed evanescent wave on the uncladded fiber core surface is able to excite the fluorescence signal more efficiently than is the nonuniform distribution of a single mode fiber. Third, the surface modification of plastic fiber improves the specificity and sensitivity of biomolecular interactions. Fourth, this setup can potentially perform multichannel detections for diverse biomolecular interactions simultaneously in real time. Finally, it is a simple setup and an inexpensive technique as an alternative to currently available techniques.

Acknowledgments

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