Film Architecture in Biomolecular Assemblies. Effect of Linker on the Orientation of Genetically Engineered Surface-Bound Proteins

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Abstract: This contribution presents strategies for the optimization of supramolecular architecture aimed at controlling the organization of biomolecules at solid surfaces. Myoglobin, modified by site-directed mutagenesis to include a unique cysteine residue, is selectively chemisorbed to self-assembled haloalkylsilylated silica surfaces of varying *n*-alkyl chain length ($n = 2, 3, 8, 11, 15$) to yield a series of surface-immobilized recombinant protein assemblies. These supramolecular assemblies are probed using tapping mode atomic force microscopy, wettability measurements, Fourier transform infrared spectroscopy, and linear dichroism spectroscopy to determine how the individual components comprising these structures (substrate, silane coupling layer, and protein) influence macromolecular protein ordering and stability. Surface roughness is found to be a minor contributor in the determination of macromolecular ordering in these assemblies. In contrast, the nature of the underlying silane self-assembled coupling layer is shown to strongly influence both the spatial and functional properties of the chemisorbed protein. Silane coupling layers with short aliphatic chain lengths ($n = 2, 3$) produce highly *trans*-conformationally ordered structures upon which differential heme prosthetic group orientation can be achieved. Long alkyl chain ($n \geq 11$) silane-derivatized surfaces also form ordered structures. The stability of myoglobin appended to long chain aliphatic silylated surfaces is poor, however. The apparent protein instability arises due to the increased hydrophobic character of these films. At intermediate alkyl chain length ($n = 8$), a conformationally disordered coupling layer with a high concentration of gauche defects is produced, regardless of the method of silane deposition or postdeposition processing. Chemisorption of myoglobin to the highly disorganized assembly yields a random orientation of the protein.

Introduction

Much attention has recently been directed toward the development of biomaterials, primarily but not exclusively derived from proteins, for a variety of technological applications, among them biosensors,[1,2] bioreactors,[3] chromatographic supports,[4] and functionalized building blocks for so-called "smart materials".[5−8] A major challenge in the development of biomaterials is to devise strategies, employing either existing synthetic technologies or novel fabrication methods, to assemble the various complex molecular species into mesoscale structures. While the selection of the appropriate biomolecule and the development of methods for its incorporation into a mesoscale structure are important, other issues pertinent to the development of protein thin films must also be considered. For example, it is necessary to control the orientation of biomolecules coupled to an active interface/surface, since well-defined protein orientation is critical in the formation of functional arrays with uniform and controllable physicochemical properties. Further considerations for materials applications include retention of biological function upon surface immobilization and long term stability of the formed bioassembly.

To date, biological recognition elements such as antibodies,[9−12] enzymes,[13−18,24] and DNA[19,21b,23] have been successfully incorporated into thin films. Few studies, however, have been directed at achieving oriented bioassemblies.[12,20,21a,22] Of these

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studies, the majority have centered on exploiting natural affinity ligands as a means of orienting functional proteins in lipid monolayers[22] or into bilayers.[21] Of particular interest in this laboratory has been the supramolecular engineering of recombinant proteins of *de novo* design and the coupling of these mutant proteins to appropriately functionalized surfaces.[25,26] Preliminary work has demonstrated the feasibility of preparing self-assembled biomolecular nanostructures by covalent attachment of genetically engineered hemeproteins to silane-functionalized substrates.[25b,26] Introduction of a unique reactive site (cysteine residue) on the cytochrome b_5 protein surface has been successfully employed to produce differential orientations of the heme prosthetic group relative to the substrate.[26d] Although differential orientation of the heme group has been achieved, the degree of orientation attained was substantially less than that predicted by molecular modeling.[26d] These results indicate the need to evaluate systematically the effect of each of the components comprising the microstructure to determine how strongly each influences the ultimate protein orientation. In this way, a fuller understanding of the physical, chemical, and biological principles which govern the film architecture of supramolecular arrays of biological macromolecules and of the relationship between the components of the biostructure and the physical properties of the supporting inorganic thin film can be established.

In this report, we examine the effect of the structural organization of the self-assembled haloalkylsiloxane coupling layer on macroscopic protein ordering. To this end, a series of haloalkyltrichloro- or haloalkyltrimethoxysilanes ($X(CH_2)_nSiCl_3$ or $X(CH_2)_nSi(OCH_3)_3$) of differing *n*-alkyl chain lengths (n = 2, 3, 8, 11, 15) are bonded to SiO_2 substrates. Trifunctional silanes are preferentially chosen over monofunctional silanes, since the former exhibit enhanced hydrolytic stablity, an essential requirement for successful protein deposition.[27,28] The microstructure (*i.e.*, alkyl chain organization) of the resultant films is characterized by contact angle measurements and transmission FT-IR spectroscopy. Tapping mode atomic force microscopy (AFM) is used to evaluate the roughness and morphology of the SiO_2 substrate and the deposited alkyl siloxane films. To determine the effect of the terminal group and chain tilt angle on average molecular orientation, a simple chromophore, 4-(dimethylamino)pyridnium halide, characterized by a single well-defined charge transfer transition dipole moment coincident with its principle molecular symmetry axis, is appended onto the formed siloxane film. UV linear dichroism measurements are used to characterize the orientation of the surface-bound chromophore. Finally, sperm whale myoglobin (Mb), modified by site-directed mutagenesis to introduce a single, specifically reactive, thiol functional group on the protein surface, is chemisorbed to the siloxane-derivatized substrates. The average orientation of the heme prosthetic group is evaluated by visible linear dichroism measurements, and the stability of the surface coupled protein is also determined.

Myoglobin, which functions as an oxygen transport protein in muscles, was chosen as the model protein in this study for several reasons. First, in general, hemeproteins are attractive candidates for incorporation into organized bioassemblies, because they possess a variety of enzymatic, optical, and electronic properties which could be exploited for various device applications. In addition, the heme prosthetic group can serve as a convenient optical marker to monitor macromolecular protein ordering. Of the hemeproteins, myoglobin is of particular interest, because it is a relatively small globular protein (∼17 kDa), possessing a single iron heme prosthetic group. In addition, the totally synthetic gene has been cloned and expressed in *Escherichia coli*, allowing facile manipulation of the amino acid sequence for mutagenesis.[29] Furthermore, myoglobin has been extensively studied, both structurally and functionally.[30,31]

Experimental Section

Materials and Reagents. (8-Bromooctyl)trimethoxysilane was purchased from United Chemical Technologies (Bristol, PA) and distilled under reduced pressure prior to use (bp 100 °C at 200 μmHg). (3-Chloropropyl)trichlorosilane was purchased from TCI America (Portland, OR) and used as received. (2-Chloroethyl)trichlorosilane was purchased from Pfaltz & Bauer (Waterbury, CT) and used as received. (11-Bromoundecyl)trimethoxysilane and (11-bromoundecyl)-trichlorosilane were obtained from Gelest (Tullytown, PA) and used without further purification. (15-Bromopentadecyl)trimethoxysilane was received as a gift from the laboratory of J. A. Katzenellenbogen (University of Illinois at Urbana—Champaign).

