Published in final edited form as:

Langmuir. 2009 March 3; 25(5): 3050–3056. doi:10.1021/la8036814.

Investigation of the Effects of Surface Chemistry and Solution Concentration on the Conformation of Adsorbed Proteins Using an Improved Circular Dichroism Method

Balakrishnan Sivaraman, Kenan P. Fears, and Robert A. Latour^{*}

Department of Bioengineering, 501 Rhodes Engineering Research Center, Clemson University, Clemson, South Carolina 29634

Balakrishnan Sivaraman: bsivara@clemson.edu; Kenan P. Fears: kfears@clemson.edu; Robert A. Latour: latourr@clemson.edu

Abstract

In this paper we present the development of methods using circular dichroism spectropolarimetry with a custom-designed cuvette to increase the signal-to-noise ratio for the measurement of the secondary structure of adsorbed proteins, thus providing enhanced sensitivity and reproducibility. These methods were then applied to investigate how surface chemistry and solution concentration influence both the amount of adsorbed proteins and their secondary structure. Human fibrinogen and albumin were adsorbed onto alkanethiol self-assembled monolayers (SAMs) on gold with CH₃, OCH₂-CF₃, NH₂, COOH, and OH terminal groups from both dilute (0.1 mg/mL) and moderately concentrated (1.0 mg/mL) solutions. An increase in surface hydrophobicity was found to cause an increase in both the amount of the protein adsorbed and the degree of structural change that was caused by the adsorption process, while an increase in solution concentration caused an increase in the amount of protein adsorbed but a decrease in the degree of conformational change, with these effects being more pronounced on the more hydrophobic surfaces. The combined use of these two parameters (i.e., surface chemistry and solution concentration) thus provides a means of independently varying the degree of structural change following adsorption from the amount of adsorbed protein. Further studies are underway to examine which of these factors most strongly influences platelet response, with the overall goal of developing a better understanding of the fundamental factors governing the hemocompatibility of biomaterial surfaces.

1. Introduction

When a biomaterial is implanted in the human body, the adsorption of plasma proteins to the surface of the implant is one of the first events to occur, subsequently influencing the cellular response to the biomaterial. As is well-known, cells generally do not have receptors that are able to recognize synthetic materials, but adhere to the surface of the implant via interactions with bioactive sites presented by the layer of adsorbed proteins. Understanding the mechanisms underlying these protein-surface interactions is important for the design of hemocompatible cardiovascular biomaterials, as the adsorption of plasma proteins influence the onset of a variety of adverse responses, such as thrombi and emboli formation and complement activation on the biomaterial surface.¹

Protein adsorption is a complex process, and is dependent on a variety of material properties such as surface charge, surface free energy, surface roughness, the balance between

^{*}To whom correspondence may be addressed: E-mail: latourr@clemson.edu (R.A.L).

hydrophobic and hydrophilic groups, and the presence of specific chemical groups on the surface.² Conventional thinking holds that surfaces that adsorb the least amount of plasma proteins support less platelet adhesion and thus exhibit lower surface thrombogenicity. However, recent studies have suggested that the critical determinant of the hemocompatibility of a surface may actually be the conformational state of the adsorbed protein rather than the total amount of adsorbed protein on the surface.^{3, 4} This likelihood is further supported by the fact that proteins do not induce adverse reactions when they are in their native soluble state in the blood. Based on this understanding, we hypothesize that adsorption processes cause the exposure of bioactive motifs that are 'hidden' within the protein in its native conformation, which then stimulate adverse cellular responses. The effect of surface chemistry in inducing post-adsorptive conformational changes in proteins may thus be an important consideration for biomaterials surface design.

Alkanethiol self-assembled monolayers (SAMs) on gold are widely used as model systems for studying the effects of surface chemistry since they are easy to prepare, form highly ordered systems, and permit a wide variety of surface functionalities to be produced.⁵⁻⁸ Numerous studies have been carried out using SAM surface chemistries on plasma protein adsorption,⁹⁻¹² platelet adhesion,^{7, 13} and leukocyte adhesion.¹⁴⁻¹⁶ While such studies have clearly shown that both the amount of protein adsorbed on a surface and the level of cellular response to the adsorbed protein layer generally increase with increasing surface hydrophobicity, it is difficult to clearly determine if cellular response is actually driven by the amount of the protein on the surface or the degree of adsorption-induced refolding of the protein on the surface. This is because it is generally true that surface chemistries that adsorb a larger amount of protein (i.e., strong protein-surface interactions) also tend to cause a higher degree of conformational change in the proteins when they adsorb. To investigate this issue, a sensitive method is needed that is able to accurately measure the degree of structural change that occurs when a protein adsorbs to a surface and experimental conditions are needed that are able to vary the degree of conformational change that occurs during adsorption in a manner that can be separated from the amount of protein that is adsorbed.

While post-adsorptive conformational changes in protein layers on surfaces have been evaluated using nuclear magnetic resonance (NMR)¹⁷ and Fourier transform infrared (FTIR) spectroscopy,^{18,19} both methods have inherent disadvantages that limit their usefulness for this application. Although NMR provides an extremely powerful technique that can potentially reveal the complete three-dimensional structure of a protein on a surface, it requires samples with very high surface area density,²⁰ while FTIR requires high protein surface concentrations, which may lead to protein aggregation and associated conformational changes.²⁰ As an alternative to these methods, circular dichroism (CD) spectroscopy can be used to analyze samples with relatively low surface area and at relatively low degrees of surface coverage. Because of these advantages, CD has been increasingly used in recent studies as a means of examining protein-surface interactions at the molecular level.^{3, 21-23}

Important considerations that should be taken into account when performing CD experiments and subsequent data analysis have been described previously.²⁴ To summarize, the protein used should be at least 95% pure and the buffer used for preparing the solution media should ideally be free of any other optically active materials that may interfere with the CD signal. The presence of such materials will substantially complicate the analysis by masking the change in the CD signal due to the protein itself. As a result of this, samples that can be prepared with nanopure water as the solvent are best suited for this method. However, this is often not an appropriate situation for biomedical studies as this does not adequately represent physiological conditions, and the absence of salts may disrupt the structure of the proteins in the solution. In addition to these factors, in order to obtain accurate data for protein adsorption

experiments, the total absorbance of the sample (including the cuvette, sample, and buffer) in the 190 nm – 200 nm wavelength range must not exceed a critical value for a given instrument beyond which the high tension (HT) voltage limits of the instrument are exceeded.²⁴ Once conditions are set that allow a CD spectrum to be successfully obtained within these designated constraints, it is then analyzed via curve fitting algorithms^{25–28} that are used to quantify the fractions of the different secondary structural components that it contains.

