Detection of biomolecular binding by Fourier-Transform SPR

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***Abstract*— Surface plasmon resonance (SPR) is a widely used label-free detection technique that has many applications in drug discovery, pharmacokinetics, systems biology and food science. The SPR technique measures the dynamics of a biomolecular interaction at a surface, yielding kinetic association and dissociation constants. Present SPR systems measure the step response of the interaction in time domain hence are subject to time-varying noise disturbances and drifts that limit the minimum-detectable mass changes. This paper presents a new synchronous SPR technique that measures the biomolecular interaction not in time domain, but in frequency domain with a high degree of rejection to uncorrelated spurious signals. The new technique was implemented using a PDMS microfluidic chemical signal modulator chip connected to a set of on-chip functionalized Au SPR sensing sites. Preliminary experimental spectral data for a model system of carbonic anhydrase binding demonstrates the feasibility of the new spectral technique.**

Keywords-surface plasmon resonance; Fourier analysis; microfluidics

I. INTRODUCTION

The measurement of interactions between biological molecules is of fundamental importance in the life sciences. The most common measured interaction is the binding of two chemical entities. For example one molecule (a ligand) can be immobilized on a surface while exposed to a dissolved analyte binding partner (denoted as ligate). Detection of binding is most commonly achieved by labeling either entity with radioactive or fluorescent markers. Label-free optical detection methods have become increasingly popular within the last decade because they avoid disturbances from conjugated markers or handling of radioactive materials. In particular, label-free optical techniques such as surface plasmon resonance (SPR) permit the study of biological interactions in real time also yielding quantitative kinetic parameters [1].

The most common type of SPR measurement is a single association-dissociation step depicted in the example sensorgram of Fig. 1 with corresponding association and dissociation constants k_a and k_d. During the cycle, the ligand-ligate complex obeys the differential equation

$$\frac{dC}{dt} = k_a \cdot (B_o - C) \cdot A - k_d \cdot C \tag{1}$$

where B_o is the surface concentration of the undisturbed immobilized ligand, A is the ligate concentration and C is the surface concentration of the ligand-ligate complex [2]. For a sudden step change in the ligate concentration of duration T_p, solutions of Eq. (1) obey the piecewise exponentials

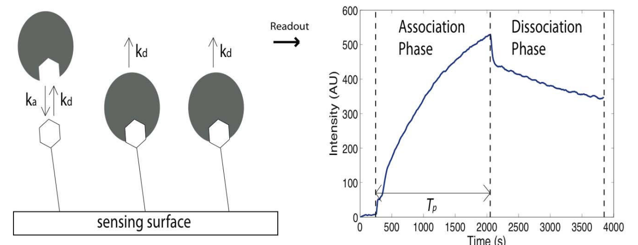

Fig. 1: Diagram of chemical reaction and sensor response to single association and dissociation steps.

$$C = \left(\frac{k_a \cdot B_o \cdot A}{k_d + k_a \cdot A}\right) \cdot \left(1 - \exp\left(-(k_d + k_a \cdot A) \cdot t\right)\right) \tag{2}$$

for $0 < t < T_p$, and

$$C = \left(\frac{k_a \cdot B_o \cdot A}{k_d + k_a \cdot A}\right) \cdot \left(1 - \exp\left(-(k_d + k_a \cdot A) \cdot T_p\right) \cdot \exp(-k_d \cdot (t - T_p))\right) \tag{3}$$

for $t > T_p$. In the step response measurement method the SPR signal is recorded as a function of time, and the signal is fitted to the exponentials to determine the kinetic constants. Because of the presence of substantial noise and systematic drifts as shown in the noisy sensorgram of Fig. 2 below, this fit scheme is only able to measure relatively coarse interactions between ligate and ligand. In drug discovery applications, this is a major limitation, as often drug targets are large proteins with masses of 10^4-10^5 Da or larger, and the interaction of interest is often triggered by the binding of a very small ligate (300 Da or less). To increase the sensitivity and response of these systems, Biacore has introduced several improvements including sophisticated referencing, temperature control and high-density

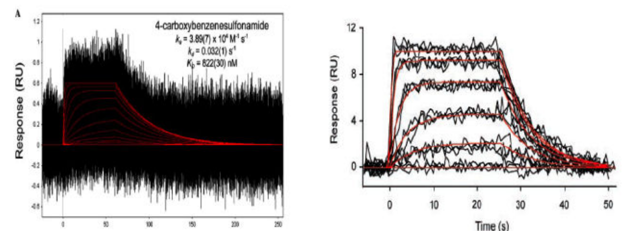

Fig. 2: Examples of noisy sensorgrams and exponential fits [2,3].

