Multifunctional Monolayer Assemblies for Reversible Direct Fluorescence Transduction of Protein−Ligand Interactions at Surfaces

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Abstract: This paper describes a convenient new method for preparing functionalizable protein-resistant monolayers that can be used to incorporate ligands and protein-sensitive fluorescent reporter groups, and the use of these monolayers for the detection of protein−ligand interactions. BODIPY X-650/665, a diode laser compatible fluorophore, and biotin, a model ligand, have been used to transduce biospecific interactions between proteins and biotin at surfaces. Silicon wafers or quartz slides were coated with (3-aminopropyl)triethoxysilane, and treated with glutaraldehyde and then 2,2′-(ethylenedioxy)bis(ethylenediamine). The resultant surface layers are resistant to nonspecific protein adsorption and contain primary amine groups that are available for subsequent derivatization. Chemical modification of the amine-terminated monolayers thus obtained was accomplished using the *N*-hydroxysuccinimide active ester of BODIPY X-650/665 and biotin activated with Woodward's reagent K. Surfaces treated only with the BODIPY dye for long periods of time to produce a near monolayer coverage of the fluorophore exhibited a dramatic attenuation of the emission of the fluore upon nonspecific adsorption of protein (e.g., albumin). Nonspecific adsorption of proteins can be minimized by diluting the fluore on the surface. Incorporation of a biospecific ligand (i.e., biotin) and the BODIPY fluore in mixed monolayers by serial chemical modification of amine-terminated monolayers yielded surfaces that can be used for fluorescence transduction of biospecific protein adsorption. Specific binding of streptavidin and anti-biotin was detected by a decrease in both the intensity and excited-state lifetime of the fluorescence of the BODIPY dye. Binding of anti-biotin to these surfaces is reversible. No significant change in the intensity was observed upon exposure of these surfaces to solutions of biotin-blocked streptavidin and anti-human IgG. Only a slight change in intensity was observed upon exposure to bovine serum albumin. Phase angle measurements obtained at a single frequency (100 MHz) were used to detect the reversible binding of anti-biotin at the monolayer surface. These observations indicate that it is possible to construct architectures containing ligands and fluores that can be used to detect binding events using lifetime-based measurements. These assemblies should be generalizable to study a wide variety of protein− and cell−surface interactions in biotechnological applications.

Introduction

The ability to detect protein−ligand interactions at surfaces is an important challenge in many scientific fields, including drug discovery,[1] the development of medical diagnostics and sensors,[2] the development of biocompatible implants and tissue engineering,[3] and the study of biomolecular recognition.[4] Fluorescence transduction is often the method of choice due to its high sensitivity, the wide availability of spectroscopic instrumentation, and the ease of introduction of fluorescent probes.[5−7] Recent developments in fluorescence instrumentation have included compact laser diode excitation sources and lifetime and phase angle measurements which are generally less sensitive to fluctuations in excitation efficiency (e.g., due to changes in background light, source fluctuation, or misalignment) and in emission efficiency (e.g., due to photobleaching, change in dye concentration, or turbidity) than intensity measurements.[8,9] Unfortunately, most of the fluorescence-based transduction systems that have been reported require the introduction of a secondary labeling reagent (e.g., in competitive or sandwich immunoassays), are often irreversible, and preclude real-time analyte monitoring, or are not generalizable to a wide range of protein and small-molecule interactions.

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Scheme 1. Structures of Biotin and the
N-Hydroxysuccinimide Active Ester of BODIPY X-650/665

BIOTIN

BODIPY X-650/665 SE
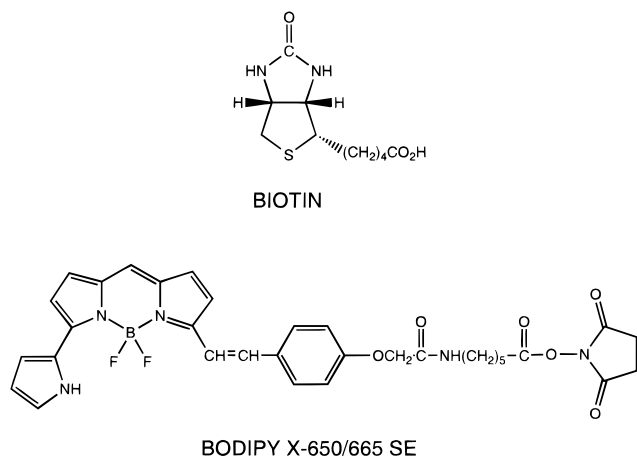
In this paper, we report (1) the preparation of molecular assemblies that present biospecific ligands and fluorescent probes on a surface that is resistant to nonspecific protein adsorption, (2) the use of a new protein-sensitive dye to transduce interactions between proteins and small molecules, and (3) a model biosensor architecture that combines both of these features for the specific and reversible detection of antibody binding to a surface. A significant aspect of this model architecture is that it should be generalizable to monitoring a wide range of protein−ligand interactions. The use of a protein-sensitive fluore to monitor nonspecific adsorption is also demonstrated. The fluore used in this study, BODIPY X-650/665,[10] exhibits quenching of its fluorescence in the presence of proteins and absorbs in the red region of the spectrum, allowing its excitation by currently available diode lasers with minimal interference from biological species.[11] These properties will be especially advantageous in the application of this dye to compact sensor devices. The model biosensor described here demonstrates the use of this protein-sensitive dye for the detection of specific and reversible binding of anti-biotin to a biotinylated surface. The structures of the BODIPY dye and biotin are shown in Scheme 1.