In this study, we present the refinement of experimental methods using CD spectropolarimetry using a cuvette that was specifically designed for protein adsorption studies to increase the sensitivity and reproducibility of our measurements by maximizing the number of surfaces that can be used in a single sample while minimizing the path-length through the buffer solution, thereby substantially improving the signal-to-noise ratio of the CD spectra. These methods were then applied to investigate the effects of surface chemistry and solution concentration on the secondary structure of adsorbed fibrinogen and albumin as a means of separately varying the amount of protein adsorbed and degree of adsorption-induced changes in the protein's secondary structure.²⁹ From these studies, we show that our refined CD methods provide a very sensitive and reproducible means to measure the conformation of adsorbed proteins. Our results indicate that for these surfaces and proteins, an increase in surface hydrophobicity for a given solution concentration results in an increase in both the amount of protein adsorbed and the degree of adsorption-induced changes in their secondary structure, while an increase in solution concentration for a given surface chemistry results in an increase in the amount of protein adsorbed but a decrease in the degree of conformational change, with this latter effect being more pronounced on the more hydrophobic surfaces.

2. Materials and methods

2.1 Protein Solutions

Human fibrinogen (FIB3, plasminogen, von Willebrand factor and fibronectin depleted) was purchased from Enzyme Research Laboratories (South Bend, IN) and human serum albumin was purchased from Sigma (Catalog No. A 9511). The buffer used for all experiments was a 25 mM potassium phosphate buffer, consisting of appropriate amounts of monobasic potassium phosphate (Sigma) and dibasic potassium phosphate (Sigma) combined as necessary to adjust the solution pH to 7.4.

2.2 Gold substrates

Quartz slides (0.375" × 1.625" × 0.0625", Chemglass) were cleaned at 50°C by immersion in a piranha solution (7:3 v/v H₂SO₄/H₂O₂) for at least 30 minutes, followed by a Radio Corporation of America (RCA) basic wash (1:1:5 v/v NH₄OH/H₂O₂/H₂O), and this procedure was repeated twice. These slides were then rinsed with 200-proof ethanol (Pharmco-Aaper; Catalog No. 11100200), followed by nanopure water and then dried under a stream of nitrogen gas.

The quartz substrates were then coated with a 30 Å chromium adhesion layer followed by 100 Å of gold via a thermal vapor deposition (TVD) evaporator (Model E 12 E, Edwards High Vacuum Ltd.), prior to SAM formation on these substrates.

2.3 Formation of self-assembled monolayers (SAMs) of alkanethiols

The following alkanethiols were used (as received) for our experiments:

1-Dodecanethiol (SH-(CH₂)₁₁CH₃; Aldrich; CH₃),

11-Mercapto-1-undecanol (SH-(CH₂)₁₁OH; Aldrich; OH),

11-Amino-1-undecanethiol, hydrochloride (SH-(CH₂)₁₁NH₂HCl; Prochimia; NH₂)

11-Mercaptoundecanoic acid (SH-(CH₂)₁₁COOH; Aldrich; COOH), and

11-(2,2,2-Trifluoroethoxy) undecane-1-thiol) SH-(CH₂)₁₁OCH₂CF₃; Aldrich; CF₃)

SAM surfaces were prepared as per established protocols.^{30, 31} Briefly, pure alkanethiol solutions were prepared in 200-proof ethanol (Pharmco-Aaper; Catalog No. 111000200) to yield a final concentration of 1.0 mM. The gold substrates were cleaned by dipping them for 1 minute each in a modified piranha wash (4:1 v/v H₂SO₄/H₂O₂), followed by an RCA basic wash (1:1:5 v/v NH₄OH/H₂O₂/H₂O), rinsed copiously with 200-proof ethanol, and then immersed in the alkanethiol solutions for 24 hours to form the SAM surfaces.

2.4 Surface characterization

2.4.1 Contact angle goniometry—Advancing contact angle measurements were measured on the SAM surfaces using a CAM 200 Optical Contact Angle/Surface Tension Meter (KSV Instruments Ltd) and CAM 200 software provided with the instrument. The surfaces were sonicated in ethanol for 10 minutes, rinsed with nanopure water and dried with flowing nitrogen gas, and mounted on the sample stage. The advancing water contact angles from six separate drops of nanopure water (pH=7.0) were measured on each surface.

2.4.2 X-ray photoelectron spectroscopy—XPS measurements of each type of surface were conducted at the National ESCA and Surface Analysis Center (NESAC/BIO, University of Washington, Seattle, WA), using a Surface Science Instrument X-Probe spectrometer (Mountain View, CA) or a Kratos-Axis Ultra DLD spectrometer, equipped with a monochromatic Al K α source (KE = 1486.6 eV), a hemispherical analyzer and a multichannel detector. The XPS spectra were collected at a nominal photoelectron takeoff angle (with respect to the sample surface normal) of 55°, at a pass energy of 80 eV for survey spectra and 20 eV for high resolution C1s and S2p spectra. The elemental composition was determined from the peak areas in the spectra, using the SSI data analysis software or Kratos Vision 2 software program.

2.4.3 Ellipsometry—Ellipsometry measurements were performed using a Sopra GES5 variable angle spectroscopic ellipsometer (Sopra Inc., Palo Alto, CA) and the accompanying GESPack software package. The substrates used were 18 mm square coverslips (VWR), coated with 50Å of chromium and 1000Å of gold via thermal vapor deposition to ensure high reflectivity. Ellipsometric measurements were performed on these gold-coated samples before and after incubation in alkanethiol, to determine the thickness of the SAM monolayer. Briefly, the spectra for five test points on each sample were scanned from 250 nm to 850 nm using an incident angle of 70° and an analyzer angle at 45°. The thickness of the SAM monolayer was calculated via the regression method in the Sopra WinElli software package (version 4.07) by setting the *n* and *k* values for the alkanethiol layer as 1.5 and 0.0, respectively. The results presented are the average of five measurements on each surface for each SAM.

