Analysis of Biomolecular Interactions Using a Miniaturized Surface Plasmon Resonance Sensor

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A commercially available miniaturized surface plasmon resonance sensor has been investigated for its applicability to biological interaction analysis. The sensor was found to exhibit excellent repeatability and linearity for high-refractive index solutions and good reproducibility for the binding of proteins. Its detection limit for the monoclonal antibody M1 was found to be 2.1 fmol, which corresponds to a surface concentration of 21 pg/mm^2. Simple surface immobilization procedures relying on biotin/avidin or glycoprotein/lectin chemistry have been explored. Equilibrium dissociation constants for the binding of the FLAG peptide to its monoclonal antibody (M1) and for the binding of concanavalin A to a glycoprotein have been determined. The close agreement of these measurements with values obtained by surface fluorescence microscopy and fluorescence correlation spectroscopy helps to validate the use of this device. Thus, this sensor shows promise as an inexpensive, portable, and accurate tool for bioanalytical applications in laboratory and clinical settings.

Surface plasmon resonance (SPR) spectroscopy is a powerful tool for the investigation of biomolecular interactions. The technique's main advantage over more conventional assays of molecular recognition, such as the enzyme-linked immunosorbent assay (ELISA), is its ability to monitor binding rapidly, in real time, and without labels. Information about specificity, affinity, and kinetics can be extracted from an SPR analysis. Diverse biomolecules, including membrane-bound and serum proteins, nucleic acids, and lipids have been examined with SPR. The principles and applications of SPR have been extensively reviewed.[1−4] Recent applications of SPR to biological systems include the functional immobilization of the G protein-coupled receptor rhodopsin,[5,6] the monitoring of binding to a DNA array by spatially resolved SPR (SPR imaging),[7,8] and the detection of protein conformational changes.[9,10] SPR has also been incorporated into hyphenated analysis systems by coupling to liquid chromatography[11,12] and to mass spectrometry.[13]

The most widely used commercial SPR instrument is the BIAcore (Biacore AB, Uppsala, Sweden).[14−16] It combines high-quality optical components, an integrated fluid handling system, and a collection of sophisticated surface chemistries. Although the BIAcore achieves excellent detection limits, stability, and automation, its size and cost restrict its use. To explore the possible application of SPR sensing in a smaller, inexpensive, and portable format, Texas Instruments (TI) developed the Spreeta sensor in the late 1990s.[17,18] According to its developers, the sensor could be used for distributed sensing for applications in environmental monitoring, quality control, and food and water safety. Since its introduction, the Spreeta has found application in nonlaboratory settings, and there have been a few reports of its use for immunosensing and toxin detection.[19,20] To date, however, the ability of the Spreeta to obtain high-quality information about biological interactions, such as protein−protein dissociation constants, has not been reported.

In this work, we have investigated the ability of the Spreeta to monitor protein−protein interactions in real time. We used the

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system to determine dissociation constants (K_d) for two systems: (1) the binding of the lectin concanavalin A to the carbohydrate moieties of the glycoprotein avidin, and (2) the binding of the anti-FLAG peptide monoclonal antibody M1 to a FLAG-β_2 adrenergic receptor fusion protein (FLAG-β_2AR). Relatively simple surface chemistries were found to be sufficient to obtain K_d values that agree with previously published values or with values determined in our laboratory by the complementary techniques of fluorescence correlation spectroscopy (FCS) and surface fluorescence microscopy.

EXPERIMENTAL SECTION

Miniature SPR Sensor. The Spreeta miniature SPR sensor (Texas Instruments, Dallas, TX) has been described in detail elsewhere by its manufacturers[17,18] and others.[19,20] Briefly, the small (1.5 × 3.0 × 4.0 cm) sensor contains a miniaturized and integrated SPR system. Near-infrared light (840 nm) from a light-emitting diode passes through a polarizer, reflects off the back of the gold sensing surface, and is directed onto a linear array of silicon photodiodes. Each detection pixel corresponds to a narrow range of incident angles, and the signals arising from the reflected light are monitored to determine the minimum signal intensity versus scattering angle, that is, the minimum of the surface plasmon resonance curve. All of the components are immobilized in an optically transparent material. The sensor was interfaced to an A/D converter that processed the signal and sent it to a PC for display and analysis.