Results

Preparation and Characterization of Amine-Terminated Oligo(ethylene glycol) Monolayers. Serial chemisorption of (3-aminopropyl)triethoxysilane (APTES), glutaraldehyde, and a hydrophilic diamine, 2,2′-(ethylenedioxy)bis(ethylenediamine) (DADOO), on quartz or on silicon wafers has been used to generate amine-terminated monolayer surfaces. Amine-terminated monolayers prepared by this approach were characterized by ellipsometry, contact angle measurements, and X-ray photoelectron spectroscopy (XPS). Monolayers formed from APTES exhibit an ellipsometric thickness on Si wafers of 4.9 ± 0.5 Å. The thickness increased to 13.2 ± 0.9 Å after the addition of glutaraldehyde and to 22.1 ± 0.7 Å on addition of DADOO. The thickness of these monolayers is somewhat less than that expected for the imine-linked, amine-terminated monolayers ($\sim$26 Å). The advancing contact angle of water on monolayers made from APTES on quartz was $45 \pm 2°$; after reaction with

(10) BODIPY = 6-(((4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-*s*-indacen-3-yl)styryloxy)acetyl)aminohexanoic acid, succinimidyl ester; Woodwards' reagent K = *N*-ethyl-5-phenylisoxazolium-3′-sulfonate.

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glutaraldehyde the contact angle was $39 \pm 1°$. The amine-terminated monolayer formed after reaction with DADOO exhibited a contact angle of $47 \pm 3°$.

X-ray photoelectron spectroscopy indicated three types of C 1s peaks, consistent with the presence of ether (CH$_2$O) and amine (CH$_2$N) (both with approximately the same C 1s binding energy) and methylene and imino (C=N) groups in the monolayers. The C/N atomic ratio for the amine-terminated monolayer was higher (7.0) than that expected for an APTES/glutaraldehyde/DADOO monolayer (4.6), indicating the possible incomplete reaction of the glutaraldehyde and/or diamine with the APTES-treated surface. A detailed analysis of the monolayers by XPS and time-of-flight SIMS and a complete documentation of their protein-resistant qualities will be published elsewhere.

Preparation and Characterization of Functionalized Monolayers. Amine-terminated surfaces can be modified readily using standard organic reactions, e.g., reaction with active esters, activated carboxylic acids, or isothiocyanates. In this study, amine-terminated monolayers were derivatized by reaction with an active ester of the BODIPY dye and with activated biotin. Biotinylation of the amine-terminated surface was accomplished using biotin that had been activated with Woodward's reagent K.[10] Monolayers on silicon wafers were used for ellipsometric measurements; those on quartz were used for fluorescence measurements.

Near monolayer coverage of the surface with BODIPY X-650/665 was accomplished by reacting the amine-terminated monolayers with the *N*-hydroxysuccinimide active ester of the dye (10 nM) for 12 h in DMF. This coupling resulted in an increase of the total film thickness from 22.1 ± 0.7 to 32.0 ± 0.2 Å. X-ray photoelectron spectroscopy indicated the presence of boron and fluorine from the BODIPY dye in the derivatized monolayer. The fluorescence emission spectrum on the surface showed that there was no change in the spectral line shape (maximum $\sim$665 nm) from that of BODIPY X-650/665 in solution. No significant change in the fluorescence intensity (at 665 nm) was observed over 20 min of constant irradiation at typical excitation fluxes (1−7 mW/mm^2).

Mixed monolayers were obtained by sequentially exposing the amine-terminated monolayers to the activated biotin and BODIPY reagents. After reaction, the films were washed repeatedly with buffer and DMF to remove unreacted biotin and dye. The biotin/fluore ratios for films prepared under a range of conditions were estimated on the basis of XPS measurements of surface sulfur (which is present only in biotin) and fluorine (which is present only in the BODIPY dye). Table 1 shows that the ratio of biotin to fluore can be varied controllably by adjusting the reaction times of the ligand and the fluore. The biotin/fluore ratio was high for long biotin reaction times, whereas the ratio was low at long fluore reaction times. Reversing the order of addition (e.g., by reacting with BODIPY first and then with biotin) resulted in low biotin/fluore ratios. The fluorescence intensity exhibited a trend consistent with the XPS data. At the lower surface coverages of the dye examined, the fluorescence intensity approached our detection limit.