2.5 Protein adsorption

Protein stock solutions were prepared by dissolving human fibrinogen (Fg) and human serum albumin (Alb) in 25 mM phosphate buffer solution (pH 7.4). All SAM surfaces were first sonicated in ethanol for 10 minutes. The NH₂, COOH and OH SAM-coated substrates were then incubated in a 25 mM potassium phosphate buffer containing 0.005 volume % Triton-X-100 (Sigma; Catalog No. T-9284) in order to block hydrophobic defect sites in the SAM surfaces (e.g., grain boundaries) and then rinsed thoroughly to remove any traces of loosely bound detergent prior to protein adsorption. Each set of SAM-coated surfaces was incubated in the phosphate buffer in a Pyrex petri dish and then a suitable amount of protein stock solution

was pipetted into the buffer, taking care to ensure that the pipette tip was below the air-water interface to avoid denaturation of the protein at this interface. Protein adsorption was conducted under two different protein solution concentrations, 0.1 mg/mL and 1.0 mg/mL, in order to investigate the effect of bulk protein concentration on adsorption-induced conformational changes. The SAMs were incubated in protein for 2 hours, after which the protein solutions over the SAM surfaces were infinitely diluted with pure buffer solution to remove the bulk protein solution and wash away any loosely adherent protein prior to removal of the SAMs from the buffer solution. Following this infinite dilution step, the SAM surfaces were able to be safely removed from the pure buffer solution without dragging the surfaces through the denatured protein film that can be expected to be present at the liquid-air interface if the protein solution had not been replaced with pure buffer prior to surface removal.

2.6 Determination of solution structure, adsorbed concentration, and adsorption-induced conformational changes of proteins using circular dichroism spectroscopy

The structure of human fibrinogen and human serum albumin in solution, the amount of protein adsorbed on each surface, and the subsequent adsorption-induced conformational changes in these proteins due to adsorption on the various SAM surfaces were determined using circular dichroism (CD) spectropolarimetry. The CD spectra (consisting of the ellipticity and absorbance values over wavelengths ranging from 190 nm to 240 nm) were obtained at room temperature using a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD). The solution structure of the proteins was determined using special high-transparency quartz cuvettes (Starna Cells Inc., Atascadero, CA) while the structure of the adsorbed proteins was determined using cuvettes that we custom-designed for maximum signal-to-noise ratio (addressed below). Prior to running the instrument, it was calibrated using a 1.0 cm path-length quartz cuvette containing a solution of (1S)-(+)-10-camphorsulfonic acid, which has an ellipticity of +190.4 millidegrees (mdeg) at a wavelength of 290.5 nm.³²

2.6.1. Solution structure—Each CD spectrum was the average of ten scans done at a scan rate of 50 nm/min, using a data pitch of 0.1 nm, in a 0.1 mm path length quartz cuvette. The background spectrum (i.e., buffer only in the cuvette) was measured first, followed by that of the protein solution. The background spectrum was subtracted from the CD spectrum of protein solution to yield the spectrum of the protein solution alone. The contribution of any protein adsorbed on the walls of the cuvette while measuring the CD spectra for the solution structure of the proteins was found to have negligible influence on the determination of the structure of the protein in solution (see Supplementary Information, Table S.1 and Fig. S.1.)

The ellipticity value (θ , in mdeg) was converted to standard units of $\text{deg}\cdot\text{cm}^2/\text{dmol}$ (designated as $[\theta]$) using the following equation:^{24, 33}

$$[\theta] = (\theta \cdot M_0) / (10,000 \cdot C_{\text{soln}} \cdot L), \quad (1)$$

where θ is the molar ellipticity in mdeg, L is the path length of the cuvette in cm, C_{soln} is the solution concentration of the protein in g/mL, and M_0 is the mean residue molecular weight of 118 g/mol.

Proteins exhibit a peptide absorbance peak at 195 nm (A_{195}), and hence we use the height of the absorbance peak at this wavelength to determine C_{soln} .³⁴ The absorbances of serial dilutions of 2.0 mg/mL stock protein solutions were measured at 195 nm, with the concentrations of the stock solutions being verified using a bicinchoninic acid assay (BCA, Pierce Biotechnology). Calibration plots of A_{195} vs. C_{soln} were first plotted for both fibrinogen and albumin, with the slope of these plots representing “ $\epsilon_{\text{protein}} \cdot L$ ” from Beer’s Law, which is expressed as:

$$A_{195} = \epsilon_{\text{protein}} \cdot C_{\text{soln}} \cdot L \quad (2)$$

Where $\epsilon_{\text{protein}}$ is the extinction coefficient of the protein in $\text{mL} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ (or cm^2/g) and L is the path length of the cuvette.

The data on the molar ellipticity as a function of wavelength that were obtained from the CD scans were deconvoluted using the SP-22X algorithm and analyzed using the SELCON and CONTIN/LL software packages^{26, 35} to yield the percentages of secondary structure components (α -helix and β -sheet). These programs analyze the ellipticity values at each wavelength and compare them with a library of proteins with known secondary structure in order to estimate the percentages of the various secondary structural components.

2.6.2. Adsorbed protein concentration—It should be noted that the term “ $C_{\text{soln}} \cdot L$ ” in Eqn. (2) has units of g/cm^2 , which is equivalent to the units of surface concentration of adsorbed protein. Under the assumption that absorbance is dependent only on the total mass of protein per unit area that the light beam passes through irrespective of whether the protein is in solution or adsorbed to a surface, the same calibration plots used for the proteins in solution along with Eqn. (2) can also be used to directly determine the amount of protein adsorbed per unit area on the SAM surfaces, Q_{ads} , by replacing $C_{\text{soln}} \cdot L$ in Eqn. (2) with Q_{ads} . The validity of using this relationship to measure the amount of protein adsorbed on the SAM surfaces was confirmed by the independent measurement of Q_{ads} using ellipsometry (see Supplemental Information, Table S.1).

