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Controlling Binding Site Densities on Glass Surfaces

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The density of surface-immobilized ligands or binding sites is an important issue for the development of sensors, array- or chip-based assays, and single-molecule detection methods. The goal of this research is to control the binding site density of reactive ligands on surfaces by diluting surface amine groups in self-assembled and cross-linked monolayers on glass prepared from solutions containing very low concentrations of (3-aminopropyl)triethoxysilane (APTES) and much higher concentrations of (2-cyanoethyl)triethoxysilane. The surface amine sites are suitable for attaching labels and ligands by reaction with succinimidyl ester reagents. Labeling the amine sites with fluorescent molecules and imaging the single molecules with fluorescence microscopy provides a means of determining the density of amine sites on the surface, which were incorporated into the self-assembled monolayer with micrometer spacings in proportion to the concentration of APTES in the synthesis. Biotin ligands were also bound to these surface amine sites using a succinimidyl ester linker, and the immobilized biotin was then reacted with either streptavidin-conjugated gold colloid particles or fluorescently labeled neutravidin. Imaging of these samples yields consistent amine and biotin site coverages, indicating that quantitative control and chemical conversion of binding sites can be achieved at very low ($<10^{-7}$) fractions of a monolayer.

Control of the density of surface-immobilized ligands or surface-binding sites is an important issue for the manipulation of surface properties and the development of sensors, array- or chip-based assays, and single-molecule detection methods. The density and chemical identity of surface-immobilized ligands can be used to control the wettability of surfaces,[1] the selective adsorption of biological molecules,[2,3] and the attachment and growth of cells on artificial surfaces.[4,5] The signal level and response sensitivity that one observes from waveguide, interfacial fluorescence, or SPR-based sensors[6] depends directly on controlling the density of reactive binding sites on the surface. For cooperative or multivalent binding of biological molecules to surface-tethered ligands, the density of ligands on the surface can significantly influence binding equilibria and energetics.[7−9]

The control of ligand density on surfaces has been addressed in a number of ways. To produce gradients in ligand density on surfaces, one can generate a diffusion gradient of silane reagent in solution so that the target glass surface is exposed to a varying concentration of reagent, leading to a gradient in the density of bound silane ligands.[1,2] This same approach can be implemented for generating gradients of organothiol ligands on metal surfaces;[10] a clever method of producing a similar ligand density gradient on thin gold films involved the reductive desorption of thiol-bound ligands by applying an in-plane potential gradient across the film.[11,12] Control of surface ligand densities[13] and the preparation of density gradients[14] can also be achieved by contact printing techniques by varying the concentration of ligand on the stamp or the contact time between the stamp and the surface.

For characterizing the reactivity of isolated ligands on surfaces at the single-molecule level, it is critical that the ligand spacing on the surface be controlled at greater than micrometer distances so that the response of individual molecules can be resolved by optical microscopy methods. Such widely spaced ligands correspond to very small ($<10^{-6}$) fractions of a full monolayer, which are challenging to generate in a controlled manner. One approach that is capable of reaching these low densities is to bind surface ligands to phospholipid molecules and dilute these into lipid bilayers deposited onto glass substrates. This technique has been successful for studies of the effects of ligand concentration on cooperative or multivalent binding[7−9] and recently for investigating lipid mobility by fluorescence correlation spectroscopy near the single-molecule limit.[15] While supported lipid bilayers can be prepared with very low concentrations of head group-bound

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ligands, the resulting bilayer substrate is fragile and must be stored under water or buffer in order to remain stable. For tracking the reactivity of specific ligands on the surface, the mobility of phospholipid molecules in the bilayer[15] allows the ligand sites to diffuse, making the characterization of a specific binding site or ligand difficult. A technique that can produce stationary populations of sparsely spaced molecules on a solid surface is controlled substrate withdrawal or dip coating from solution.[16,17] This method of dosing has produced surface populations at predictable surface coverages as small as 5×10^{-8} of a monolayer; however, the molecules are only physisorbed to the substrate and leave the surface upon exposure to solvent.

The approach taken in this work to immobilize ligands or binding sites at very low densities on a surface is to incorporate reactive sites at low concentrations within a cross-linked alkoxysilane monolayer self-assembled onto glass. Self-assembled silane monolayers have been widely applied in materials science, separations, and analysis, due to their versatility in modifying surfaces to produce stable and uniform films with a variety of interfacial properties.[18−26] From the earliest studies of self-assembled organosilane monolayers, there have been considerable efforts directed to the production of mixed monolayers to control wetting and adsorption behavior.[18,21−26]

While previous studies investigated mixed self-assembled monolayers composed of major fractions of two different silanes, our goal in this work is to use mixed silanes to generate binding sites with very wide spacing on the surface. The general idea is to incorporate a target silane reagent, having a reactive group to provide a binding site for a ligand, at very low concentrations into the self-assembled and cross-linked film, composed primarily of an inert silane that is compatible with the solution interface and reactions of the immobilized ligand. Specifically, we test the incorporation of very dilute amine sites into a nitrile-terminated alkylsilane, where the dipolar character of the nitrile group is compatible with aqueous solution but otherwise inert to reactions with the amine binding sites or immobilized ligands. A previous study in the literature has reported dispersing 10% *n*-alkylamine sites into an *n*-alkylsilane monolayer bound to oxidized silicon surfaces,[25] producing mixed monolayers that were homogeneous and suitable for binding DNA.