Interactions of Proteins with Monolayers. 1. Amine-Terminated DADOO Monolayers. Exposure of these films to solutions of bovine serum albumin (BSA) or streptavidin (1 mg/mL of protein in phosphate buffer for 2 h) resulted in no detectable adsorbed protein as measured by ellipsometry. These results suggest that the films prepared by this approach are resistant to nonspecific adsorption of proteins.[12] In contrast, the use of a hydrophobic diamine (1,7-diaminoheptane) led to mono-

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Table 1. XPS and Fluorescence Data for Mixed Monolayers of Biotin and BODIPY X-650/665 Produced by Various Reaction Conditions[a]

| reaction time (h)		biotin/ fluore ratio[e]	fluore intensity[c] (cps × 10³)	change in intensity[d] (cps × 10³)	
biotin[a]	fluore[a]			streptavidin	biotin-blocked streptavidin
2[b]	12[b]	0.15	121	61	28
2	2	0.78	67	55	2
4	2	2.09	32	12	1
8	2	2.16	9	7	0.7
12	2	14.6	4	1	0.01

[a] Amine-terminated monolayers were derivatized by first reacting with biotin for the specified time (see the Experimental Section for additional details) and then with the fluore. [b] Amine-terminated monolayers were derivatized by first reacting with fluore followed by biotin. [c] At λ_{max}. The power density of the laser used was 1.7 mW/mm². [d] Samples were exposed to protein solution (1 mg/mL in phosphate buffer) for 2 h. [e] From XPS ratio of sulfur/fluorine.
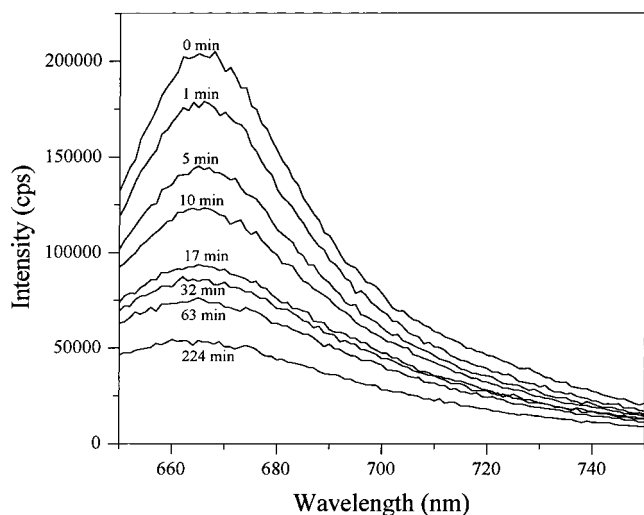
Figure 1. Transduction of nonspecific adsorption of BSA to BODIPY X-650/665-terminated monolayers through measurement of the emission spectrum. The surface was exposed to a 1 mg/mL solution of BSA in phosphate-buffered saline (pH 7.4, 0.1 M). A He−Ne laser (633 nm, 6.9 mW/mm²) was used to excite the fluore.

layers that exhibited nonspecific adsorption of BSA (ellipsometric thickness increase of 21.0 ± 0.6 Å after a 2 h exposure to a 1 mg/mL solution) and streptavidin (26.9 ± 0.3 Å).

2. BODIPY-Terminated Monolayers. Surfaces with high concentrations of the BODIPY dye were formed by treating the amine-terminated monolayers with the fluorescent reagent for long periods of time (10 nM in DMF for 12 h). In contrast with the amine-terminated monolayers, which exhibited no measurable adsorption of proteins, these monolayers on silicon wafers were observed to adsorb BSA, as evidenced by an increase in ellipsometric thickness from 32.0 ± 0.2 to 53.0 ± 0.3 Å after a 2 h exposure to a 1 mg/mL solution of BSA in phosphate buffer. Furthermore, exposure of the dye-terminated monolayers on quartz to a 1 mg/mL solution of BSA resulted in a time-dependent quenching of the fluorescence by the protein as shown in Figure 1. A similar, albeit much less dramatic, quenching was observed when other dyes (i.e., rhodamine and Cy-5) coupled to the amine-terminated monolayers were exposed to proteins. These results suggest that the BODIPY dye exhibits protein-mediated quenching of its fluorescence as

(12) We have shown that these monolayers are resistant to the adsorption of a wide range of proteins and to the attachment of bacterial cells. The details of these studies will be published elsewhere.
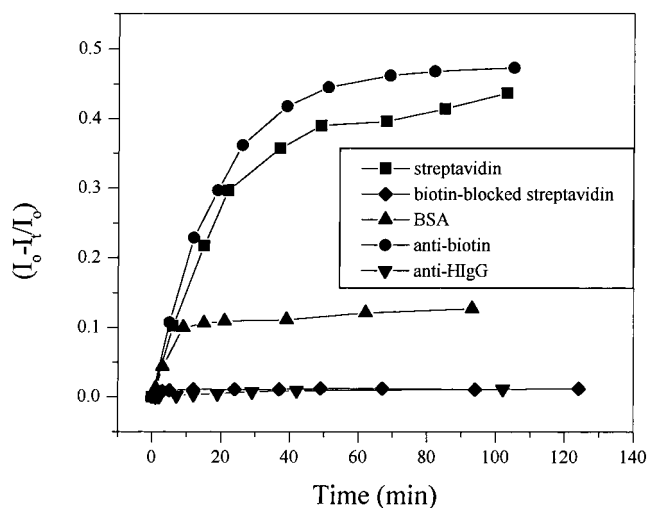
Figure 2. Intensity-based transduction of biospecific adsorption of streptavidin and anti-biotin to monolayers incorporating approximately equimolar amounts of surface-bound biotin and BODIPY X-650/665. The y axis reflects the change in fluorescence emission intensity at 665 nm. BSA (1 mg/mL), streptavidin (1 mg/mL), anti-biotin (0.1 mg/mL), and anti-human IgG (0.1 mg/mL) were dissolved in phosphate buffer (0.1 M, pH 7.4).

a protein adsorbs to the surface, and that protein-mediated quenching is useful in detecting protein binding (nonspecific or specific) to a surface.