Physical Methods. Contact angle measurements were carried out using a custom-built contact angle goniometer. The contact angle of deionized water (Milli-Q; 18 MΩ cm) was measured on a series of 5 μL sessile (free standing) drops applied to the surface with a blunt cut syringe. Data were averaged over 3−5 spots for a given sample. Transmission FT-IR spectra for N_2-dried double-sided monolayer films on SiO_2 substrates were recorded using unpolarized light over the frequency range 3500−2500 cm^{-1} on a Bio-Rad FTS-60A instrument equipped with a liquid-nitrogen-cooled MCT detector. This experimental configuration does not permit detection of vibrational frequencies less than 2500 cm^{-1} due to strong absorption in this region by the bulk fused quartz substrate. Spectra were recorded under N_2 at 2 cm^{-1} resolution and averaged over 1024 scans to provided a high signal-to-noise (S/N) ratio. UV−visible absorption spectra were recorded using a Cary 3 spectrophotometer at a spectral resolution of 3 nm. Each point in a typical absorption spectrum was averaged 50 times.

Linear dichroism (LD) spectroscopy measurements were made on double-sided films by insertion of a sample rotation stage into the Cary 3 spectrophotometer as described previously.[32] Linear polarization of the source beam was achieved with a Glan-Taylor (Melles Griot, Boulder, CO) polarizing cube located in the incident beam path. The absorption spectra were obtained by subtracting the signal of a silane-derivatized slide from the spectrum of the chromophore/protein films at various angles. The absorption band was fit to a Gaussian and the

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integrated peak intensity was obtained using SpectraCalc software (Galatic Industries, Salem, NH).

Atomic force images of glass and silanized substrates were obtained under ambient conditions using a Nanoscope III scanning probe microscope (Digital Instruments, Santa Barbara, CA). The images were collected in the tapping mode, an operational mode in which the tip makes discontinuous surface contact, thereby eliminating lateral forces exerted by the scanning tip. The instrument was operated using a 0.7 μm scanner with 125 μm microfabricated Si tips (Digital Instruments) with resonant frequencies between 270 and 350 kHz. Images were acquired with an average peak-to-peak voltage of 0.6 V. The image sizes were 1 μm by 1 μm with a height of 10 nm. The glass substrates were scanned at a rate of 0.5 μm/s and the silanized glass substrates at a rate of 0.25 μm/s. Each sample was scanned at a range of angles and sample locations to ensure that the features were real and sample-related, not tip-induced artifacts.

Silane Coupling Layer Preparation. Fused silica (Hereaus Amersil, Duluth, GA) and fused quartz (Quartz Scientific, Fairport Harbour, OH) substrates were cleaned by sonication in aqueous detergent (30 min) and in deionized H_2O (30 min), followed by a 45 min immersion into hot "piranha" solution (70:30 (v/v) H_2SO_4:H_2O_2). After cooling to room temperature, the glass substrates were rinsed copiously with deionized H_2O and then with methanol, a methanol:toluene or methanol:chloroform mixture (1:1 v/v), and finally with toluene or chloroform. Self-assembled ultrathin films of silane were prepared by immersing the fresh, hydrophilic SiO_2 substrates into a 2−4% (v/v) solution of the organosilane in freshly distilled (from CaH_2) toluene or chloroform. The deposition was carried out with stirring for 24−48 h under flowing N_2. Upon completion of the reaction, the substrates were rinsed with either chloroform or toluene and then with acetone:chloroform (1:1 v/v) or acetone:toluene (1:1 v/v) and, finally, with acetone, followed by 20 min of air curing at 110 °C. All substrates were stored under N_2 prior to subsequent treatment/reaction.

Chromophore Chemisorption. The chromophore quarternization was carried out by immersion of the silanized substrate in an *n*-propanol solution of the chromophore precursor, 4-(dimethylamino)pyridine (**I**, 3.6×10^{-2} M) (Aldrich), and refluxing at 90 °C for 11 h. Upon completion of the reaction, the substrates were rinsed with fresh *n*-propanol and dried under flowing N_2.

Protein Chemisorption. A totally synthetic gene of sperm whale myoglobin (the plasmid used was pMb 122−9), which had been previously constructed, allowed for facile manipulation of the amino acid sequence of the protein using site-directed mutagenesis.[29] The mutant, Mb A126C, was constructed such that alanine, amino acid residue 126, had been replaced with cysteine to provide a unique sulfhydryl group on the protein surface. The mutant was expressed in *E. coli* and isolated according to reported procedures.[33] The protein was purified by ion exchange chromatography on a carboxymethylcellulose (Whatman CM-52) column in 10 mM phospate buffer, pH 6.4. The protein was concentrated under N_2 using an ultrafiltration cell fitted with a YM-10 filter and stored frozen until further use. Protein purity was determined by SDS−PAGE. Protein chemisorption to the prefunctionalized substrates (*vide supra*) was carried out by diluting the Mb A126C in 20 mM phosphate buffer, pH 8.0, with an excess of dithiothreitol (DTT). The protein was separated from DTT by size exclusion chromatography using a Sephadex G-25 (Pharmacia) column equilibrated with phosphate buffer, pH 7.8, and 1 mM EDTA. This resulted in a final protein solution of 1−5 μM, determined spectrophotometrically on the basis of the Soret band ($\epsilon = 157$ mM^{-1} cm^{-1}), which was used for subsequent surface derivatization. The prepared silylated surfaces were immersed in the protein solution (1−5 μM Mb in 10 mM KPi, 1 mM EDTA buffer, pH 7.8−8.0) for 4−5 h at room temperature. (This procedure produced substrates with protein immobilized on both sides of the glass slide). After treatment, the substrates were rinsed in 10 mM KCl solution, repeatedly washed in phosphate buffer and MilliQ water, and finally dried under flowing N_2. Samples were immediately taken for spectroscopic characterization.

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Results and Discussion

In this study, we examine the factors which control the structural organization (*i.e.*, conformational order, alkyl chain packing, and orientation) of covalent self-assembled protein nanostructures. In particular, we consider the influence of the microscopic orientation of the head groups (or molecular chain) of the alkylsilane coupling layers on the ultimate macroscopic protein ordering.

