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Real-time association rate constant measurement using combination tapered fiber-optic biosensor (CTFOB) dip-probes

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Abstract

This document reports a novel method of measuring association rate constant (k_a) for antibody-antigen interaction using evanescent wave-based combination tapered fiber-optic biosensor (CTFOB) dip-probes. The method was demonstrated by measuring association rate constant for bovine serum albumin (BSA) and anti-BSA antibody interaction. “Direct method” was used for detection; goat anti-BSA “capture” antibodies were immobilized on the probe surfaces while the antigen (BSA) was directly labeled with Alexa 488 dye. The probes were subsequently submerged in 3 nM Labeled BSA in egg albumin (1 mg/ml). The fluorescence signal recorded was proportional to BSA anti-BSA conjugates and continuous signal was acquired using a fiber optic spectrometer (Ocean Optics, Inc.). A 476 nm diode laser was used as an excitation source. Association constant was estimated from a plot of signal as a function of time. Measured association rate constant k_a for the binding of BSA with anti-BSA at room temperature is $(8.33 \pm 0.01) \times 10^4 M^{-1} s^{-1}$.

Keywords

association constant; BSA; anti-BSA; fiber-optic; biosensor; real-time

1. INTRODUCTION

Our ability to understand the role of proteins in many immunologically relevant processes is limited by our ability to quantify the many qualities of specific proteins in solution. An ideal biosensor would not only be able to accurately detect concentrations, but also shed light on various factors that would affect the kinetic properties of proteins in a serum solution. Said biosensors would be of great use in the study of acute phase proteins, such as Interleukin-1 and Tumor necrosis factor- α , where their low yet dynamic concentrations are most readily found in human blood. Real-time kinetic analysis of these factors could aid greatly in patient diagnosis. In-house produced combination tapered fiber-optic biosensor (CTFOB) dip-probes has the potential to meet most of these requirements.^{1,2} These probes use evanescent wave induced fluorescence and fluorescent labeling techniques to safely detect their environment with great sensitivity and specificity. The probes are electrically isolated and the exponentially decaying energy of evanescent waves ensures that the affected system is

limited to the surface of the probes. When compared to small diameter fibers ($<100\text{ }\mu\text{m}$), CTFOB's ($300\text{ }\mu\text{m}$) are also far more easily handled than similar biosensors being developed, due to the use of combination, rather than continuous tapering, and provide greater re-coupling of surface fluorescence.^{1,3}

In the present study, the CTFOB fluorescence-based technique was used along with direct labeling of the antigen bovine serum albumin (BSA) and a real-time signal collection modality to find the association rate constant for BSA and goat anti-BSA. First antibody is immobilized on the probe surface. The corresponding antigen, having been directly labeled with a fluorescent dye, was then put in solution to react with the antibody-strewn surface. The probe surface was subsequently excited by the evanescent wave technique, which has an effective range that decreases exponentially with distance from the probe surface. This ensured that only articles associated with the antibody-antigen bond would fluoresce. Fluorescence was directly proportional to the number of labeled antigens attached to the immobilized (detection) antibodies.² Low volumes (ng/ml) of labeled protein were used in a greater volume (mg/ml) of non-specific protein to simulate the serum solutions of mammalian blood.⁴ This method produced more fluorescence than sandwich immunoassay setups, due to the increased proximity of fluorescent dye molecules to the probe surface, however, was suitable mainly as a proof of concept as it would not be practical to label only the protein of interest in patient blood serum samples.

2. MATERIALS AND METHODS

2.1 Protein-dye conjugation

Two labeled solutions were prepared for the experiment, an antigen, bovine serum albumin (BSA), and labeled egg albumin (EA), used as the non-specific negative control protein. First a 0.1M NaHCO_3 solution (sodium bicarbonate, pH 8.3) was prepared in phosphate buffered saline (PBS). EA was prepared in PBS using egg albumin powder (1 mg/ml), while BSA was diluted from stock to 1 mg/ml in PBS. Labeled BSA was prepared with $51.5\text{ }\mu\text{L}$ of 1 mg/ml BSA, $1\text{ }\mu\text{L}$ of stock Alexa Fluor 488 fluorophore (Molecular Probes, Eugene, OR), and $1\text{ }\mu\text{L}$ of 0.1M NaHCO_3 . Labeled EA was prepared with $52.5\text{ }\mu\text{L}$ of 1 mg/ml EA, $1.5\text{ }\mu\text{L}$ of stock Alexa 488, and $1\text{ }\mu\text{L}$ of 0.1M NaHCO_3 . The two serums were incubated simultaneously for 1 hour at room temperature with constant agitation. Each serum was subsequently filtered with two gel filtration spin columns (Princeton Separations, Adelphia, NJ) for 5 minutes at 2000 rpm .