In this work, mixed silane monolayers were self-assembled onto glass from solutions containing very low concentrations of (3-aminopropyl)triethoxysilane (APTES) and much higher concentrations of (2-cyanoethyl)triethoxysilane (CETES). Alkoxysilane reagents were chosen for this application because they are compatible with a terminal amine group and are less susceptible than chlorosilanes to polymerization due to traces of water in solution.[24,26] The densities of aminesilane groups were characterized using epifluorescence imaging of individual dye molecules covalently attached to the surface amine groups. The surfaces were also characterized by bright-field plasmon-resonance imaging of streptavidin-conjugated 10-nm gold colloids bound to biotin molecules immobilized on the amine binding sites. The density of the immobilized biotin molecules was also assessed using total internal reflection fluorescence (TIRF) imaging of fluorescently labeled neutravidin. All three of these methods give consistent amine coverage results and indicate that quantitative control of binding site densities can be achieved at very low ($<10^{-7}$) fractions of a full monolayer.

EXPERIMENTAL SECTION

Chemicals and Materials. Spectrophotometric-grade solvents, toluene, methanol, *n*-heptane, and *N*,*N*-dimethylformamide (DMF), were obtained from Fisher Scientific (Hampton, NH). Toluene and *n*-heptane were dried over sodium for 24 h and filtered through a Millipore (VWR, West Chester, PA) PTFE 0.2-μm filter prior to use; methanol and DMF were used as received. Both 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester (TMR-SE) and 6-(6-((biotinoyl)bis(aminohexanoyl))succinimidyl ester (biotin-xx-SE) were purchased from Biotium inc. (Hayward, CA) and were used as received. Tetramethylrhodamine-labeled neutravidin and 5-(and-6)-carboxy-Alexafluor-514 succinimidyl ester (Alexa-SE) were purchased from Molecular Probes (Eugene OR). CETES and APTES were acquired from Gelest (Morrisville, PA). Streptavidin-conjugated gold colloid particles having a mean diameter of 9.7 nm, a particle density of 1.7×10^{13} particles mL^{-1}, and an average of 20 streptavidin/gold colloid were acquired from Ted Pella (Redding, CA). Coverslips (No. 1, 22 × 22 mm) were purchased from VWR (West Chester, PA).

Surface Derivatization. Surface preparation began with cleaning of the glass coverslips to remove contamination. Cleaning of coverslip surfaces was accomplished by rinsing the slides with methanol, allowing them to dry, and then placing them in a UV−ozone cleaner (Jelight Co. model 342) for 25 min on each side. Slides were checked using water contact-angle measurements to determine cleanliness; a contact angle of <5° indicated that slides were sufficiently clean to produce uniform silane monolayers with minimal fluorescence background.

Fresh stock solutions of 1 M CETES and 1 nM APTES were prepared in dry toluene; small aliquots of these solutions were diluted into dry *n*-heptane producing reaction solutions consisting of 2 mM CETES and the desired concentration (0.04−0.105 nM) of APTES. Cleaned coverslips were placed in the reagent solutions and allowed to react for a period of 2 h, after which, they were rinsed 4 times each with toluene and methanol. Surface-modified coverslips were annealed in a 120 °C oven for 30 min to promote reaction with the surface and cross-linking of the silanes. Coverslips were then stored under methanol for up to one week until used.

Reaction with amine reactive sites was accomplished through the use of succinimidyl ester binding chemistry,[27,28] where the

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labeling reagent will react with surface amines to form a peptide bond to the surface. Succinimidyl ester binding in phosphate buffer (pH 9.3) to a primary amine linker for a cyanine dye has been shown to be complete, 87% isolated yield.[27] The succinimidyl ester reagent is susceptible to rapid hydrolysis in aqueous solution, with a half-life of 10 min at pH 8.6 and 4 °C.[28] To produce higher yields for a binding reaction, one can avoid hydrolysis of the succinimidyl ester reagent by carrying out the reaction in an organic solvent such as DMF or DMSO, allowing the effective reaction time to be increased.[28] Based on this strategy, a stock solution of 3 mg of TMR-SE in 5 mL of DMF was prepared and kept at −20 °C until use. An aliquot of the TMR-SE stock solution was diluted 1:125 in DMF (7.5 μM), and the amine-modified surfaces were reacted in this solution for 1 h and then rinsed twice in DMF and four times in methanol for 20 min each. The derivatized coverslips were stored in methanol in the dark prior to their examination by fluorescence microscopy (see below). Reaction of amine-modified slides with Alexa-SE was carried out under identical conditions.

Surface-amine sites were also tagged with a biotin label in order to test the binding site density using streptavidin-conjugated gold colloid and fluorescently labeled neutravidin. A succinimidyl ester reaction with the surface-amine groups was again employed, in this case using biotin-xx-SE at 7.5-μM in DMF reacted for 1 h, followed by rinsing twice in DMF and four times in methanol for 20 min each. Once excess biotin-xx reagent was removed, the biotinylated coverslips could be reacted with streptavidin-conjugated gold particles or fluorescently labeled neutravidin. For the gold labeling of the biotin sites, slides were submerged in a glass petri dish containing streptavidin-conjugated gold colloids at a concentration of 2.1×10^{11} particles/mL, suspended in 20 mM phosphate buffer saline (pH 7.5, ionic strength of 100 mM with sodium chloride). Coverslips were allowed to react for a period of 24 h, rinsed with buffer and methanol, and allowed to dry prior to imaging. For fluorescence labeling of the biotin sites, biotinylated coverslips were introduced into a microscopy flow cell[29] and illuminated with 528.7-nm laser radiation on the TIRF microscope for 20 min, to photobleach any fluorescence spots from the glass substrate. The substrate was then reacted with a 6.7 pM TMR-labeled neutravidin in 20 mM phosphate buffer saline for 50 min in the flow cell, after which the surface was rinsed with multiple flow-cell volumes of phosphate buffer and immediately imaged using TIRF microscopy (see below).

