Simultaneous Detection of Multiple Proteins that Bind to the Identical Ligand in Supported Lipid Bilayers

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ⓢ *Supporting Information*

ABSTRACT: Herein, we developed a new separation-based detection method that is capable of simultaneously identifying multiple competitively binding proteins for the same ligand on supported lipid bilayers (SLBs). This strategy used unlabeled target analyte proteins that bind to fluorescently tagged, lipid-conjugated ligands within the SLB. The protein−ligand binding complexes were then focused under an applied potential to different locations within the SLB based on each protein's size and charge. Both protein identity and relative surface concentration information could be obtained, simultaneously. Specifically, the competitive binding of streptavidin and goat anti-biotin for biotin-conjugated lipids was explored. It was found that streptavidin could inhibit the binding of goat anti-biotin antibodies for biotin-cap-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2−1,3-benzoxadiazol-4-yl)(biotin-cap-NBD-PE) lipids and that streptavidin more effectively outcompeted the anti-biotin antibody at lower protein concentrations. Also, modulating the chemical composition of the membrane helped control the ultimate focusing position and separation of the streptavidin-bound biotin, anti-biotin-bound biotin, and free biotin-conjugated lipid bands. The assay developed herein provides a simple and convenient strategy for simultaneously monitoring target analytes that bind to the identical ligand and may ultimately be useful in developing assays that help overcome problems associated with cross-reactivity.
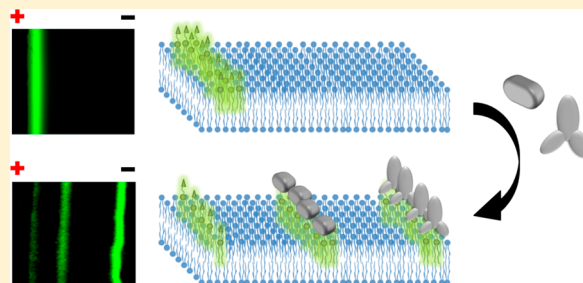
Competitive binding is common *in vivo* in cellular processes such as secretion, proliferation, differentiation, apoptosis, migration, and protein transport/insertion.[1−8] Moreover, the cross-reactivity of antibodies for multiple cellular targets is a significant challenge in biotechnology.[9−11] In the past decade, numerous label-free techniques have been developed based on ligand−receptor binding, but the detection of proteins that competitively bind to the identical ligand remains challenging.[5,12−21] In addition, the detection of biomarkers from biological fluids requires the capability of simultaneously detecting multiple low concentration analytes in a background of myriad other high abundance proteins and interfering compounds.[5,16]

Previously developed detection techniques for ligand−receptor binding have successfully exploited optical, mechanical and electrical effects caused by protein binding. Such techniques involve surface plasmon resonances (SPR), surface acoustic waves (SAW), electrical signals from functionalized electrodes and nanowires, nanoparticle-based bio-barcodes, microcantilevers, optical microcavity resonators, nanocalorimetry, interferometry, and environmentally sensitive fluorophores among other techniques.[5,12−21] Unfortunately, the signals generated from molecules competing for the same binding sites as well as nonspecific adsorption were challenging to discern from one another in these systems because such signals

are not readily distinguished from one another. Indeed, chemically specific information about the target analyte would be typically required.

There are, of course, more chemically specific methods to distinguish competitively binding ligands. For example, one can employ gel electrophoresis in conjunction with immunoprecipitation assays to separate proteins which bind to the same ligand.[22,23] Spectroscopic methods like infrared,[24] Raman,[25,26] and NMR,[27,28] as well as mass spectroscopy[29−31] can also potentially be employed. None of these methods are specific to the detection of analytes on lipid membranes and most of them have additional limitations if the mixture is complex or the analytes are in low concentration. As such, it would be beneficial to develop a separation-based method as part of the detection scheme that would allow one to quantify the amount of each competitively binding analyte that is attached to a lipid membrane interface.

