

Specific Adsorption of Histidine-Tagged Proteins on Silica Surfaces Modified with Ni²⁺/NTA-Derivatized Poly(ethylene glycol)

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Silica surfaces modified with nitrilotriacetic acid (NTA)-polyethylene glycol (PEG) derivatives were used to immobilize hexahistidine-tagged green fluorescent protein (His₆-GFP), biotin/streptavidin-AlexaFluor555 (His₆-biotin/SA-AF), and gramicidin A-containing vesicles (His₆-gA). Three types of surface-reactive PEG derivatives—NTA-PEG3400-Si(OMe)₃, NTA-PEG3400-vinylsulfone, and mPEG5000-Si(OMe)₃ (control)—were grafted onto silica and tested for their ability to capture His₆-tag species via His₆/Ni²⁺/NTA chelation. The composition and thicknesses of the PEG-modified surfaces were characterized using X-ray photoelectron spectroscopy, contact angle, and ellipsometry. Protein capture efficiencies of the NTA-PEG-grafted surfaces were evaluated by measuring fluorescence intensities of these surfaces after exposure to His₆-tag species. XPS and ellipsometry data indicate that surface adsorption occurs via specific interactions between the His₆-tag and the Ni²⁺/NTA-PEG-grafted surface. Protein immobilization was most effective for NTA-PEG3400-Si(OMe)₃-modified surfaces, with maximal areal densities achieved at 45 pmol/cm² for His₆-GFP and 95 fmol/cm² for His₆-biotin/SA-AF. Lipid vesicles containing His₆-gA in a 1:375 gA/lipid ratio could also be immobilized on Ni²⁺/NTA-PEG3400-Si(OMe)₃-modified surfaces at 0.5 mM total lipid. Our results suggest that NTA-PEG-Si(OMe)₃ conjugates may be useful tools for immobilizing His₆-tag proteins on solid surfaces to produce protein-functionalized surfaces.

Introduction

Supported lipid membranes are widely studied for the biophysical characterization of lipid membranes and are now emerging as an important platform for integral membrane protein biosensors.^{1–5} This ambitious goal will require sensors that have been designed with (1) stable and fluid membranes; (2) polymer-grafted surfaces to prevent membrane protein inactivation via adsorption onto the solid support; and (3) ligands that enable specific binding and orientation of the membrane protein because their transport and/or catalytic functions are vectorial in nature. The development of methods that improve the stability and controlled orientation of proteins will have a direct impact on the performance of membrane protein-based devices.

The first challenge in stabilizing supported lipid membranes is required to block the opportunities for bilayer membrane delamination^{5b} in areas of the surface where defects can promote membrane instability. The second goal is being addressed by covalently anchoring the membrane to the surface using hydrophilic tethers to increase the adhesion and stability of

supported lipid membranes.^{2–8} An alternative strategy has been the use of polysaccharide-, polyacrylamide-, and polyethylene glycol (PEG)-based lipopolymers to provide a hydrophilic cushion between the supported membrane and the solid support.^{9–18} Studies with PEG-grafted surfaces have shown that nanometer-scale aqueous reservoirs could be formed beneath the supported membrane, allowing for enhanced stability of the lipid membrane.^{13,14,18} This strategy, however, can decrease lipid lateral mobility because of membrane–solid substrate contact and promote protein adsorption and inactivation as a result of surface-mediated unfolding.^{19,20}

These advances, however, have not fully addressed the third requirement of introducing membrane protein components into the supported membrane in an oriented fashion. Asymmetric orientation of the His-tag molecules has been achieved through metal-mediated complexation of nitrilotriacetic acid (NTA) conjugates and hexahistidine-tagged (His₆-) proteins on silica^{21–24}

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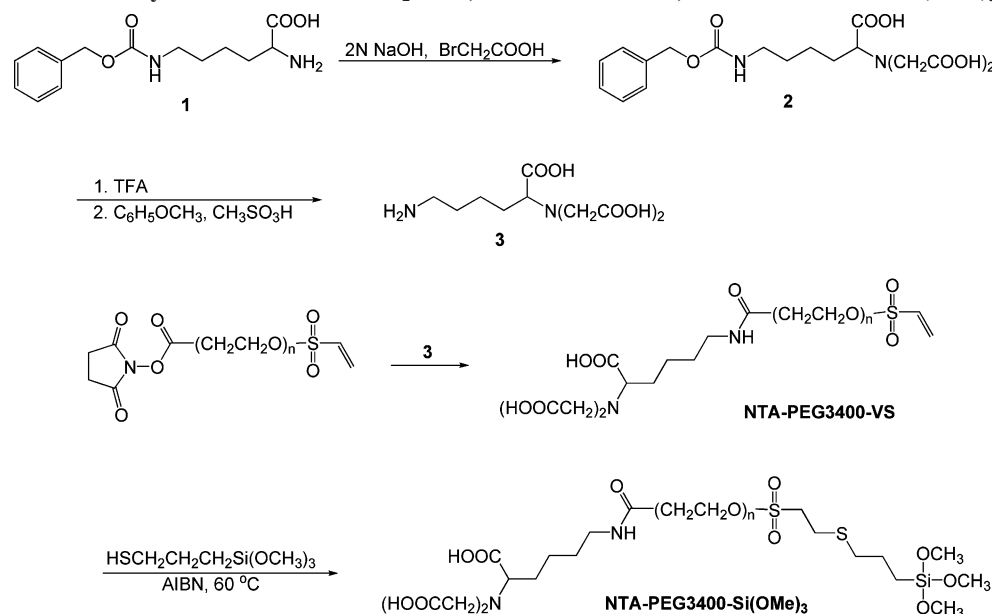
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Scheme 1. Synthesis Scheme for NH₂-NTA, NTA-PEG3400-VS, and NTA-PEG3400-Si(OMe)₃

and gold^{25–31} surfaces. The alkyl-NTA and oligoethylene glycol-NTA derivatives reported thus far, however, may not be large enough to retain the activity of ABC transporters^{31b} and other membrane proteins with extensive water-soluble domains.¹⁸ We sought to develop materials that would entrap aqueous reservoirs of controlled dimensions between the membrane and the solid support while providing control over protein orientation, aspects that will be essential for the successful development of membrane protein-based biosensors.