Microscopy Measurements. Fluorescence imaging of surface-immobilized tetramethylrhodamine and Alexa-514 dye molecules was performed using epifluorescence microscopy. The fluorescence microscope was described previously;[30,31] several modifications and improvements to this microscope have been made. Briefly, the 514.5-nm line from an argon ion laser (Spectra-Physics) is used as the excitation source and is passed through a Pellin-Broca prism and an aperture to remove plasma lines. The beam is shuttered by an acoustooptic deflector (AOM, Crystal Technology) to control sample illumination. The switched beam is passed through a quarter wave plate to transform the linearly polarized light into circular polarized light, so that excitation of molecules that are attached to surfaces is independent of their orientation. The beam is then passed on a roughened glass disk to create an incoherent spot source for excitation; the disk is rotated at several hundred rotations per minute in order to average out the speckle pattern on the time scale of the experiment. The spot on the glass disk is then reimaged with a 55-mm focal length, *f*/1.2 camera lens (Canon) into the back of the microscope (Nikon). The beam is then passed through a Chroma 514.5-nm band-pass filter (10-nm bandwidth), reflected off of a 545-nm dichroic beam splitter and directed into a Nikon plan fluor 100×, 1.3 NA, oil immersion objective. The beam overfills the collection cone of the objective, creating a nearly uniform intensity profile over the observation area; the laser power coupled into the objective ~17 mW. Fluorescence emission was collected back through the same objective, transmitted by the 545-nm dichroic beam splitter, through a 530-nm long-pass filter and imaged on a Photometrics CoolSNAP$_{HQ}$ 12-bit CCD camera with a low-bandwidth sensitivity of 3.4 photoelectrons/bit. MetaMorph (Universal Imaging) software was used to control the camera, collect images (1-s integration times), and analyze the image data. The length scales for all microscopy experiments were calibrated with a USA-1951 standard resolution test target (Newport).

Imaging of surface-attached, streptavidin-conjugated gold colloid particles was accomplished using an Olympus IX71 inverted microscope. The samples were imaged in bright field using a 0.30 NA overhead illuminator; light transmitted by the sample was collected through an Olympus plan apo 60×, 1.45 NA, oil immersion objective, passed through a dichroic beam splitter and bandpass emission filter (Chroma Z514RDC and HQ560/50, respectively), and imaged on a Photometrics CoolSNAP$_{HQ}$ CCD camera. MetaMorph Imaging software was again used to collect and analyze the bright-field images acquired in 10-ms integrations. Surface-bound neutravidin were also imaged using the same Olympus inverted microscope, operated in TIRF mode. Excitation of the sample was achieved using an argon ion laser (Coherent, model Innova 300) operated at 528.7 nm and coupled into the microscope using a single-mode optical fiber. Total internal reflection was achieved by translating the fiber vertically, which in turn moved the position of the incoming laser beam (25 mW) to the edge of the objective until internal reflection was observed at the interface between the coverslip surface and the buffer solution. TIRF images were acquired with 200-ms integration times.

RESULTS AND DISCUSSION

Single-Molecule Fluorescence Counting of Surface Binding Sites. Reactive amine functional groups were immobilized at very low surface densities on glass by self-assembly of mixed silane monolayers from solutions containing very low concentrations of APTES and much higher concentrations of CETES. The surface derivatization chemistry is illustrated in Figure 1, where self-assembly from dry *n*-heptane solution promotes adsorption from solution of the reactants onto the glass surface. The surfaces were rinsed in toluene and methanol to eliminate excess silane reagent, following which they were heated to 120 °C for 30 min to promote condensation reactions with the surface and cross-linking of monolayer film.[18−23] The concentrations of APTES (0.04−0.105 nM) to CETES (2 mM) corresponded to a concentra-

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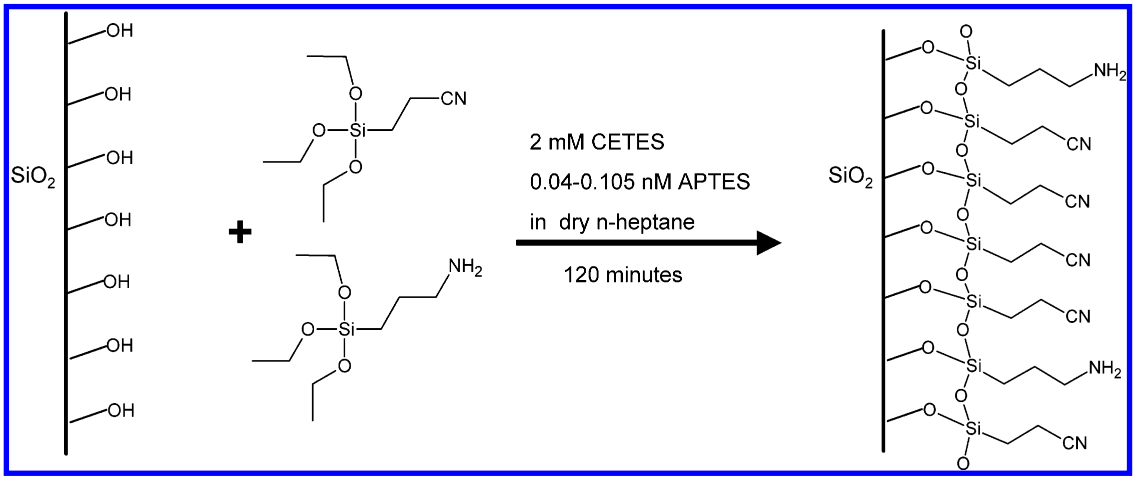

Figure 1. Immobilization and cross-linking of dilute aminopropylsilane groups in a cyanoethylsilane self-assembled monolayer on glass.

tion ratio of 2×10^{-8}–5.25×10^{-8}. If the amine-bindng site concentrations in the monolayer corresponded directly to these dilution factors, then one would expect the amine sites to be spaced between 2.1 and 3.4 μm based on molecular density of self-assembled and cross-linked alkylsiloxane monolayers of ~0.23 (±0.02) nm^2/silane determined by infrared absorption[21,32,33] and X-ray reflection measurements.[34] The expected distances between highly diluted amine binding sites, therefore, could be easily resolved within the diffraction limits of optical microscopy.