Such a method might be analogous to traditional separation-based detection methods in bulk solution, such as Western blot analysis and isoelectric focusing.[32−35] In these techniques, proteins are first separated by gel electrophoresis and then
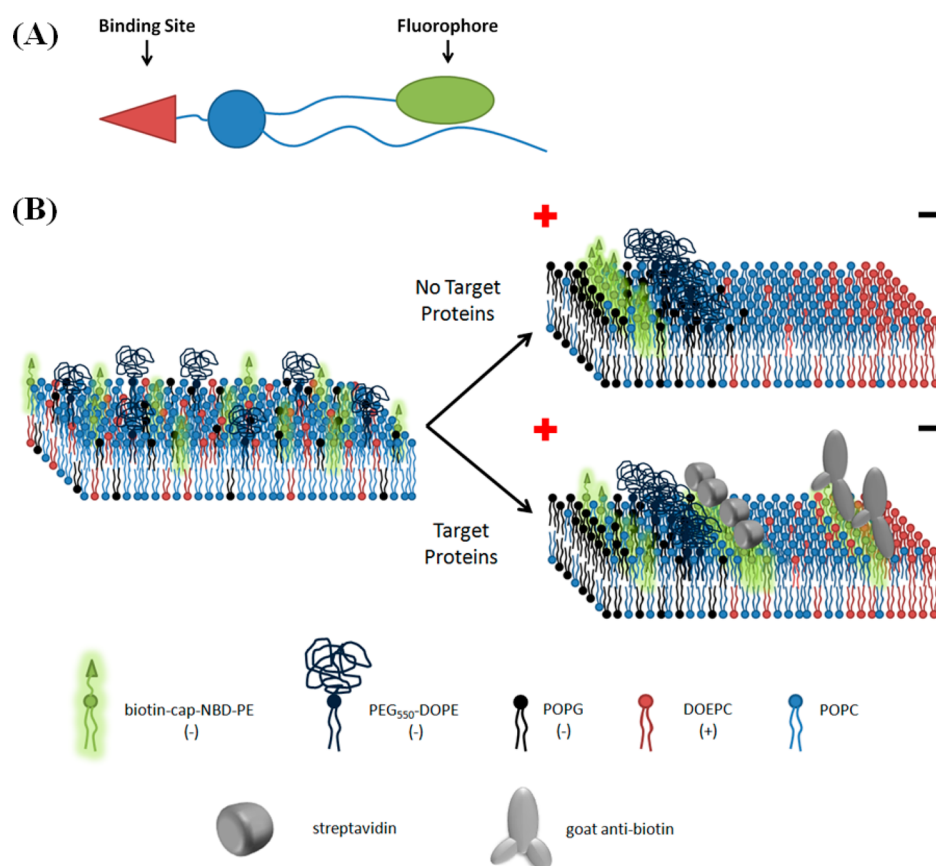
Figure 1. (A) Schematic diagram of a fluorescently labeled lipid with a protein binding site linked to the headgroup. (B) Schematic diagrams of a supported bilayer containing biotin-cap-NBD-PE (green). Without target proteins, the tail-labeled biotin-cap-NBD-PE should form a single band near the positive electrode upon application of an electric field (top right). Upon binding of two distinct target proteins, three separated bands will be formed, i.e., one for each of the targeted proteins and a third one from any ligand that remains unbound (bottom right). The schematic diagram is not drawn to scale, as the protein molecules would be larger than shown.

transferred to a membrane carrier. Target proteins are detected and identified by applying a specific antibody or a series of antibodies, which can be fluorescently, radioactively or enzymatically active. By analogy to this type of strategy, we have developed a novel electrophoretic-based detection method on SLBs by monitoring the steady-state focusing position of fluorescently labeled ligands. This method is based on our previously developed electrophoretic−electro-osmotic focusing (EEF) method.[36,37]

Using the EEF technique, it is possible to separate and concentrate distinct proteins bound to the same ligand type on an SLB based on the size and charge of the macromolecules (Figure 1). Herein, a fluorescently labeled ligand-conjugated lipid (shown schematically in Figure 1A) is incorporated into an SLB and its EEF behavior is monitored in a DC electric field. As illustrated in Figure 1B, upon binding of target proteins, the fluorescently labeled ligands in the SLB experience a change in both their electrophoretic and electro-osmotic forces. This causes the focusing position of these fluorescently labeled ligands to migrate. For different proteins, the changes in the electrophoretic and electro-osmotic forces on fluorescently labeled ligands are distinct. Because different types of proteins will focus to different positions, multiple types of proteins that bind to the same ligand can be detected simultaneously. As such, we reasoned that this method should be particularly useful for quantitatively detecting protein cross-reactivity and

even monitoring protein competition for the same ligand as a function of protein concentration.

By monitoring the focusing behavior of fluorescently labeled ligands in SLBs, several unique advantages can be realized. First, the technique strongly discriminates against nonspecifically bound species. Indeed, the interactions between nonspecifically adsorbed proteins and the lipid bilayer are expected to be sufficiently weak that the focusing position of the ligands should not be affected. Second, so long as the concentration of dye-conjugated lipid ligands is sufficiently low,[38] the fluorescence intensity should reflect their relative concentrations at the interface and ultimately the concentration ratios of bound proteins. In fact, the fluorescence intensity ratios between various protein-bound ligands and free ligands can be directly related to the fraction of ligands which is free as well as protein-bound. Finally, when the ratios of fluorescence intensities are employed to obtain information on competitive binding, intensities of all components are effectively normalized, so errors from photobleaching effects are mitigated. To show the efficacy of this assay, we demonstrate the ability to distinguish the binding of goat anti-biotin and streptavidin to fluorescently tagged biotin-cap-NBD-PE lipids, as shown schematically in Figure 1B.