Sample Handling and Flow System. A flow cell was attached to the front of the sensor to allow the introduction of solutions to the gold sensing surface. Two different flow cells were used. In each case, a rectangular channel cut into a rubber gasket defines the volume of the cell.. The flow cell provided by TI with the Spreeta had a total volume of ~15 μL (neglecting volume changes resulting from compression of the gasket). A smaller flow cell was designed in-house. In this design, the flow cell volume was reduced to ~3.5 μL (before compression) by using a thinner rubber mat and changing the position of the liquid inlet and outlet ports.

Solutions were delivered to the surface either with or without flow. For static experiments (without flow), the attached needle of a 0.5-mL tuberculin syringe was fitted with two concentric lengths of PE tubing, the larger piece of which fit into the inlet port of the flow cell. Buffer or protein-containing solution was then manually injected into the flow cell. Approximately 25 μL of protein solution was injected to ensure that the entire sensing surface was covered. Refractive index (RI) changes of the solution adjacent to the surface were monitored over time. Protein solutions were allowed to remain in contact with the surface until a stable RI value indicated that the binding had reached equilibrium. Protein-free buffer solutions (0.25 mL) were then injected to rinse off non-specifically bound proteins. For the flow-based experiments, a Harvard Apparatus PHD 2000 programmable syringe pump (Holliston, MA) was used to continually flow buffer over the sensing surface. Protein solutions were loaded into the 100-μL sample loop of a 2-position, 6-port Cheminert switching valve (VICI, Houston, TX) and then injected into the flow cell. Temperature was not actively controlled.

Protein Immobilization for SPR Measurements. The general immobilization scheme consisted of depositing layers of BSA−biotin, avidin (or streptavidin), then various proteins of interest. BSA−biotin was purchased from Pierce (Rockford, IL). Streptavidin, avidin, concanavalin A type V (Con A), human IgG, goat anti-human IgG, and M1 anti-FLAG antibody were obtained from Sigma (St. Louis, MO). All proteins were divided into aliquots and stored at −20 °C without further purification. For experiments involving Con A, all samples were prepared in Con A buffer [phosphate buffered saline (PBS) with 5 mM $MgCl_2$, 5 mM KCl, and 2 mM $CaCl_2$, pH 7.4], which was also used as the run buffer. Control experiments on Con A included 200 mM methyl αD mannopyranoside (Sigma, St. Louis, MO). For the binding experiments using β_2AR, all samples were prepared in HS/DDM/Ca buffer [0.5 M NaCl, 20 mM Tris, 0.1% *N*-dodecyl-β-D-maltoside (DDM; Anatrace, Maumee, OH), 2 mM $CaCl_2$, pH 7.4], which was also the run buffer. In some control experiments for the FLAG-β_2AR system, FLAG peptide (sequence DYKDDDDK, synthesized at the Stanford University PAN facility, Stanford, CA), EDTA (Fisher Scientific, Pittsburgh, PA), or both were added to the run buffer and all sample solutions.

Deposited protein on the sensor surface was removed in one of two ways: (1) by rinsing with 100 mM NaOH in Milli-Q water containing 1% Triton X-100 detergent followed by rinsing several times with water or (2) by gently wiping the gold surface with a Kimwipe wetted with 70% ethanol, followed by flushing with water. Both cleaning procedures effectively removed all the layers of immobilized and bound protein, as indicated by a return of the measured RI of pure water to 1.3330.

Expression, Purification, and Labeling of β_2AR. Expression of human β_2AR in Sf-9 insect cells, membrane preparation, solubilization, and purification were performed as described by Ghanouni et al.[21] As expressed, β_2AR includes the FLAG peptide at its N terminus, and the names β_2AR and FLAG-β_2AR will be used interchangeably. For SPR measurements, purified, unlabeled receptor was used in HS/DDM/Ca buffer. For fluorescence labeling, purified β_2AR (1 μM) was mixed with an equimolar concentration of tetramethylrhodamine-5-maleimide (TMR; Molecular Probes, Eugene, OR) in HS/DDM (0.5 M NaCl, 20 mM Tris, 0.1% DDM, pH 7.4) to produce TMR-labeled receptor (TMR−5-β_2AR). After incubating for 2 h at room temperature, the labeling reaction was terminated by adding a 100-fold excess of cysteine. ALP-affinity chromatography was used to separate functional from nonfunctional β_2AR, as previously described.[22] The eluent from the ALP-resin was purified by Ni chromatography using nickel-chelating Sepharose from Pharmacia (Uppsala, Sweden) then dialyzed against HS/DDM buffer.