To measure the surface density of aminopropylsilane molecules immobilized in the cyanoethylsilane monolayer, the amine groups were reacted with TMR-SE in DMF for 60 min, according to the reaction scheme outline in Figure 2A. The reacted slides were rinsed in DMF and methanol and then imaged by epifluorescence microscopy, which was previously shown to yield reliable (>96%) detection of rhodamine 6G molecules adsorbed to glass surfaces;[30] example data are shown in Figure 3A. The threshold for counting single-molecule spots was determined from the background intensity and noise level for a sample where no TMR molecules were immobilized, which produced an equivalent background as regions between bright spots on TMR-labeled surfaces. This background level was μ_B ~17 photoelectrons, while the pixel-to-pixel variation in background counts had a standard deviation, σ_B = 12 photoelectrons, which is 3 times greater than the photoelectron shot noise. The threshold for counting molecules[35] was set conservatively at Lc = 85 photoelectrons, which is 5.7 times σ_B above μ_B, making the probability of false positive counts arising from the variation in the dark background negligible, $< 10^{-8}$. The fluorescent spots had an average diameter (fwhm) of 320 nm, which is equivalent to the diffraction limit of the 1.3-NA objective at the average wavelength of TMR fluorescence emission of 560 nm, where the diffraction-limited Gaussian spot[36,37] should exhibit a fwhm = 0.72λ/NA = 310 nm. The spots exhibited a distribution of intensities above the background with an average peak intensity μ_P = 414 photoelectron counts and a standard deviation that was ~3 times larger than the photoelectron shot noise, σ_P = 67, probably due to differences in molecular orientation, local environment of the surface, or photobleaching. The intensity threshold for counting molecules, Lc = 85 photoelectrons, is $4.9\sigma_P$ below the average peak intensity, so the probability of missing a TMR molecule on the surface is small, $<10^{-6}$.

Setting the threshold, Lc, at 85 photoelectrons, one can plot the positions of the fluorescence spots on the surface that exceed this level, which result in the image shown in Figure 3B. The locations of the molecular spots appear at first glance to exhibit patterns of molecules bound in curved lines with large open areas between them. While one might be tempted to attribute these "patterns" to inhomogeneities in the binding of amine-terminated silanes in the self-assembled monolayer, they are actually characteristic of a random distribution in two dimensions. Examples of 2-D random distributions show very similar patterns (see Supporting Information), where a random distribution clearly does not lead to a uniform spacing of bound molecules.

To determine the surface concentration of amine-bound tetramethylrhodamine molecules on the surface, the number of spots above threshold were counted in five different areas on three separate coverslips for each amine concentration. This operation was carried out for four concentrations of aminopropylsilane in the cyanoethylsilane monolayer, including "blank" where no APTES was present in the self-assembly step. The blank slides generated an average background spot density of 0.033 (±0.006) spots/μm^2, which is similar to a previously reported background for blank glass slides (0.021 spots/μm^2)[30] and apparently due to luminescent impurity sites in the glass; coverslips fresh from the UVO cleaning exhibit the same background spot density without a CETES monolayer or exposure to TMR-SE. The blank spot density was subtracted, and the resulting amine site densities versus APTES concentrations are plotted in Figure 4. In addition to the tetramethylrhodamine labeling of amine sites, these sites were also labeled with 5-(and 6)-carboxy-Alexafluor-514 succinimidyl ester. This probe carries a double negative charge from two sulfonate groups so that it is more water soluble and less prone to nonspecific adsorption to glass than tetramethylrhodamine. The Alexa-514 label produces somewhat weaker fluorescence than tetramethylrhodamine in single-molecule imag-

