Quantification of protein interaction kinetics in a micro droplet

L. L. Yin,^{1,2} S. P. Wang,^{1,a)} X. N. Shan,¹ S. T. Zhang,² and N. J. Tao^{1,a)}

¹Center for Bioelectronics and Biosensors, Biodesign Institute, Arizona State University, Tempe, Arizona 85287, USA

²College of Chemistry and Chemical Engineering, Chongqing University, Chongqing 400044, China

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Characterization of protein interactions is essential to the discovery of disease biomarkers, the development of diagnostic assays, and the screening for therapeutic drugs. Conventional flow-through kinetic measurements need relative large amount of sample that is not feasible for precious protein samples. We report a novel method to measure protein interaction kinetics in a single droplet with sub microliter or less volume. A droplet in a humidity-controlled environmental chamber is replacing the microfluidic channels as the reactor for the protein interaction. The binding process is monitored by a surface plasmon resonance imaging (SPRi) system. Association curves are obtained from the average SPR image intensity in the center area of the droplet. The washing step required by conventional flow-through SPR method is eliminated in the droplet method. The association and dissociation rate constants and binding affinity of an antigen-antibody interaction are obtained by global fitting of association curves at different concentrations. The result obtained by this method is accurate as validated by conventional flow-through SPR system. This droplet-based method not only allows kinetic studies for proteins with limited supply but also opens the door for high-throughput protein interaction study in a droplet-based microarray format that enables measurement of many to many interactions on a single chip. © 2015 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4934802>]

I. INTRODUCTION

Proteins are the machines of life processes at the molecular level.¹ Typically, proteins carry out their functions through interactions with other proteins by creating complexes. Proteins must associate with each other to create these active complexes and then dissociate to stop the functional activity. Characterization of these complex interactions is fundamental to the understanding of life processes and is essential to the discovery of cancer biomarkers, development of diagnostic assays, and screening for therapeutic drugs.

Conventional methods for detecting and characterizing protein-protein interactions either have low throughput or are limited to measuring steady-state, high-affinity protein interactions.² They include end-point methods such as co-immunoprecipitation (Co-IP),³ far western blots,⁴ various two-hybrid methods,⁵ and tandem affinity purification (TAP) prior to mass spectrometry.⁶ These methods provide little information about binding affinity and no information about the kinetics, but such information is crucial for a complete understanding of the dynamic proteome. Another limitation of these methods is that most of them are based on fluorescent, radiation, or nanoparticle labeling approach. These labeling tags could cause some inconsistent or even contradictory results.^{7,8}

Surface plasmon resonance (SPR) has become an important technique for characterizing the protein interaction over the past decade, as it is a label-free method and provides substantial binding kinetics information.⁹ However, most SPR

systems require a solution containing the analyte protein flowing over the sensor chip coated with target protein during the entire association phase. This process often lasts several minutes and even hours, which consumes a large amount of protein samples. The sample volume requirement often makes the measurement cost inhibitive, because preparation of protein samples is usually labor intensive and involves multiple experimental steps (i.e., expression, extraction, and purification). This problem will be prominent for proteins that are difficult to express on the bacterial or to obtain in a general protocol. In addition, microfluidic based measurement has low throughput due to the limited number of flow channels, and it also suffers from clotting of the fluidic channels by bubbles and impurities in the sample solution.

A series of SPR related technologies have been developed in our lab to solve different practical problems for the measurement of bimolecular interactions.^{10–14} Here, we present a novel droplet-based SPR imaging approach that measures the protein interaction kinetics with significantly reduced sample consumptions. Through this method, we can save the protein sample for hundreds of times while obtain all kinetics constants for protein interaction same as the conventional flow-through SPR system. Furthermore, this novel approach does not need any microfluidic device and thus opens the door for measurement of multiplexed many to many molecular interactions on a single chip.