Biotinylation of M1 Antibody. A portion of 50 μM M1 antibody was incubated with a 12-fold molar excess of EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL) in 100 mM NaCl, 10% glycerol, 50 mM Hepes, pH 8.0 for 1 h at room temperature. Excess labeling reagent was removed by dialysis against HS buffer (0.5 M NaCl, 20 mM Tris, pH 7.4).

Comparison with Fluorescence Microscopy and Fluorescence Correlation Spectroscopy. To validate the SPR measurements obtained with the Spreeta, we have carried out fluorescence studies. The details of the fluorescence setup and the description

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of how the fluorescence measurements were recorded have been placed in Supporting Information.

Fitting of Surface Binding Data. In treating the SPR data for the Con A/glycoprotein system, we used a previously published equation that is based on the equilibrium sensor response and accounts for the multivalence of the Con A in solution.[23]

In the treatment of the data for the FLAG/M1 system, we assumed that there is no cooperativity between the two binding sites of the M1 antibody. When the antibody is immobilized, all binding sites on the surface are treated equally. The dissociation constant is, therefore, given by

$$K_d = \frac{[R_f][B_f]}{[RB]} = \frac{([R_t] - [RB])([B_t] - [RB])}{[RB]} \quad (1)$$

where $[R_f]$ and $[B_f]$ are the free and $[R_t]$ and $[B_t]$ are the total FLAG-β_2AR and antibody binding site concentrations (twice the antibody concentration), respectively. [RB] represents the concentration of receptor−antibody complexes formed. In SPR and surface fluorescence microscopy experiments, the measured response is assumed to be proportional to [RB]. The measured response, I, depends on the concentration $[R_t]$ and can be expressed as

$$I([R_t]) \propto [RB] = \frac{1}{2}([B_t] + [R_t] + K_d - \sqrt{([B_t] + [R_t] + K_d)^2 - 4[B_t][R_t]}) \quad (2)$$

This binding curve accounts for the depletion of FLAG-β_2AR from solution and is used to fit the surface data (SPR and fluorescence) using weighted least-squares. In the SPR experiments when the sample solution is continually flowed over the surface, the free FLAG-β_2AR concentration can be assumed to be constant and equal to the injected concentration, $[R_{inj}]$, and eq 2 simplifies to

$$[RB] = \frac{[R_{inj}][B_t]}{K_d + [R_{inj}]} \quad (3)$$

RESULTS AND DISCUSSION

Linearity and Repeatability of the SPR Sensor Response. A series of aqueous KCl solutions were injected into the flow cell, and the sensor response was monitored. Because KCl solutions have a higher refractive index than water, the position of the surface plasmon resonance curve shifts when the sensing surface is exposed to KCl. The sensor response was linear with concentration over a range from 10 mM (RI = 1.3332) to 1 M KCl (RI = 1.3447). Subsequent injection of water through the flow cell led to a recovery of the baseline water signal (RI = 1.3330). This is consistent with the expectation that the refractive index change from KCl results entirely from bulk solution effects and not from binding of analytes to the gold sensing surface.

To assess the repeatability of the sensor response, water was continually flowed over the sensor at 50 μL/min, and 100 μL of PBS was injected into the flow cell. These injections were repeated 10 times, and the sensor response was recorded. Over the course of 2500 s, the refractive index of the baseline increased by 1×10^{-4}. After correcting for baseline drift, the mean peak response was calculated to be 1.80×10^{-3} with a standard deviation of 1×10^{-5} , or 0.5% of the mean, indicating good repeatability for injections of high refractive index solutions that do not bind to the surface.

Reproducibility of the Sensor Response to Protein Binding. To immobilize proteins, the gold sensing surface is first saturated with bovine serum albumin labeled with biotin (BSA−biotin). BSA−biotin has several advantages as an initial layer: it adsorbs spontaneously to gold, is stable during buffer washes, prevents nonspecific binding of subsequent protein layers, leaves a layer of biotin residues available for reaction with avidin (or streptavidin), and can be completely removed by rinsing with NaOH or ethanol. Addition of excess avidin creates a stable layer of avidin that can then bind another layer of biotinylated protein or interact with a lectin, such as Con A, through its carbohydrate moieties. We have employed both biotin/avidin and lectin/glycoprotein interaction to immobilize the proteins of interest.