This study examines the effectiveness of different PEG-NTA conjugation strategies in specifically binding and orienting His₆-tagged proteins. His₆-tag green fluorescence protein (His₆-GFP), His₆-tag biotin/streptavidin-AlexaFluor555 (His₆-biotin/SA-AF), and lipid vesicles containing His₆-tag gramicidin A (His₆-gA) were used as probes to monitor the adsorption of His₆-tag proteins on PEG-modified surfaces in the presence and absence of NTA derivatives. Protein adsorption on Ni²⁺/NTA-PEG-modified surfaces in the absence of His₆-tags was also examined as a control. Our experiments show that specific and reversible binding to PEG-modified silica surfaces occurred only when the PEG molecule contained a Ni²⁺-activated NTA ligand and the protein possessed a hexahistidine interaction with the Ni²⁺/NTA-PEG-grafted surface.

Experimental Methods

Materials. *N*-Hydroxysuccinimidyl-PEG3400-vinylsulfone (NHS-PEG3400-VS) and methoxy-PEG5000-silane (mPEG5000-Si(OMe)₃) were purchased from Nektar Therapeutics (Huntsville, AL). *N*-Carboxy-L-lysine was purchased from Aldrich (St. Louis, MO). Gramicidin A was purchased from Fluka (St. Louis, MO). His₆-gA was prepared by custom peptide synthesis and supplied in C-terminal hexahistidine form by Abgent, Inc. (San Diego, CA). Egg yolk phosphatidylcholine (EPC) and 1- α -dioleoylphosphatidylethanolamine (DOPE) were obtained from Sigma (St. Louis, MO). AlexaFluor 555-labeled streptavidin (SA-AF) and 1- α -dipalmitoylphosphatidylethanolamine-*N*-NBD (NBD-DPPE) were purchased from Molecular Probes (Eugene, OR). His₆-biotin was purchased from Biopeptide Co. (San Diego, CA), and (3-mercaptopropyl)trimethoxysilane (MPTS) was from Gelest Inc. (Morrisville, PA). All other solvents and reagents were purchased from Aldrich. ¹H NMR spectra were recorded using a Bruker ARX300 spectrometer operating at 300 MHz. Chemical shifts are reported in parts per million relative to the residual solvent peak as internal standard.

Synthesis of NTA Intermediate. Lysine nitrilotriacetic acid (NTA) was synthesized as shown in Scheme 1 using a method that has been previously reported.^{32,33}

Synthesis of NTA-PEG3400-Si(OMe)₃. *NTA-PEG3400-VS.* NHS-PEG3400-VS (0.85 g, 0.25 mol) and NTA (0.33 g, 1.25 mmol) were dissolved in 8.5 mL of DMSO. Triethylamine (TEA) (2.00 mL, 14.30 mmol) was added, and the solution was stirred at room temperature for 24 h before quenching with 2 N HCl (28.3 mmol). The solution was then dialyzed for 24 h against 1 L of distilled water using a dialysis membrane (Spectra/Por, MWCO 1000), and the retentate was lyophilized. The NTA-PEG3400-VS product was stored at -20 °C until use. Yield: 50.3%. ¹H NMR (CDCl₃): δ 3.11–2.91 (br, CH₂CH₂O), 2.45 (t, *J* = 6.0 Hz, NHCH₂), 1.88–1.53 (br, NHCH₂(CH₂)₃CHCOOH).

NTA-PEG3400-Si(OMe)₃. Silanation of NTA-PEG3400-VS was performed by free radical addition of mercaptopropyltrimethoxysilane (MPTS) to the vinyl sulfone substituent as shown in Scheme 1.^{34,35} NTA-PEG3400-VS and an 8-fold excess of freshly distilled MPTS were added to toluene in a round-bottomed flask, followed by the addition of a 0.4-fold excess of 2,2'-azobisisobutyronitrile (AIBN)

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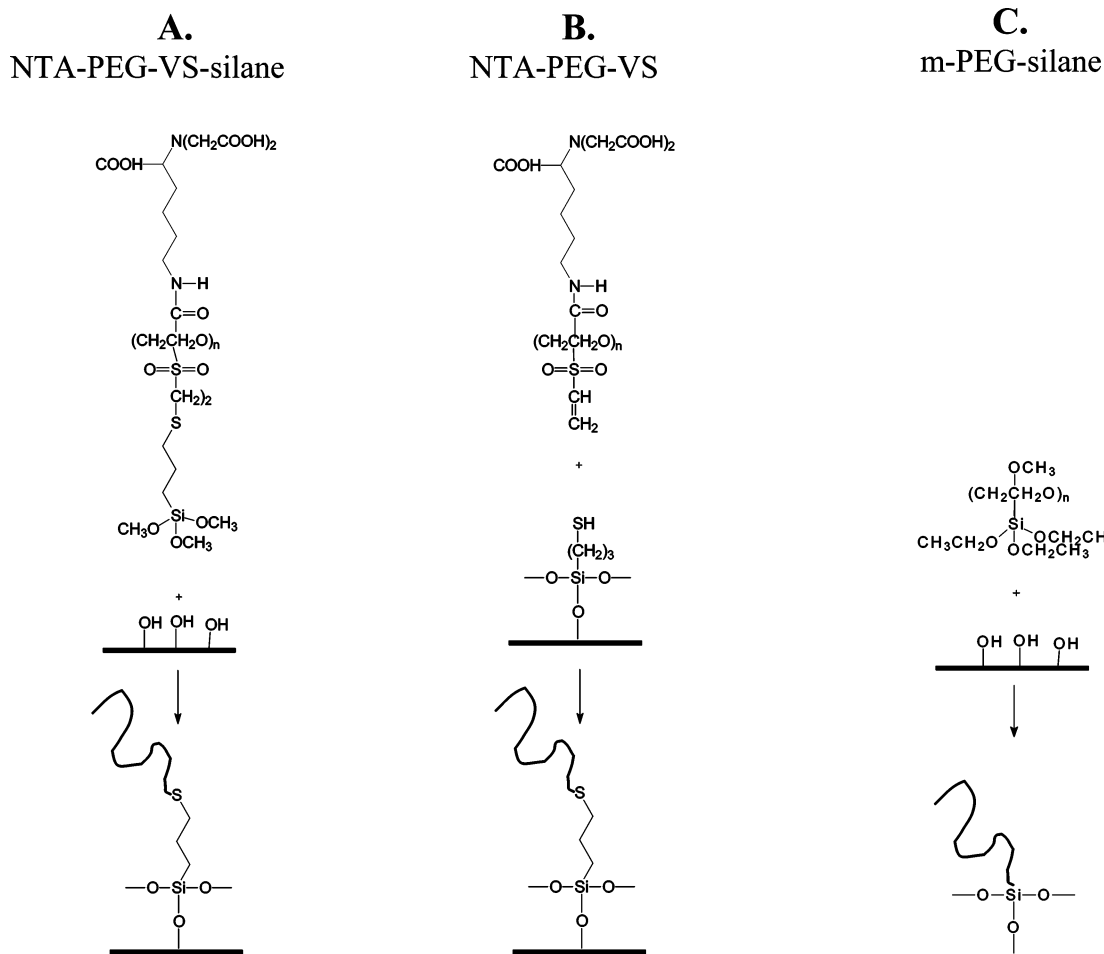
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Scheme 2. Methods for Modifying Silica with NTA-PEG3400-Si(OMe)₃, NTA-PEG3400-VS, and mPEG5000-Si(OMe)₃