II. EXPERIMENTAL SECTIONS

A. Materials

Phosphate-buffered saline (PBS, pH = 7.4) was purchased from Thermo Fisher (Waltham, MA). Dithiolalkane aromatic

^{a)}Authors to whom correspondence should be addressed. Electronic addresses: shaopeng.wang@asu.edu and njtao@asu.edu

PEG3-OH (Dithiol-PEG-OH) and dithiolalkane-aromatic-PEG6-COOH (Dithiol-PEG-COOH) were purchased from SensoPath Technologies (Bozeman, MT) (see Figure S1 of the supplementary material for the molecular structures²⁵). Sodium acetate (NaOAc), N-hydroxysuccinimide (NHS), N-ethyl-N'-[3-(dimethylaminopropyl)carbodiimide]hydrochloride (EDC), Immunoglobulin G (IgG) from human serum, and anti-human IgG (Fab specific) antibody (anti-IgG) were obtained from Sigma-Aldrich (St. Louis, MO). Lyophilized IgG and Anti-IgG were dissolved in deionized water as 10 mg/ml stock solution and stored at -20°C in 10 μl aliquots.

B. SPRI setup

A prism-based SPRI setup was used for the present study (Figure 1). In the setup, the sensor chip immobilized with Anti-IgG was placed on an equilateral SF-11 prism with a drop of BK7 index matching oil. A *p*-polarized light from a 670 nm light-emitting diode (LED, L7868-01, Hamamatsu, Japan) was directed through the prism onto the chip. The reflected beam produced the SPR image, which was captured by a CCD camera (Pike F032B, Allied Vision Technologies, Newburyport, MA) with a 12X variable zoom lens (Navitar, Inc., Rochester, New York). The LED was mounted on a temperature-controlled mounting socket and powered by a laser diode driver and a temperature controller (LDM 21, LDC201CU, and TED200C, respectively, Thorlabs, Newton, NJ).

C. Humidity control chamber

A humidity control chamber was designed to minimize droplet evaporation. The chamber had double layers of polydimethylsiloxane (PDMS) cells and a PDMS lid as shown in Figure 2(a). The gap between the inner and outer cells was filled with deionized water to increase the humidity level inside the chamber. After a droplet was spotted on the sensor surface, the chamber was sealed with the lid immediately to prevent sample evaporation. The sealed chamber eliminated the evaporation induced SPR signal drift (due to increase of bulk refractive index), as shown in Figure 2(b). A 1 μl micro syringe with 0.02 μl precision was used to dispense the IgG droplet onto the sensor chip.

D. Immobilization of anti-IgG

Major steps of surface immobilization are shown in Figure S1.²⁵ The SPR sensor chips were BK-7 glass coverslips coated with 2 nm thick of chromium followed with 47 nm thick of gold. Prior to the surface functionalization, the chips were rinsed by deionized water and ethanol, blown dry by nitrogen, and cleaned by hydrogen flame. Next, the cleaned chips were incubated overnight in 1 nM mixed dithiol ethanol solution containing 50:1 PEG-OH/PEG-COOH (Figure S1b²⁵).¹⁵ The mixed self-assembled monolayer (SAM) coated chips were washed by deionized water and ethanol and blown dry by nitrogen before immobilizing the ligand protein. 0.5 ml freshly prepared deionized water solution containing 0.1 M NHS and 0.4 M EDC was deposited onto the chip surfaces to convert the-COOH group into active NHS ester receptors, which will react with the amino group of ligand protein to form an amide bond. After 20 min, the chips were thoroughly cleaned by deionized water, and then 10 μl of 20 $\mu\text{g}/\text{ml}$ anti-IgG dissolved in 20 mM NaOAc (pH = 5.5) was immediately applied to the chip surfaces and kept for 1–1.5 h to allow the protein bound. Finally, the chips were cleaned by deionized water again.

E. Conventional flow-through SPRI experiment

To validate the kinetics parameters obtained from the droplet-based measurement, we also measured the binding kinetics of IgG to anti-IgG with a conventional flow-through system on the same setup. A gravity-based multi-channel drug perfusion system (SF-77B, Warner Instruments, CT) was used to control the local solution on the sensing area, with a flow rate of 350 $\mu\text{l}/\text{min}$.

III. RESULTS AND DISCUSSIONS

A. Measure the association of IgG to anti-IgG in a droplet

The first challenge for droplet-based measurement is the evaporation of the droplet in the open air. The evaporation not only increases the salt concentration in the droplets but also changes the temperature of the sensing area. Since SPR is sensitive to both salt concentration and temperature, the evaporation causes significant signal drift and affects the

FIG. 1. Schematic illustration of the droplet-based measurement of protein interaction kinetics.