■ EXPERIMENTAL SECTION

Materials. Fibrinogen and streptavidin were purchased from Sigma (St. Louis, MO, U.S.). Goat anti-biotin was purchased

Scheme 1
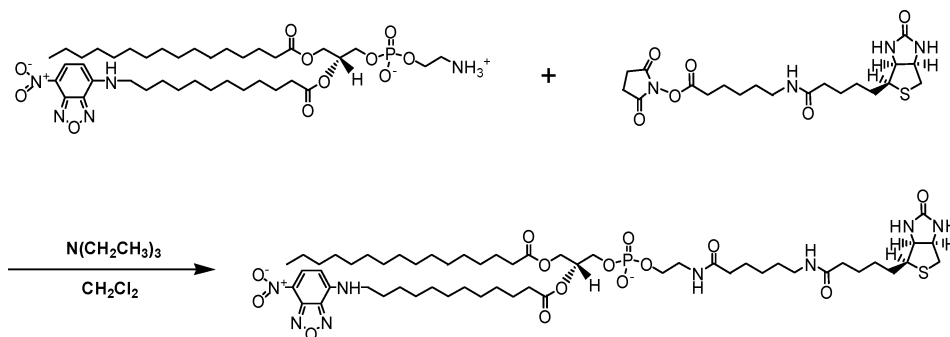
from Rockland Immunochemicals, Inc. (Gilbertsville, PA, U.S., #600-101-098). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (DOEPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (POPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy-(polyethylene glycol)-550] (PEG$_{550}$-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.). Succinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) was purchased from ProteoChem (Denver, CO, U.S.). Polydimethylsiloxane (PDMS) was obtained from Dow Corning (Midland, MI, U.S., Sylgard 184 silicone elastomer kit).

Synthesis of Biotinylated NBD-PE (biotin-cap-NBD-PE). 1 mg of 18:1−12:0 NBD-PE and 0.62 mg of succinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) were dissolved and mixed in 200 μL of anhydrous dichloromethane. Next, 0.15 μL of triethylamine was added to the solution and the mixture was stirred at room temperature for 48 h. The reaction is shown in Scheme 1. As pictured, the NHS-LC-biotin and 18:1−12:0 NBD-PE were linked together through a peptide bond. The products were separated and purified on a silica gel TLC (thin layer chromatography) plate with mixed solvents (CH$_2$Cl$_2$ and methanol 9:1). An electrospray ionization (ESI) mass spectrum of the purified sample was also taken, and used to verify the formation of the target molecule (Figure S1 in the Supporting Information). It should be noted that the biotinylation of zwitterionic NBD-PE lipids removes the positive charge on the primary amine of the NBD-PE headgroup. As such, the biotin-cap-NBD-PE has a charge of −1 near neutral pH, which can be exploited in the electrophoretic separation experiments. Similar tail-labeled, ligand-conjugated lipids have been previously employed to detect protein binding on SLBs when the protein binding caused fluorescently labeled lipids to self-quench[39,40] or cause a decrease in the lipid diffusion coefficient.[41]

SLB Patterning and Lipids Composition. SLB patterns were formed by using a PDMS stamping method on clean glass coverslips (Corning, NY, U.S., 22 × 22 mm, No. 2). The detailed procedures have been described in our previous work.[36,37] The SLB patterns consisted of a series of 380 μm wide parallel SLB lines with 200 μm wide fibrinogen protein monolayer spacers separating the SLB patches from one another. The SLBs were formed by the vesicle fusion method.[42,43] Small unilamellar vesicles (SUVs) with 10% DOEPC, 3% POPG, 1% PEG$_{550}$-DOPE, 0.1% biotin-cap-NBD-PE, and 85.9% POPC were prepared by several freeze−thaw cycles and vesicle extrusion through a polycarbonate filter (GE Healthcare Bio-Sciences, Pittsburgh, PA, U.S., 0.2 μm pore

size) with 100 nm pores. A 1 mg/mL lipid vesicle solution was introduced onto the glass coverslip, and SLBs formed spontaneously on the area without the fibrinogen monolayer.[44]

Role of Additional Lipids. It should be noted that the DOEPC and PEG$_{550}$-DOPE lipids were added to the SLBs to facilitate the separation of the membrane bound protein bands. Specifically, positively charged DOEPC lipids were found to prevent the clustering of negatively charged streptavidin molecules bound on the SLB surface during incubation under high ionic strength buffer conditions (Figure S2 in the Supporting Information). Moreover, the DOEPC lipids could form a positive charge density gradient upon the application of a potential along the membrane. This prevented the accumulation of membrane bound proteins at the far negative edge of the SLB. PEG$_{550}$-DOPE lipids were used to improve the separation of the bands from free biotin-cap-NBD-PE and streptavidin bound biotin-cap-NBD-PE. PEG$_{550}$-DOPE has one negative charge and a hydrophilic PEG headgroup, which is larger than the lipid head groups in the SLB, but smaller than the membrane-bound proteins. As such, according to EEF theory, the focusing position of the PEG$_{550}$-DOPE band should be between the focusing positions of the negatively charged lipids and the protein bound lipids (Figure S3 in the Supporting Information). In fact, without PEG$_{550}$-DOPE, the bands from free biotin-cap-NBD-PE and streptavidin-bound biotin-cap-NBD-PE could not be well separated (Figure S4 in the Supporting Information).