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binding sites (as the SPR sensor detect changes roughly within 100 nm off the surface). In spite of the commercial availability of Biacore systems (with over 1000 systems deployed worldwide), these systems have not been able to sense interactions of small ligates, presently 100 Da being the minimum detectable mass.

Key to improvements in SPR based systems is the development of techniques that reject experimental noise and disturbances.

II. FOURIER TRANSFORM SPR

An improved detection scheme can be implemented in linear systems if the excitation signals and the system responses are highly correlated [4]. If the test signal has a sharp autocorrelation the input signal and the system output are nearly orthogonal to all signals except themselves hence rejecting noise sources. An example of such approach is the Fourier-transform detection scheme shown in the block diagram of Fig. 3. In this method a periodic excitation signal of constant frequency given by clock ϕ is used to excite the biochemical system. The corresponding association and dissociation cycles produce a highly correlated sensorgram output of the same frequency, and at steady state of constant amplitude. The correlated sensorgram thus can be easily detected even in the presence of high levels of additive uncorrelated disturbances and noise. The scheme can be interpreted in frequency domain by plotting the power spectrum of the SPR response as shown in Fig. 4.

While the random noise and disturbances have a broadband spectra, the dissociation and association kinetics are modulated at a single frequency showing as a spike in the spectrum that can be easily discriminated. By repeating the procedure at different discrete frequencies one may obtain the transfer function of the biochemical system

$$H(j\omega) = \frac{Y(j\omega)}{X(j\omega)} \quad (4)$$

If the biochemical interaction is determined by Eq. (1) the transfer function is directly related to the kinetic constants k_a and k_d. It is easily shown that $H(j\omega)$ has a characteristic roll-off pole behavior

$$H(j\omega) = \frac{k_a \cdot B_o}{(k_d + k_a \cdot A_o)} \cdot \frac{1}{(1 + j\omega / p_1)} \quad (5)$$

with pole p_1

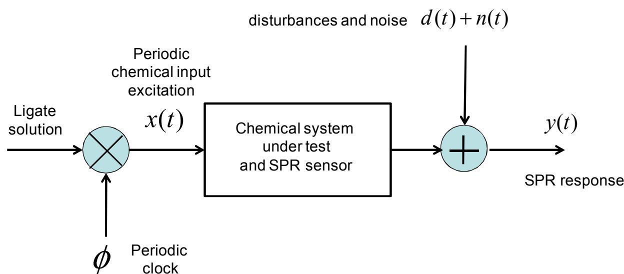

Fig. 3: FT-SPR detection scheme. The input is a periodic cycle of association and dissociation steps determined by clock ϕ.

$$p_1 = k_d + k_a \cdot A_o \quad (6)$$

Therefore we can directly measure the kinetic constants by repeating the transfer function measurements at different ligate concentrations. This is the basis for the FT-SPR methodology.

The FT-SPR technique requires sweeping of the clock ligate modulation frequency and the generation of a stream of high-frequency ligate plugs exposed to the SPR surface. In order to create such short fast plugs it is necessary to use microfluidic chips which permit the transport of time-dependent chemical signals with low dispersion [5]. We implemented such system as discussed below.

III. MATERIALS AND METHODS

A. Surface Plasmon Resonace system

A GWC Technologies SPRimager®II system was used to collect the data. The system was modified by the manufacturer to be used in a horizontal configuration, with a custom built microfluidic mounting cell. We further modified the instrument with an 805 nm laser, a Navitar Zoom 6000 lens system, and a Hamamatsu EMCCD camera.