To assess the reproducibility of protein binding using our immobilization scheme, solutions of BSA−biotin, streptavidin, and M1−biotin were injected above a clean gold sensing surface. In replicate experiments ($n = 8$), the mean refractive index changes caused by protein binding were $(1.4 \pm 0.3) \times 10^{-4}$ for BSA−biotin, $(6 \pm 1) \times 10^{-4}$ for streptavidin, and $(1.4 \pm 0.8) \times 10^{-4}$ for M1−biotin. The standard deviation is around 20% of the mean for BSA−biotin and streptavidin. Variations in the amount of refractive index change is assumed to reflect differences in the amount of protein immobilized and can be attributed to different initial states of the gold surface, to aggregation, and to nonspecific binding of the proteins. For avidin and streptavidin in particular, we observed that solutions that had been stored at 4 °C for more than 1 day exhibited large amounts of nonspecific binding. Fluorescence microscopy of these solutions indicated significant protein aggregation.

Detection Limits. To determine the Spreeta sensor's limit of detection for a particular protein, we first measured a blank sample by injecting PBS into the flow cell and recording the response for 45 min. The signal was stable over this time with a standard deviation of 5.6×10^{-6}. This deviation in the baseline signal is comparable to the short-term noise levels previously reported for an integrated SPR sensor of this kind.[18] A calibration curve was prepared by measuring the refractive index changes caused by the injection of a series of dilute M1−biotin solutions above a sensing surface that had been prepared with BSA−biotin and streptavidin. Owing to the high affinity of the biotin/streptavidin interaction ($K_d = 1 \times 10^{-15}$ M) and the low concentration of M1−biotin in the injected sample (from 14 pM to 0.14 nM), we assume that all the M1−biotin that is injected becomes attached to the surface. The slope of the linear region of the calibration curve is 1.18×10^5 RI/M. Using the 3σ definition of detection limit,[24] the lowest detectable protein concentration is calculated to be 0.14 nM. This value corresponds to a surface coverage of 21 pg/mm^2 and an absolute detection limit of 0.32 ng or 2.1 fmol of a 150 kDa protein. This limit of detection is ~100 times higher than

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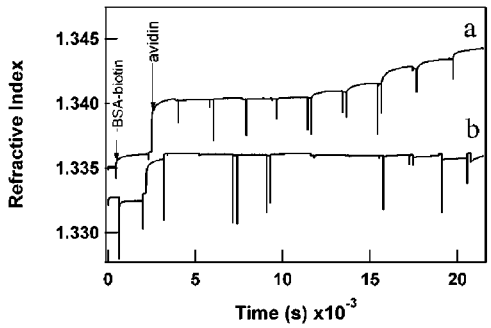

Figure 1. Sensorgrams showing (a) the refractive index changes caused by the nonspecific deposition of BSA−biotin followed by the binding of avidin and then the binding of Con A in nine progressively more concentrated solutions and (b) the introduction of the same solutions as in sensorgram a, but with excess sugar to saturate Con A's carbohydrate binding sites. For both a and b, an unmodified gold sensing surface was treated with the following solutions: BSA−biotin (0.2 mg/mL), avidin (0.5 mg/mL), and Con A (38, 110, 190, and 380 nM and 1.1, 1.9, 3.8, 11.3, and 18.9 μM).

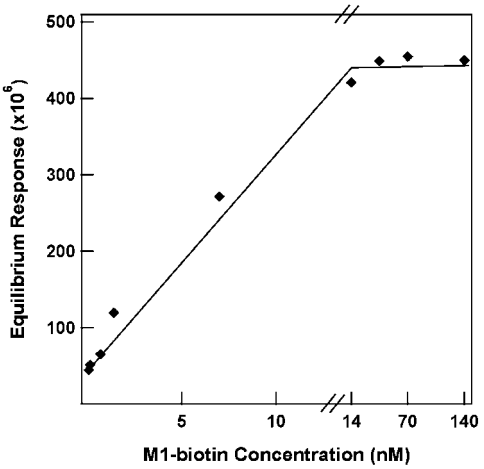

Figure 2. Binding curve showing the saturation of a surface prepared with BSA−biotin and streptavidin when a series of progressively more concentrated M1−biotin solutions is added. The axis has been broken at 14 nM, and the low-concentration region of the curve has been expanded to show more clearly the linear increase in refractive index in response to M1−biotin binding. An unmodified gold surface was exposed to the following solutions: BSA−biotin (0.2 mg/mL), avidin (0.5 mg/mL), and M1−biotin (0.014, 0.07, 0.14, 0.7, 1.4, 7, 14, 35, 70, and 140 nM).