Introduction of Target Proteins. Goat anti-biotin and streptavidin were serially diluted from a 1 mg/mL stock solution by a 1 mg/mL BSA solution in 10 mM Tris buffer at pH 8.0 with 100 mM NaCl. A 20 mL sample of diluted protein solution was prepared at each concentration. A 200 μL aliquot of this solution was incubated above the patterned SLB for ~2 h. During this time, the solution in contact with the patterned SLB was repeatedly refreshed by pipetting fresh protein solution into the system and pipetting old solution out. This helped ensure that the bulk protein concentration remained constant as protein molecules became bound to the surface. After incubation, the sample was incorporated into a flow cell device. As a control experiment, patterned SLBs were incubated with 1 mg/mL BSA solution in 10 mM Tris buffer at pH 8.0 with 100 mM NaCl for ~2 h. This led to no discernible shift in the lipid-conjugated ligand position upon EEF focusing.

Fluorescence Imaging and Electrophoretic−Electroosmotic Focusing. Epifluorescence images were obtained using a Nikon E800 fluorescence microscope with a Roper Scientific MicroMAX 1024B charge-coupled device (CCD) camera (Princeton Instruments, Trenton, NJ, U.S.). The experimental conditions were controlled in EEF experiments

using a flow cell device that has been previously described,[45] and the experiments are based on electrophoresis in supported lipid bilayers.[36,38,46] The pH, ionic strength, and temperature of the SLB system were regulated by continuously flowing a buffer solution through the flow cell channels at 120 mL/hour per channel during electrophoresis. The buffer contained 10 mM Tris with 100 mM NaCl at pH 8.0. The EEF separation could be affected by the buffer pH and ionic strength.[36] 100 mM NaCl and pH 8.0 were chosen to achieve an optimal separation (Figure S5 in the Supporting Information). A 75 V/cm electric potential was applied across the bilayers for 60 min during the experiments.

■ RESULTS AND DISCUSSIONS

Migration of biotin-cap-NBD-PE in SLBs. We monitored the migration of 0.1 mol % biotin-cap-NBD-PE in supported lipid bilayers. These membranes were made with 85.9 mol % POPC, 3 mol % POPG, 1 mol % PEG$_{550}$-DOPE, and 10 mol % DOEPC. As can be seen in Figure 2A, the biotin-cap-NBD-PE
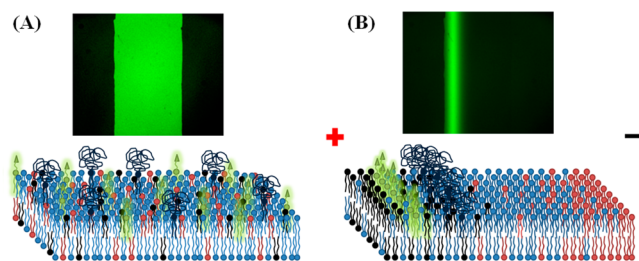

Figure 2. Fluorescence micrographs and corresponding schematic images (A) before and (B) after focusing of biotin-cap-NBD-PE lipids with a 75 V/cm electric field for 60 min. The pH value was continuously maintained at 8.0 by using a 10 mM Tris buffer with 100 mM NaCl.

lipids were initially evenly distributed throughout the lipid bilayer patch. However, after the application of a 75 V/cm electric field parallel to the bilayer for about 30 min, these lipids were focused near to the positive electrode side of the membrane (Figure 2B). Simultaneously, the negatively charged POPG lipids should also migrate toward the positive electrode and form a negatively charge density gradient in the SLB.[36−38] Moreover, the positively charged DOEPC should form a gradient against the negatively charged electrode. Neither the DOEPC nor POPG were directly observed, as they were not fluorescently labeled. It should be noted that the biotin-cap-NBD-PE band did not migrate all the way to the barrier on the positive electrode side. This is due in part to an electro-osmotic effect,[36] which pushes these lipids toward the negative electrode. Moreover, there is a lipid demixing effect[38] caused by the competition between POPG and biotin-cap-NBD-PE for space adjacent to the positive electrode. The formation of a single band for biotin-cap-NBD-PE demonstrated that there is only a single fluorescent lipid component in the membrane. As such, it serves as evidence that the reaction between the NBD-PE and biotin-LC-NHS (shown in Scheme 1) went to completion. When unreacted NBD-PE lipids remained in the SLB, they increased the fluorescence intensity uniformly across the bilayer after EEF, because NBD-PE is zwitterionic and does not focus under an applied field (Figure S6 in the Supporting Information).