The chemisorbed protein biostructures were prepared by self-assembly methods in a two-step process. The first step involved functionalization of the SiO_2 substrates with the selected organosilane. It is believed that the alkylsiloxane films are formed by hydrolysis of the trichlorosilane or trimethoxysilane groups with trace water which is present on the substrate surface.[34−37] The silanols formed then condense with surface hydroxyl groups to form stable linkages to the substrate. Inclusion of a postdeposition curing step is then used to condense unreacted adjacent silanols, thereby forming a cross-linking network on the substrate. This was followed by covalent binding of the genetically engineered myoglobin mutant to the preformed organosilane layer. This binding proceeds via reaction of the alkyl halide head group of the chemisorbed organosilane with the thiol anion produced on the protein surface.

Evaluation of Substrate Roughness. The orientation of proteins in self-assembled films is a complicated function of several factors, including (1) substrate roughness both before and after linker derivatization, (2) the conformational order of the coupling layer, (3) the location of the genetically engineered attachment site on the protein surface, and (4) the efficiency of chemisorption (*i.e.*, reduction of non-specifically bound protein). The first of these issues, the effect of substrate roughness, was addressed by comparing the substrate RMS (root mean square corrugation) topographic variability to the dimensions of the protein[38] (*ca.* 25 Å × 35 Å × 45 Å). If the substrate roughness is equal to or greater than the size of myoglobin, it could lead to an "averaging-out" or apparent randomization of the protein orientation. The substrate roughness before silane derivatization was evaluated by tapping mode AFM of freshly cleaned SiO_2 substrates prior to silane deposition (Figure 1). The image shows the grain structure of the SiO_2 surface. Surface roughness analysis indicates an RMS value of 1.48 nm.

Evaluation of Silane Coupling By FT-IR Spectroscopy. The transmission FT-IR spectrum of (2-chloroethyl)trichlorosilane chemisorbed to silica is presented in Figure 2. The disappearance of the free Si−OH vibrational mode, within the limits of detection, in the derivatized substrates indicates the successful covalent anchoring of the trichloro/trimethoxy functional groups to the silica substrate, via hydrolysis with water and subsequent formation of a three-dimensional alkylsiloxane network by intralayer polymerization.[39] The weak absorbance band at 2958 cm^{-1} has been assigned to the νCH_2(Cl) stretch.[40,41] The peaks located at 2851 and 2918 cm^{-1} cor-

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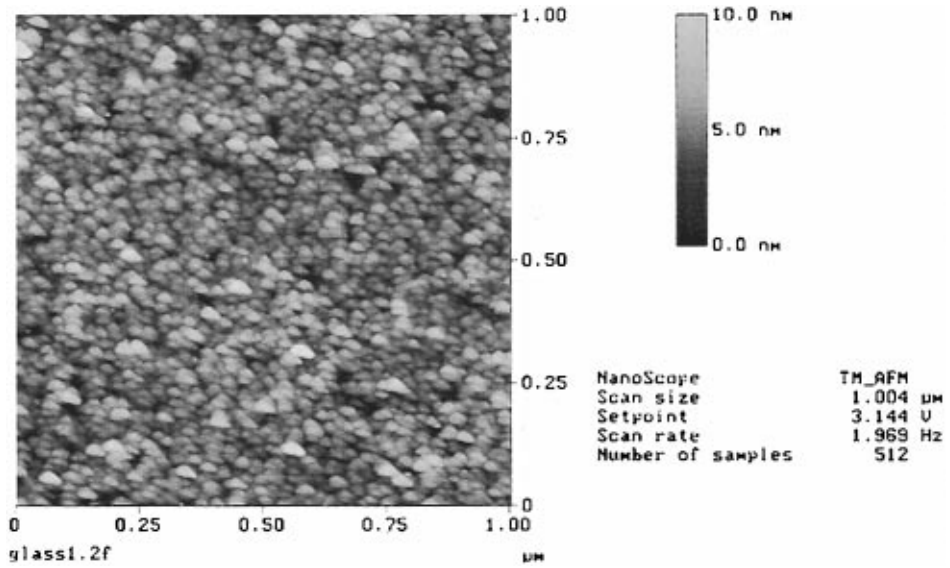

Figure 1. Tapping mode atomic force image of a freshly cleaned quartz substrate.

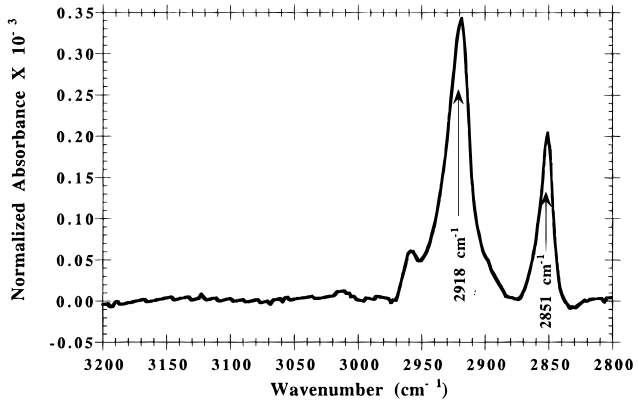

Figure 2. Transmission FT-IR spectrum of a $Cl(CH_2)_2SiCl_3$-derivatized SiO_2 substrate with signature absorption modes indicated (full explanation provided in the accompanying text).

Table 1. Peak Positions and Line Width for Transmission FT-IR Spectra Collected on Halo-Terminated Alkylsiloxane Films Deposited on SiO_2

methylene carbon chain length, *n*	$\nu(CH_2)_{as}$ (cm^{-1})	FWHM $\nu(CH_2)_{as}$ (cm^{-1})	$\nu(CH_2)_s$ (cm^{-1})	FWHM $\nu(CH_2)_s$ (cm^{-1})
C_2	2918	19	2851	14
C_3	2920	22	2851	15
C_8	2928	20	2856	15
C_{11}	2925	23	2854	16
C_{15}	2925	23	2854	16

respond to the symmetric, $\nu_s(CH_2)$, and asymmetric, $\nu_{as}(CH_2)$, methylene stretching modes.[40,42] The position and width of these peaks have been previously shown to be sensitive probes of two-dimensional alkyl chain packing and order in monolayer films.[41] In ordered solid or crystalline hydrocarbons with all *trans* conformation the $\nu_s(CH_2)$ and $\nu_{as}(CH_2)$ peaks are located at 2850 and 2918 cm^{-1}. In highly disordered liquid alkanes, however, the methylene stretches are blue-shifted to 2856 and 2928 cm^{-1}. As shown in Table 1, as the alkyl chain length of the silane coupling agent increases from C_2, a shifting of the $\nu(CH_2)$ modes to higher energy and band broadening are observed. These changes are indicative of an increase in conformational disorder (*i.e.*, a higher concentration of gauche defects) of the alkyl chains with increasing chain length. It is