2.2 CTFOB dip-probe fabrication

Each probe started as a 7.5 cm length of a multimode optical silica/silica fiber (Polymicro Technologies, LLC, and Phoenix, AZ). The fibers have a core diameter of $600\text{ }\mu\text{m}$ (attenuation 4 dB/km @ 800 nm), a cladding diameter of $661\text{ }\mu\text{m}$ and a polyimide buffer diameter of $702\text{ }\mu\text{m}$. Each length that was cut was examined on both ends for imperfections with a fiber inspection scope (CL-200, Thorlabs Inc., Newton, NJ). The plastic buffer was removed to precisely 2 cm with a natural gas open flame. The burned fiber tips were wiped free of ash, sonicated for 4 minutes in distilled water and dried.

In order to create an even, combination taper, probes were placed in a plastic beaker filled with 10 cm deep 10% HF (hydrofluoric acid). This ensured that the acid reached past the 2 cm polyimide buffer height so capillary ascent would occur. The probes were monitored until the tips had reached a thickness of 300 μm (measured on multiple probes with digital calipers) and the HF had ascended under the polyimide buffer, creating an even taper. The probes were then sonicated for 4 minutes in distilled water. After drying, the rest of the polyimide cover was burned off and then cleansed in distilled water with sonication for 4 minutes. The probes were then transferred to a solution of 1M NaOH and sonicated for 4 minutes. Finally, the probes were sonicated for 4 minutes in 50% non-dry ethanol.

2.3 Goat anti-BSA immobilization

The combination tapered probes were first kept for 1 minute in 2% APTS (3-aminopropyltriethoxysilane) solution in dry acetone to derivatize the glass surface with primary amines ($-\text{NH}_2$). These amines are necessary to react with the immobilization reagent Sulfo-SMCC (Sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate). This forms a maleimide-activated probe surface that can react with the [chemically exposed] sulfhydryl groups on the primary antibodies (Harmanson, 1996). The 4.5 mM Sulfo-SMCC solution is prepared with 1.4 mg Sulfo-SMCC, 30 μL DMSO (Dimethyl sulfoxide), and 670 μL of 10 mM EDTA (ethylenediaminetetra-acetic acid) in PBS. The APTS-coated probes were incubated in the Sulfo-SMCC solution for 1 hour at room temperature with constant agitation. To expose the sulfhydryl group of the capture antibody (affinity pure goat anti-bovine serum albumin), the antibodies were reduced in a solution containing 14.4 μL of antibody stock, 1.8 μL of MEA (β -Mercaptoethylamine HCL) and 1.8 μL EDTA for 90 minutes at 37 $^\circ\text{C}$. Following incubation, excess MEA salts were removed using a gel filtration spin column. The remaining solution was diluted to 10 $\mu\text{g/mL}$ in PBS-EDTA. The maleimide-activated probes were then incubated in the reduced antibody for 4 hours at room temperature. Afterwards, the probes were washed in PBS-EDTA and a PBS solution containing 0.02% of Tween20 soap solution.

2.4 Real-time detection setup

A schematic of the portable signal detection setup developed for use with CTFOB's in real-time acquisition is shown in fig. 1. The objective of the experimental setup was to provide the probes with a constant source of excitation light and record evanescent wave feedback due to fluorescence on the probe surfaces. A laser diode of wavelength 476 nm was used as the excitation source, and output was rectified with a collimation tube, and a dichroic band pass filter (to prevent red tail emission; max transmission 476 nm, 10 nm bandwidth). The probes were mated to the rest of the collection chamber via an SMA bare fiber terminator. The short focal length focusing/collection lens (FCL) was used both to focus the laser beam onto the probe and collect/collimate the beam as it travel back from the fluorescent dye. The dichroic short pass filter was mounted at a 45-degree angle and allowed for the laser light to pass through while fluorescence to reflected back towards the collection port. A redundant long pass filter was added in front of the collection port to further remove scattered laser light and improve the signal to noise ratio. The setup allowed for quick exchange of probes and was mounted vertically so that the probe could be dipped into a sample aliquot during the real-time detection sequences.