that reported for a BIAcore instrument in the detection of myoglobin (17 kDa) using an antibody sandwich assay.[25]

Con A Immobilization onto Avidin. The application of SPR spectroscopy to the study of protein−protein interactions requires reliable protein immobilization procedures. Ideally, the immobilization procedure can easily be applied to many different proteins and creates a uniform and stable bound protein layer that can freely interact with proteins in solution. Many proteins, both serum and membrane-bound, contain oligosaccharide units, and these sugars can be recognized by carbohydrate-binding proteins known as lectins. The interaction between the lectin concanavalin A (Con A) and various natural and synthetic ligands has been investigated previously using SPR.[23,26] Because all known lectins are multivalent, containing two or more carbohydrate binding sites, they could be used as an intermediate layer in a sandwich-style immobilization scheme. Con A, which is a tetramer at physiological pH, was investigated as a possible bridging molecule to immobilize glycoproteins above the sensing surface.

The surface was prepared by injections of BSA−biotin and avidin (streptavidin, a bacterial analogue of the avian glycoprotein avidin may not be used in this application because it is not glycosylated.). Injected Con A interacted with the avidin layer as a result of Con A's affinity for the mannose component of the avidin carbohydrate moiety. Figure 1 shows a sensorgram in which BSA−biotin, avidin, and multiple concentrations of Con A are introduced next to an unmodified gold surface. These data were analyzed to find the equilibrium dissociation constant for Con A/sugar binding (see below). Once a layer of Con A was immobilized on the surface, glycoprotein was introduced. We successfully immobilized human immunoglobulin G (IgG) to Con A through the IgG's carbohydrate moieties and then immobilized a layer of goat anti-human IgG using antigen−antibody recognition. These interactions were continually monitored by SPR (data not shown).

β_2 Adrenergic Receptor Immobilization onto M1. Another immobilization scheme we investigated used the high-affinity binding between the FLAG peptide fusion tag and M1, a monoclonal antibody that recognizes the N terminus of the FLAG peptide in a Ca^{2+}-dependent manner. The FLAG tag is used to purify fusion proteins by immunoaffinity chromatography. In our SPR experiments, an unmodified gold sensing surface was prepared with BSA−biotin and streptavidin as described earlier. Biotinylated M1 antibody (M1−biotin) was then injected. To determine the appropriate amount of M1−biotin to inject, the streptavidin-treated surface was titrated with successively more concentrated solutions of M1−biotin. Figure 2 shows that the refractive index increases rapidly between 1 and 10 nM M1−biotin, after which more concentrated solutions do not lead to further binding. These data indicate that all of the available binding sites on streptavidin are saturated by an M1−biotin concentration between 1 and 10 nM. As a result, subsequent immobilization procedures used 14 nM M1−biotin to ensure uniform surface coverage.

Once a layer of M1−biotin was immobilized through interaction with streptavidin, a FLAG-labeled protein was introduced. In these studies, the protein used was the β_2 adrenergic receptor (β_2AR). This well-characterized protein has been used as a model for the family of ligand-activated G protein-coupled receptors. We recently demonstrated the functional immobilization of this receptor using SPR and fluorescence spectroscopy studies.[27] For studies of receptor function, such as interaction with agonists, antagonists, kinases, and G proteins, it is important to immobilize the receptor with consistent orientation. A nonuniformly oriented population of receptors would give rise to a population of responses that would be impossible to interpret without information on the accessibility of the reacting sites. The N-terminal FLAG tag, which is used to purify the receptor, can also be used as an immobilization handle by allowing the peptide to be bound by the M1

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Table 1. Comparison of K_d Values Determined by SPR, FCS, and Surface Fluorescence Microscopy for the M1/FLAG and Avidin/Con A Systems and Comparison with Published Values

	M1/FLAG	avidin/Con A
Spreeta	5 ± 1 nM ($n = 6$; no flow)	(assume bivalent) 6.0 ± 0.9 μM (assume tetravalent) 12 ± 2 μM
FCS	2.4 ± 0.7 nM	n/a
surface fluorescence	4 ± 2 nM	n/a
literature values	412 nM[a]	(assume bivalent) 4.0 ± 0.1 μM[b] (assume tetravalent) 17 μM[c]

[a] From kinetic rate constants determined on a BIAcore; FLAG-GFP binding to M1.[32] [b] From equilibrium analysis on a BIAcore; Con A binding to carboxymethyldextran.[23] [c] From affinity capillary electrophoresis; Con A binding to dextran.[28]

antibody. This immobilization scheme leaves the receptor oriented with its cytosolic C terminus facing away from the surface. The refractive index change resulting from FLAG-β_2AR binding to immobilized M1−biotin was monitored by SPR.