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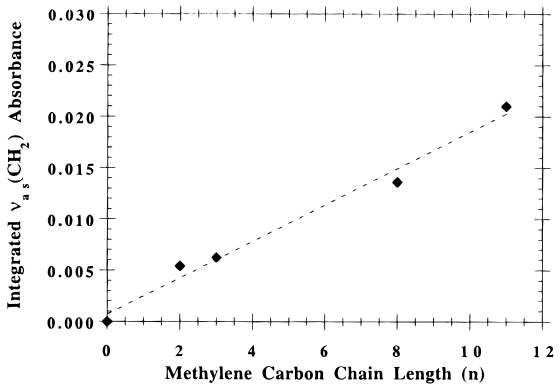

Figure 3. Integrated absorbance (measured for the $\nu_{as}(CH_2)$ stretch) as a function of methylene carbon number for SiO_2 alkyl halide siloxane-derivatized SiO_2 substrates. The line represents a linear least-squares fit to the experimental data.

essential to evaluate the level of surface coverage since diminished coverages would also lead to a partially disordered microstructure.[43] Figure 3 shows a plot of the integrated peak area for the asymmetric methylene stretch as a function of the alkyl chain length of the adsorbate molecule. As can be seen, the plot is approximately linear, which, in the absence of gross conformational changes among different length linkers, would imply that an equivalent level of surface coverage was achieved for all adsorbates.

The peak frequencies do not monotonically increase with increasing chain length but rather reach a maximum at C_8. This finding is in accord with a recent external reflection IR spectroscopic study of a homologous series of methyl-terminated alkylsiloxane films of C_{17}−C_{13} and C_{10} formed on silicon,[44] in which both the symmetric and asymmetric methylene stretches were found to blue-shift with decreasing chain length (*i.e.*, C_{17}−C_{10}). Furthermore, molecular dynamics simulations of the conformation and mobility of *n*-alkyl ligands immobilized onto amorphous silica surfaces predict that the highest gauche dihedrals will occur for C_8.[45] Thus, it appears that a transitional region may exist between short chain ($<C_8$) and longer chain

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Table 2. Static Contact Angle Measurements Collected on Halo-Terminated Alkylsiloxane Films Deposited on SiO_2 Substrates

methylene carbon chain length, *n*	Θ_{H_2O} (deg)	methylene carbon chain length, *n*	Θ_{H_2O} (deg)
C_2	79	C_{11}	84
C_3	75	C_{15}	88
C_8	78		

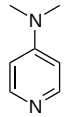
($>C_8$) coupling layers in which the film microstructure is highly disordered. It has been postulated that, at alkyl chain lengths greater than C_8, steric restrictions on chain geometries are imposed by neighboring ligands, whereas at shorter chain lengths restrictions are imposed, instead, by the surface.[45]

Evaluation of Silane Coupling by Wettability Measurements. Contact angle/wettability measurements have been shown to be a sensitive technique for examining the collective nature of surface functional groups.[46,47] As shown in Table 2, the water contact angles observed for the silylated substrates pass through a minimum region and then increase with increasing alkylsilane chain length. A similar trend has recently been reported for a series of *n*-alkyl silanes ($n = 1, 4, 8, 18$) chemisorbed to borosilicate glass.[48] This observation may be attributed to the interplay of several factors. First, the degree of silanization (*i.e.*, silane surface coverages) and/or the efficiency of alkyl chain packing (*i.e.*, concentration of gauche defects) determine the ability of the water molecules to interact with unreacted hydrophilic silanol groups which remain on the underlying glass. Therefore, a highly ordered, densely packed siloxane film, by restricting access of the water molecules to the surface, should yield a high contact angle. The higher contact angle observed for the C_2 monolayer relative to the C_3 is consistent with the FT-IR data outlined above which indicated that the (2-chloroethyl)siloxane films were more highly ordered than the films formed from (3-chloropropyl)silane. Beyond a chain length of about C_6, another factor must be considered when attempting to correlate contact angles with the efficiency of the silanization reaction. It has recently been demonstrated that water probes the formed monolayer film to a depth of only 10 Å or less.[49] Therefore, with increasing chain length, water contact angles become a less complete measure of alkyl chain packing density and more a measure of the terminal functional groups. Nevertheless, both the C_{15} and C_{11} silanes produce a substantially more hydrophobic surface environment than that generated using the C_8, C_3, or C_2 silane films.

Evaluation of Silane Film Morphology. The film morphology/surface topography of the silane-derivatized SiO_2 substrates was also studied by tapping mode AFM. Figure 4A shows the 2-D image of an uncured SiO_2 substrate onto which $Br(CH_2)_{11}SiCl_3$ was deposited by immersion in a 3% (v/v) toluene solution of the C_{11} silane for 24 h followed by washing (*vide supra*) and drying under flowing N_2. The most prominent feature of the image is clusters, presumably of silane, on the film surface. The average surface roughness (RMS) is 2.02 nm. This value is substantially higher than that of the bare SiO_2 substrate, a result of the large diameter of the surface clusters. The lateral dimension of the adsorbed clusters range from 50 to 100 nm (It should be emphasized, however, that lateral AFM image sizes are exaggerated due to tip convolution effects.[10]) Such aggregates could arise, for example, from polymerization of the silane in the reaction solution prior to its deposition onto the surface.[50,51]

Figure 4B shows a 2-D image for the same C_{11} silane deposited on SiO_2 in an identical fashion but with inclusion of a postdeposition curing step (20 min at 110 °C in air). This image clearly shows a smoother surface, which is substantiated by a reduction of the RMS to only 0.95 nm. This change in film morphology can be attributed to the curing process, which is believed to act to condense adjacent silanols of the organosilane into a surface-attached cross-linked siloxane network. A similar observation has recently been described for multilayer polymer films comprised of poly(dimethylsiloxane) on polyurethane.[52] Most importantly, the atomic force microscopy reveals that the surface roughness is reduced to more than a factor of 2 less than the smallest dimension of the protein.