3. **Mixed Monolayers Containing BODIPY and Biotin**. Initial experiments with monolayers containing mixtures of covalently bound BODIPY and biotin used ellipsometry and steady-state fluorescence spectroscopy to monitor protein adsorption. To examine the specificity of protein adsorption to biospecific recognition between biotin and its receptors, monolayers were prepared by reacting amine-terminated monolayers first with biotin (2 h) and then with the BODIPY dye (1 h). The resultant films contained approximately equal molar quantities of biotin and the fluore (as indicated by XPS).

Figure 2 displays the change in fluorescence emission intensities (at the emission maximum of 665 nm) as a function of time for mixed monolayers prepared by the above approach that have been exposed separately to BSA, biotin-blocked streptavidin, streptavidin, anti-biotin, and anti-human IgG. Exposure to anti-human IgG or streptavidin that had previously been exposed to biotin showed no significant change in fluorescence intensity. Only a slight decrease in fluorescence intensity was observed when the mixed monolayers were exposed to BSA. Exposure of the films to either streptavidin or anti-biotin resulted in a significant time-dependent decrease in fluorescence intensity (Figure 2). Similar films fabricated on silicon wafer substrates (with 2 h biotin and 1 h BODIPY reaction times) exhibited an increase in thickness of 20.2 ± 2.0 Å when exposed to streptavidin, 13.1 ± 0.7 Å when exposed to anti-biotin, and 8.0 ± 0.9 Å after exposure to BSA. No significant increase in the thickness was observed upon exposure of the samples to biotin-blocked streptavidin and anti-human IgG. These experiments indicate that (1) the mixed monolayers exhibit considerable resistance to nonspecific protein adsorption and (2) the decrease in fluorescence intensity was due to quenching by streptavidin and anti-biotin specifically bound to the biotin in the mixed monolayers.

The reversibility of this system was demonstrated by adding free biotin to mixed monolayers previously exposed to anti-biotin. An increase in fluorescence intensity was observed upon
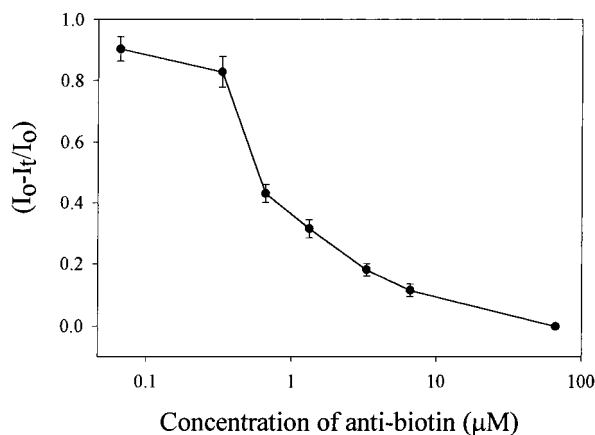
Figure 3. Change in fluorescence intensity versus anti-biotin concentration for anti-biotin binding to monolayers incorporating approximately equimolar amounts of surface-bound biotin and BODIPY X-650/665. The *y* axis reflects the change in fluorescence emission intensity at 665 nm. The surfaces were exposed to solutions of the antibody in phosphate buffer for 2 h.
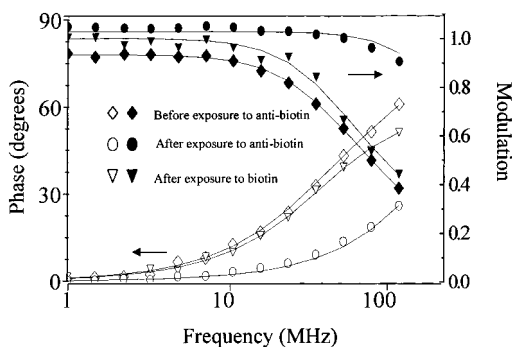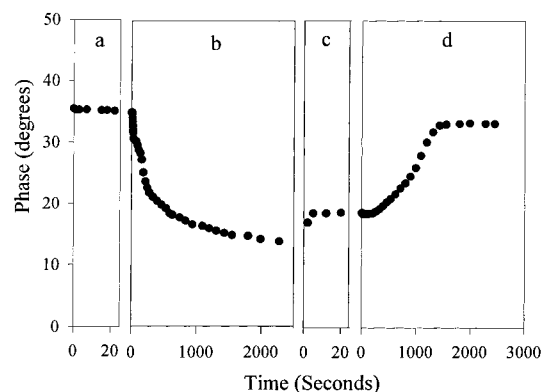

Figure 4. Lifetime-based transduction of biospecific adsorption and desorption of anti-biotin to a monolayer incorporating surface-bound biotin and BODIPY X-650/665. Data collection was initiated after 2 h of incubation of the film with anti-biotin (0.1 mg/mL) and biotin (1.0 mM) solutions in phosphate buffer. The curves represent least-squares fits to the frequency response of the phase angle and modulation depth that were used to estimate the fluorescence lifetimes.

the addition of biotin (data not shown), consistent with the displacement of anti-biotin from the monolayer surface. This reversibility of anti-biotin binding was confirmed with time-resolved fluorescence measurements that are described below.