relative to NTA-PEG3400-VS. The mixture was stirred for 24 h at 60 °C under a N₂ atmosphere before precipitation of the product with anhydrous Et₂O. The precipitate was filtered, washed with excess Et₂O, and dried under reduced pressure to give NTA-PEG3400-Si(OMe)₃ as a white powder in 53% yield. ¹H NMR (CDCl₃): δ 3.9–3.15 (br, CH₂CH₂O), 2.45 (t, *J* = 6.0 Hz, NHCH₂), 1.68–1.14 (br, NHCH₂(CH₂)₃CHCOOH), and 0.6 (CH₂Si).

Surface Preparation and Characterization. Silicon wafers or cover glasses were immersed in fresh piranha solution for 1 h and rinsed by bath sonication in deionized water. Piranha solution was prepared by mixing pure sulfuric acid and hydrogen peroxide (30% solution) in a 7:3 volume ratio. (**Caution!** Piranha solutions are potentially explosive and highly corrosive. Handle with extreme caution.) The clean substrates were dried under N₂ gas and then immersed in a toluene solution of mPEG5000-Si(OMe)₃ or NTA-PEG3400-Si(OMe)₃ (10 mg PEG derivative/mL) for 16 h at 60 °C.³⁵ The NTA-PEG3400-VS-modified surfaces were prepared as follows: piranha-cleaned surfaces were immersed in 1% MPTS in toluene for 4 h and washed with toluene. The dried MPTS-modified surface was then incubated in NTA-PEG-VS (10 mg/mL) in 0.01 M phosphate-buffered saline (PBS) for 16 h at room temperature.³⁶

Ellipsometric angles Δ and Ψ were measured on the PEG-grafted surfaces with an Auto ELII automatic null ellipsometer (Rudolph Research Corporation, Flanders, NJ) at two incident angles, 70 and 60°, using two wavelengths, 633 and 405 nm. Typically, five measurements were taken, and the values were averaged. First, the complex refractive index of the silicon wafer, *n*-*ik*, and the thickness and refractive index of the native oxide layer were obtained by fitting the Δ–Ψ values from the bare substrate at both angles.

Details of the algorithm can be found in Cuypers et al.³⁷ For silicon, the optimal values were 3.84–0.018i and 5.36–0.300i at 633 and 405 nm, respectively. For silicon oxide, the refractive indices used were 1.465 and 1.470, respectively, for an oxide layer thickness of 3.1 nm. A four-layer model was then used to determine the thickness, *d*₁, and refractive index, *n*₁, of the dry polymer layer from the measured values of Δ and Ψ. The surface density, Γ, was determined from *d*₁ and *n*₁ at each angle and each wavelength by using the equation

$$\Gamma = d_1 \left(\frac{M}{A_r} \right) \left(\frac{n_1^2 - 1}{n_1^2 + 2} \right) \quad (1)$$

where *M* is the molecular weight and *A_r* is the molar refractivity of the polymer. From *d*₁ and the density ρ (assumed to be 1 g/cm³), the average distance, *L*, between the grafted PEG chains was determined from the de Gennes equation

$$L = \left(\frac{M}{\rho d_1 N_A} \right)^{1/2} \quad (2)$$

where *N_A* is Avogadro's number.

X-ray photoelectron spectroscopy (XPS) experiments utilized a Kratos Axis ULTRA XPS instrument. All XPS spectra were obtained at 10^{−9} Torr with monochromatized Al Kα radiation as the X-ray source. A hemispherical analyzer with a multichannel detector was used with a takeoff angle of 90°. For the survey, the dwell time was 0.1 s, and the pass energy was 160 eV. For high-resolution spectra,

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Table 1. Analysis of PEG-Modified Surfaces by Ellipsometry and Contact Angle Measurements

Φ (deg)		mPEG5000-Si(OMe) ₃		NTA-PEG3400-Si(OMe) ₃		NTA-PEG3400-VS + MPTS
		70°	60°	70°	60°	70°
633 nm	d_1 (nm)	14 ± 3	16 ± 1	17 ± 3	16 ± 1	4 ± 0
	n_1	1.436 ± 0.176	1.362 ± 0.057	1.346 ± 0.119	1.361 ± 0.064	1.703 ± 0.255
	Γ ($\mu\text{mol}/\text{m}^2$)	2.9 ± 0.2	2.8 ± 0.2	4.3 ± 0.2	4.2 ± 0.3	2.0 ± 0.3
	L (Å)	8 ± 1	7 ± 0	6 ± 1	6 ± 0	12 ± 0
405 nm	d_1 (nm)	13 ± 1	14 ± 0	13 ± 1	14 ± 1	3 ± 0
	n_1	1.529 ± 0.124	1.478 ± 0.042	1.545 ± 0.113	1.472 ± 0.074	1.747 ± 0.287
	Γ ($\mu\text{mol}/\text{m}^2$)	3.2 ± 0.3	3.2 ± 0.2	4.8 ± 0.4	4.6 ± 0.4	1.7 ± 0.1
	L (Å)	8 ± 1	8 ± 0	7 ± 0	6 ± 1	14 ± 0
contact angle (deg)		48 ± 2°		51 ± 1°		51 ± 2°

a pass energy of 40 eV and a dwell time of 0.3 s were used for Ni 2p, O 1s, and C 1s, and 0.5 s was used for N 1s.

The surface wettability of the PEG-modified surfaces that had been dried in a desiccator overnight was determined under static conditions using a Kruss contact angle goniometer (Hamburg, Germany).