Evaluation of Silane Coupling Layer by UV Linear Dichroism Spectroscopy. To determine the effect of the orientation of the terminal group of the alkyl linker upon the orientation of an attached moiety, 4-(dimethylamino)pyridine (**I**, DMAP) was covalently bound to the series of substrates



I

derivatized with the C_2, C_3, C_8, and C_{11} silanes (*vide supra*) and examined by UV LD spectroscopy. The use of the 4-(dimethylamino)pyridine as a molecular probe to evaluate alkyl chain structure/disorder, rather than the myoglobin mutant, in the initial stages of this work was prompted by several factors. First, successful attachment of this simple, linear molecule to the preformed silane film is accompanied by a ca. 20 nm shift in its UV absorption maximum, making monitoring of the progress of the reaction straightforward and eliminating the problems of discriminating between physisorbed and chemisorbed material. In addition, the absorbance of a double-sided film of chemisorbed protein is weak (and the S/N ratio is poor) due to the large size of the protein and the low achievable surface coverages. (Surface coverages were calculated to be on the order of 25% of a monolayer by measurement of the Soret absorption band at 409 nm after reaction and comparison of the value obtained to that of the close-packed monolayer, $\Gamma_{theoretical}$). Next, a simple chromophoric tag should not be strongly affected by electrostatic interactions, as would be the case for large, highly charged adjacent proteins.[53] Finally, use of a simple, 1-dimensional chromophore tag having a single dominant transition moment permits direct measurement of the orientation of the head group in the silane monolayer to which it is attached.

In linear dichroism measurements the ratio of absorption associated with a known transition dipole (μ) under orthogonally polarized light is monitored.[54] For experiments in which light absorption by the chromophore is detected in the transmission

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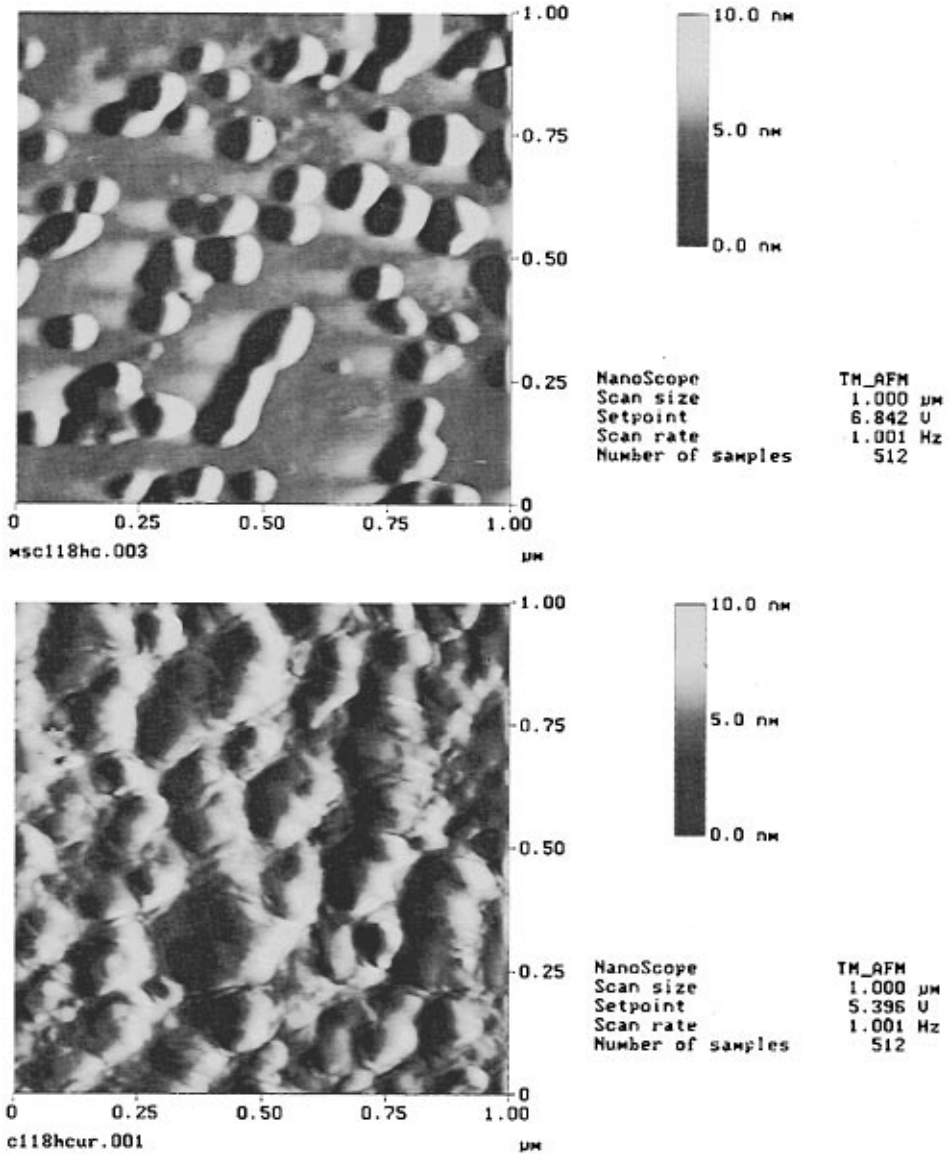

Figure 4. (A, top) Tapping mode atomic force image of an uncured $Br(CH_2)_{11}SiCl_3$ deposited onto SiO_2. (B, bottom) Tapping mode atomic force image of cured (15 min at 110 °C in air) $Br(CH_2)_{11}SiCl_3$ deposited onto SiO_2.

mode, the dichroic ratio, ρ, is defined as

$$\rho = \frac{\int \Delta T_s \, d\lambda}{\int \Delta T_p \, d\lambda} \tag{1}$$

where ΔT_s and ΔT_p are the difference in transmission between the blank and sample for *s* and *p* polarized light. For small ΔT_s and ΔT_p values (<1%), ΔT may be replaced by the absorbance in *s* and *p* polarization (A_s and A_p).[32] For an ideal azimuthally isotropic monolayer comprised of molecules which have a linear transition moment at a flat dielectric surface, it has been shown[55] that

$$\rho = \frac{\int A_s \, d\lambda}{\int A_p \, d\lambda} = \frac{1}{\cos^2 \alpha + 2 \sin^2 \alpha \cot^2 \beta} \tag{2}$$

where α is the angle of incidence and β is the average orientation angle of the transition moments relative to the surface (Figure 5A). Therefore, measurement of the dichroic ratio gives the angle between the surface normal and the average orientation of the transition dipole moment of the adsorbed chromophore.