FIG. 2. Humidity control chamber: (a) Schematic illustration of the chamber; (b) SPR response for a droplet of PBS buffer with or without humidity control.

accuracy of protein interaction measurement. Furthermore, the evaporation will eventually dry out the droplet and terminate the binding reaction. Smaller droplets dry out faster. A $1\ \mu\text{l}$ droplet typically dries out in minutes, which are insufficient to complete the kinetic measurement. To solve this problem, we designed a humidity-controlled chamber to minimize sample evaporation (Figure 2). The droplet remains hydrated for over 2 h in the chamber, sufficient to complete the kinetic measurement.

After solving the evaporation challenge, we carried the binding experiment using a drop on a drop protocol as illustrated in Figure 1. The ligand protein (Anti-IgG antibody) was immobilized onto the sensor chip prior to the experiment. The functionalized sensor chip was assembled onto the prism, and a droplet of $0.5\ \mu\text{l}$ PBS was deposited onto the anti-IgG functionalized area, in order to activate the surface and to measure the baseline. The SPR angle was tuned to near the resonance angle for the buffer, and the droplet was shown as a dark spot on the chip (Figure 3(a)). After a stable baseline was recorded, the second droplet containing the analyte protein (IgG) was placed on top of the buffer droplet. The two droplets mixed together, and the association of IgG to the anti-IgG was measured. A control experiment (see Section 1 of the supplementary material for details²⁵) shows that the mixed dithiol PEG-OH/PEG-COOH SAM effectively eliminated the non-specific adsorption of analyte protein (IgG), since no measurable analyte binding was found on a ligand free PEG surface.

B. Calculating binding kinetics constants from the association phase

Conventional kinetic measurements need both the association phase and dissociation phase of the binding data to obtain all kinetic constants. To simplify the liquid handling requirement for droplet-based interactions, we eliminated the washing step and obtained all kinetic constants from the association curves at multiple analyte concentrations.

1. Equilibrium analysis for dissociation constant (K_D)

Figure 3(b) shows the SPR responses for different concentrations of analyte protein using averaged intensity in the center area of the droplet (yellow square in Figure 3(a)). The dissociation constant can be obtained from the equilibrium

SPR signal (R_{eq}) at steady state.¹⁶ For a given concentration of analyte protein, R_{eq} can be expressed as

$$R_{eq} = \frac{c * R_{max}}{c + K_D}, \quad (1)$$

where c is the concentration of the analyte protein, R_{max} is the maximum SPR signal obtained when all surface binding sites are occupied, and K_D is the equilibrium dissociation constant. Because of the limited volume of the droplet, we need to consider the depletion of analyte protein in the droplet during the binding process, i.e., the reduced concentration of analyte protein at equilibrium condition compared to that at the initial binding phase. Our analysis shows that smaller droplets have larger depletion (see Section 2 of the supplementary material for the analysis²⁵). For the droplet size in our test, the concentration of analyte at the end of association phase drops a few percent from the initial value (Table S1²⁵) and it is corrected before equilibrium analysis.¹⁷ The equilibrium constant (K_D) can be calculated from the equilibrium SPR signals through Equation (1) after correcting the analyte concentration (see supplementary material for the correction detail²⁵). As shown in Figure 3(b), $K_D = 74.9 \pm 4.0\ \text{nM}$ and $R_{max} = 103.1 \pm 16.9\ \text{mDeg}$ were obtained for the IgG/anti-IgG interaction. However, K_D is a static parameter describing the equilibrium state of the binding and does not provide any kinetic information, which is quantified by association rate constant (k_{on}) and dissociation rate constant (k_{off}).

2. Calculating association/dissociation rate constants from observed binding affinity (k_{obs})

During the association phase, the increase of SPR signal over time (R_t) can be expressed as

$$R_t = R_{eq} \left[1 - e^{-(k_{on} * c + k_{off})t} \right], \quad (2)$$

where t is the elapsed time. It is obvious that the association response curve contains information for both k_{on} and k_{off} . To extract the kinetics information, we define the observed binding constant (k_{obs}) as

$$k_{obs} = k_{on} * c + k_{off}. \quad (3)$$

Therefore, R_t can be expressed as

$$R_t = R_{eq} [1 - e^{k_{obs} * t}]. \quad (4)$$

FIG. 3. Extracting the equilibrium dissociation constants. (a) SPR image of the droplet. (b) Equilibrium analysis for the SPR responses at different concentrations. (Corrected droplet concentrations were used here to extract the equilibrium dissociation constant.) (c) The association curves for droplets with different concentrations of IgG binding to anti-IgG immobilized on the sensor surface. The color dots are experiment results, and the black curves are fitting results for observed binding affinity, k_{obs} . (d) Linear fitting of k_{obs} of all concentrations gives: k_{on} (slope), k_{off} (y-intercept), and $K_D = k_{off}/k_{on}$.