2.6.3. Adsorbed protein structure—As noted above, the CD spectra for the analysis of adsorbed protein structure on the different SAM surfaces were obtained using a specially modified quartz cuvette that we custom-designed to enhance the signal-to-noise ratio for these experiments by increasing the number of surface samples and reducing the path-length of buffer through which the beam of light passes (as seen in Figure 1). The cuvette was designed to hold four SAM surfaces with 1/16” (0.159 cm) as the total path-length of the buffer through which the CD beam passes. By reducing this path-length, we were able to keep the total absorbance and HT voltage below their critical values while maximizing the number of surfaces being scanned, thereby minimizing the signal-to-noise ratio to obtain a high level of sensitivity for the measurement of the CD spectra of the adsorbed protein layers.

To evaluate the structure of the adsorbed protein layer on each SAM surface, the background spectra for the SAM-coated quartz slides were first obtained prior to protein adsorption. After the completion of the protein adsorption process, the modified cuvette was filled with 1.0 mL of plain buffer and the protein-coated SAM surfaces were inserted into the slots provided for the slides, taking care to ensure that the protein layer remained wetted with buffer solution during the entire time (to avoid any drying-induced conformational changes). The spectra for the SAM-coated quartz slides with the adsorbed protein layer were then taken.

The collected spectra were prepared for analysis by subtracting the background spectrum taken for each SAM without the adsorbed protein from the corresponding spectrum of the same SAM surface after protein adsorption, with this difference then representing the contribution to the overall spectrum from the adsorbed protein layer alone. The absorbance of the adsorbed protein at 195 nm (A_{195}) was measured and the amount of protein adsorbed on the SAM surface was obtained from the calibration curve of A_{195} vs. Q_{ads} . The resulting ellipticity values (θ , in mdeg) were then converted to molar ellipticity values ($[\theta]$, in standard units of $\text{deg} \cdot \text{cm}^2/\text{dmol}$) using the following equation:^{24, 33}

$$[\theta] = (\theta \cdot M_0) / (10,000 \cdot Q_{\text{ads}}), \quad (3)$$

This is the same as Eqn.1, with the “ $C_{\text{soln}} \cdot L$ ” term being replaced by Q_{ads} , which represents the surface concentration of the adsorbed protein. The molar ellipticity data was then analyzed using the SP-22X algorithm and analyzed using the SELCON and CONTINLL algorithms to determine the secondary structural composition of the adsorbed protein layer.

2.7 Statistical analysis

The results we present are the mean values with 95% confidence interval (CI). The statistical significance of differences between mean values for different samples/conditions was evaluated using Student's t-test, with values of $p < 0.05$ considered as statistically significant.

3. Results and Discussion

3.1 Surface characterization

The contact angle and ellipsometry data for the SAM surfaces is presented in Table 1 and is in excellent agreement with previously reported values for these types of surfaces.^{30, 36} The contact angle for the $\text{OCH}_2\text{-CF}_3$ -terminated SAM (CF_3) was approximately 90° , which was lower than that observed for a $\text{CH}_2\text{-CH}_2\text{-CF}_3$ -terminated SAM (estimated to be around 100°), but was consistent with a previous study.³⁷ It has been shown previously that water exerts its influence on atomic layers at a distance of approximately $5\text{\AA}$ from the upper surface of the thiol layer, penetrating these layers and forming hydrogen bonds.³⁸ The ether group of the $\text{OCH}_2\text{-CF}_3$ SAM is located at a distance of $\sim 3\text{\AA}$ from the surface, as a result of which water can form hydrogen bonds with the oxygen atoms of the ether groups. This explains the lower contact angle observed for the $\text{OCH}_2\text{-CF}_3$ SAM, compared to the $\text{CH}_2\text{-CH}_2\text{-CF}_3$ -terminated SAM. The XPS data for the SAM surfaces is presented in Table 2, with these data also providing values within the expected range for each surface chemistry.

3.2 Determination of conformational changes using circular dichroism spectroscopy

The CD spectra for human fibrinogen (Fg) and human serum albumin (Alb) in solution are shown in Figure 2. As seen in this figure, both Fg and Alb show a shoulder at 222 nm and a negative minima at 208 nm, as well as a positive maxima at 193 nm, which are characteristic of α -helical proteins.^{4, 24} The α -helix and β -sheet for native Fg and Alb was quantified using the CDPro software package, and the results are tabulated in the Table 3. These values agree well with those reported previously in literature,³⁹⁻⁴² and in the Protein Data Bank (PDB) files.^{43, 44}

The CD data quantifying the secondary structural components of the adsorbed Fg and Alb on the SAM surfaces are shown in Figures 3 and 4, respectively, and are compared with the α -helix and β -sheet content of native Fg and Alb in solution. As clearly shown in these plots, the degree of adsorption-induced conformational changes is dependent on the SAM surface chemistry, with a prominent decrease in α -helix content ($p < 0.05$) for both Fg and Alb with increasing hydrophobicity of the surfaces (as indicated by the water contact angle values presented in Table 1). This is consistent with observations made by other groups suggesting that proteins undergo greater conformational changes as they adsorb on hydrophobic surfaces.^{45, 46}

This appears to be a characteristic response from adsorption-induced protein unfolding; or since the β -sheet content increases it would be more appropriate to refer to this as adsorption-induced protein refolding. We believe that this overall behavior is caused by the

thermodynamic driving force of the reduction in free energy of the system, which causes the hydrophobically associated secondary structures of the protein to separate from each other to form hydrophobic interactions with the nonpolar functional groups of the SAM surface, thus reducing the overall solvent-exposed surface area of the hydrophobic functional groups in the system. This apparently destabilizes the α -helix structures of the protein, causing them to unravel, with the relatively flat SAM surface then acting as a template that favors the formation of β -sheet structure in their place.