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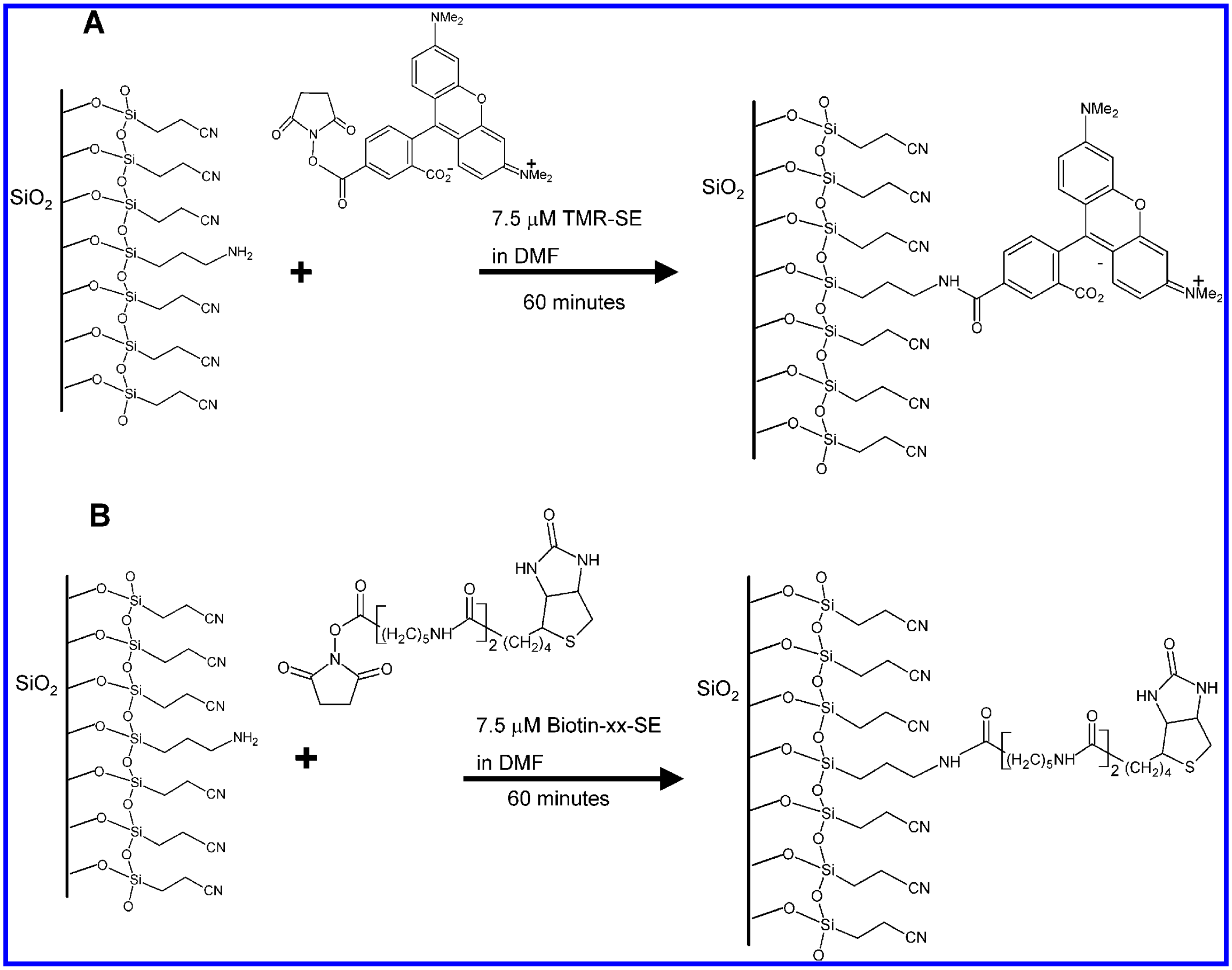

Figure 2. Labeling of amine binding sites. (A) Immobilization of tetramethylrhodamine succinimidyl ester to aminopropylsilane sites on the glass surface. (B) Immobilization of 6-(6-((biotinoyl)bis(aminohexanoyl))succinimidyl ester to aminopropylsilane sites on the glass surface.

ing experiments ($\mu_P = 170$ photoelectrons), and therefore a lower threshold (Lc = 41 photoelectrons) was used to read out the single-molecule spots. The lower threshold increased the probability of false positive counts from the background; Lc is 2.9 times σ_B above μ_B in this case, making the probability of false positive counts $\sim 2 \times 10^{-3}$/pixel. To reduce the potential impact of false positives on the spot count, an additional criterion was applied, where at least three connected pixels must be above the threshold for the spot to be counted; this criterion lowers the false positive probability from background fluctuations to a negligible level. Alexa-514 labeling results at two concentrations of APTES are included in the plot in Figure 4, and these closely match the molecular spot counts determined from tetramethylrhodamine labeling. Both results show a consistent, linear dependence of the surface density of labeled amine sites on the concentration of APTES in the self-assembled monolayer.

Biotin−Avidin Labeling of Amine Binding Sites. While the single-molecule labeling of amines with two different fluorescent probes gave consistent spot densities that varied linearly with the APTES concentration, the single-molecule fluorescence measurement is also sensitive to the population of background spots from the glass substrate, the number of which is comparable to the amine site densities produced at the lowest concentrations of APTES. It is important to confirm the reactive amine site density by a technique that is insensitive to luminescent impurity spots. To that end, the surface site density was characterized in a series of APTES concentration-dependent experiments, where biotin was immobilized to the amine sites and then biotin−streptavidin binding[38] was used to capture streptavidin-conjugated gold particles to the surface site. Surface amine sites were tagged with biotin using a condensation reaction to immobilize biotin-xx-SE to the aminopropylsilane sites on the surface; see Figure 2B. The excess biotin-xx-SE reagent was removed, and the biotinylated coverslips were reacted with streptavidin-conjugated gold (10-nm diameter) and imaged under bright-field illumination; this gold-colloid labeling and imaging approach has been used previously to observe and track the motions of single protein molecules in cells and membranes.[39]

To increase the contrast for detecting the absorption and scattering by the gold particles, a bandpass filter restricts the wavelength of the transmitted radiation to 530−580 nm; this range

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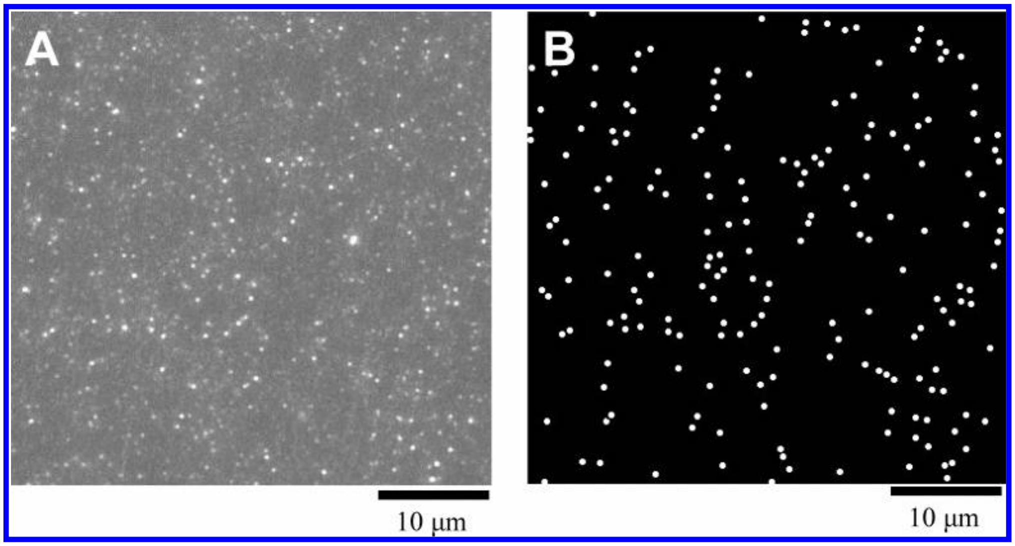