B. Microfluidics

The PDMS microfluidic channels use a two-layer system to create valves for switching between buffer and analyte [6]. First the polymer is molded over photolithographically defined patterns using SU-8 and AZ9260 photoresists. Then each layer is cleaned and exposed to oxygen plasma for 12 seconds at a pressure of 400 mTorr in a March Plasmod system. The layers are finally aligned and bonded together using an in-house built alignment tool. There are two sets of channels in each chip, one to act as a reference (control) and the other for detection of the analyte of interest (sensor).

C. Gold/glass substrate

SF10 glass substrates in a 2x2" square were purchased from Schott glass. These were chosen to match the SF10 prism used in the GWC system. The glass is cleaned by piranha etch (3:1, H_2SO_4:H_2O_2) for 20 minutes, rinsed for 5 min in 18 MΩ deionized (DI) water, and dried under N_2. The substrates are placed in an oven at 80 °C for 10 minutes before being transferred to a TMV sputter deposition system. A 3 nm adhesion layer of Ti/W is deposited, followed by 40 nm of gold. The metal layers on the substrate are patterned using photolithography to make an array of 140 x 180 µm rectangles. The metals are etched and the samples rinsed thoroughly with 18 MΩ DI water after each etch step to prevent contamination.

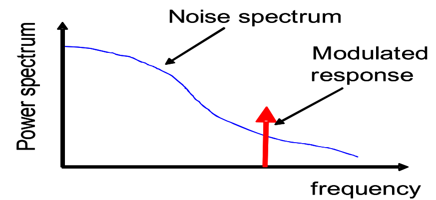

Fig. 4: Power spectra of FT-SPR sensorgram. The discrete frequency modulated response can be discriminated from the broadband noise spectrum.

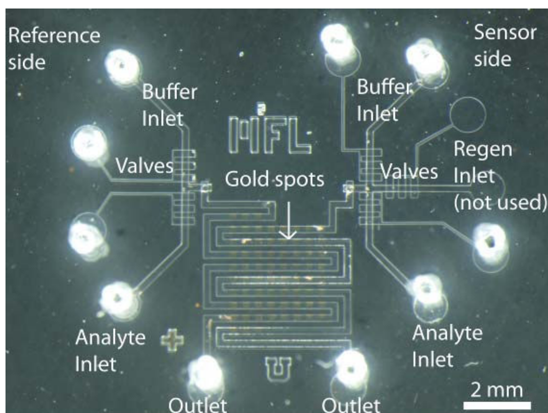

Fig. 5: Image of the assembled FT-SPR chip, with the patterned gold on the SF10 glass substrate.

Finally, the photoresist is removed and the patterned substrates stored in a desiccator until used.

D. SPR microfluidic chip assembly

Before assembly of the FT-SPR microfluidic chips the patterned glass substrates are further cleaned by flame annealing with a butane microtorch. This is followed with ultrasonic cleaning in methanol for five minutes. The methanol is replaced with fresh solution and the substrate sonicated a total of three times. The dual layer PDMS channels are drilled to make through holes for fluid access, and then cleaned with acetone, IPA, and DI water. The PDMS is dried with an air gun and then baked for 10 minutes on a hot plate at 100 °C, followed by a cooling step for 10 minutes. At this point, both the glass substrate and PDMS channels are placed in the oxygen plasma cleaner for 12 seconds at the same settings mentioned previously. Alignment is performed to coordinate the channels with the patterned gold surface, and then the assembled chip is baked, again for 10 minutes at 100 °C.

E. Gold spot functionalization

Surface chemistry modification is performed *in situ* to enable the bonding of the PDMS and glass substrate. The assembled chip is connected to a syringe pump and flushed with 0.01 M HCl for 5 minutes to remove any contaminants or oxide, followed by a 15 minute rinse with DI water. A mixture of long and short chain polyethylene glycol (PEG) molecules is used to provide both a capture surface and one that is protein resistant, following the protocol described by Uchida [7]. A 5 kDa carboxymethyl-PEG-thiol molecule from Laysan Bio is introduced to the flow channels for 20 minutes, followed by a 0.05 M NaOH rinse. Then a 2 kDa methoxy-PEG-thiol is passed through the channels and rinsed similarly. The smaller PEG is applied a total of three times to backfill any gaps in the initial PEG layer and prevent protein adsorption on the gold surface. The surface is again treated with 0.01 M HCl and rinsed with DI water to ensure the proper chemical functionality for the next step.