To obtain a qualitative view of the detection limit of this system for measurement of antibody adsorption, varying amounts of the antibody were titrated into phosphate buffer in which a quartz substrate was immersed. As shown in Figure 3, when sub-micromolar quantities of the antibody were added, there were only small changes in the fluorescence intensity ($\sim$10% for 0.1 μM). However, the fluorescence intensity increases rapidly in the micromolar range, reaching a plateau at approximately 100 μM.

Lifetime and phase angle measurements were also used to transduce binding interactions between anti-biotin and surface biotin groups. Figure 4 shows the frequency domain phase angle and modulation depth curves of the mixed monolayers on a quartz substrate. The excited-state lifetime of the BODIPY fluore in the mixed monolayers was estimated by least-squares fitting of both the phase angle and modulation depth as a function of excitation frequency (double exponential fit). Prior to exposure to protein, the two lifetime components obtained through this procedure were 1.20 ns (60%) and 0.85 ns (40%). After exposure of the mixed monolayers to 0.1 mg/mL anti-biotin


Figure 5. Phase angle-based transduction (excitation frequency 100 MHz) of biospecific adsorption and desorption of anti-biotin to a monolayer incorporating surface-bound biotin and BODIPY X-650/665: (a) Sample in phosphate buffer (prior to antibody exposure); (b) sample exposed to 0.1 mg/mL anti-biotin in phosphate buffer; (c) sample after being washed three times with phosphate buffer; (d) sample exposed to a 1 mM solution of biotin in phosphate buffer.

for 2 h and washing to remove free antibody, the average lifetime decreased (0.54 ns (90%) and 1.24 ns (10%)) consistent with the quenching observed in intensity measurements. When the anti-biotin-treated monolayers were placed in a solution of biotin (1 mM), the frequency domain curve returned to essentially the same position as that of the original mixed monolayers. This indicates that the quenching of the fluorescence resulting from binding of anti-biotin to the surface is reversible.

Figure 5 shows a plot of the phase angle at 100 MHz vs time for mixed monolayers that have been exposed to anti-biotin and subsequently with biotin. Binding of anti-biotin to the mixed monolayer (Figure 5b) results in a dramatic decrease in the phase angle due to the diminished fluorescence lifetime on anti-biotin binding to the monolayer surface. Only a modest increase in the phase angle (Figure 5c) was observed on washing with buffer, possibly due to displacement of weakly bound anti-biotin from the surface. On addition of a solution of biotin to the mixed monolayers, the phase angle increased with time (Figure 5d) as anti-biotin was displaced from the monolayer surface by reaction with the free biotin.

Subsequent experiments examined the effect of varying the relative amounts of fluore and ligand on the surface on the degree of specificity of binding and the change in fluorescence intensity associated with protein adsorption. Table 1 summarizes the different reaction times for biotin and the BODIPY fluore, the resultant relative levels of biotin and fluore obtained, and the response of the surfaces to specific and nonspecific adsorption of streptavidin. The degree of nonspecific adsorption was assessed by measuring the fluorescence response of the surfaces to biotin-blocked streptavidin. Monolayers with high biotin/fluore ratios exhibit highly specific adsorption toward streptavidin (i.e., little change in the fluorescence intensity upon exposure to biotin-blocked streptavidin). Monolayers with low biotin/fluore ratios showed nonspecific adsorption of biotin-blocked streptavidin, which is likely due to interaction of the protein with the hydrophobic fluore. Intermediate biotin/fluore ratios (e.g., 0.78) showed optimum responses, with a substantial change in fluorescence intensity (e.g., $\sim$80%) upon exposure to unblocked streptavidin, yet little change in intensity (e.g., <5%) upon exposure to biotin-blocked streptavidin. These results indicate that surfaces containing approximately equimolar amounts of biotin and fluore are optimal for detection of biospecific adsorption.
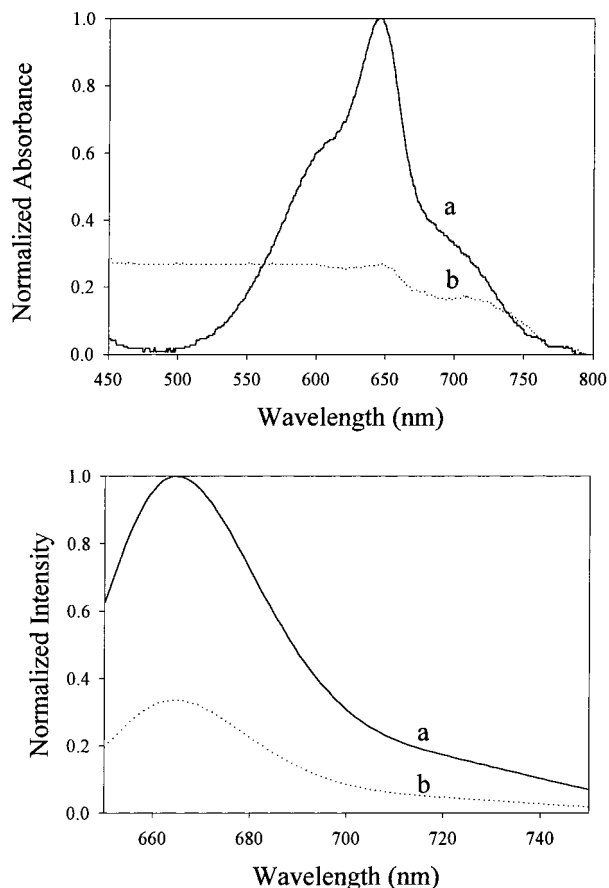
Figure 6. Paired absorption (top) and emission (bottom) spectra of (a) 10 nM BODIPY X-650/665 and (b) 10 nM BODIPY X-650/665 mixed with 2.8 μM streptavidin in phosphate buffer/DMF (99/1 v/v) solution. The mixtures of protein and dye were incubated for 1 h before the spectra were taken.