Detection of Goat Anti-biotin. In a next set of experiments, we tested the binding of goat anti-biotin to the

membrane described in Figure 2. In this case, electrophoresis was performed after incubating 333 pM goat anti-biotin over the bilayer for 2 h and then washing away excess protein molecules with fresh buffer. A 75 V/cm field was applied until steady state focusing of the biotin-cap-NBD-PE fluorescent molecules was achieved (Figure 3A). As can be seen, the
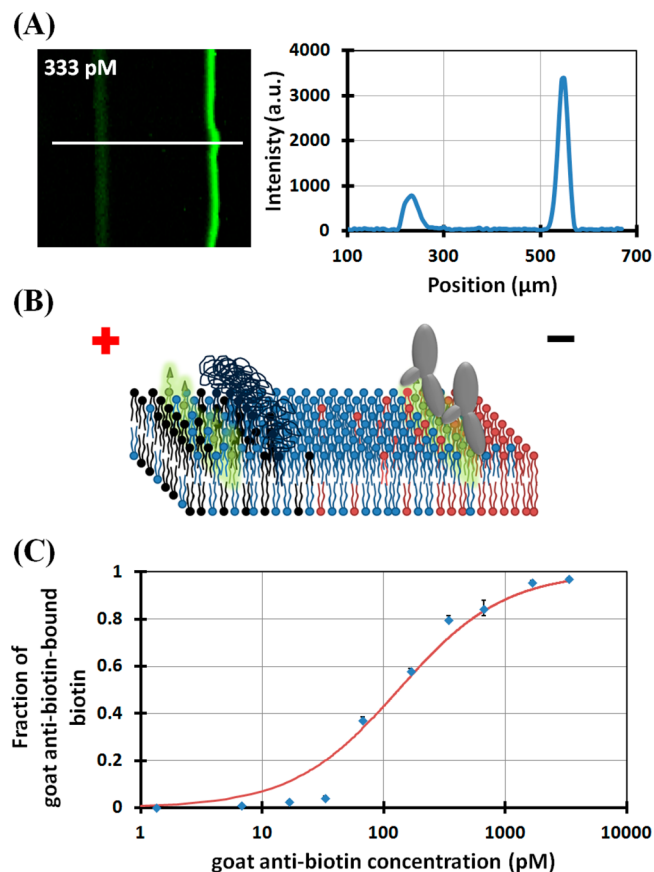

Figure 3. Focusing a band of goat anti-biotin bound to biotin-cap-NBD-PE in a charged lipid bilayer with a 75 V/cm electric field for 60 min. (A) Fluorescence micrograph of the focused bands after incubating with 333 pM goat anti-biotin (left), and the corresponding line profile from this micrograph (right). The band on the left side of the micrograph represents free biotin-cap-NBD-PE lipids, whereas the band on the right side of the micrograph is from goat anti-biotin-bound biotin-cap-NBD-PE lipids. (B) Schematic representation of the focused biotin-cap-NBD-PE bands after goat anti-biotin binding. The schematic is not drawn to scale. (C) Dose−response curve of goat anti-biotin. The blue squares are experimental data points. The red line is a Langmuir isotherm fit to the data.

fluorescent dye molecules were focused into two distinct bands. The one closest to the positive electrode was found at almost the exact same position as the one in Figure 2B. As such, this peak should represent unbound biotin. By contrast, the second peak nearer to the negative electrode represents the bound antibody−ligand complex. It is known that goat anti-biotin has a pI value between 7 and 8.[26] Therefore, this protein is not highly charged under the experimental conditions employed here. However, the radius of the goat anti-biotin molecule is about 4.5 nm[36] and the protein−lipid binding complex should protrude well above the plane of the lipid bilayer surface. As such, it will be subjected to strong electro-osmotic forces.[36] These forces will cause the protein to focus closer to the negative electrode edge of the membrane where the buildup of

DOEPC lipids was significant. The result is depicted schematically upon steady state focusing in Figure 3B.

The fraction of goat anti-biotin-bound biotin-cap-NBD-PE lipids should increase with the surface-bound concentration of goat anti-biotin up to the ligand saturation point. It should be noted that goat anti-biotin proteins can bind to a maximum of two biotin-cap-NBD-PE lipids because the antibody has two binding sites.[47] A unique advantage of the current assay is that the percentage of both free ligands and protein-bound ligands can be read out directly at each incubation concentration. By integrating the fluorescent intensities of each peak in Figure 3A, we found that approximately 80% of the biotin-cap-NBD-PE lipids became bound to goat anti-biotin upon the introduction of 333 pM of protein. Moreover, incubation with a higher (or lower) concentration of protein for the same amount of time led to a greater (or lesser) fraction of antibody-bound ligands (Figure 3C and Figure S7 in the Supporting Information). In fact, we found that almost all the ligands (97% of all ligands) became protein bound when incubated with 3.3 nM goat anti-biotin antibody for 2 h. By repeating these experiments at various concentrations, a limit of detection value for goat anti-biotin was established (Figure S7 in the Supporting Information). This value was ~1.3 pM, at the 99% confident interval (3 times the noise level). Moreover, measuring the ratio of bound to unbound fluorophores obviates any effects that photobleaching might have on the measurement, assuming that the protein bound and unbound forms of the fluorophore-conjugated lipid bleach at the same rate.