Expression of Hexahistidine-Tagged Green Fluorescent Protein (His₆-GFP). Ampicillin-resistant *E. coli* that had been transfected with a gene to express wild-type His₆-GFP under L-(+)-arabinose induction were grown in Luria broth (25 g of LB/L of deionized water) containing ampicillin (0.05 mg/mL) for 24 h at 37 °C. L-(+)-Arabinose (0.2 mg/mL) was added, and the incubation continued for 24 h at 30 °C. After centrifugation, the cell pellet was lysed with BugBuster (Novagen, San Diego, CA) protein-extraction reagent, and the supernatant was concentrated by ultrafiltration (MWCO = 10 kD). The concentrated protein solution was then purified using a Ni²⁺-activated IMAC column (Amersham Biosciences, Piscataway, NJ) and eluted with imidazole. Imidazole was removed by three sequential centrifugal desalting steps using 10 kD cutoff Centricon filter units to ensure the complete removal of imidazole. The final concentration of His₆-GFP was determined by Bradford assay using BSA standards (Bio-Rad, Hercules, CA), and the purity was confirmed by SDS-PAGE electrophoresis.

Calibration Curves for His₆-GFP and Streptavidin. Stock solutions of His₆-GFP and SA-AF were diluted in 10 mM HEPES buffer containing 20% glycerol to produce concentrations of 100 μM –1 mM and 100 nM–1.8 μM , respectively.³⁸ The diluted protein solutions were spotted on epoxy-terminated hydrophobic glass slides (Arrayit, Sunnyvale, CA) using a manual microarrayer (V&P Scientific Co., SD). The delivery volume of the manual microarrayer pin was 50 nL, and the diameters of the deposited solutions were 300 μm . Fluorescence intensities at each GFP or SA-AF spot were measured using confocal laser scanning microscopy (CLSM, Bio-Rad MC 1024, Bio-Rad Laboratories, Hercules, CA). The fluorescence intensities of 10 individual spots were measured using the same instrument settings and the values averaged for each protein concentration. Surface density determinations were based on the delivery of the calibrated volume to a spot of known diameter, the fluorescence intensity observed within the spot area, and the concentration of the protein applied to the spot.

Adsorption of His₆-GFP and His₆-biotin/SA-AF on NTA-PEG-Modified Surfaces. Surfaces modified with NTA-PEG3400-Si(OMe)₃, NTA-PEG3400-VS, or mPEG5000-Si(OMe)₃ were activated by treating them with 50 mM NiCl₂ for 1 h. To prime the surfaces for SA-AF binding, the Ni²⁺-chelated surfaces were immersed in His₆-biotin solutions at concentrations ranging from 0.2 to 10 mg/mL in 0.01 M phosphate-buffered saline at pH 7.4 (PBS) for 1 h at room temperature. The samples were then washed with PBS, and the His₆-biotin-modified surface was incubated in SA-AF solutions for 2 h at room temperature. A final wash with PBS/0.1% Tween 20 was used to remove unbound streptavidin molecules.

Solutions containing varying concentrations of His₆-GFP were prepared in HEPES buffer. Ni²⁺-chelating NTA-PEG surfaces were

incubated in His₆-GFP solutions for 2 h and then rinsed with HEPES buffer. The fluorescence intensities of the protein-immobilized surfaces were then measured using CLSM as described above. Tests for specific binding were performed by incubating the surfaces with 500 mM imidazole in HEPES buffer for 10 min, followed by a HEPES buffer wash to remove the Ni²⁺/NTA-bound His₆-protein that was desorbed via Ni²⁺ stripping by excess imidazole. The fluorescence intensity was measured before and after the surfaces were exposed to imidazole, and the difference was attributed to nonspecific binding.

Lipid Vesicle Preparation and Immobilization on NTA-PEG3400-Si(OMe)₃-Modified Surfaces. A CHCl₃ solution of EPC/DOPE/NBD-DPPE (64:35:1 mol %) was evaporated under a gentle N₂ flow and dried under a 100 μm Hg vacuum for 4 h. The dried lipid mixture was then hydrated in HEPES buffer, suspended by vigorous vortex stirring, and dispersed by probe sonication (three cycles of 10 min duration at 60 W). The sample was immersed in an ice bath during sonication to prevent overheating. The resulting solution of small unilamellar vesicles (SUV) was clarified by centrifugation for 5 min at 5000 rpm and diluted to the desired concentrations. The average diameter of the lipid vesicles measured by dynamic light scattering (DLS) ranged between 90 and 130 nm.

His₆-gA and wild-type gA solutions were prepared at various concentrations using a stock solution of the peptides in methanol (0.5 μM). These solutions (100 μL) were introduced by slow addition to 1 mL of a 1 mM SUV solution with gentle stirring. Lipid vesicles containing His₆-gA or wild-type gA were incubated for 1 h on Ni²⁺/NTA-PEG-modified surfaces, and the fluorescence intensity was determined by CLSM as previously described. Similar experiments were performed using SUV with a constant protein/lipid ratio at concentrations ranging from 0.1 to 10 mM.

Results and Discussion

Surface Preparation and Characterization. Two NTA-PEG derivatives, NTA-PEG3400-Si(OMe)₃ and NTA-PEG3400-VS, were prepared by lysine-NTA substitution of *N*-hydroxysuccinimide-activated-PEG-VS. These materials were grafted onto glass using either (1) direct chemisorption in toluene at 60 °C (NTA-PEG3400-Si(OMe)₃ and mPEG5000-Si(OMe)₃) or (2) conjugation with thiol-terminated, MPTS-modified glass in aqueous solution at room temperature (NTA-PEG3400-VS). Because the surface-modification schemes using NTA-PEG3400-Si(OMe)₃ and NTA-PEG3400-VS ultimately produce the same surface-grafted molecules, we used both approaches to determine which procedure would generate the most densely PEGylated surfaces for NTA-mediated His₆-protein adsorption.

The thicknesses (d_1), refractive indices (n_1), surface densities (Γ), and average distances (L) between grafted chains of the PEG-grafted surfaces were determined by ellipsometry and contact angle measurements (Table 1). Generally, the ellipsometry results obtained using two different measurement angles were the same within experimental error for surfaces that were probed at $\lambda = 633$ nm. At $\lambda = 405$ nm, the calculated thicknesses at both measurement angles were slightly lower than those determined at $\lambda = 633$ nm, but the calculated refractive indices were higher,

(38) Glycerol was added as a humectant to prevent protein aggregation and dehydration during transfer and measurement of the surfaces, which could have affected the photophysical properties of the microcontact-printed chromophores.

resulting in slightly higher surface densities. For NTA-PEG3400-VS-grafted surfaces, only the data at 70° are shown. Our experiments show that the thicknesses and surface densities of NTA-PEG3400-VS-grafted surfaces are considerably smaller than those of NTA-PEG3400-Si(OMe)₃- and mPEG5000-Si(OMe)₃-grafted surfaces, apparently because of lower grafting densities in the NTA-PEG3400-VS system. The thicknesses of the chemisorbed PEG layers were the same within experimental error at 70°, where the observed thicknesses were 14 ± 3 and 17 ± 3 nm for mPEG5000-Si(OMe)₃- and NTA-PEG3400-Si(OMe)₃-modified surfaces, respectively, whereas the NTA-PEG3400-VS-grafted surface thicknesses (4 ± 0 nm) were less than 25% of the chemisorbed PEG layer thickness. The differences in Γ and L for the three dried PEG layers had no effect on the water contact angles, which were 48 ± 2°, 51 ± 1°, and 51 ± 2° for mPEG5000-Si(OMe)₃, NTA-PEG3400-Si(OMe)₃, and NTA-PEG3400-VS, respectively. The contact angles were 30° for all the PEG-grafted surfaces upon storage for 1 day in contact with the normal laboratory atmosphere. All protein absorption experiments used surfaces immediately after the surface modification reactions were complete.