To ascertain the specificity of binding, β_2AR was introduced to the bound M1−biotin in two different buffers, one containing Ca^{2+} and the other containing Ca^{2+} and excess FLAG peptide. For replicate measurements ($n = 4$), the mean refractive index change for the binding of 62.5 nM β_2AR under each set of buffer conditions was $(6.4 \pm 1.5) \times 10^{-5}$ in buffer with Ca^{2+} and $(1.8 \pm 1.8) \times 10^{-5}$ in buffer with Ca^{2+} and FLAG. The presence of excess FLAG peptide is able to significantly inhibit binding, presumably by saturating FLAG binding sites on the M1. Nonspecific binding in this case was ~28%.

Determination of Dissociation Constants by SPR. One popular and powerful application of SPR in bioanalysis is the determination of equilibrium association and dissociation constants (K_a and K_d) for the interactions of biomolecules. We have used the Spreeta sensor with and without sample flow to determine equilibrium dissociation constants for two sets of biomolecules: Con A binding to avidin and the FLAG epitope on the β_2 adrenergic receptor binding to M1. We have compared the K_d values determined by SPR experiments to literature values (determined by SPR and other methods) and to values obtained in our laboratory by two independent methods, FCS and surface fluorescence microscopy.

Determination of Dissociation Constants by SPR: Con A and Avidin. All studies on the binding of Con A to immobilized avidin were performed without sample flow. According to Winzor and co-workers,[23] solute multivalence imposes limits on the applicability of a kinetic analysis to data that deviate from pseudo-first-order kinetics. Instead, the equilibrium biosensor response and an expression including the valence of the free solute can be used to calculate K_a or K_d values. To allow adequate time for the attainment of equilibrium between the free solute (Con A) and the complex of solute bound to ligand (Con A bound to avidin), we injected a plug of Con A and allowed the sample to incubate without flow over the sensing surface for 20 min. Subsequent injection of buffer washed away any nonspecifically bound protein. The sensor response immediately after buffer injection was taken as the equilibrium sensor response. The sensor responses resulting from the injection of 11 samples of Con A, with concentrations ranging from 38 nM to 113 μM, were recorded, corrected for nonspecific binding, and plotted as a function of injected Con A concentration. K_d values were determined as described by Winzor and co-workers.[23]

Table 1 shows the value of K_d determined by equilibrium binding analysis on the Spreeta and shows a literature value measured on a BIAcore instrument. If in fitting the data we assume that Con A is bivalent, we find a K_d of 6.0 ($\pm$ 0.9) μM. This value agrees well with the value of 4.0 ($\pm$ 0.1) μM reported by Winzor and co-workers[28] who made the same assumption. If instead we assume that Con A is tetravalent at the pH used in this study, we find a K_d of 12 ($\pm$2) μM. This value agrees qualitatively with values from affinity capillary electrophoresis (ACE) studies reported by Novotny and co-workers[28] for the binding of Con A to dextran (17 μM) and dextrin (34 μM). In those ACE studies, differences in the linkages between the glucose residues were found to change the affinity of Con A by a factor of 2. The sugar residue recognized by Con A in our investigation was not glucose, but mannose. The presence of a different sugar might account for the differences between the K_d values reported by Novotny and co-workers[28] and those reported here. The main point of the Con A study in this investigation, however, was to demonstrate that a miniaturized SPR sensor can provide reasonable information about low affinity, multivalent protein−protein interactions and that further characterization of the sensor for these kinds of systems is warranted.

Although BSA is not glycosylated,[29] unpurified samples of BSA can contain a glycoprotein that can result in false positive responses in studies involving lectins.[30] It was therefore important to characterize the possible interaction between Con A and the undetermined glycoprotein impurity in the BSA−biotin layer. Binding of a progressively more concentrated series of solutions of Con A to a layer of BSA was monitored by SPR. The calculated value of K_d was the same as that determined when the layer of avidin was also present (data not shown). These data indicate that the K_d values determined in the Con A system reflect exclusively the interaction of Con A with the carbohydrate groups on a glycoprotein and that the particular glycoprotein used does not affect the observed binding.