We first determined k_{obs} for each concentration by fitting the association response curve using Equation (4) (the black curves in Figure 3(c)). Next, we linearly fitted the observed binding constant (k_{obs}) for different concentrations of IgG (Figure 3(d)). According to Equation (3), the values of k_{on} and k_{off} were obtained from the slope and y-intercept of the fitted line, respectively.^{18,19} The obtained association and dissociation rate constants are $2.7 \pm 0.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $1.7 \pm 0.6 \times 10^{-3} \text{ s}^{-1}$, respectively, and a K_D value of 61.5 nM was obtained by $K_D = k_{off}/k_{on}$. This value is closely matched to K_D the equilibrium analysis described in the last paragraph, suggesting this fitting model is valid for our case.²⁰ See Section 4 of the supplementary material for additional details of the derivations.²⁵

3. Validating the result with conventional flow-through SPR

To validate the droplet based kinetic measurement results, we also measured the IgG/anti-IgG binding kinetics using the conventional flow-through method on the same SPR setup, with both association and dissociation phases, as shown in Figure 4. The kinetics parameters were calculated by global fitting of all sensorgrams based on a monovalent model for protein interaction. The binding kinetics obtained from the conventional flow-through method matches those from the droplet-based method as shown in Table I. The small differences in the rate constants are due to mass transport

limited reaction in the droplet and will be discussed in detail in Sec. III C.

The consumption of analyte protein in the droplet-based method is reduced about 7000 folds compared to that in the conventional SPR system. The amount of ligand protein needed for the droplet-based method can also be reduced

FIG. 4. Conventional flow-through SPR kinetic measurement results. Sensorgrams for different concentrations of IgG binding to anti-IgG immobilized on the sensor surface. The values of $(3.1 \pm 1.0) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $(1.1 \pm 0.3) \times 10^{-3} \text{ s}^{-1}$, and $(34.3 \pm 6.2) \text{ nM}$ for k_{on} , k_{off} , and K_D , respectively, were obtained according a 1:1 model of protein interaction (the black line was the fitting result for each concentration).

TABLE I. Comparison of the binding kinetics obtained by conventional flow-through and droplet-based SPR.

	Conventional SPR	Droplet-based SPR
$k_{on}(M^{-1}s^{-1})$	$(3.1 \pm 1.0) \times 10^4$	$(2.7 \pm 0.7) \times 10^4$
$k_{off}(s^{-1})$	$(1.1 \pm 0.3) \times 10^{-3}$	$(1.7 \pm 0.6) \times 10^{-3}$
K_D (nM)	34.3 ± 6.2	61.5 ± 26.9 (74.9 ± 4.0) ^a
Sample consumption (μg)	280	0.04

^aThe value in the bracket is from equilibrium analysis.

to similar level when the ligand immobilization is also using a droplet-based approach, which can be realized by integrate the SPR system with an ultra-low volume liquid dispensing device.^{14,21,22}

C. Mass transport limited reaction in the droplet

As shown in Table I, after correcting the average concentration of analyte protein at equilibrium condition, the droplet-based approach still gives higher K_D value. We believe that this effect is due to mass transport limited local-depletion of free analyte near the sensor surface, $[A]_{free}$, as illustrated in Figure 5(a). For a given R_{eq} , the average analyte concentrations in the droplet (blue line) used for kinetics analysis are higher than analyte concentration near the sensor surface calculated from the kinetic constants obtained from conventional flow-through system (red dashed line).

Another evidence of local-depletion is that the binding rate within the droplet becomes location-dependent as the analyte concentration drops. Figure 5(b) displays the binding signal distribution in the droplet of 33.3 nM IgG at the equilibrium condition, and Figure 5(c) (red circles) shows the binding signal profiles along the center lines (dashed lines in 5(b)) of

the droplet. These plots clearly show that the center of the droplet has higher IgG binding signal than the edge.