A chemical modification of the carboxy-terminated PEG chains is performed using sulfo-*N*-hydroxysuccinimide (S-NHS) and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC) to create an amine reactive surface. S-NHS at 100 mM and EDC at 400 mM in water are mixed 1:1 and passed through both the reference and sensing channels for 30 minutes. The system is rinsed with phosphate buffered saline (PBS) for two minutes, and then the capture molecule is immobilized to the sensor surface. For this project we have chosen to immobilize aminoethyl-benzenesulfonamide (ABS), which will react with the analyte, carbonic anhydrase (CA), similar to the chemistry described by Lahiri [8]. This molecule is passed over the sensing surface, while only the buffer is passed over the reference surface, illustrating the need for the dual channel system. Finally, both surfaces are blocked with ethanolamine at pH 9 for 30 minutes and rinsed with PBS.

F. Experiments

With the chemistry in place the assembled chip is mounted in the SPR system cell. Refractive index matching fluid with a value of 1.72 is used to couple between the prism and glass substrate. Microfluidic connections are made to the valves and flow channels, and then the valves filled with water, due to the gas permeability of PDMS.

The valves are actuated by a pressure system at 25 psi and controlled with in-house code. Syringes containing analyte, CA, at 6.64 µM (200 µg/mL) concentration and buffer are pressurized as well with a constant source at 10 psi which corresponds to a flow rate of 16 cm/s. Experiments are performed by running multiple cycles of a square wave input at different frequencies, from 5 minutes down to 1 minute. A total of 10 cycles is collected at each frequency. Wasabi camera control software from Hamamatsu is used to analyze the collected data by selecting multiple sensing and reference spots in the image. The mean intensity value in each spot is determined for all of the frames in the experiment. Fourier transform analysis of the data is performed in MatLab.

IV. RESULTS AND DISCUSSION

Fig. 6 shows example sensorgrams of a 5 minute analyte/5 minute buffer cycle which corresponds to an excitation frequency of 1.7 mHz. The reference surface signal grew slightly, due to nonspecific adsorption of protein on the surface. However, this was very minimal compared to the signal generated on the sensor surface. Also, the reference surface did not show any decay when buffer was flushed through the system during the dissociation phase of the initialization. This verifies the occurrence of nonspecific adsorption, compared to the sensor surface where there was a much larger signal change indicative of a reversible reaction.

Fourier transform analysis is used to determine the magnitude of the transfer function $\|H(j\omega)\|$ at the excitation frequency from the magnitude of the measured power spectrum (Fig. 7). $\|H(j\omega)\|$ is next plotted versus each excitation frequency as shown in the Bode plot of Fig. 8. From this plot we can determine the characteristic roll-off pole which corresponds to the kinetics of the biochemical reaction under test. For this experiment the pole p occurs near a frequency of 2.1 mHz. This matches very well with the pole calculated from the single-step determined values of k_a and k_d for this surface of 1.3×10^2 $M^{-1}s^{-1}$ and 1.4×10^{-3} s^{-1} respectively, which would

yield a value of p = 2.3 x 10^{-3} s^{-1} at this concentration. However, the experimental pole is lower than its calculated values based on other reported values of k_a and k_d for this system [8], which assuming a concentration, A, of 6.64 µM, ranges from 15 to 0.033 s^{-1}. The shorter pole value we determined could be attributed to the different surface chemistry used from those previously reported [7,8].