Determination of Dissociation Constants by SPR: FLAG-β_2AR and M1. The equilibrium dissociation constant for the FLAG-β_2AR/M1 system was determined both in static and flow experiments. In the static experiments, the surface was prepared by injections of BSA−biotin, streptavidin, and M1−biotin. A series of solutions of β_2AR ranging in concentration from 2.0 to 250 nM were injected and allowed to incubate for 20 min, after which

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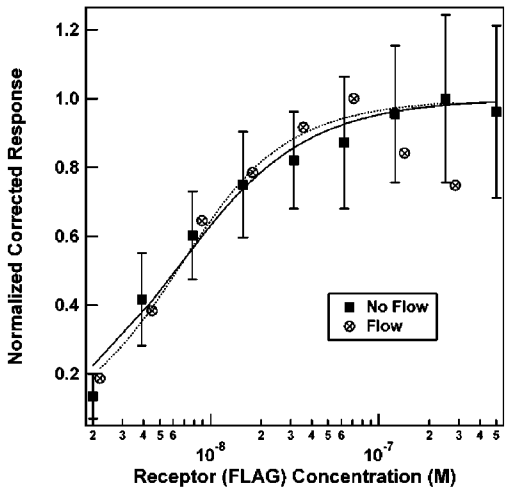

Figure 3. Binding curves for the M1/FLAG system with (open crossed circles) and without (solid squares) flows of samples and buffer solutions over the sensor. For the static measurements, an unmodified gold surface was exposed to the following solutions: BSA−biotin (0.2 mg/mL), streptavidin (0.5 mg/mL), M1−biotin (14 nM), and FLAG-β_2AR (2.0, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, and 500 nM). The binding curves were fit using eq 2. For the flow measurements, solutions (buffer and sample) were flowed at 50 μL/min. The same protein solutions were used as in the static experiments, except that the concentrations of FLAG-β_2AR were 2.2, 4.5, 8.9, 17.85, 35.7, 71.4, 142.8, and 285.6 nM. Flow data were fit using eq 3. The flow measurements were gathered once. The error bars on the static data represent one standard deviation of the mean with six replicate measurements.

sample-free buffer solution was injected to rinse away any nonspecifically bound protein. Refractive index changes resulting from receptor binding were recorded, and the K_d was calculated as described in the Experimental Section. It was particularly important in the static experiments, in which ligand depletion above the sensing surface might occur to an appreciable extent, to use the fitting isotherm that makes no assumptions about the concentration of free ligand. For the flow experiments, 100 μL of sample was injected at a flow rate of 50 μL/min through a flow cell with a volume of 3.5 μL. Proteins were injected in the same order as in the static experiments. The equilibrium binding response was taken to be the difference in the refractive index before and after sample injection through the flow cell. Figure 3 shows two binding curves for the FLAG tag on β_2AR to immobilized M1, one collected with flow and the other collected without flow. The binding curves overlay very well, with respective K_d values of 3 ± 2 nM (error of the fit) and 5 ± 1 nM (error calculated as the standard deviation from 6 replicate measurements). These data indicate that, in this high-affinity system, equilibrium data obtained with and without flow give comparable results. Values for nonspecific binding were subtracted from the total refractive index change at each point to give information about specific binding. The apparent deviation of the flow-based binding curve from expected values at high concentrations of injected FLAG-β_2AR can be attributed to a large amount of nonspecific binding at those concentrations.

The dissociation constant for M1/FLAG was determined without flow, using two different surface coverages of M1−biotin. A surface was prepared by injecting BSA−biotin and streptavidin, then either 1.4 nM or 14 nM M1−biotin. The refractive index change resulting from M1−biotin binding was ~10-fold lower for the 1.4 nM solution than for the 14 nM solution. For replicate measurements (n = 3), the surface prepared with 1.4 nM M1−biotin gave a K_d of 5 ± 3 nM for the M1/FLAG interaction, whereas the surface prepared with 14 nM M1−biotin gave a K_d of 5 ± 1 nM (n = 6). These data indicate that the density of antibodies on the surface is low enough that rebinding to the surface is not affecting the measured binding or the calculated K_d values.