The rigid rod type chromophore precursor (**I**) becomes an effective chromophore accompanied by an approximately 20 nm blue shift in λ_{max} upon pyridine-N-quarternization. Figure 6 shows the results of dichroic ratio measurements at a range of incident angles for 4-(dimethylamino)pyridine chemisorbed on a C_3-silanized surface. In this plot the solid lines represent the theoretical curves predicted by eq 1 for a range of tilt angles, θ. From this data, the average orientation angle of the transition dipole of the chromophore was found to be $61 \pm 4°$ with respect to the surface normal (Table 3).[56] Using a simple geometrical model of a single, *trans* conformationally oriented molecule (with bond angles of 109.5° within the alkyl chain), an angle of 70.5° between the principal chromophore transition moment and the surface normal is predicted for (3-chloropropyl)silane tagged with DMAP. Previous studies of methyl-terminated alkylsiloxane films, however, have found that the hydrocarbon

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(56) The presence of surface roughness, even at size scales smaller than the protein dimensions, complicates the interpretation of orientations obtained by linear dichroism measurements. Modeling of the features obtained by AFM for derivatized and cured organosilane films resulted in a roughness-induced spread of *ca.* ±6°. This should be interpreted as a lower bound on the width of the orientation distribution function.

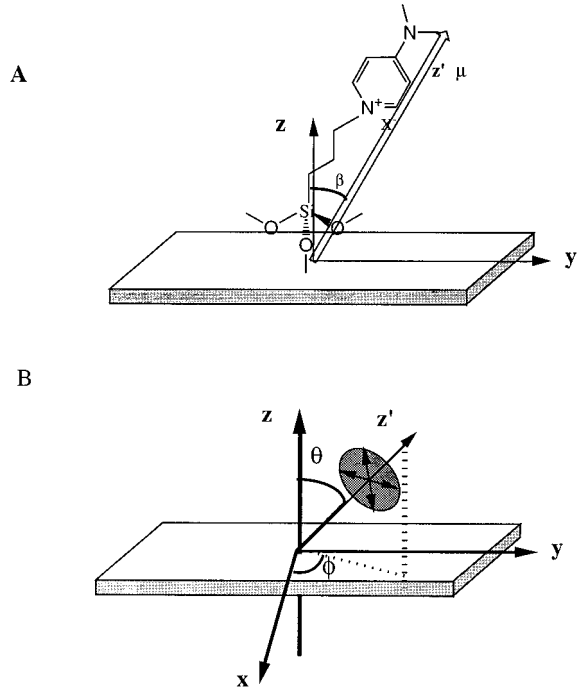

Figure 5. (A) Geometric model of a noninteracting molecule with a transition moment coincident with the principle molecular axis. β is the spherical polar angle between the molecular transition moment, z', and the surface normal, z. (B) Geometric model of a ring oscillator (e.g., heme prosthetic group). θ is the spherical polar angle between the heme normal and the surface normal.

Table 3. Average Orientation of Chromophore with Respect to the Surface Normal for DMAP Tagged Silane-Derivatized Films and Average Orientation of the Heme Normal with Respect to the Surface

Chromophore Data			
n	average orientation angle, β (deg)	n	average orientation angle, β (deg)
2	36 ± 2	11	62 ± 2
3	61 ± 4	15	60 ± 6
8	54 ± 4		

Heme Data					
n	θ (deg)	ϕ (deg)	n	θ (deg)	ϕ (deg)
2	69 ± 5	21 ± 5	11	*a*	*a*
3	44 ± 2	46 ± 2	15	*a*	*a*
8	40 ± 6	50 ± 6			

[a] Protein denatures upon chemisorption.

chain is canted by an average of $8° \pm 3°$ toward the surface normal, corresponding to an average orientation angle of $62° \pm 3°$, a value in excellent agreement with the results obtained here. The simple geometrical model also predicts that if the chromophore is appended to an even number chain, its transition dipole moment will be nearly coincident with the surface normal. The dichroic ratio measured at a range of incident angles for 4-(dimethylamino)pyridinium chloride chemisorbed onto a C_2-silanized surface (not shown) was found to correspond to an average orientation angle of $36° \pm 2°$. Although this angle represents an obvious improvement in chromophore orientation over the C_3, odd chain length linker, it is larger than that anticipated on the basis of the simple geometric arguments above. It is, however, consistent with recent second-order nonlinear optical measurements on a variety of even chain monolayers terminated with NLO-active functionalities, which indicated that, on average, the chromophore dipoles were oriented at an angle of 33−37° from the surface normal.[57] This

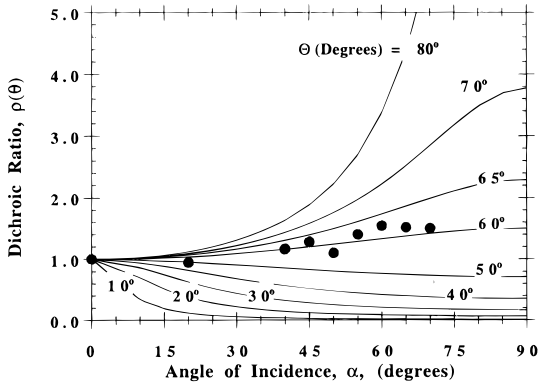

Figure 6. Linear dichroism data for 4-(dimethylamino)pyridinium on a (chloropropyl)siloxane surface. The solid lines correspond to the theoretically predicted, based on eq 2, dichroic ratio as a function of incident angle, α, for a range of molecular tilt angles, β, for a linear oscillator.

result clearly demonstrates that while the alkyl chain length is an important factor in determining the orientation of an appended molecule, it is not the sole factor.

Having demonstrated that highly ordered films with oriented chromophore tags can be readily assembled using the short aliphatic silane linkers (C_2 and C_3) and that the degree of orientation of the chromophore is primarily dictated by the orientation of the underlying methylene carbon chain, the effect of chemisorbing the same chromophore onto longer chain silanes (C_8 and C_{11}) was explored. Linear dichroism measurements performed on an (8-bromooctyl)siloxane-derivatized surface tagged with the DMAP chromophore yielded an average orientation angle of $54° \pm 4°$, which is consistent with a random distribution of chromophores. This observation is consistent with the FT-IR results summarized above, which indicated a "liquidlike" alkylsilane layer, *i.e.*, a liquidlike (and, therefore, highly disorganized) coupling layer would not be expected to produce an oriented chemisorbed chromophore layer. Finally, linear dichroism measurements on chromophore tagged C_{11} and C_{15} films yield tilt angles of $62° \pm 2°$ and $60° \pm 6°$, respectively, a result which agrees favorably with that found experimentally for the shorter, odd number carbon (*i.e.*, C_3) chain.