2.5 Signal generation and acquisition

Fluorescent signal was recorded from one probe at a time using the OOIBase32 software (Ocean Optics, Inc., Dunedin, FL). Probes not immediately used were preserved in a 0.02% sodium azide solution and refrigerated to keep the immobilized antibodies stable. During each acquisition, the probes were either recorded in a solution of the true antigen, 3 nM labeled BSA, or 4 nM labeled EA in the case of negative control probes. Both solutions were diluted from the original labeled quantities using 1 mg/mL unlabeled EA. A strict protocol was followed in installing the sample on the detection setup to limit where the reagent contacted each probe. First, narrow glass tubes were used to contain 200 μ L aliquots of each sample and were mounted on a level guide that positioned them directly beneath and centered to the probe. Second, the probe tips were carefully submerged to the depth of the bottom limit of the taper. Together, these two precautions were necessary to prevent capillary climb caused probe proximity to walls, which could result in either labeled protein or free dye adhesion to sections of the probe not shielded by the immobilized antibodies. Ten seconds before each acquisition, the signal was zeroed for the ambient light. After each signal acquisition, the laser light was blocked to prevent the bleaching of dye molecules. The process was repeated for approximately 3 hours in the true antigen samples, until the data showed that the probes were saturated. For the negative control probes only 1 hour of acquisition was necessary in order to show that the signal was not increasing. The computer automatically recorded a time log and the magnitude of each acquisition (in seconds and a.u., respectively) and simultaneously streamed data to an excel-compatible text file that was analyzed periodically to monitor the probe interactions and determine when each series had reached saturation. From the recorded profiles, signals were extracted using least square fitting method¹.

3. RESULTS AND DISCUSSION

3.1 Sensitivity and Specificity

None of the negative control probes recorded significant fluorescence due to non-specific binding of either free dye or labeled EA. Prior to sample incubation, auto-fluorescence background signals were found to be same for both negative control and true antigen probes. The negative control probes were tested, in real time, in a solution of 4 nM labeled EA. After only one hour, it became apparent that no specific binding was taking place and that any signal due to non-specific binding could be regarded as negligible. The non specific signal is the sum of labeled protein fluorescence and auto fluorescence from proteins. The nonspecific auto-fluorescence signal can be subtracted from the total signal using least square fitting method described in our earlier work¹. Variation of extracted negative control probe signal with time is shown in fig. 2. Variation of anti-BSA/BSA interaction signal with time is plotted in fig. 3. It can be seen that compared to the antigen probes signal (fig. 3), the signal due to non-specific bindings was insignificant. This indicates highly specific signal recording with CTFOB probes.

3.2 Data Analysis

In case of CTFOB probes the number of antibodies N_{ab} immobilized on the probe surface was much smaller than the number of labeled antigens N_{ag} in the sample solution. In such

case the antigen-antibody interaction signal, $s(t)$ as a function of time t can be given⁵ by eqn. 1.

$$s(t) \propto \frac{N_{ab}}{1+k_d/k_a N_{ag}} (1 - \exp[-(k_a N_{ag} + k_d)t]) \quad (1)$$

Where k_a is association constant and k_d is dissociation constant. When we use very high concentration or number of antigen N_{ag} , such that $k_a N_{ag} \gg k_d$ eqn. 1 can be rewritten as

$$s(t) \propto \frac{N_{ab}}{1+k_d/k_a N_{ag}} (1 - \exp[-(k_a N_{ag}t)]) \quad (2)$$

Eqn. 2 clearly indicates that by fitting an exponential growth function to the experimentally recorded antibody-antigen interaction signal as a function of time, can obtain the value of association constant k_a for that particular antibody-antigen interaction. Anti BSA-BSA association constant was obtained by fitting an exponential growth function to curve shown in fig. 3. The value obtained was $k_a = 8.33 \pm 0.01 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. This value is quite close to other reported values by surface plasmon resonance (SPR) method⁶.

4. CONCLUSIONS

The aforementioned experiment have successfully demonstrated that combination tapered fiber-optic biosensor dip-probes, under a real-time modality, can be successfully used to determine the association rate constant of antigen-antibody interactions in serum-like solutions. As a proof of principle association constant for BSA and goat anti-BSA interaction was measured. The results are comparable to the other reported values obtained by SPR-based kinetic analysis. The reported method is adaptable to most proteins compatible with immunological assays. Future studies are aimed at verifying the label free BSA/anti-BSA reaction.

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Figure 1.

A block diagram of the experimental setup; LD, laser diode; BF, band pass filter; ND, neutral density; SF, short pass filter; FL, focusing lens; LF, long pass filter; FCL, focusing/collection lens.

Figure 2.
Signal as a function of time from negative control probe incubated in 4 nM labeled EA.

Figure 3.

Exponential growth function fits to data with $R^2 = 0.998$. Value of association constant $k_a = 8.33 \pm 0.01 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ was obtained by dividing value of coefficient K by the 3 nM concentration of the labeled BSA solution.