To gain insight into the structure of the grafted PEG layer, we compared the Flory diameter ($2R_F$) and the average distance (L) between PEG grafting sites among the different surface types.³⁹ The Flory radius, R_F , of PEG was estimated for a polymer composed of N units, each of size a , from the following equation:

$$R_F = aN^{3/5} \quad (3)$$

The average distances between the PEG chains calculated from the ellipsometric thicknesses at 70° were 6 Å for NTA-PEG3400-Si(OMe)₃, 8 Å for m-PEG5000-Si(OMe)₃, and 12 Å for NTA-PEG3400-VS (Table 1). The Flory diameters are 4.0 nm for PEG3400 and 5.1 nm for PEG5000 at a unit length of 3 Å.⁴⁰ A comparison between the average PEG grafting distances and the Flory diameters suggests that the PEG molecules are in the brush regime for the chemisorbed PEG derivatives, as has been described for many other PEG-grafted surfaces.^{13,24,41} The PEG molecules on the NTA-PEG3400-VS-grafted surfaces are less dense than the surfaces produced by direct trimethoxysilane-mediated chemisorption and are likely in the mushroom configuration, a finding that is consistent with other studies employing thiol-vinyl sulfone reactions for grafting PEG onto glass.^{36,42} Because the PEG grafting density depends on temperature, solvent, and synthesis pathway,^{42–44} the conditions chosen for the NTA-PEG3400-Si(OMe)₃ and mPEG5000-Si(OMe)₃ grafting reactions (60 °C in toluene) were below the PEG critical solution temperature. The repulsive forces of the PEG chains, which are lower at higher temperatures, lead to greater chain packing densities at the surface because of a smaller excluded volume.^{44,45} The observed PEG layer density is also dependent on the concentration of pinning sites available at the surface.⁴⁴ The surface density of pinning sites is likely lower for the NTA-PEG3400-VS derivative than for the NTA-PEG3400-Si(OMe)₃ and mPEG5000-Si(OMe)₃ precursors because (1) the MPTS activation step required for NTA-PEG3400-VS grafting consumes two surface silanol groups for each MPTS molecule deposited

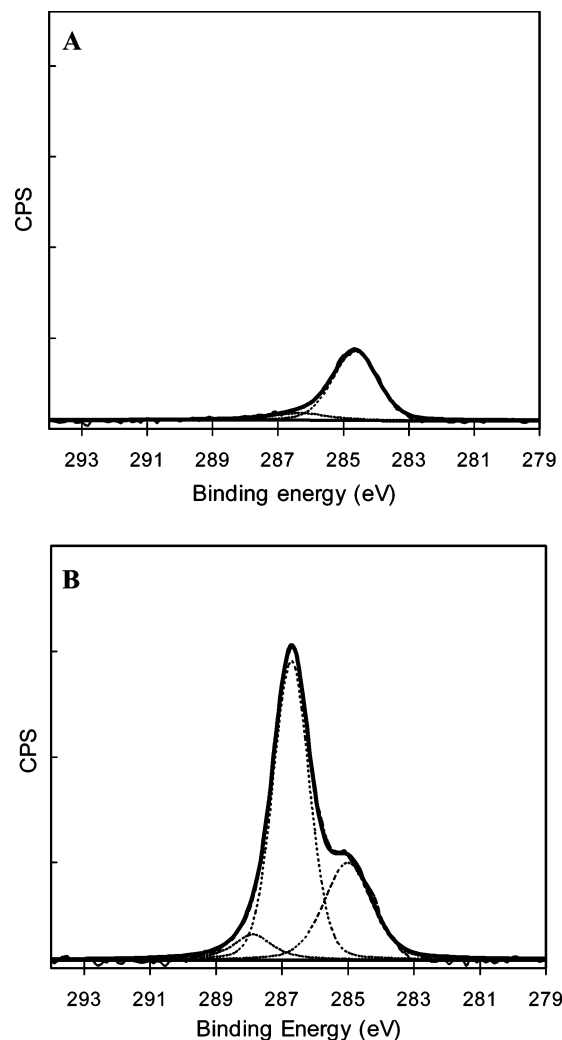


Figure 1. C 1s XPS spectra of (A) a bare silicone oxide surface and (B) an NTA-PEG3400-Si(OMe)₃-modified surface.

and (2) some of the chemisorbed MPTS may become inactivated due to air oxidation during rinsing and handling.

NTA-PEG3400-Si(OMe)₃-modified surfaces were scanned using high-resolution XPS analysis of C 1s and O 1s binding energies (Figure 1). Contributions from the ether carbon component at 286.5 eV, the C–H bond at 285 eV, and the COOH groups at 288 eV were observed in the C 1s peaks of NTA-PEG3400-Si(OMe)₃-modified surfaces. A large contribution from the ether carbon component in the C 1s peak was observed relative to the peak of the bare silicone oxide surface, thus providing clear evidence of PEG on these surfaces.^{39,46} Surfaces modified with NTA-PEG3400-Si(OMe)₃ and mPEG5000-Si(OMe)₃ were then treated with a Ni²⁺ solution and dried prior to XPS analysis. We were unable to detect a Ni signal from these samples at the expected energy of 856 eV, even though the presence of Ni²⁺ has been observed on NTA-functionalized self-assembled monolayers.^{47,48} Because the surface density of NTA ligands is much lower on our PEG grafted surfaces than the previously reported NTA side chain polymers⁴⁷ and NTA self-assembled monolayers,⁴⁸ we infer that the Ni²⁺ concentrations on these surfaces is too low to be detected.