The fraction of ligand-bound goat anti-biotin as a function of protein concentration is shown in Figure 3C. Protein solutions were incubated over the surface for 2 h and then washed away as an EEF measurement was performed. The data points (shown in blue) form a curve reminiscent of a binding isotherm, and the red line represents the best fit to a Langmuir isotherm. As can be seen, the points fit the curve fairly well down to 50 pM, at which point they are too low. This is expected, as 2 h is not long enough for the proteins to diffuse to the substrate surface at the lowest concentrations. Moreover, because the EEF measurements were made after rinsing protein away, they do not represent true equilibrium. Nevertheless, protein molecules bound at the interface do not readily diffuse back into the bulk solution as they undergo rebinding after desorption.[48] The apparent equilibrium dissociation constant, K_{Dapp}, which can be abstracted from the red line, is 133 pM. This value is slightly tighter than previous measurements of this antigen–antibody binding pair.[49]

Detection of Streptavidin. In Figure 4A, a focusing experiment was performed with an SLB incubated with 83 pM streptavidin. Like in Figure 3A, the fluorescently tagged biotin-cap-NBD-PE lipids were focused into two distinct bands, which represent free and protein-bound ligand fractions, respectively. Compared to goat anti-biotin-bound biotin-cap-NBD-PE, streptavidin-bound biotin-cap-NBD-PE lipids focused closer to the positive electrode side of the SLB, where the negative charge density was higher. This result is depicted schematically in Figure 4B and is expected based on EEF focusing theory.[29] Indeed, streptavidin has a pI of about 5.0[50] and at pH 8.0, each streptavidin should have a charge of about −2, which makes it more negatively charged than goat anti-biotin, which is close to neutral. Moreover, the radius of streptavidin is approximately 2.5 nm,[51] which is much smaller than that of goat anti-biotin. As such, streptavidin-bound biotin-cap-NBD-PE lipids should focus closer to the positive electrode side of the SLB compared
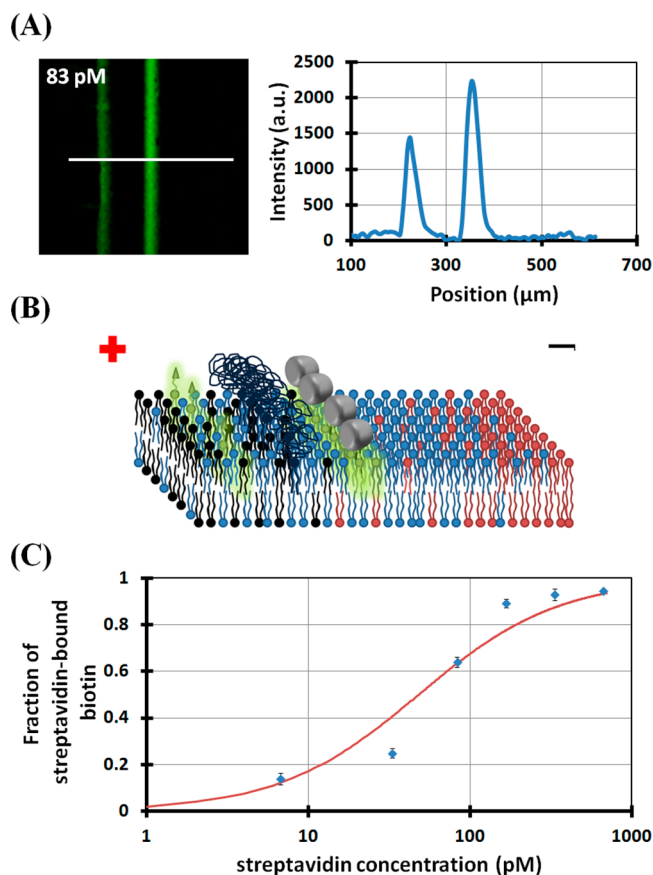

Figure 4. Focusing a band of streptavidin bound to biotin-cap-NBD-PE in a charged lipid bilayer with a 75 V/cm electric field for 60 min. (A) Fluorescence micrograph of the focused bands after incubating with 83 pM streptavidin (left), and the corresponding line profile from the micrograph (right). The band on the left side of the micrograph represents free biotin-cap-NBD-PE lipids, whereas the band on the right side of the micrograph is from streptavidin-bound biotin-cap-NBD-PE lipids. (B) Schematic diagram of the focused biotin-cap-NBD-PE bands with streptavidin binding. The schematic diagram is not drawn to scale. (C) Dose–response curve of streptavidin. The blue squares are experimental data points. The red line is a Langmuir isotherm fit to the data.

to goat anti-biotin-bound lipids on grounds of both its smaller size and more negative charge.