Figure 3. Images of tetramethylrhodamine immobilized to amine binding sites on glass, self-assembled from a solution of 0.06 nM APTES and 2.0 mM CETES. (A) Gray scale image of fluorescence from surface-immobilized tetramethylrhodamine. (B) Threshold image marking the locations of molecules on the surface.

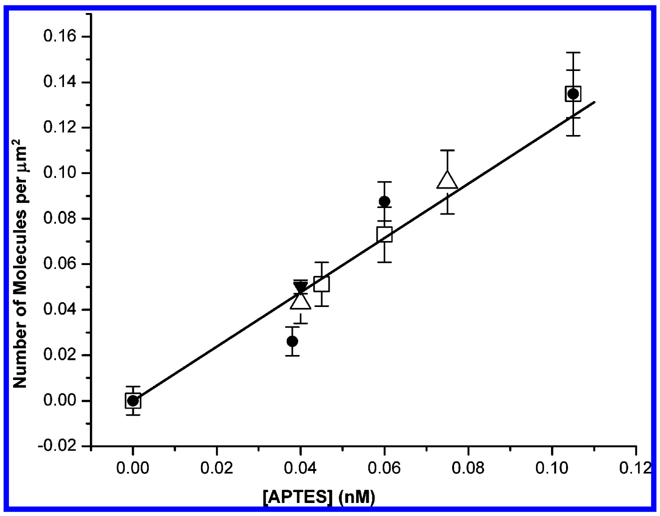

Figure 4. Amine binding site density versus the concentration of APTES in the self-assembly reaction, where the CETES concentration (2 mM) is constant. Surface densities are determined by counting single TMR molecules (□), single Alexa-514 molecules (Δ), steptavidin-conjugated gold colloid particles (●), and TMR-labeled neutravidin (▼).

overlaps with the plasmon resonance extinction of the streptavidin-conjugated gold particles, which peaks at ~535 nm and drops to only half intensity by 580 nm. An example bright-field image of the gold particles are shown in Figure 5A. The diameter of these dark spots is larger than the diffraction-limited size of single molecules imaged in fluorescence; the poorer spatial resolution is not due to the gold particles being "out of focus" but is rather due to the limited numerical aperture (0.3 NA) of the bright-field illuminator. The predicted diffraction-limited diameter for 0.3 NA illumination is ~1.3 μm, which is slightly larger than the size of the Airy disk pattern produced in the bright-field image, probably due to particle scattering at higher angles than the incident radiation; see Supporting Information. These spots exhibit a readily detected change in intensity, relative to the bright background, and provide a reliable area to threshold and count the gold particles bound to the glass surface. The threshold for counting the gold particle spots was determined from the average level, $\mu_B = 230$ photoelectrons, and variation, $\sigma_B = 18$ photoelectrons, of the bright background. The threshold for counting a negative-going spot was set at Lc = 165, 65 photoelectrons or 3.6 times σ_B below the bright background, so that the probability of false positive results is small, $\sim 2 \times 10^{-3}$; to reduce the likelihood of false positives, again an additional criterion was applied where at least three connected pixels must be below the threshold for the spot to be counted. The intensity at the center of the dark spots decreases to 118 photoelectrons on average with a standard deviation of ~14 photoelectrons, so that Lc = 165 is 3−4 standard deviations above the average dark spot, making the probability of missing a spot $\sim 3 \times 10^{-3}$.

Application of this threshold to the data produces a plot of the locations of the gold particles on the slide, as shown in Figure 5B, giving a similar random pattern of spots as observed with single-molecule fluorescence imaging. The spot densities based on counting streptavidin-labeled gold particles are included with the single-molecule fluorescence counting results in Figure 4. In this case, no background spot count was subtracted from the measured spot densities, based on a control experiment where a cyanoethylsilane surface was prepared without amine-binding sites and allowed to react with streptavidin-conjugated gold colloid. After rinsing, this surface exhibited no detectable spots below the bright-field background threshold. The measured spot densities from streptavidin-labeled gold particles closely follow the single-molecule fluorescence results and confirm the reactive amine site densities by a technique that is insensitive to luminescent impurity spots.

The relationship between the amine site density, determined from single-molecule counting of fluorescent labels and imaging of bound gold particles, and the concentration of APTES concentration in the synthesis of the self-assembled monolayer (Figure 4) can be examined to determine relative binding efficiencies of the two silanes. A linear least-squares fit of the data in Figure 4 has a slope of 1.2 (±0.1) amine sites/μm² nM. Using this result in a specific example, a 0.1 nM solution of APTES, which