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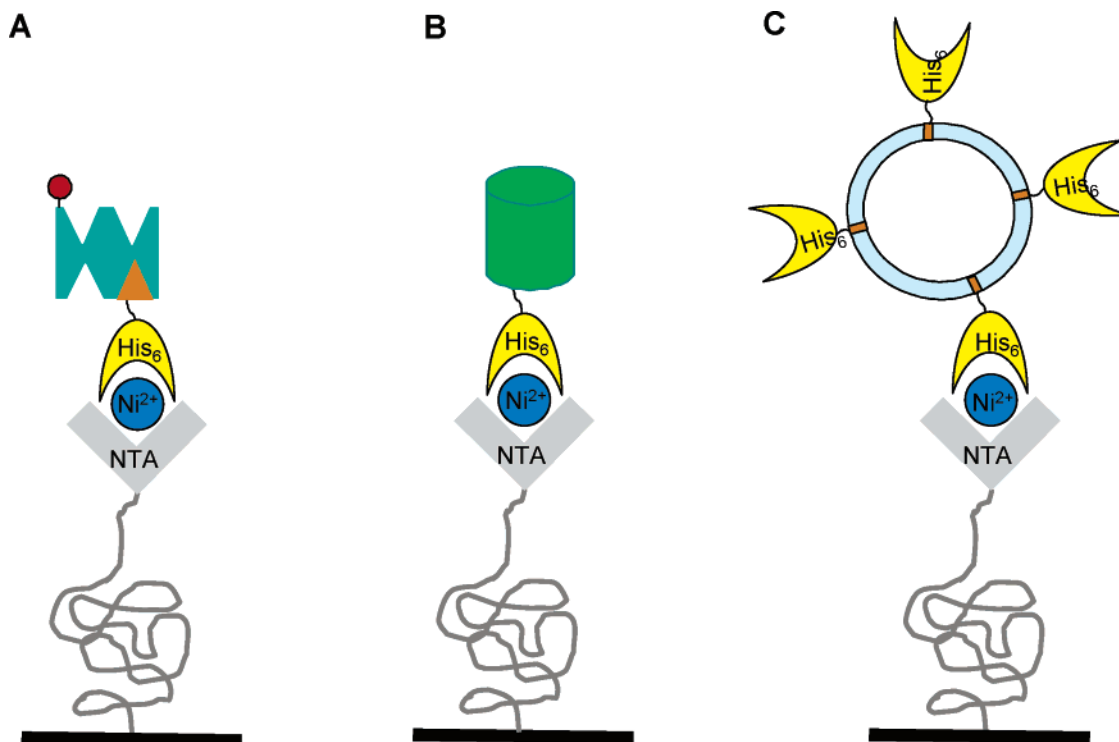


Figure 2. Interaction between a PEG-containing layer and a protein or a lipid vesicle: (A) Ni²⁺/NTA-PEG-containing layer interacting with His₆-biotin and streptavidin-AlexaFluor555 (SA-AF); (B) Ni²⁺/NTA-PEG-containing layer interacting with His₆-GFP; and (C) Ni²⁺/NTA-PEG-containing layer interacting with a His₆-gA-containing lipid vesicle.

His₆-GFP and His₆-biotin/Streptavidin Immobilization.

Three model systems were used in this study to evaluate the immobilization properties of freshly prepared Ni²⁺/NTA-PEG surfaces toward His₆-tag substrates—direct adsorption of His₆-GFP, sequential adsorption of His₆-biotin/SA-AF, and adsorption of His₆-gA, a hydrophobic peptide that was imbedded within fluid-phase lipid vesicles (Figure 2). The surface densities of His₆-GFP and His₆-biotin/SA-AF immobilized on Ni²⁺/NTA-PEG surfaces were monitored using fluorescence microscopy. Glycerol-containing solutions of His₆-GFP and SA-AF were prepared over the 1 nM–1.25 μM concentration range, spotted onto epoxy-coated glass cover slips, and the calibration curve of normalized fluorescence intensity versus the surface density of GFP and SA-AF was determined (Supporting Information). The resulting CLSM fluorescence images showed homogeneous distributions of both fluorescent proteins, with no evidence of crystallization or precipitation on the time scale of the measurements. The fluorescence intensities within 10 spotted zones of equivalent area and concentration were then averaged using identical CLSM imaging conditions and converted to surface densities using the known amounts of His₆-GFP and SA-AF transferred by microcontact printing. The normalized fluorescence intensities thus obtained were linearly dependent on the surface densities of both proteins, enabling the use of these calibration curves for the determination of His₆-GFP and SA-AF surface concentrations under His₆-protein capture conditions.

To evaluate the ability of the different PEG-grafted surfaces with respect to SA-AF capture, NTA-PEG3400-Si(OMe)₃, NTA-PEG3400-VS, and mPEG5000Si(OMe)₃ modified surfaces were sequentially immersed in 50 mM Ni²⁺, His₆-biotin (0.05–10 μM), and 1 μg/mL SA-AF. The surface density of immobilized streptavidin increased as the concentration of His₆-biotin solution increased up to 1 μM (Figure 3A). A second set of PEG-modified surfaces were sequentially immersed in solutions of 50 mM Ni²⁺, 1 μM His₆-biotin, and SA-AF solutions of increasing concentra-

tion to probe the effects of streptavidin surface loading at constant biotin concentration (Figure 3B). The maximum streptavidin surface density achieved was 90 fmol/cm² on surfaces that were modified with NTA-PEG3400-Si(OMe)₃.

His₆-GFP adsorption on PEG-grafted surfaces was also determined using the same approach (Figure 4). As observed for the His₆-biotin/SA-AF system, we found that the His₆-GFP surface densities increased on NTA-PEG-grafted surfaces as the His₆-GFP solution concentration increased. The maximum His₆-GFP surface density achieved was 45 pmol/cm² on surfaces that were modified with NTA-PEG3400-Si(OMe)₃.