The fraction of bound sites as a function of streptavidin concentration was generated by incubating the SLB with multiple streptavidin concentrations (Figure 4C and Figure S8 in the Supporting Information). As with anti-biotin, the incubation time was 2 h at each concentration. The limit of detection of streptavidin was determined to be ~600 fM within the 99% confident level (3 times the noise level) (Figure S8 in the Supporting Information). Even using a best fit to this curve, only a few data points fall on the line (Figure 4C, the red curve represents a Langmuir isotherm fit). The K_{Dapp} value in this case was 48 pM, which is quite weak for biotin–streptavidin binding. This is not surprising, as it would take an extraordinarily long incubation time with fM quantities of protein to approach equilibrium for this system. Nevertheless, it should be noted that the 600 fM LOD value that was found in the measurements described above could almost certainly be improved upon by waiting ever longer periods of time for protein to bind to occur.

Simultaneous Detection of Multiple Proteins and Competitive Binding. In a next set of experiments, we detected goat anti-biotin and streptavidin mixtures simultaneously in a single SLB patch. The total concentration of protein was held constant at 1 nM, while the concentration ratio of the two proteins was varied. Three protein concentration ratios were used: 833/167, 333/667, and 167/833 pM (streptavidin/goat anti-biotin). The proteins were premixed in solution and then incubated with the SLBs. Figure 5 shows the three distinct bands that were formed upon steady
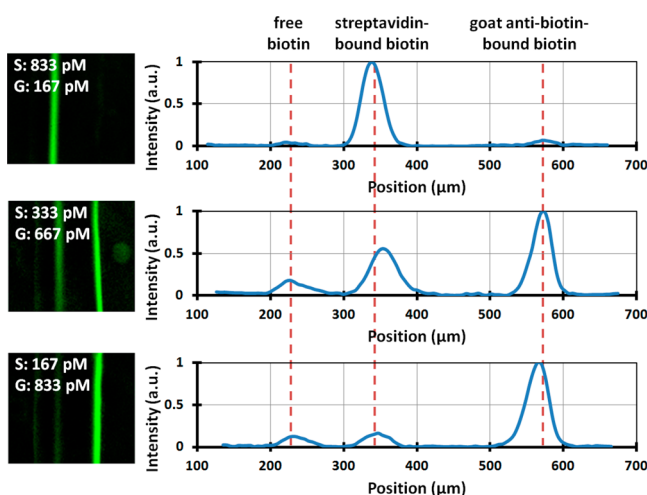

Figure 5. Simultaneous detection of streptavidin and goat anti-biotin mixtures using biotin-cap-NBD-PE in a charged lipid bilayer with a 75 V/cm electric field. The fluorescent images of SLBs with different protein solution concentrations are shown on the left (S denotes streptavidin and G denotes goat anti-biotin), whereas the corresponding fluorescence line profiles are shown on the right.

state focusing from these mixtures. All three bands can be readily identified as the protein ratio is varied. The peaks from left to right correspond to free biotin-cap-NBD-PE, streptavidin-bound biotin-cap-NBD-PE and goat anti-biotin-bound biotin-cap-NBD-PE, respectively. The focusing positions of streptavidin-bound biotin-cap-NBD-PE and goat anti-biotin-bound biotin-cap-NBD-PE were unchanged within experimental error compared to the focusing positions in their individual experiments.

Competitive Binding Experiments. In a final set of experiments, competitive binding between streptavidin and goat anti-biotin was explored. For experiments in which the bulk concentration of goat anti-biotin was held constant, an increase in the bulk concentration of streptavidin was expected to attenuate the amount of goat anti-biotin bound to the SLB surface. To test this idea, two series of experiments were conducted with 333 pM and 667 pM goat anti-biotin. In each series of experiments, the bulk concentration of goat anti-biotin was kept constant, while the bulk concentration of streptavidin was varied from 0 to 833 pM. The proteins were introduced above the bilayer surface from premixed solutions and the incubation time was 2 h. The results are shown in Figure 6A. As can be seen, the goat anti-biotin-bound biotin-cap-NBD-PE fraction decreased with increasing bulk streptavidin concentration. However, this decrease slowed down and began to level off even as the bulk streptavidin concentration was increased. In fact, there was a substantial fraction of binding sites that were occupied by the antibody even at 833 pM
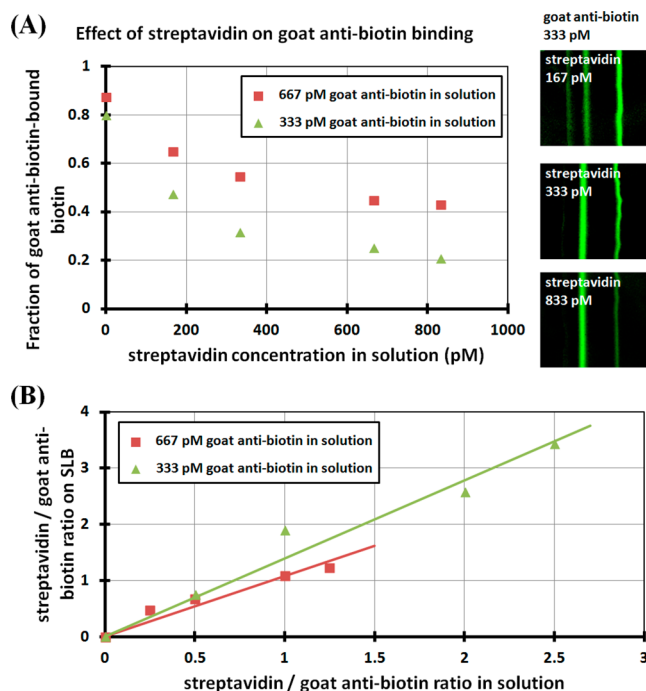