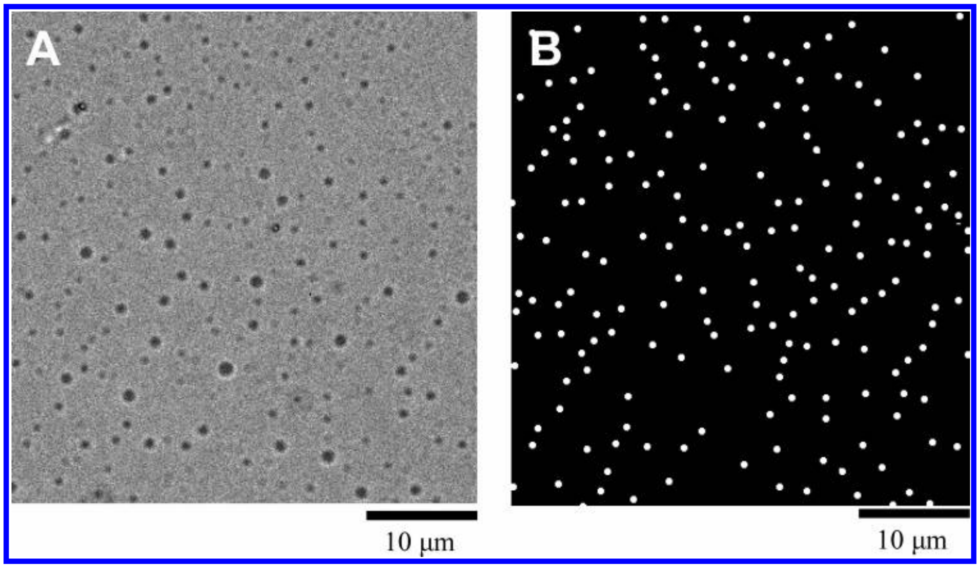

Figure 5. Images of surface-bound, streptavidin-conjugated gold colloid immobilized to biotin sites on the glass surface. The biotin sites were prepared by reaction of biotin-xx-SE with the amine binding sites, self-assembled from a solution of 0.06 nM APTES and 2.0 mM CETES. (A) Bright-field, gray-scale image of the plasmon resonances of surface-immobilized gold particles. (B) Threshold image marking the location of gold particles on the surface.

corresponds to a 5×10^{-8} dilution, relative to the constant 2 mM CETES in the synthesis, produces a surface concentration of amine sites of 0.12 amine/μm^2. Compared to the molecular density of a self-assembled and cross-linked alkylsiloxane monolayers of ~4.4 (±0.4) silanes/nm^2, determined by infrared absorption[21,32,33] and X-ray reflection measurements,[32] this surface concentration of amine sites (0.12 amine/μm^2) corresponds to a surface dilution of 2.7×10^{-8}, or about half the concentration of amine sites expected if the binding of the two silanes were statistical and depended only on the relative concentrations of the two silanes in the self-assembly reaction solution.

Several possible explanations could be offered for a smaller relative concentration of labeled sites on the surface compared to the ratio of silane reagents in solution. First, there could be molecules missed in the counting of labeled sites due to overlap between single-molecule fluorescence or single-particle extinction spots. With a diffraction-limited spot size of 320 nm for single-molecule fluorescent spots and a maximum spot density of 0.14 μm^{-2}, the probability of spot overlap is less than 1.4% based on a Poisson statistical model,[30] so that an apparent 50% discrepancy is not due to this phenomenon. There is a possibility that the succinimidyl ester reaction that binds labels to the amine sites was incomplete. As mentioned in the Experimental Section, however, this reaction is reported to be complete (87% isolated product yield[28]) even under conditions less favorable[29] than those employed in this study. Furthermore, the yield was consistent among the binding of three different succinimidyl ester probes to the surface. We are left with the conclusion that the densities of surface amine groups reported by these experiments are correct, and that the cyano-terminated silane exhibits preferential self-assembly to the glass surface. A higher concentration of bound cyano groups would be expected if the free energy of the self-assembled monolayer were raised slightly by substituting an amine-terminated silane for a cyano-terminated silane. This free energy cost could derive from the disruption of strong dipole–dipole head-to-tail interactions[40] between adjacent C/N groups on the surface, which would dominate the monolayer structure at the high surface coverage of cyanoethylsilane.

An important application of chemically modified surfaces with controlled and widely spaced binding sites would be for immobilizing and studying individual biological molecules. The possibility of using these surfaces for such applications was tested using biotin–avidin binding, which is commonly employed to immobilize antibodies, enzymes, liposomes, and even biological cells to surfaces.[38] Neutravidin (a deglycosylated form of avidin) was chosen for this study because it exhibits lower nonspecific adsorption to surfaces than avidin,[41] while retaining a high affinity for biotin.[42] Biotinylated coverslips were prepared by immobilizing biotin-xx-SE to aminopropylsilane sites on the glass surface, as above. The biotinylated coverslips were assembled into the microscopy flow cell and then illuminated with 528.7-nm laser radiation on the TIRF microscope for 20 min to photobleach any fluorescence spots from the glass substrate. The biotinylated coverslips were then exposed to a 6.7 pM solution of TMR-labeled neutravidin in phosphate buffer in a microscopy flow cell for 50 min, after which the surface was rinsed with multiple flow-cell volumes of buffer and imaged immediately using TIRF microscopy.