NTA-PEG3400-Si(OMe)₃ surfaces displayed the highest surface densities of immobilized His₆-biotin/SA-AF and His₆-GFP compared to surfaces modified with either NTA-PEG3400-VS or mPEG5000-Si(OMe)₃ (Figures 3B and 4). Given the well-known capability of mPEG-coated surfaces to minimize protein adsorption,⁴⁹ it was expected that the surface densities of His₆-GFP and His₆-biotin/SA-AF would be low on mPEG5000-Si(OMe)₃-modified surfaces that lack the Ni²⁺/NTA affinity ligand. Indeed, the binding of His₆-GFP and His₆-biotin/SA-AF on NTA-PEG surfaces is the result of competition between PEG repulsion of the protein adsorbate and Ni²⁺/NTA attraction of the protein via the His₆ affinity ligand. Grafted polymer chains are known to adopt conformations in which steric hindrance between neighboring polymer chains and apposed surfaces, such as bound proteins and lipid vesicles, is avoided. Szleifer and co-workers have determined the minimum free energy of grafted polymers over a wide range of grafting densities.^{50,51} They have also described the attractive forces of polymer end groups that determine the maximum adsorption possible via specific interactions when the grafting density has been optimized.⁵¹ Our results support the predictions that protein adsorption is highly dependent on the magnitude of the attractive forces of the end groups and

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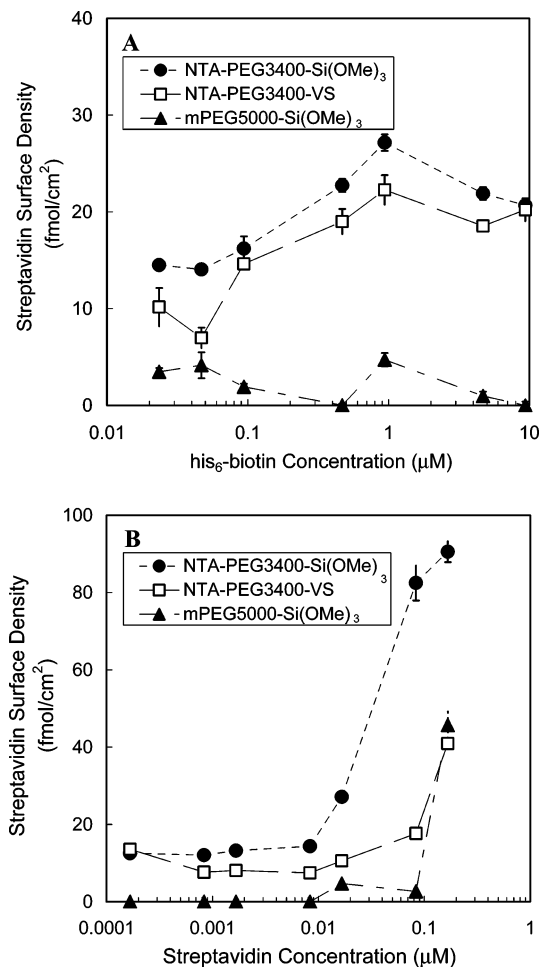


Figure 3. Surface density of streptavidin as a function of (A) His₆-biotin concentration or (B) SA-AF concentration. PEG-modified surfaces were sequentially exposed to (A) 50 mM Ni²⁺ solution, different concentrations of His₆-biotin solution, and finally 1 μg/mL SA-AF solution or (B) 40 mM Ni²⁺ solution and 1 mg/mL His₆-biotin, followed by different concentrations of SA-AF solution. The lines serve as a guide to the eye.

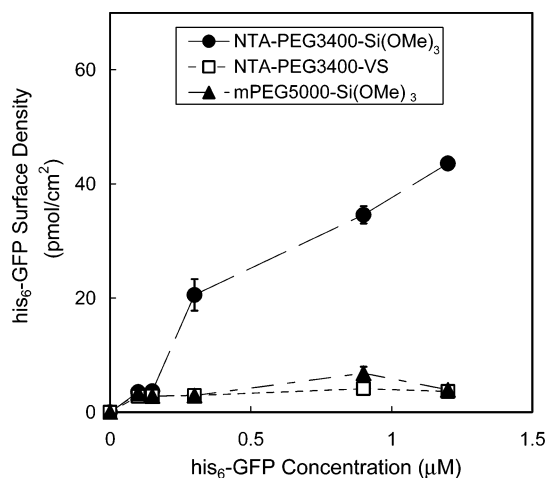


Figure 4. Immobilization of His₆-GFP on PEG-modified surfaces, after activation with 50 mM Ni²⁺, as a function of His₆-GFP concentration. The lines serve as a guide to the eye.

the grafting density of PEG. These findings underscore the importance of optimizing both the strength and surface density of affinity ligands, as well as the underlying structure of the grafted polymer layer, to provide precise control over a bound protein layer via specific interactions.

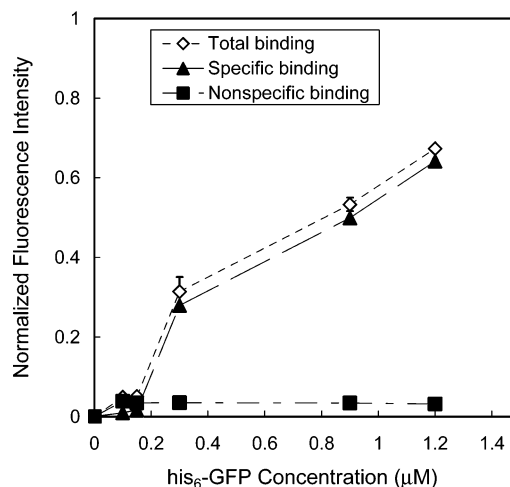


Figure 5. Immobilization of His₆-GFP on NTA-PEG3400-Si(OMe)₃-modified surfaces, represented as normalized fluorescence intensity, as a function of His₆-GFP concentration. The total binding and nonspecific binding (i.e., after washing the His₆-GFP-loaded surface with 500 mM imidazole) on the NTA-PEG surface were measured. Specific binding was calculated as the difference between total binding and nonspecific binding.

Because we were unable to detect the presence of nickel ions on Ni²⁺/NTA-PEG3400-modified surfaces directly, we performed a ligand competition experiment to establish the presence of this chelated metal ion indirectly. Reversible binding of His₆-proteins to the Ni²⁺/NTA-modified surface was probed by the addition of a large excess of imidazole, which removes Ni²⁺ from the His₆-protein/Ni²⁺/NTA complex via ligand competition. As the data in Figure 5 show, His₆-GFP immobilized on the NTA-PEG3400-Si(OMe)₃-grafted surface was fully displaced by the addition of 500 mM imidazole. We infer from the significant reduction in GFP fluorescence intensity after imidazole addition that the NTA-PEG3400-Si(OMe)₃-grafted surface contained ligated Ni²⁺ ions that were responsible for specific binding of His₆-GFP.