Figure 6. (A) Competitive binding between streptavidin and goat antibiotin on biotin-cap-NBD-PE in charged lipid bilayers. (B) Concentration ratio of streptavidin to goat antibiotin bound on the SLB versus the bulk concentration.

streptavidin. For the 333 pM goat anti-biotin data, the fraction of goat anti-biotin-bound biotin-cap-NBD-PE decreased from 0.80 to 0.20 over this streptavidin range. When equal bulk concentrations of goat anti-biotin and streptavidin were employed, the site fractions of goat anti-biotin and streptavidin were 0.32 and 0.60, respectively, with 0.08 free ligands. The streptavidin would be expected to have higher site occupation not only because it binds more tightly, but also because it is smaller and can diffuse to the surface more quickly.[52−54] It also has twice as many binding sites, although only two of these can face toward the surface at any given time.[47,55] These factors should also help facilitate faster binding. Finally, streptavidin has a higher negative charge density,[50,56−59] which should lead to an attraction between it and the positively charged DOEPC lipids in the bilayer. Nevertheless, this electrostatic attraction is presumed to be quite small because of the relatively high ionic strength of the buffer.[56,57]

With 667 pM goat anti-biotin, the fraction of goat anti-biotin-bound biotin-cap-NBD-PE decreased from 0.88 to 0.43 as the streptavidin concentration was increased from 0 to 833 pM. At equal goat anti-biotin and streptavidin bulk concentrations, goat anti-biotin and streptavidin occupied 0.45 and 0.49 site fractions, respectively, with only a 0.06 site fraction of free ligand. This result clearly demonstrates the role that kinetics plays in the relative fraction of sites occupied by the proteins rather than just equilibrium. Indeed, as the total concentration of protein is increased, the sites appear to be simply occupied by the first protein to arrive rather than controlled by thermodynamic equilibrium under the conditions of these experiments.

Using the data from Figure 6A, the ratio of streptavidin to goat anti-biotin bound on the surface can be plotted against the ratio of the protein concentrations in the bulk solution (Figure 6B). As can be seen, the ratio varied linearly, but with a slope of

1.4 with 333 pM goat anti-biotin, but only 1.1 with 667 pM goat anti-biotin. These results indicate that the more tightly binding protein, streptavidin, can inhibit the more weakly binding protein, goat anti-biotin, more effectively at lower total protein concentration. Also, there are other factors determining the ratio of competitive binding proteins at a surface, such as steric hindrance or electrostatic repulsions between protein molecules. Moreover, the linear variation of the ratios indicates that the surface concentration varies in a straightforward fashion with bulk protein concentration.

CONCLUSIONS

We have developed a supported lipid membrane-based separation technique that is capable of simultaneously detecting multiple proteins interacting with the identical ligand on an SLB without labeling the analytes. The protein causes the fluorescently tagged ligands in the bilayer to migrate to a unique position upon the application of an electric field lateral to the membrane interface. Free and protein-bound ligand populations could be simultaneously identified, and the limit of detection for the binding proteins could be readily determined. Moreover, it was shown that anti-biotin antibodies competed more effectively for binding sites with streptavidin at lower protein concentrations.

Using the methods described in this paper, it is anticipated that membrane—ligand interactions with multiple proteins can be facilely monitored on lipid bilayers and quantitative information about complex competition events could be rapidly extracted. Two particular classes of lipids that could be explored are gangliosides and phosphatidylinositides. Both lipids bind with a variety of different proteins, and the mechanisms of specificity have not been well explored.[60−76] Therefore, assays analogous to the ones described above could be designed to tease out the varying interactions. Such assays would require the tail-labeling of the target lipids, which could interfere with the binding process in some cases, and the validity of such assays will need to be explored.

Of course, any ligand of interest that can be linked to a fluorescently labeled lipid could potentially be used to evaluate protein binding. Multiple ligands could also be tested in one experiment, when they are conjugated to lipids with different fluorophores. Fluorescent membrane proteins could also be used for studying protein—protein interactions on SLBs. In fact, in combination with microfluidic devices, this method has the potential to become a high-throughput detection method with high sensitivity and selectivity for competitive surface binding without labeling the protein of interest.[77,78]

ASSOCIATED CONTENT

ⓢ Supporting Information

Additional information concerning the clustering of ligands upon protein binding, detection under different experimental conditions and the EEF focusing of PEG$_{550}$-NBD-PE. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b00999.

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Notes
The authors declare no competing financial interest.

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