To understand the differences of kinetics constants between two methods and the location-dependent phenomenon in the droplet, we developed a two-dimensional model using COMSOL Multiphysics to simulate the change of IgG concentration in the droplet during the binding process. The model includes two modules: transport of diluted species and surface reactions. The former is to simulate the diffusion of IgG molecules in the droplet, and the latter is to simulate the IgG/Anti-IgG binding reaction on the sensor surface. These two parts were coupled by the following equation:²³

$$\frac{\partial c}{\partial t} + \nabla \cdot (-D \nabla c) = k_{on} * c * ([B] - c_s) - k_{off} * c_s, \quad (5)$$

where c is the concentration of IgG for the binding reaction, which changes over time. D is the diffusion coefficient of IgG in the media, $[B]$ is the concentration of anti-IgG immobilized on the sensor surface, and c_s is the surface concentration of IgG indicating the amount of IgG associated to anti-IgG. And t , k_{on} , and k_{off} were defined previously. See Section 5 of the supplementary material for further details of the simulation.²⁵

Through this model, we simulated the concentration gradient of IgG in the droplet and on the sensor surface. The results confirmed the local-depletion of analyte due to a mass transport limited binding kinetics, which depends on the binding rate, diffusion rate of analyte molecules, and the shape and size of the droplet. As shown in Figures 5(d) and 5(e), when the binding rate is faster than the diffusion rate, especially at the beginning of association phase (e.g., $t = 100$ s), probe molecules near the surface and at the edge will deplete faster than those at the center of the droplet, resulting in lower local-concentration of the analyte near the sensor surface that in the bulk, and lower edge concentration than the center area. The former resulted in the larger K_D value obtained from

FIG. 5. Edge effect in the droplet-based reaction. (a) The actual analyte concentration near the sensor surface (red dashed line) is lower than the averaged droplet concentration used to calculate the binding kinetic constants for the droplet-based method (blue line) due to the limit of mass transportation rate. (b) Differential SPR image for the 33.3 nM IgG droplet at the end of the 900 s association phase. Scale bar: 500 μm . (c) Normalized SPR intensity profile along the central lines (dashed lines in (b)) of the droplet image, where the red circles are the raw data points and the blue line is the simulation result by a COMSOL program. These intensity are normalized by the one in the central point where $x = 0$. (d) and (e) Concentration distribution of analyte protein in the droplet after association of 100 s (d) and 900 s (e) shows local-depletion of the analyte near the sensor surface and in the edge. The corrected analyte concentrations in the center area at the maximum binding state ($t = 900$ s) are used to extract the binding affinity for droplet-based approach.

the droplet reaction, and the later induced the weaker binding response at the droplet edge. Comparing Figures 5(d) and 5(e), we can see that as the reaction reaching equilibrium state ($t = 900$ s), the local-depletion effect becomes less pronounce in the center area. Therefore, we use only the center area to calculate the rate constants to minimize the mass transport limited reaction caused error.

Based on above analysis, the difference between the binding kinetics obtained from the droplet-based method and those from conventional flow-through method is caused by two factors: (1) the reduction of the average concentration of analyte protein in the droplet after binding due to limited droplet volume and (2) the local analyte depletion near the sensing surface, due to mass transport limited reaction in the droplet. The former could be corrected with the binding signal at equilibrium condition (see Section 3 of the supplementary material for details²⁵). The latter is binding rate dependent and would vanish with smaller droplets (see Section 6 of the supplementary material for details²⁵) or decreased contact angle of the droplet on the sensor chip.²⁴

IV. CONCLUSION

We demonstrated a novel method to measure protein interaction kinetics in a single droplet that reduces the sample volume requirement by 3 orders of magnitudes than conventional flow-through kinetic measurement. A droplet with sub microliter or less volume in a humidity-controlled environmental chamber was replacing the microfluidic channels as the reactor for the protein interaction. The binding process in the droplet is measured by a SPRi setup. All kinetic constants of IgG/anti-IgG interaction were obtained from the association phase of the interaction at multiple concentrations of the analyte. The results were validated by conventional flow-through kinetic measurements using the same setup. The slightly larger dissociation constant obtained from the droplet method was caused by volume limited analyte depletion and mass transport limited surface analyte depletion. The formal was corrected

with calibration and the later would diminish with smaller size droplet or decreased droplet contract angle. This droplet-based method opens the door for high-throughput protein interaction study in a droplet-based microarray format. Both target and probing proteins can be delivered to the sensor surface via an integrated liquid dispenser, and many to many interactions in a single microarray chip can be realized easily.

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