Although the OH and COOH SAMs exhibited similar levels of hydrophilicity ($p > 0.05$), as inferred from the contact angle data in Table 1, there was a significant difference ($p < 0.05$) in the way both fibrinogen and albumin adsorbed and rearranged on these different surfaces, with the COOH SAM surfaces causing both a significantly greater loss of α -helix and a significantly greater increase in β -sheet in the protein structure compared to the OH SAM. As shown in Figures 3A and 3B (fibrinogen) and Figures 4A and 4B (albumin), the structures of the proteins adsorbed on the OH SAM surfaces were closest to their native structures, with no statistical difference ($p > 0.05$) between the α -helix values for fibrinogen adsorbed on the OH SAM surface at both protein concentrations. This may be attributed to the fact that the internal hydrophobic interactions within the proteins dominate over their tendency to unfold and form contacts with the polar groups of the OH surface, thereby helping in preserving the native structure of fibrinogen and albumin.⁴⁷ In other words, due to the hydrophilicity of the surfaces (i.e. the lack of a hydrophobic driving force to cause protein refolding), the OH groups on the SAM surface primarily interact with the proteins by forming hydrogen bonds and van der Waal's interactions with the hydrogen-bondable groups present on the polar and charged amino acid residues presented on the outer surface of the protein. As a result of this, the thermodynamic stability of the proteins adsorbed on the OH-surfaces is minimally perturbed, thereby largely causing it to retain its native structure following adsorption to the surface. On the other hand, the proteins adsorbed on the COOH-SAM surface showed significantly greater ($p < 0.05$) structural rearrangement (i.e., loss of α -helix accompanied by gain of β -sheet) compared to their native structures. We attribute this behavior to the negatively charged carboxyl groups on the SAM surface ($pK = 7.4$)⁴⁸ interacting with positively charged amino acid residues on the protein's surface, or possibly salt-bridges formed by oppositely charged amino acid residue pairs within the core of the protein. We believe that these types of interactions apply new external forces on the proteins, leading to a situation where the native states of the Fg and Alb no longer represent the lowest free energy states of the protein-SAM-solution complexes, with the proteins then subsequently refolding to the new lowest free energy state conformations.

As also clearly evident from the results presented in Figures 3 and 4, the NH_2 -SAM surface resulted in a significantly greater degree of structural changes in the adsorbed protein ($p < 0.05$) than the COOH-SAM surface. One explanation for this is that Fg (pI 5.5)⁴⁹ and Alb (pI 4.7)⁴⁹ should both have a greater number of negatively charged amino acid residues on their surface compared to the number of positively charged residues for a buffer solution of pH 7.4. This should then cause the positively charged NH_2 -SAM surface (surface pK 8.9)⁴⁸ to have a stronger electrostatic interaction with these proteins than the COOH-SAM (surface pK 5.0).⁴⁸ In addition to these electrostatic effects, the NH_2 -SAM surface also exhibits a much lower degree of attraction to water molecules (as evident by its relatively high contact angle, as presented in Table 1), with this tending to minimize the free energy penalty associated with dehydrating the functional groups on the SAM surface as the protein adsorbs.

These results nicely illustrate that the hydrophobicity of the surface is not the sole factor that governs protein adsorption behavior, but that the characteristics of the specific functional groups involved also play a significant role.

Finally, statistical comparisons between the data shown in Figures 3A versus 3B, and 4A versus 4B, indicates that the amount of loss in α -helix structure and the increase in β -sheet structure in both Fg and Alb were significantly greater when these proteins were adsorbed from 0.1 mg/mL solution concentration than from the 1.0 mg/mL solution concentrations ($p < 0.05$), with the exception of fibrinogen adsorbed on the OH SAM. These results can be explained by kinetic arguments based on the relative kinetics of mass transport of the protein to the SAM surfaces compared to the kinetics of the refolding of the protein on the SAM surfaces. When the proteins adsorb from a dilute solution, mass transport to the surface will be substantially slower than from a concentrated solution, thus giving the protein molecules more time to unfold, refold, and spread out over the surface before the entire surface becomes saturated, which inhibits further spreading of the protein over the surface.^{29, 46} Further evidence is provided for this type of adsorption behavior in the following subsection.

3.3 Quantification of protein adsorption on the SAM surfaces

Figures 5 and 6 illustrate the amount of fibrinogen and albumin, respectively, adsorbed on the SAM surfaces from bulk solutions of 0.1 mg/mL and 1.0 mg/mL solutions.

Fibrinogen has dimensions of about $5.0 \times 5.0 \times 47.0 \text{ nm}^3$,⁵⁰ yielding a surface coverage of $2.26 \mu\text{g}/\text{cm}^2$ when it is adsorbed end-on (25 nm^2 per adsorbed molecule of Fg), whereas a side-on adsorption configuration yields a surface coverage of $0.24 \mu\text{g}/\text{cm}^2$ (235 nm^2 per adsorbed molecule of Fg). On the basis of the albumin molecule having dimensions of approximately $4.0 \times 4.0 \times 14 \text{ nm}^3$,⁵⁰ it was estimated⁴⁷ that the area occupied per adsorbed molecule of Alb for end-on adsorption would be 16 nm^2 , thereby yielding a coverage of $0.72 \mu\text{g}/\text{cm}^2$, and the surface area for side-on adsorption was similarly estimated as 56 nm^2 , yielding a surface coverage of $0.21 \mu\text{g}/\text{cm}^2$.

Our results (shown in Figures 5 & 6) suggest that our surfaces are saturated with protein and these protein molecules are arranged on the surface in a mixture of side-on and end-on configurations, as the values for surface coverage, with one exception, lie between the theoretical values for side-on and end-on protein adsorption for both of these proteins.

An interesting result from the quantification of protein adsorption is the fact that the levels of protein adsorption were much lower for both fibrinogen and albumin on the OCH_2CF_3 SAM compared to the CH_3 -terminated SAM, despite the fact that both of these types of surfaces are considered to be strongly hydrophobic. This can be attributed to the role of water, which can be expected to be able to interact with the subsurface ether group of the OCH_2CF_3 -SAM via hydrogen bonding to a much greater extent than a hydrogen-bondable group from the protein because of the much smaller size and increased mobility of a water molecule,^{29, 52} thus providing a thermodynamic mechanism to resist the dehydration of this surface by an adsorbing protein. In fact, the OCH_2CF_3 -SAM surface even adsorbed a significantly lower amount ($p < 0.05$) of each protein than the NH_2 -SAM surface, even though the NH_2 -SAM is a much more hydrophilic surface. The higher protein adsorption on the NH_2 -SAM than OCH_2CF_3 -SAM is attributed to the favorable attractive forces between the positively charged SAM surface and the negatively charged proteins combined with the ability of the subsurface ether group of the OCH_2CF_3 -SAM to preferentially hydrogen bond with water compared to an adsorbing protein.