Taken together, the UV linear dichroism and FT-IR data indicate that a high degree of chromophore orientation is achievable on the SiO_2 substrates used in these studies, regardless of the roughness of the underlying substrate. Although the independence of chromophore orientation and substrate roughness has been previously postulated (*i.e.*, as the substrate decoupling mechanism[58−60]), this data represents initial evidence in support of this hypothesis for siloxane thin films. The order in these films arises from the nearly vertical alignment of the methylene chains. This alignment is driven, in longer linkers, by intermolecular interactions and made possible by the flexible geometry assumable by the Si−O−Si bonds, which have been found to deviate to 86° (versus 152° for idealized geometry), and the O−Si−O bond, which can range from ideal tetrahedral geometry to 94°.

Evaluation of Surface-Bound Protein Monolayers. Up to this point, we have considered a simple model system in which a linear chromophore is appended to the terminus of aliphatic

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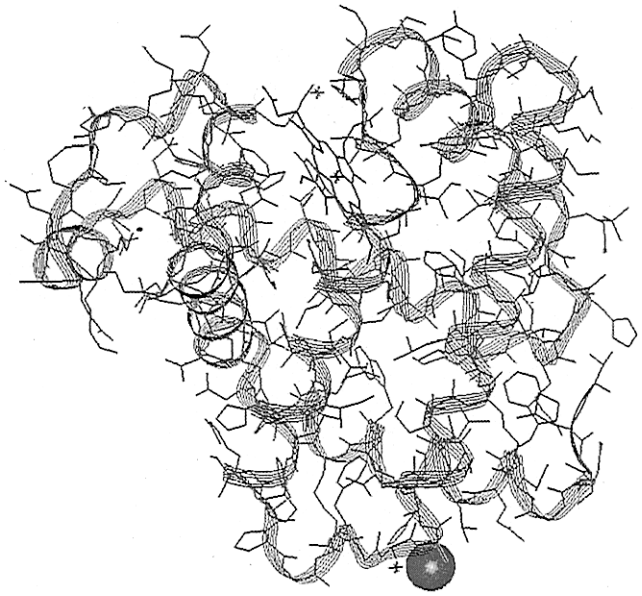
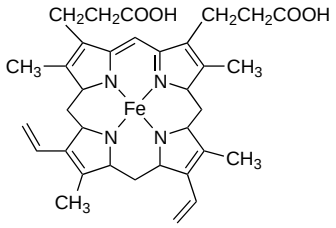


Figure 7. Insight II molecular graphics image of MbA126C. The ribbon represents the polypeptide chain. The heme prosthetic group is shown as a stick structure. The sphere represents the γ sulfur of the engineered cysteine residue.

chains of varying length. This treatment is now extended to a more complex system involving an appended globular protein. Ordered attachment of a protein to a silane monolayer requires that the protein possess a unique functionality capable of reacting with the alkyl halide of the silane. The myoglobin mutant MbA126C, designed specifically to possess a unique sulfhydryl group (cysteine residue) on a highly exposed loop on the protein surface, facilitates the oriented coupling of the protein to the formed silane film (Figure 7). The high reactivity of the cysteine residue has previously been demonstrated by its rapid dimerization in air.[61] In addition, a mutation at this position orients the heme plane nearly parallel to the point of attachment, thereby producing alignment of the heme prosthetic group essentially coincident with the surface normal. The exact angle of the heme plane relative to the site of surface attachment was modeled by using Insight II software (Version 2.1.0, Biosym Technologies, San Diego, CA). This software, which uses as input the X-ray crystallographic coordinates of wild type Mb, permits substitution of a cysteine on the 126th residue and calculation of an angle between two *trans* imidizole nitrogens comprising the heme and the γ sulfur of cysteine. Such an analysis predicts an angle of 158.5° between the heme plane and the C−S bond of the cysteine binding site.

Hemeproteins possess a characteristic Soret absorbance at 408 nm ($\epsilon \geq 10^5$ M^{-1} cm^{-1}) which arises from the heme prosthetic group, a complex of protoporphyrin IX and ferrous iron. The Soret band results from a x−y degenerate $\pi-\pi^*$ transition which lies in the plane of the iron protoporphyrin IX[62,63] (**II**) and can serve as a definitive spectroscopic probe from which the average orientation of the heme can be directly monitored. Furthermore, since heme position/orientation is constrained by protein scaffolding, these measurements can be used to monitor protein orientation.



II

In a manner similar to that described for the case of a simple chromophore, the measured dichroic ratio, ρ, is proportional to the tilt angle of the heme plane relative to the glass substrate. Specifically, for an ideal azimuthally isotropic monolayer comprised of molecules which are ring oscillators (*i.e.*, degenerate *x*,*y* transition moments), it has been shown that[64]

$$\rho = \frac{\int A_s \, d\lambda}{\int A_p \, d\lambda} = \frac{1}{\cos^2 \alpha + \dfrac{2 \sin^2 \alpha \sin^2 \theta}{2 - \sin^2 \theta}} \qquad (3)$$

where α is the angle of incidence and θ is the angle between the heme plane normal, z', and the substrate normal, z (Figure 5B).

Shown in Figure 8 are the experimentally determined dichroic ratios at a range of incident angles for Mb A126C chemisorbed on a C_3-silanized substrate. The data indicate that on average, the heme normal is oriented 44° ± 2° with respect to the substrate normal. This corresponds to the heme plane being positioned 46° ± 2° relative to the surface normal (Table 3). This value is in excellent agreement with results obtained in molecular modeling studies (*vide supra*), which predict that, for an isolated (*i.e.*, single, non interacting) protein tethered to an odd chain silane linker, the heme normal should be oriented at ca. 42° ± 3° with respect to the surface normal. The dichroic ratio measured at a range of incident angles for Mb A126C chemisorbed onto a C_2 surface was found to correspond to an angle between the heme plane and the surface normal of 21° ± 5°. This value too is in excellent agreement with that predicted on the basis of molecular modeling, 20.0° ± 3.0°.

On a C_8 siloxane surface, chemisorbed MbA126C was found to yield an angle between the heme plane and the surface normal of 50° ± 6°, a value close to that which would result from a random distribution of protein orientations, 54.7°. This result indicates once again that the microstructure of an intermediate alkyl chain length silylated surface is too disorganized/liquidlike to effect an ordered condensed structure.