Effect of Proteins on BODIPY. To obtain a qualitative basis for the origin of protein-mediated quenching of BODIPY fluorescence, absorption spectra of the dye in the presence of various proteins were collected and compared to the native spectrum of BODIPY. Figure 6 (top) shows the absorption spectra of the BODIPY dye in the absence (a) and presence (b) of streptavidin in solution. The bottom panel in Figure 6 depicts the corresponding emission spectra of the native BODIPY (a) and BODIPY/streptavidin (b) solutions. In the presence of protein, a dramatic change in the absorption spectral line shape of BODIPY is concomitant with the reduction in emission quantum yield. Similar changes were observed when the BODIPY was placed in solution with other proteins (e.g., anti-biotin).

Discussion

Nonspecific Adsorption of Proteins. The amine-terminated monolayers used in this study were assembled by the serial chemisorption of APTES, glutaraldehyde, and a diamine on quartz or silicon substrates. A similar approach has been reported by Lev and co-workers[13] who derivatized cystamine-treated gold films with glutaraldehyde and a hydrophobic diamine. The monolayers prepared using DADOO as the diamine yielded monolayers that were resistant to the nonspecific binding of proteins. The thickness of the amine-terminated monolayers (~22.1 Å) is significantly less than that anticipated for the bis-imine-linked monolayer composed of APTES, glutaraldehyde,

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and DADOO (ca. 26 Å). The reduced thickness may be due to incomplete reaction of the amine/aldehyde groups during the chemisorption of the monolayers or to inefficient packing of the APTES−glutaraldehyde−DADOO chains in the monolayer. XPS measurements were consistent with this view of the structure of these monolayers. An important aspect of these monolayers is that, while they are resistant to nonspecific adsorption of proteins, they can be functionalized with fluores, ligands, and receptors. Whitesides et al. have developed self-assembled monolayers (SAMs) incorporating high densities of oligo(ethylene glycol) groups that are highly resistant to the adsorption of proteins and the attachment of cells.[14] This group has also recently described SAMs containing carboxylic acid terminated oligo(ethylene glycol) groups which can be converted to active esters for their facile reaction with amines.[15]

The amine termini of the monolayers prepared by our approach can be readily modified using active esters, isothiocyanates, or carboxylic acids that have been activated using Woodward's reagent K[16] or other activating agents. These surfaces were readily derivatized with the *N*-hydroxysuccinimide active ester of the BODIPY dye. This dye, with excitation and emission maxima at wavelengths of 650 and 665 nm, respectively, is compatible with excitation by HeNe and diode lasers and is extremely photostable. Monolayers reacted with the fluore for long periods of time (12 h) exhibited nonspecific binding of protein that resulted in a dramatic decrease in the fluorescence intensity (Figure 1).

Fluorescence Transduction of Biospecific Adsorption. Biotinylation of the amine-terminated monolayers was accomplished through reaction with biotin activated with Woodward's reagent K. A similar derivatization of amine-terminated surfaces through the use of 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide, was reported by Willner et al.[17] to prepare electrodes modified with ferrocene. By serially reacting the amine-terminated monolayers with the active ester of BODIPY and the activated biotin and by controlling the reaction time of each, the relative amounts of fluore and biotin on the surface can be easily controlled.

The biotin/fluore ratio is crucial in the use of these monolayers for the detection of protein binding. The ratio could be varied from 0.1 to 15 either by changing the reaction order of biotin and BODIPY or by increasing the reaction time (Table 1). When the biotin/fluore ratio is high, the surface exhibits little nonspecific binding of streptavidin, but the fluore intensity is too low (near the noise level) to allow transduction of protein binding. When the biotin/fluore ratio is too low, the surface nonspecifically adsorbs protein due to the large amount of hydrophobic dye on the surface, which readily interacts with proteins.