As clearly shown in Figures 5 and 6, the amount of protein adsorbed is significantly influenced by solution concentration. At the lower solution concentration (0.1 mg/mL), the rate of arrival of the protein molecules at the surface by diffusion is slower than from the solution with higher concentration (1.0 mg/mL). This provides a condition where an adsorbed protein has more time to undergo adsorption-induced refolding and spreading before it “bumps” into proteins adsorbed to neighboring sites, thus enabling each adsorbed protein molecule to occupy a larger amount of surface area and subsequently saturate the surface with a lower total amount of

adsorbed protein. As a direct result of this phenomenon, and as we observed in this study, surface saturation occurred with a lower amount of adsorbed protein per unit area ($p < 0.05$) for all SAM surfaces when the protein was adsorbed from the more dilute solution, with the exception of albumin adsorbed on the OH SAM, which exhibited similar levels of surface coverage for both 0.1 mg/mL and 1.0 mg/mL solution concentrations. Most importantly for our interests, this decrease in the amount of adsorbed protein with increased solution concentration coincides with an increase in the degree of protein refolding, as shown by the CD results of the adsorbed protein structure in Figures 3 and 4. This then provides conditions where the degree of structural change in the proteins following adsorption is no longer directly proportional to the amount of protein adsorbed to the surface, which will subsequently enable us to investigate which of these two parameters most strongly influences platelet adhesion behavior in planned upcoming studies.

4. Conclusions

This study examined the use of CD spectropolarimetry for measuring the adsorption-induced conformational changes in fibrinogen and albumin as a function of surface chemistry and solution concentration. As an important component of these studies, we developed a custom-made sample-holding cuvette that was specifically designed in such a manner to minimize the path length of the buffer solution through which the CD beam passes while providing multiple surfaces with adsorbed protein. The use of this new cuvette was found to substantially improve the signal-to-noise ratio for these types of measurements compared to our previous studies.³

Based on the results from our present studies, we conclude that both surface chemistry and the concentration of the protein solution play a significant role in influencing the amount of protein adsorbed to the surfaces and the degree of conformational change that these proteins undergo when they adsorb. Furthermore, these results show that by varying both surface chemistry and solution concentration, we can uncouple the relationship between the amount of protein adsorbed and the degree of adsorption induced refolding of the protein. We also conclude that the specific characteristics of the functional groups presented by a surface, and not simply their relative degrees of hydrophobicity, influence the amount of protein adsorbed and the degree of protein refolding that occurs following adsorption.

Recent studies^{3, 4, 7} have shown that the conformation of the adsorbed protein layer, and not just the amount of adsorbed protein, is an important determinant of cellular response (e.g., platelet response) to biomaterial surfaces. CD spectropolarimetry provides an excellent means to quantitatively investigate these types of interactions. Future studies are planned to correlate cellular response to both the amount of protein adsorbed and the degree of adsorption-induced structural changes in proteins in order to develop a more comprehensive understanding of how protein adsorption influences the biocompatibility of implanted biomaterials; and most importantly, to gain further insights into how surfaces can be engineered to control protein adsorption behavior and thereby direct biological response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by NIH Grant Number P20 RR-016461 from the National Center for Research Resources and its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. We are also grateful to Dr. James E. Harriss at Clemson University for fabrication of the gold-coated surfaces used in our studies, and Ms. Megan Grobman, Dr. Lara Gamble and Dr. David Castner at the University of Washington for assistance with the characterization of our surfaces using XPS.

References

1. Gorbet MB, Sefton MV. Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. *Biomaterials* 2004;25(26):5681–5703. [PubMed: 15147815]
2. Horbett TA, Brash JL. Proteins At Interfaces - Current Issues And Future-Prospects. *ACS Symposium Series* 1987;343:1–33.
3. Hylton DM, Shalaby SW, Latour RA. Direct correlation between adsorption-induced changes in protein structure and platelet adhesion. *J Biomed Mater Res Part A* 2005;73A(3):349–358.
4. Tanaka M, Motomura T, Kawada M, Anzai T, Kasori Y, Shiroya T, Shimura K, Onishi M, Mochizuki A. Blood compatible aspects of poly(2-methoxyethylacrylate) (PMEA) - relationship between protein adsorption and platelet adhesion on PMEA surface. *Biomaterials* 2000;21(14):1471–1481. [PubMed: 10872776]
5. Goncalves IC, Martins MCL, Barbosa MA, Ratner BD. Protein adsorption on 18-alkyl chains immobilized on hydroxyl-terminated self-assembled monolayers. *Biomaterials* 2005;26(18):3891–3899. [PubMed: 15773038]
6. Margel S, Vogler EA, Firment L, Watt T, Haynie S, Sogah DY. Peptide, Protein, And Cellular Interactions With Self-Assembled Monolayer Model Surfaces. *J Biomed Mater Res* 1993;27(12):1463–1476. [PubMed: 8113233]
7. Rodrigues SN, Goncalves IC, Martins MCL, Barbosa MA, Ratner BD. Fibrinogen adsorption, platelet adhesion and activation on mixed hydroxyl-/methyl-terminated self-assembled monolayers. *Biomaterials* 2006;27(31):5357–5367. [PubMed: 16842847]
8. Tidwell CD, Ertel SI, Ratner BD, Tarasevich BJ, Atre S, Allara DL. Endothelial cell growth and protein adsorption on terminally functionalized, self-assembled monolayers of alkanethiols on gold. *Langmuir* 1997;13(13):3404–3413.
9. Hirata I, Hioko Y, Toda M, Kitazawa T, Murakami Y, Kitano E, Kitamura H, Ikada Y, Iwata H. Deposition of complement protein C3b on mixed self-assembled monolayers carrying surface hydroxyl and methyl groups studied by surface plasmon resonance. *J Biomed Mater Res Part A* 2003;66A(3):669–676.
10. Kidoaki S, Matsuda T. Adhesion forces of the blood plasma proteins on self-assembled monolayer surfaces of alkanethiols with different functional groups measured by an atomic force microscope. *Langmuir* 1999;15(22):7639–7646.
11. Martins MCL, Ratner BD, Barbosa MA. Protein adsorption on mixtures of hydroxyl- and methylterminated alkanethiols self-assembled monolayers. *J Biomed Mater Res Part A* 2003;67A(1):158–171.
12. Rixman MA, Dean D, Macias CE, Ortiz C. Nanoscale intermolecular interactions between human serum albumin and alkanethiol self-assembled monolayers. *Langmuir* 2003;19(15):6202–6218.
13. Sperling C, Schweiss RB, Steller U, Werner C. In vitro hemocompatibility of self-assembled monolayers displaying various functional groups. *Biomaterials* 2005;26(33):6547–6557. [PubMed: 15939466]
14. Barbosa JN, Barbosa MA, Aguas AP. Adhesion of human leukocytes to biomaterials: An in vitro study using alkanethiolate monolayers with different chemically functionalized surfaces. *J Biomed Mater Res Part A* 2003;65A(4):429–434.
15. Barbosa JN, Barbosa MA, Aguas AP. Inflammatory responses and cell adhesion to self-assembled monolayers of alkanethiols on gold. *Biomaterials* 2004;25(13):2557–2563. [PubMed: 14751741]
16. Tegoulia VA, Cooper SL. Leukocyte adhesion on model surfaces under flow: Effects of surface chemistry, protein adsorption, and shear rate. *J Biomed Mater Res* 2000;50(3):291–301. [PubMed: 10737870]
17. Pelton JT, McLean LR. Spectroscopic methods for analysis of protein secondary structure. *Analytical Biochemistry* 2000;277(2):167–176. [PubMed: 10625503]
18. Haynes CA, Norde W. Structures And Stabilities Of Adsorbed Proteins. *J Colloids Interface Sci* 1995;169(2):313–328.
19. Chittur KK. FTIR/ATR for protein adsorption to biomaterial surfaces. *Biomaterials* 1998;19(4-5):357–369. [PubMed: 9677150]

