Adsorption-Induced Conformational Changes in Fibronectin Due to Interactions with Well-Defined Surface Chemistries

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Protein adsorption onto synthetic materials influences cell adhesion and signaling events that direct cell function in numerous biomedical applications. Adsorption of fibronectin (FN) to different surfaces alters protein structure and modulates integrin binding, cell adhesion, cell spreading, and cell migration. In the present study, self-assembled monolayers of alkanethiols on Au were used to analyze the effects of surface chemistry (CH₃, OH, NH₂, and COOH) on the adsorption of a recombinant fragment of FN, FNIII7−10, that incorporates both the synergy and RGD cell binding motifs. Surface chemistry potentiated differential FNIII7−10 adsorption kinetics and adsorbed structure as determined by surface plasmon resonance spectroscopy and antibody binding assays. FNIII7−10 functional activity, determined by cell adhesion strength, was modulated in a fashion consistent with these structural changes (OH > NH₂ > COOH > CH₃). However, these changes in protein parameters did not correlate simply to differences in surface wettability, indicating that additional surface parameters influence protein adsorption. These results demonstrate that surface chemistry modulates adsorbed protein structure and activity and establish a relationship between surface-dependent changes in structural domains of FNIII7−10 and functional activity.

Introduction

Protein adsorption plays a critical role in numerous biomedical and biotechnological applications. Adsorption of proteins onto synthetic surfaces is a thermodynamically driven process. Due to the diverse circumstances in which proteins and surfaces come in contact, an understanding of protein adsorption is fundamental to fields as varied as bioseparation, development of biosensors, food processing, and implant technology. In addition to activating blood clotting and inflammatory responses, adsorbed proteins mediate cell adhesion to synthetic surfaces. Cell adhesion to adsorbed proteins is particularly important in cell function, host responses to implants, and design of tissue engineering substrates.

Protein adsorption is a complex, dynamic process involving noncovalent interactions, including hydrophobic interactions, electrostatic forces, hydrogen bonding, and van der Waals forces. Protein parameters including primary structure, size, and structural stability as well as surface properties such as surface energy, roughness, and chemistry have been identified as key factors influencing the adsorption process. In particular, surface chemistry influences adsorbed protein type, quantity, and conformation. For example, adsorption of the extracellular matrix protein fibronectin (FN) on different surfaces alters protein structure and modulates cell adhesion, spreading, and migration. Although these adsorption studies provide insights into the relationship between surface chemistry and protein adsorption, the role of protein structure and function in the adsorption process has been less well understood.

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between surface properties and protein adsorption, many of these experimental systems lack surface homogeneity or have indeterminate surface properties. Recent studies have focused on using model surfaces, such as self-assembled monolayers (SAMs) of alkanethiols on Au, that allow the systematic investigation of the effects of surface chemistry on protein adsorption without altering other surface properties.

Current models for protein adsorption indicate that the adsorption process induces a partial unfolding of protein as determined by Fourier transform infrared (FTIR) spectroscopy, NMR, atomic force microscopy (AFM), and total internal reflectance fluorescence (TIRF) spectroscopy. Although protein activity is a crucial consideration for analyzing protein adsorption, many of these studies document changes in protein structure/conformation, the only surface characteristic studied was the protein footprint area during adsorption due to a partial unfolding or reorientation of the protein, and the rate of this conformational change was dependent on surface hydrophobicity.24

We previously demonstrated that upon adsorption onto alkanethiol SAMs presenting different chemistries, FN undergoes changes in structure that modulate α5β1 integrin binding and cell adhesion.25 The objective of the present study was to analyze adsorption-induced conformational changes in the central cell binding region of FN as a function of surface chemistry. A recombinant fragment of FN (FNIII7-10) that incorporates both the PHSRN synergy and RGD binding motifs was used to model the full plasma FN molecule and isolate the central cell binding domain. SAMs of alkanethiols on Au presenting hydrophobic (CH3), neutral hydrophilic (OH), positively charged (NH2), and negatively charged (COOH) surfaces were used to examine the effects of surface chemistry on FNIII7-10 adsorption. Using kinetic and quasi-equilibrium assays, we demonstrate significant surface-chemistry-dependent structural changes in FN binding domains and adhesive activity. These findings establish a relationship between surface-dependent changes in structural domains of FN and functional activity.

Materials and Methods

Reagents. LB agar, LB broth, ampicillin, and IPTG used for bacteria culture and protein production were obtained from Invitrogen (Carlsbad, CA). Chemical reagents, Cell Lytic B-Clear II, and DNase I used for bacterial lysis and FNIII7-10 purification were obtained from Sigma Chemical (St. Louis, MO). Hitrap Q Sepharose Fast Flow anion exchange chromatography columns were obtained from Amersham Pharmacia (Piscataway, NJ). Centrifugal concentration devices were purchased from Gelman Laboratory (Ann Arbor, MI), and Slide-A-Lyzer dialysis cassettes used in protein purification were purchased from Pierce Chemical Co. (Rockford, IL). Bolton-Hunter Reagent for FNIII7-10 iodination was purchased from NEN Life Science Products (Boston, MA), and Calcein-AM used in cell adhesion detection was acquired from Molecular Probes (Eugene, OR). Cell culture reagents, DPBS, PBS without Ca2+ or Mg2+, and human plasma fibronectin (pfFN) were purchased from Invitrogen. Newborn calf serum was obtained from HyClone (Logan, UT).