For Mb A126C chemisorbed on the C_{11}-silane-derivatized surface, measurement of an average orientation angle was not possible due to the apparent instability of the protein on the surface. This instability was indicated by a reduction in the Soret absorbance band intensity with time and the concomitant appearance of a weak band at ca. 390 nm, indicative of an irreversible change in the heme pocket and production of free heme. The temporal stability of the Soret absorbance band was also monitored for MbA126C chemisorbed onto the preformed C_{15} siloxane film. As observed for the C_{11} film, a rapid loss of the Soret band intensity was observed over a 1 h period, indicating loss of heme and/or protein denaturation. The instability of Mb A126C on both the C_{11} and C_{15} surfaces may be due to the unfavorable interactions which arise from binding the polar protein surface to a nonpolar siloxane interface. Such unfavorable interactions can cause perturbations in the Mb structure and disruption of the noncovalent forces that maintain a stable protein structure. For example, it has recently been

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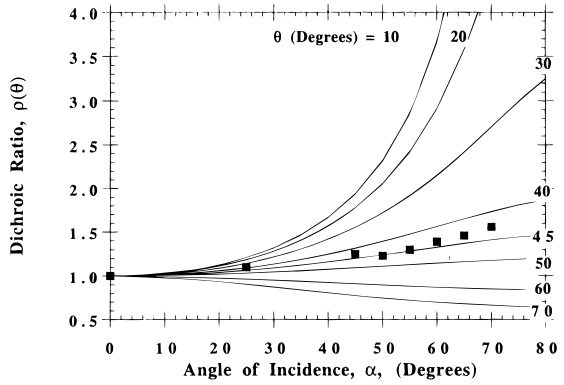

Figure 8. Linear dichroism data for MbA126C on a (chloropropyl)-siloxane surface. The solid lines correspond to the theoretically predicted, based on eq 3, dichroic ratio as a function of incident angle, α, for a range of molecular tilt angles, θ, for a ring oscillator.

reported that two globular proteins undergo denaturation upon adsorption onto the surface of hydrophobic polystyrene microspheres.[65] Also, wild type Mb confined to the surface of poly(dimethylsiloxane) has been reported to undergo loss of heme, as revealed by a significant decrease in the Soret absorbance.[66]

Interestingly, no evidence of protein degradation was observed for Mb A126C chemisorbed onto the shorter chain (*e.g.*, C_2, C_3, C_8) silane monolayers used in this study within the time frame of the linear dichroism experiments (ca. 3 h). No reduction in the Soret band intensity was observed over a 2 h period in the C_8 system, despite a difference of only three carbons in the length of the methylene chain *versus* C_{11}. This effect may be the result of the loosely packed alkyl chain structure producing a more hydrophilic surface in the C_8 film (*cf.* Table 2).

Conclusions

The influence of molecular architecture on the properties of genetically engineered hemeprotein supramolecular assemblies, constructed by deposition of a (haloalkyl)silane coupling layer followed by covalent attachment of myoglobin through a single cysteine mutation, has been evaluated. Specifically, the formation and structure of the silylated silica surface and the effect of incremental alkyl chain length variations on chemisorbed macromolecular protein ordering and stability has been examined. FT-IR spectroscopy and UV linear dichroism spectroscopy measurements of the average orientation of chromophore tagged (haloalkyl)silane self-assembled films indicate that the aliphatic chain length strongly influences the configuration/structure of the formed assemblies. For both short ($n = 2, 3$) and long ($n \geq 11$) alkyl chain lengths, a close-packed, predominately *trans*-conformationally ordered structure is produced. The short chain alkyl organosilanes behave much like rigid rod molecules with restricted conformational freedom, perhaps due to interactions with the surface. For long-chain organosilanes, the van der Waals interactions between chains appear to sustain an ordered structure by interactions between methylene groups.

The average molecular orientation of the simple chromophore appended to the chain termini is shown to track well the orientations predicted employing simple geometric arguments. That is, the chromophore transition moment associated with the principle charge transfer band is aligned more coincident with the surface normal for even aliphatic chain length silylated surfaces than those of odd chain length. Interestingly, such a high degree of structural organization in the short- and long-chain heterogeneous superlattice assemblies has been achieved despite the roughness of the underlying substrate, a finding which provides evidence confirming the recently proposed substrate decoupling mechanism. At intermediate chain lengths (C_8), however, film organization is substantially diminished as evidenced by a high concentration of gauche defects. In addition, a more nearly isotropic distribution of chromophore orientations is produced upon covalent attachment to the C_8 silane layer.

Visible linear dichroism experiments measuring differential orientation of the heme prosthetic group relative to the substrate shows that a myoglobin mutant can be selectively oriented. Our findings reveal that orientation is affected not only by genetic manipulation of the locus of the surface exposed sulfhydryl attachment site, but also by the underlying film microstructure. Vectorial orientation of the protein relative to the surface normal compares favorably with the average orientation angles predicted by molecular modeling for self-assembled monolayers constructed with the short chain alkylsilanes. As found for the simple appended chromophore films, alignment of the heme plane nearly coincident with the surface normal was achieved by covalent coupling to the C_2 functionalized surface. That the heme prosthetic group orientation tracks the theoretically predicted values indicates that protein physisorption, which would act to lower protein ordering, plays only a minor role.

Although the linear dichroism spectroscopy used in the present study provides consistent information regarding the average heme prosthetic group tilt angle with expected molecular orientation, it gives no information concerning the distribution about that average. Information regarding the angular distribution is currently under investigation for these mesoscopic bioassemblies employing fluorescence anisotropy measurements and will be presented elsewhere.[67]

Spectroscopic measurements monitoring the Soret band as a function of time reveal that no gross conformational changes occur in the holoprotein structure upon chemisorption to the C_2-, C_3-, and C_8-silylated substrate. Upon covalent binding to the C_{11} and C_{15} silane-derivatized surface, however, essentially complete loss of the heme group occurs within 1 h after deposition.

The immobilization and interfacial behavior of biomolecules is complicated by a number of additional factors, among them the variable contribution from hydrophobic, electrostatic interactions, and van der Waals forces. Nonetheless, careful consideration of all factors which impact the structure of both the coupling layer and the biomolecule, for example, the use of engineered proteins in conjunction with well-ordered silylated surfaces, should prove a powerful means by which biomolecular thin films can be constructed for a variety of biomedical and protein-based materials

Acknowledgment. The authors wish to dedicate this paper to Professor Nelson J. Leonard on the occasion of his 80th birthday and in recognition of the legacy of excellence established by Professor Leonard and his students during his long career at the University of Illinois. We thank NIH (Grant GM31756) and NSF (Grant CHE 9420211) for support of this research and Ms. Aretta Weber for assistance in preparation of the manuscript.

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