20. Baker BR, Garrell RL. g-Factor analysis of protein secondary structure in solutions and thin films. *Faraday Discuss* 2004;126:209–222. [PubMed: 14992408]
21. Blondelle SE, Ostresh JM, Houghten RA, Perezpaya E. Induced Conformational States Of Amphipathic Peptides In Aqueous Lipid Environments. *Biophys J* 1995;68(1):351–359. [PubMed: 7711261]
22. Herrick S, Blanc-Brude O, Gray A, Laurent G. Fibrinogen. *Int J Biochem Cell B* 1999;31(7):741–746.
23. McMillin CR, Walton AG. Circular-Dichroism Technique For Study Of Adsorbed Protein Structure. *J Colloids Interface Sci* 1974;48(2):345–349.
24. Greenfield N. Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. *Nature Protocols* 2006;1(6):2527–2535.
25. Sreerama N, Venyaminov SY, Woody RW. Estimation of protein secondary structure from circular dichroism spectra: Inclusion of denatured proteins with native proteins in the analysis. *Anal Biochem* 2000;287(2):243–251. [PubMed: 11112270]
26. Sreerama N, Woody RW. Estimation of protein secondary structure from circular dichroism spectra: Comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal Biochem* 2000;287(2):252–260. [PubMed: 11112271]
27. Sreerama N, Woody RW. Analysis of membrane protein CD spectra. *Biophys J* 2004;86(1):102A–102A.
28. Sreerama N, Woody RW. On the analysis of membrane protein circular dichroism spectra. *Protein Sci* 2004;13(1):100–112. [PubMed: 14691226]
29. Latour, RA. Biomaterials: Protein-Surface Interactions. In: Wnek, GE.; Bowlin, GL., editors. *The Encyclopedia of Biomaterials and Bioengineering*. Vol. 1. Informa Healthcare; 2008. p. 270-284.
30. Bain CD, Troughton EB, Tao YT, Evall J, Whitesides GM, Nuzzo RG. Formation Of Monolayer Films By The Spontaneous Assembly Of Organic Thiols From Solution Onto Gold. *J Am Chem Soc* 1989;111(1):321–335.
31. Gooding JJ, Mearns F, Yang WR, Liu JQ. Self-assembled monolayers into the 21(st) century: Recent advances and applications. *Electroanalysis* 2003;15(2):81–96.
32. Cassim JY, Yang JT. A Computerized Calibration Of Circular Dichrometer. *Biochemistry* 1969;8(5):1947–&. [PubMed: 5785216]
33. Akaike T, Sakurai Y, Kosuge K, Senba Y, Kuwana K, Miyata S, Kataoka K, Tsuruta T. Study on the Interaction between Plasma-Proteins and Polyion Complex by Circular-Dichroism and Ultraviolet Spectroscopy. *Kobunshi Ronbunshu* 1979;36(4):217–222.
34. Goncalves S, Santos NC, Martins-Silva J, Saldanha C. Fluorescence spectroscopy evaluation of fibrinogen-beta-estradiol binding. *J Photoch Photobio B* 2007;86(2):170–176.
35. Sreerama N, Woody RW. Computation and analysis of protein circular dichroism spectra. *Numerical Computer Methods, Pt D* 2004;383:318–351.
36. Faucheux N, Schweiss R, Lutzow K, Werner C, Groth T. Self-assembled monolayers with different terminating groups as model substrates for cell adhesion studies. *Biomaterials* 2004;25(14):2721–2730. [PubMed: 14962551]
37. Coelho MAN, Vieira EP, Motschmann H, Mohwald H, Thunemann AF. Human serum albumin on fluorinated surfaces. *Langmuir* 2003;19(18):7544–7550.
38. Wagner R, Richter L, Wu Y, Weissmuller J, Kleewein A, Hengge E. Silicon-modified carbohydrate surfactants VII: Impact of different silicon substructures on the wetting behaviour of carbohydrate surfactants on low-energy surfaces - Distance decay of donor-acceptor forces. *Appl Organomet Chem* 1998;12(4):265–276.
39. Budzynski AZ. Difference In Conformation Of Fibrinogen Degradation Products As Revealed By Hydrogen Exchange And Spectropolarimetry. *Biochim Biophys Acta* 1971;229(3):663–&. [PubMed: 4252126]
40. Kloczewiak M, Wegrzynowicz Z, Matthias FR, Heene DL. Studies On Chemically Modified Fibrinogen .2. Physicochemical Properties Of Maleylated Fibrinogen. *Thromb Res* 1976;9(4):359–368. [PubMed: 982356]