Our results indicate that an approximately equimolar amount of biotin and fluore on the surface is optimal for transduction of biospecific protein adsorption. These monolayers specifically bind streptavidin and anti-biotin as evidenced by an increase in the ellipsometric thickness and a reduction in the intensity and

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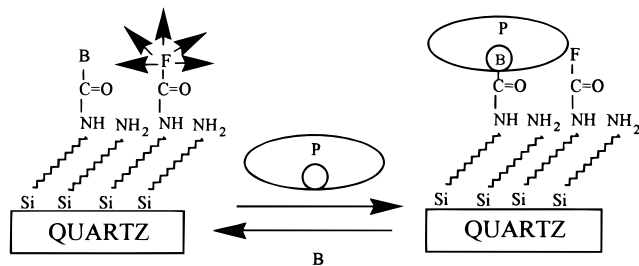
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Scheme 2. Proposed Transduction Mechanism of Protein (P)-Mediated Quenching of the BODIPY Fluore on Mixed Biotin (B)- and Fluore (F)-Containing Monolayers

lifetime of the fluorescence on exposure of these proteins to the monolayer. Both biotin-blocked streptavidin and nonspecific antibodies (e.g., anti-human IgG) result in no significant change in the monolayer thickness or in the fluorescence intensity.

For biosensing applications, it is desirable to have a spectroscopic signal that directly reflects the degree to which proteins are bound to an active surface (e.g., containing immobilized ligands). Reversibility of binding is also an important hallmark of real-time biosensing which requires the minimization of irreversible nonspecific protein−surface interactions. In our system, changes in the fluorescence intensity and lifetime of surface fluores reflect the binding and dissociation of proteins from the surface. Time-dependent fluorescence intensity measurements were first used to detect the specific adsorption of streptavidin and anti-biotin. In fluorescence lifetime-based approaches, it is only necessary to measure the phase angle at one frequency as a function of time to get rapid, real-time detection.[18] At 100 MHz frequency, association and dissociation of the anti-biotin have been demonstrated using time-dependent phase angle measurements. In both the intensity-based measurements and the lifetime-based measurements the attenuation of the fluorescence signal (intensity or phase angle) due to protein binding was slower than expected for the kinetics of binding of streptavidin and anti-biotin to a biotinylated surface. For example, we have observed the kinetics of adsorption of streptavidin to a biotinylated SAM to be diffusion-limited in surface plasmon resonance studies.[19] For high streptavidin concentrations such as those used here (1 mg/mL), a saturation of specific binding is typically observed within a few minutes. Likewise, during the dissociation of anti-biotin in the presence of free biotin, there was a significant induction period of about 20 min in the fluorescence response, after which the response was fast. These results are due to the fact that we are not directly measuring the binding of these proteins to surface-bound biotins, but their interaction with surface-bound fluores.

Implications for Real-Time Chemical Sensing. It is gratifying to note that the presence of two very different protein species (i.e., streptavidin and anti-biotin) can be transduced when held in place by a specific ligand proximal to the dye (Scheme 2). This finding is significant in that it lays the essential groundwork for a generalizable approach to the detection of a wide range of ligand−protein interactions. To completely evaluate the potential of the current scheme (or similar schemes) for real-time, reversible biosensing, it is necessary to understand the nature of the interaction of the protein with the BODIPY dye and to optimize the interaction for this purpose. While the exact nature of the interaction of the protein dye and the BODIPY

dye used here is not completely understood, investigation toward this end is underway. Preliminary absorption experiments indicate that a variety of proteins form nonfluorescent (or less fluorescent) molecular complexes with the BODIPY dye. Other researchers have reported the protein sensitivity of other BODIPY derivatives,[20] although all previous studies have dealt with BODIPY derivatives that excite at shorter wavelengths than the one used here (absorption maximum ∼650 nm). In addition to the possible slow association kinetics for the formation of the nonfluorescent complex, the possible nonoptimal distribution of the fluores and ligands in the assemblies used here may contribute to the slow time response of the sensors described. We are currently conducting experiments to elucidate the kinetics and equilibrium constant for formation of protein/BODIPY complexes.

In the molecular assemblies studied here, the presumed random distribution of ligand and reporter fluore on the surface is unlikely to be optimum for the best time response and detection limit. Ideally, one would like one ligand−protein binding event to result in the fast, intimate contact and quenching of one (or several) fluores. If the distribution of ligand and fluore on the surface is random, it may be that a ligand binding event results in either no fluore quenching or very slow fluore quenching. Hence, the slow fluorescent response and the detection limit observed (Figure 3) may be due, at least in part, to a nonoptimal distribution of fluore and biotin on the surface. These considerations may also lead to a significant dependence of the kinetic response and detection limit of the sensor on the size of the analyte protein. We are currently conducting experiments that are designed to optimize the correspondence of fluorescence quenching with ligand binding. Toward this goal, we have synthesized a series of molecules containing one BODIPY fluore, one ligand, and an active ester that will allow its incorporation into amine-terminated surfaces.