Lipid Vesicle Immobilization via His₆-gA. gA, a pentadecapeptide composed of alternating D and L-amino acids, forms a β helix that spans one monolayer of bilayer membranes. When two gA helices present in opposing monolayer leaflets hydrogen bond via their N termini, they can dissipate ion gradients across the membrane by transporting positive monovalent ions through the dimeric channel. This property of gA has been exploited as the responsive element of an ion-sensitive supported membrane biosensor.^{9,52} In this study, gA was modified with a hexahistidine affinity tag at its C terminus⁹ because it is known that this modification does not interfere with the activity of gA.^{9,53} Wild-type gA and His₆-gA were then inserted into preformed lipid vesicles by the slow addition of methanolic solutions of the peptides to the vesicle solutions with stirring. Because His₆-gA was assumed to be incapable of flip-flop into the inner layer of the lipid bilayer from the outer membrane monolayer because transport of the hydrophilic hexahistidine sequence through the hydrophobic core of the lipid bilayer would be required, we expected that His₆-gA is associated only with the outer leaflet of the bilayer lipid vesicles.

His₆-gA-containing lipid vesicle adsorption was then examined using Ni²⁺/NTA-PEG3400-Si(OMe)₃-grafted surfaces. Changes in adsorption as a function of the gA/lipid ratio, *R*, was probed

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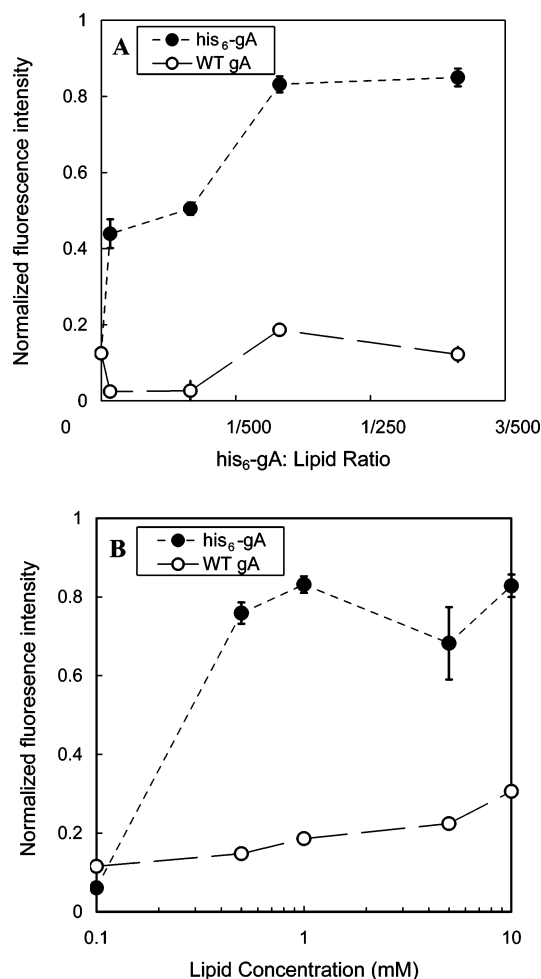


Figure 6. Normalized fluorescence intensity as a function of (A) the gA/lipid ratio at 1 mM lipid vesicle concentration or (B) the lipid vesicle concentration at a constant gA/lipid ratio of 1:375.

by measuring the fluorescence intensity of Ni²⁺/NTA-bearing coverslips that were exposed to lipid vesicles composed of 1 mM EPC/DOPE/NBD-DPPE (64:35:1) in HEPES buffer. Fluorescence intensity from the surface increased as the His₆-gA composition in the membrane increased as a result of His₆-gA/Ni²⁺/NTA-mediated vesicle adsorption. Control experiments using gA-free and wild-type gA vesicles produced very little fluorescence from the Ni²⁺/NTA-PEG3400-Si(OMe)₃-grafted surfaces, indicating that the nonspecific adsorption of lipid vesicles onto these surfaces is not significant. Specific vesicle adsorption was optimized using lipid vesicles containing 1:375 His₆-gA/lipid (Figure 6A). Exposure of various lipid concentrations ranging from 0.01 to 10 mM total lipid at 1:375 His₆-gA/lipid ratios was also examined to establish the maximum surface loading of vesicles at this composition. Our results show that His₆-gA lipid vesicle adsorption increases with increasing vesicle concentration (Figure 6B), with surface saturation occurring at 0.5 mM lipid.

In summary, we evaluated the protein capture efficiencies of the NTA-PEG-grafted surfaces by measuring the fluorescence intensities of PEGylated surfaces after exposure to His₆-tag

species. Protein immobilization was most effective for NTA-PEG3400-Si(OMe)₃-modified surfaces when direct His₆-protein/Ni²⁺/NTA ligation was used; the areal densities achieved when multiple adsorption steps were required (e.g., His₆-biotin/SA-AF) led to greatly reduced levels of adsorbed protein. Lipid vesicles containing His₆-gA in a 1:375 gA/lipid ratio could also be immobilized on Ni²⁺/NTA-PEG3400-Si(OMe)₃-modified surfaces. These experiments show that specific and reversible binding to PEG-modified silica surfaces occurred only when the PEG molecule contained a Ni²⁺-activated NTA ligand and the protein possessed a hexahistidine interaction with the Ni²⁺/NTA-PEG-grafted surface.

Conclusions

A novel NTA-PEG derivative that is capable of efficiently immobilizing His₆-proteins on silica surfaces has been synthesized using two different reaction pathways. XPS, contact angle, ellipsometry, and fluorescence confocal laser scanning microscopy show that areal densities up to 45 pmol/cm² His₆-GFP and 95 fmol/cm² His₆-biotin/SA-AF can be immobilized on silica that has been modified using a simple NTA-PEG3400-Si(OMe)₃ chemisorption procedure. Reversible binding was observed on Ni²⁺/NTA-PEG3400-Si(OMe)₃-modified surfaces for His₆-GFP and His₆-biotin/SA-AF. Taken together, our results show that NTA-PEG conjugates appear to provide an ideal blend of characteristics for promoting the specific adsorption of His₆-proteins or vesicles containing His₆-tag species via the distal NTA moiety without compromising the intrinsic property of PEG to reduce the nonspecific adsorption of proteins or vesicles onto the underlying substrate. Our results also suggest that NTA-PEG-modified surfaces may provide a general and powerful method for immobilizing soluble and integral membrane proteins with control over 1) orientation relative to the solid support, 2) areal density and 3) spacing between the immobilized protein and the solid substrate. Control over these features is essential for the practical realization of membrane protein-based biosensors. Further development of these NTA-PEG molecules and grafting techniques are in progress for monitoring the activity of Ste14p,⁵⁴ an isoprenylcysteine carboxyl methyltransferase involved in Ras signal transduction.

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Supporting Information Available: Calibration curve of normalized fluorescence intensity as a function of both SA-AF and His₆-GFP surface density. QCM measurement of His₆-GFP adsorption onto NTA-PEG3400-Si(OMe)₃-modified surfaces in the presence and absence of chelated Ni²⁺. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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