41. Lee DC, Haris PI, Chapman D, Mitchell RC. Determination Of Protein Secondary Structure Using Factor-Analysis Of Infrared-Spectra. *Biochemistry* 1990;29(39):9185–9193. [PubMed: 2271587]
42. He XM, Carter DC. Atomic-Structure And Chemistry Of Human Serum-Albumin. *Nature* 1992;358(6383):209–215. [PubMed: 1630489]
43. 1EI3, chicken fibrinogen. Protein Data Bank.
44. 1AO6, human serum albumin. Protein Data Bank.
45. Andrade JD, Hlady V. Protein Adsorption And Materials Biocompatibility - A Tutorial Review And Suggested Hypotheses. *Adv Polym Sci* 1986;79:1–63.
46. Roach P, Farrar D, Perry CC. Interpretation of protein adsorption: Surface-induced conformational changes. *J Am Chem Soc* 2005;127(22):8168–8173. [PubMed: 15926845]
47. Wertz CF, Santore MM. Effect of surface hydrophobicity on adsorption and relaxation kinetics of albumin and fibrinogen: Single-species and competitive behavior. *Langmuir* 2001;17(10):3006–3016.
48. Fears KP, Creager SE, Latour RA. Determination of the surface pK of carboxylic- and amine-terminated alkanethiols using surface plasmon resonance spectroscopy. *Langmuir* 2008;24(3):837–843. [PubMed: 18181651]
49. Werner C, Eichhorn KJ, Grundke K, Simon F, Grahert W, Jacobasch HJ. Insights on structural variations of protein adsorption layers on hydrophobic fluorohydrocarbon polymers gained by spectroscopic ellipsometry (part I). *Colloid Surf A* 1999;156(1-3):3–17.
50. Sigal GB, Mrksich M, Whitesides GM. Effect of surface wettability on the adsorption of proteins and detergents. *J Am Chem Soc* 1998;120(14):3464–3473.
51. Le MT, Dejardin P. Simultaneous adsorption of fibrinogen and kininogen at a silica/solution interface. *Langmuir* 1998;14(12):3356–3364.
52. Latour RA. Thermodynamic perspectives on the molecular mechanisms providing protein adsorption resistance that include protein-surface interactions. *J Biomed Mater Res A* 2006;78A(4):843–854. [PubMed: 16832826]

Figure 1.

Top-view of the custom-designed cuvette used for measuring the CD spectra of adsorbed protein on the SAM surfaces.

Figure 2. Representative CD spectra for human fibrinogen (Fg) and human serum albumin (Alb) in solution.

Figure 3.

Changes in secondary structure of adsorbed human fibrinogen (Fg) adsorbed at 0.1 mg/mL (A) and 1.0 mg/mL (B) on SAM surfaces, determined by CD compared to its native conformation. (n=6, mean $\pm$ 95% CI). (NS denotes not significant, all other values are significantly different from one another; $p < 0.05$)

Figure 4.

Changes in secondary structure of adsorbed human serum albumin (Alb) adsorbed at 0.1 mg/mL (A) and 1.0 mg/mL (B) on SAM surfaces, determined by CD compared to its native conformation. (n=6, mean $\pm$ 95% CI). Note: NS denotes not significant, all other values are significantly different from one another; $p < 0.05$)

Figure 5. Fibrinogen (Fg) adsorption (Q_{ads}) from bulk solutions of 0.1 mg/mL and 1.0 mg/mL on SAM surfaces. ($n=6$, mean $\pm$ 95% CI).

Figure 6.

Albumin (Alb) adsorption (Q_{ads}) from bulk solutions of 0.1 mg/mL and 1.0 mg/mL on SAM surfaces. ($n=6$, mean $\pm$ 95% CI). Note: NS denotes not significant, all other values are significantly different; $p<0.05$)

Table 1

Contact angle and ellipsometry data for the SAM surfaces. (Mean $\pm$ 95% CI, n =7)

Parameter	CH ₃	OCH ₂ CF ₃	COOH	NH ₂	OH
Contact Angle (°)	100.9 $\pm$ 1.9	90.2 $\pm$ 0.8	17.9 $\pm$ 1.3	47.6 $\pm$ 1.8	17.6 $\pm$ 1.9
Thickness (Å)	11.5 $\pm$ 2.2	22.1 $\pm$ 4.9	15.8 $\pm$ 1.9	14.7 $\pm$ 2.5	12.1 $\pm$ 1.4

Table 2

Atomic composition of Au-alkanethiol SAM surfaces as determined via XPS (mean $\pm$ 95% C.I., n = 3)

Surface moiety	C (%)	S (%)	N(%)	O (%)	F (%)
CH ₃	64.9 $\pm$ 2.6	2.8 $\pm$ 0.9	Negligible	Negligible	Negligible
OCH ₂ CF ₃	44.1 $\pm$ 1.8	1.7 $\pm$ 0.2	Negligible	6.2 $\pm$ 1.0	13.0 $\pm$ 0.4
NH ₂	54.0 $\pm$ 0.8	2.0 $\pm$ 0.2	4.0 $\pm$ 0.3	3.3 $\pm$ 0.3	Negligible
COOH	47.6 $\pm$ 1.6	1.6 $\pm$ 0.1	Negligible	7.6 $\pm$ 0.3	Negligible
OH	56.7 $\pm$ 3.0	2.8 $\pm$ 2.2	Negligible	7.5 $\pm$ 0.9	Negligible

Table 3

Structural composition of human fibrinogen and human serum albumin in solution, as determined by CD spectra (mean $\pm$ 95% CI, n = 6).

Sample	α -helix (%)	β -sheet (%)
Human fibrinogen (Fg)	39.9 $\pm$ 1.1	9.5 $\pm$ 1.1
Human albumin (Alb)	65.1 $\pm$ 1.3	2.6 $\pm$ 1.1