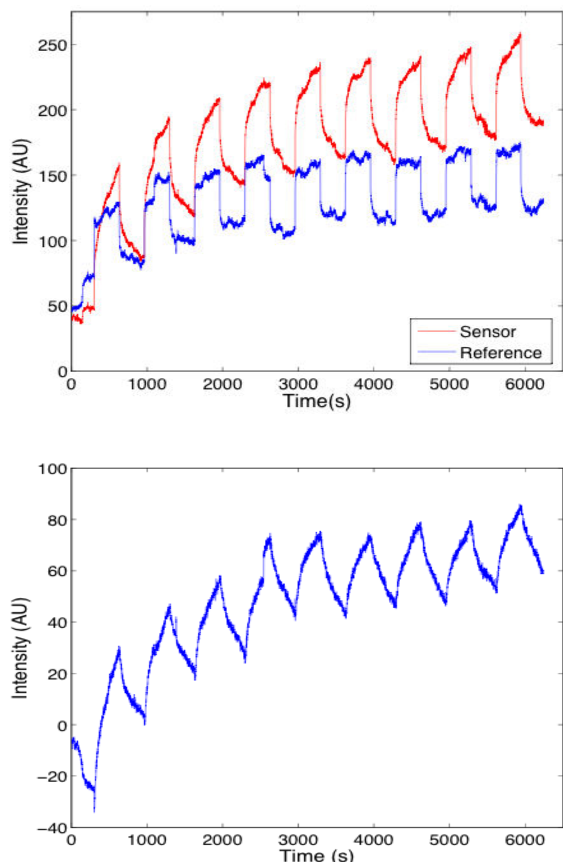

Fig. 6: Sensorgrams of the 5 minute, or 1.7 mHz, buffer and analyte cycles. Top: The average of reference (control) and sensor spots. Bottom: The difference of the averages.

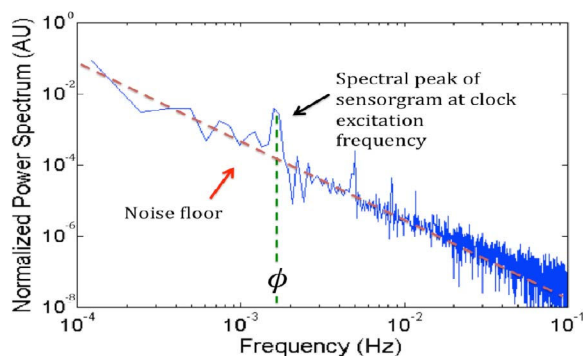

Fig. 7: Power spectrum of sensorgram when excited by periodic clock signal at 1.7 mHz for 100 min. The amplitude of the transfer function can be determined from the sensorgram spectra at the excitation frequency ϕ.

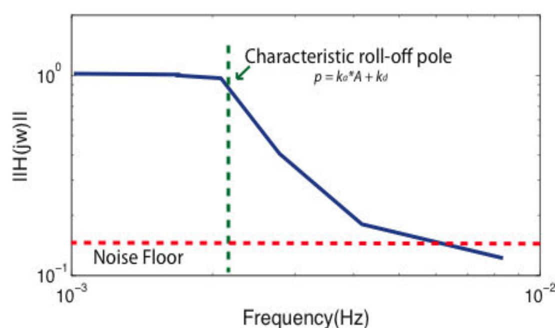

Fig. 8: Bode plot of transfer function magnitude versus excitation frequency for the experimental data. The pole corresponds to the values of the dissociation constant, k_d, the association constant, k_a, and the concentration of analyte, A, as shown in Eq. (6).

V. CONCLUSION

We have demonstrated a new SPR detection method that measures kinetic binding constants based on periodic chemical excitation or repeated association and dissociation cycles at a specific frequency. This method permits the measurement of the binding kinetics from the Fourier power spectrum of the recorded response at the excitation frequency. The new frequency-domain technique is immune to the effects of broadband noise and disturbances that adversely affect conventional step-response time-domain methods. The chemical excitation is provided via a PDMS microfluidic chip signal generator. Using this methodology we measured the binding kinetics of a CA-reactive PEG test surface with characteristic role-off pole of 2.1 mHz consistent with the single step calculated value of p = 2.3 x 10^{-3} s^{-1} for the concentration used in the experiment.

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