The fluorescent spots from the labeled neutravidin were bright (see Figure 6A), averaging $\mu_p = 416$ photoelectrons in 200 ms, which is 0ne-fifth of the exposure time used in the epifluorescence microscope to image single tetramethylrhodamine labels. This brighter intensity is due in part to the more efficient excitation from the evanescent wave in the TIRF mode. In addition, the multiple fluorescent labels (~2.3/neutravidin) lead to brighter spots, but also increase the relative spot-to-spot variation in the

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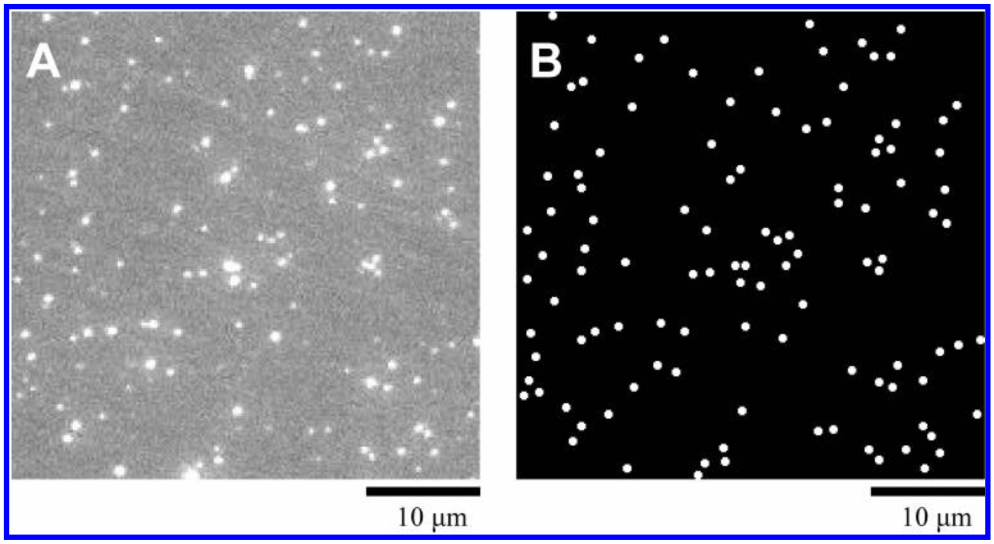

Figure 6. Images of surface-bound, TMR-labeled neutravidin immobilized to biotin sites on the glass surface. The biotin sites were prepared by reaction of biotin-xx-SE with the amine binding sites, self-assembled from a solution of 0.06 nM APTES and 2.0 mM CETES. (A) Gray-scale image of the fluorescence from surface-immobilized TMR-labeled neutravidin. (B) Threshold image marking the locations of molecules on the surface.

peak intensity (σ_p = 102 photoelectrons) due to differences in the number of labels per probe molecule. A threshold for counting bound molecules was determined using data from a blank slide, which produced a background, μ_B = 16 photoelectrons with standard deviation, σ_B = 10 photoelectrons, comparable to the values observed on the epifluorescence microscope. The threshold, Lc, was set at 85 photoelectrons or 7 times σ_B above the background, which leads to a negligible probability of false positives from the fluctuations in the dark background (see Figure 6B). Because of the pre-exposure of the substrate to laser radiation and photobleaching of impurity spots in the glass, a zero background from the substrate was indeed achieved in this case; after the photobleaching step and prior to exposure to labeled neutravidin, the substrates exhibited no detectable spots. False positives could also arise from nonspecific binding of neutravidin to the cyanoethylsilane surface; this possibility was tested by allowing the labeled neutravidin to interact with a CETES-derivatized surface with no immobilized biotin. This surface was allowed to interact with neutravidin under identical conditions and was rinsed with buffer prior to imaging. This experiment produced no spots above threshold, indicating that nonspecific adsorption of neutravidin to the CETES-derivatized surface yields no false positive counts. The surface density of bound neutravidin was determined from this experiment for two different slides prepared with 0.04 nM APTES in the original self-assembled monolayer synthesis yielded an average of 0.050 (±0.003) biotin molecules/μm^2. This point is included in the plot of the site density versus APTES concentration (Figure 4) and agrees well with the predicted site densities from the previous experiments.

Discussion. The goal of this research was to control the binding site density of reactive ligands on surfaces by diluting surface amine groups in self-assembled and cross-linked monolayers composed principally (>99.99999%) of cyanoethylsilane on glass. The cyanoethylsilane surface is strongly dipolar, compatible with aqueous solution, and it shows little nonspecific binding of a water-soluble protein (neutravidin) from aqueous solutions. The extreme dilution of amine binding sites allows them to be spaced over micrometer distances that can be resolved by optical microscopy. The surface amine sites are suitable for attaching labels and ligands by reaction with succinimidyl ester reagents. Labeling the amine sites with fluorescent molecules and imaging the surface provided a means of determining the density of amine sites on the surface and their dependence on the concentration of the amine reagent (APTES) in the original self-assembled monolayer synthesis. Amine sites were incorporated into the self-assembled monolayer in proportion to the concentration of APTES in the synthesis, but the mole ratio of aminopropylsilane to cyanoethylsilane in the monolayer was about half the ratio in the synthesis, indicating that the cyanoethylsilane forms a more stable monolayer, perhaps through head-to-tail dipole−dipole interactions between adjacent nitrile groups.

Using single-molecule counting to determine the surface density of amine sites on the surface suffers from a background of luminescent spots from the glass substrate. Two different methods were employed to confirm the number of amine sites independent of the substrate background. Both approaches involved the binding of biotin to the surface amine sites using 6-(6-((biotinoyl)bis(aminohexanoyl))succinimidyl ester. The immobilized biotin was then labeled with streptavidin-conjugated gold colloid particles, followed by imaging of the plasmon resonance absorption with bright-field illumination. A second approach to counting immobilized biotin molecules was to react them in a microscopy flow cell with fluorescently labeled neutravidin and image them with TIRF microscopy, where the background of the substrate could be photobleached with laser excitation prior to the labeling step. Both of these approaches produced no detectable background spots, and furthermore, the quantitative spot densities agreed with the single-molecule counting results corrected for substrate background. The results of all three imaging methods give consistent amine and biotin site coverages, indicating that quantitative control of binding site densities and their chemical transformation can be achieved at very low (<10^{-7}) fractions of a full monolayer. Applications of these substrates for investigating single-molecule binding equilibria and reaction kinetics at individual ligand sites on surfaces are currently being developed in our laboratory.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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