Conclusions

This research demonstrates that it is possible (1) to tailor surfaces that are resistant to nonspecific protein adsorption so that they display both small-molecule ligands and fluorescent dyes and (2) to utilize these mixed monolayers to detect the binding of proteins using fluorescence intensity, lifetime, and phase measurements. The model biosensor system described in this paper should be generalizable, since it allows for the detection of antibody binding without the need to introduce a reagent and could be extended to a wide range of protein−small-molecule interactions. The synthetic technique described herein can be easily extended to utilize many other protein-sensitive dyes including those that increase their quantum yield in the presence of proteins.[21] Through fluorescence imaging, the use of similar molecular monolayers may be used to study a wide variety of protein−surface (e.g., recognition, catalysis, and diffusion) and cell−surface (e.g., formation of focal contacts, protein excretion, and gliding) interactions.

Experimental Section

Reagents. (3-Aminopropyl)triethoxysilane (Sigma), glutaraldehyde (Aldrich), 2,2′-(ethylenedioxy)bis(ethylenediamine) (Aldrich), and Woodward's reagent K (WRK; Aldrich) were used as obtained. The fluorescent dye BODIPY X-650/665 and biotin were obtained from

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Molecular Probes, Inc. (Portland, OR). Bovine serum albumin (Molecular Probes, Inc., 1 mg/mL), streptavidin (Molecular Probes, Inc., 1 mg/mL), monoclonal anti-biotin (Sigma clone bn-34, 0.1−0.0001 mg/mL), and goat anti-Human IgG (Sigma, 0.1 mg/mL) were dissolved in phosphate buffer (0.1 M, pH 7.4) for protein adsorption measurements.

Preparation of Monolayers. Quartz substrates were cleaned with piranha solution (70/30 v/v H$_2$SO$_4$/30% H$_2$O$_2$) for 30 min at 60 °C, and rinsed thoroughly with deionized water. *Warning: piranha solution reacts violently with most organics and must be handled with extreme care.* Monolayers were constructed by immersing a quartz substrate in a 0.1 M solution of (3-aminopropyl)triethoxysilane in toluene for 2 h, followed by treatment for 2 h with a 25% glutaraldehyde solution in ethanol and, finally, treatment of the aldehyde-terminated monolayer with a 10 mM solution of 2,2′-(ethylenedioxy)bis(ethylenediamine) (DADOO) in ethanol. After exposure to glutaraldehyde and DADOO, the films were washed with ethanol and dried under a stream of nitrogen.

Derivatization of Monolayers. Unless otherwise indicated, mixed monolayers containing oligo(ethylene glycol) groups, surface-bound biotin, and BODIPY fluores were prepared on quartz by reacting the amine-terminated monolayers with a 1 mM solution of biotin (activated with WRK) in phosphate buffer for 2 h followed by treatment with a 10 nM solution of the active ester of the BODIPY dye in DMF for 1 h. After exposure to the *N*-hydroxysuccinimide active ester of BODIPY X-650/665, the films were washed with DMF, sonicated in DMF for 5 min, rinsed with DMF, and dried under a stream of nitrogen.

Surface Characterization. Advancing contact angles were measured by using a Rame-Hart Model 100 contact angle goniometer. The reported values are the average of three measurements taken at different locations on the monolayer surface. The average monolayer thickness was measured by using a multiwavelength (Model M-44, J. A. Woollam Company, Inc.) ellipsometer, assuming the refractive index of the organic layer is 1.45. XPS analyses were conducted on an AXIS-HSi instrument from Kratos Analytical, Inc. (Ramsey, NY). An Al Kα$_{1,2}$

monochromatized X-ray source ($h\nu$ = 1486.7 eV) with an emission power of 150 W (15 kV and 10 mA) was used to stimulate photoelectron emission. The residual pressure in the analysis chamber was on the order of 10^{-9} Torr or lower during spectral acquisition. All spectra were acquired with a constant pass energy of 40 eV. The spectral envelopes were resolved into Gaussian peaks to fit the spectra, and the hydrocarbon C 1s peak was referenced at 284.6 eV.

Protein Adsorption. Mixed monolayers on Si wafers or quartz plates were placed in vials containing phosphate buffer. A protein solution of appropriate concentration was added to the vial. Silicon wafer substrates were used for ellipsometric measurements; quartz substrates were used for fluorescence measurements. Unless otherwise indicated, the samples were exposed to protein for 2 h, and then the vial containing sample and protein was rinsed with buffer solution. Protein adsorption monitored by fluorescence experiments was accomplished by holding the quartz plate coated with the monolayer in a cuvette containing the buffer and protein solutions.

Fluorescence Measurements. Fluorescence spectroscopy was performed on a SPEX phase fluorimeter (Model Fluorolog-3 with Tau-2 phase fluorimetry attachment from Instruments S.A., Edison, NJ) equipped with a He−Ne (633 nm) laser excitation source. Colloidal silica (LUDOX SK, Sigma, 25 wt % in water) was used as a reference in time-resolved fluorescence measurements, for which the lifetime was assumed to be zero. Absorption spectra were recorded by using a Shimadzu UV/vis spectrophotometer (Model UV-1601).