For surface fluorescence microscopy experiments, M1 was immobilized on a glass slide as described earlier. TMR−5-β_2AR solutions with concentrations ranging from 0 to 920 nM were then added to the surface. After rinsing, the fluorescence intensity was measured at 5 different sites on the surface and was found to increase with increasing receptor concentration from 103 ± 4 to 1156 ± 65 counts/pixel. The results were fitted with eq 2, which resulted in a dissociation constant of K_d = 4 ± 2 nM.

All FCS measurements were calibrated with measurements of TMR in solution. The diffusion coefficient for TMR is 2.8×10^{-10} m^2/s.[31] The correlation time was measured to be τ_D = 64 ± 5 μs. The correlation time of TMR−5-β_2AR was measured to be τ_D = 371 ± 57 μs. When M1 antibody in concentrations between 0.1 and 300 nM was added to a 1 nM solution of TMR−5-β_2AR, the correlation time increased and peaked at concentrations of M1 around 10 nM at τ_D = 691 ± 17 μs. For higher concentrations of M1, the correlation time decreased again (τ_D = 498 ± 39 μs) but was always higher than the correlation time measured for TMR−5-β_2AR alone. The FCS data were fit as described in the Supporting Information section, yielding a dissociation constant of K_d = 2.4 ± 0.7 nM.

Figure 4 shows a binding curve determined on the Spreeta and a binding curve determined by surface fluorescence microscopy. The two curves overlay well, with a K_d value of 5 ± 1 nM for the Spreeta and 4 ± 2 nM for the fluorescence measurement. Table 1 summarizes these K_d values and shows how the K_d values obtained with the miniaturized SPR sensor compare to literature values and to values obtained in our laboratory with FCS and surface fluorescence microscopy. Because information about the binding and dissociation constants of the M1/FLAG system is limited,[32] we were motivated to explore alternative methods of determining K_d using both surface-bound and free-solution approaches. As shown in Table 1, the values obtained by FCS and surface fluorescence microscopy corroborate the data gathered by the Spreeta sensor. All methods find that the M1/FLAG affinity is in the low nanomolar range. The difference between our value in Table 1 and that determined by Einhauer and Jungbauer[32] using kinetic measurements on a BIAcore instrument might be attributable to the presence of a different protein attached to the FLAG tag. The agreement between the values determined using the Spreeta sensor and the values determined by FCS and fluores-

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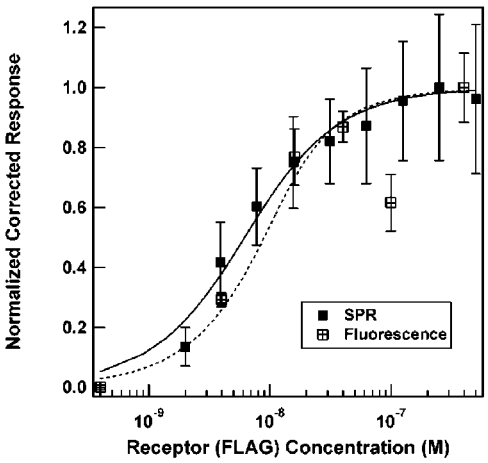

Figure 4. Binding curves for the M1/FLAG system determined by surface plasmon resonance spectroscopy (solid squares) and surface fluorescence microscopy (open crossed squares). Experimental conditions for the static SPR experiment are the same as in Figure 3. For the fluorescence experiment, a clean glass slide was incubated with BSA−biotin, streptavidin, and M1−biotin, followed by TMR−5-β_2AR in the following concentrations: 0.4, 4, 16, 40, 100, and 400 nM. The binding curves were fit using eq 2. The error bars on the SPR data represent one standard deviation of the mean with six replicate measurements. The error bars on the fluorescence data were calculated using the fluorescence intensity values from five different spots on the surface.

cence surface microscopy validate the use of the Spreeta for the measurement of protein−protein and protein−peptide binding.

CONCLUSIONS

The miniaturized and integrated Spreeta SPR sensor has been demonstrated to provide accurate thermodynamic binding information. We have been able to measure binding affinity constants for two widely used biomolecular systems (M1/FLAG and Con A/glycoprotein) that have previously not been completely characterized. The close agreement with available literature values is encouraging. The simple Spreeta SPR sensor is less sensitive than the more sophisticated BIAcore system, but its lower cost and footprint recommend its use in many practical applications.

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SUPPORTING INFORMATION AVAILABLE

Supporting Information Available: The details of the fluorescence setup and the description of how the fluorescence measurements were recorded. This material is available free of charge via the Internet at http://pubs.acs.org.

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