Antibodies and Cells. Several antibodies (Ab's) were used as structural probes for adsorbed FN in enzyme-linked immunosorbent assays (ELISA). Primary monodonal Ab HFN7.1 (Developmental Hybridoma, Inc., Iowa City, IA) directed against the flexible linker between the 9th and 10th type III repeat, FnI-11 directed against the 9th type III repeat, and mAbAβ37 (Chemicon, Temecula, CA) directed against the 8th type II repeat were used.30,45,46 An alkaline phosphatase-conjugated donkey anti-mouse IgG Ab (Jackson Immunoresearch, West Grove, PA) was used as a secondary Ab against all three primary antibodies. Murine NIH3T3 fibroblasts (CRL-1658, ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum and 1% penicillin/streptomycin.

Surface Preparation and Characterization. SAM surfaces were prepared and characterized as previously described.29 Alkanethiols 1-dodecanethiol (HS-(CH2)11-CH3), 11-mercapto-1-undecanethiol (HS-(CH2)11-OH), and 11-mercaptoundecanoic acid (HS-(CH2)10-COOH) were purchased from Aldrich Chemical (Milwaukee, WI) and used as received. The amino-terminating alkanethiol 12-aminol-1-mercapto decane (HS-(CH2)12-NH2) was synthesized and characterized by our group.29 The assembled SAMs of their respective alkanethiols are referred to hereafter as CH3, OH, COOH, and NH2.

SAMs were assembled on Au-coated glass chamber slides (16 well Lab-Tek Chamber Slides, Nalgene Nunc International, Naperville, IL) for equilibrium conformation and cell adhesion studies. Au-coated glass coverslips (9 mm square, Belco Glass, Vineland, NJ) were used as SAM substrates for radiolabeling and kinetic adsorption experiments. Glass chamber slides were cleaned by O2 plasma etching in a Plasma Pren II-862 Plasma Cleaner (Plasmatic Systems, Inc., North Brunswick, NJ), and coverslips were cleaned with 70% H2SO4 and 30% H2O2 at 90 °C for 1 h, rinsed with deionized H2O (diH2O), rinsed with 95% ethanol, and dried under a stream of N2 prior to metal deposition. Au-coated substrates were prepared by deposition of thin films of Ti (150 Å) followed by Au (150 Å) using an electron beam evaporator (Thermionics Laboratories, Hayward, CA) at a deposition rate of 2 Å/s and a chamber base-pressure of approximately 2 × 10−6 Torr.

Freshly prepared Au surfaces were immersed in alkanethiol solutions (1 mM in absolute ethanol), and SAMs were allowed to assemble overnight (16 h). SAMs were rinsed in 95% ethanol, dried under N2, and allowed to equilibrate in DPBS for 15 min prior to incubation in FNIII7-10 solutions. Surfaces were validated by contact angle measurements. Ambient air–water substrate contact angle measurements (5 μL of deionized H2O were taken with a Rame-Hart model no. 100-00 goniometer (Mountain Lakes, NJ) fitted with a digital camera and analyzed using in-house image analysis software.

**Recombinant Fibronectin Fragment.** FNIII\textsubscript{7-10} was produced and purified as described previously.\textsuperscript{31} Escherichia coli transformed with cDNA coding for human FNIII\textsubscript{7-10} and ampicillin resistance were streaked onto an LB agar plate containing 100 µg/mL ampicillin and incubated overnight at 37 °C. A single colony was isolated and expanded in 5 mL of LB broth with 50 µg/mL ampicillin for 3–5 h at 37 °C. The starter culture was added to 500 mL of LB + 50 µg/mL ampicillin, 1.0 M IPTG to induce FNIII\textsubscript{7-10} expression. The culture was grown overnight at 28 °C. Bacteria pellets were collected at 25 000g for 10 min and frozen at −80 °C. Upon thawing, bacteria were lysed and cytosolic proteins were exposed by addition of Celllytic B-Clear II (5 mL/g) and DNAse I (5 µg/mL) for 30 min. The lysate was centrifuged at 25 000g for 15 min. Proteins were precipitated by centrifugation in 40% ammonium sulfate for 25 min at 25 000g. The pellet was resuspended in 15 mL of start buffer (0.2 M Tris, pH 7.7, 50 µM glycine, pH 12) and dialyzed overnight against CAPS buffer (10 mM CAPS, 150 mM NaCl, pH 11.0) and flash frozen for storage at −80 °C.

**Surface Plasmon Resonance Spectroscopy.** Protein adsorption kinetics was quantified via surface plasmon resonance (SPR) spectroscopy using a Biacore instrument (Biacore, Piscataway, NJ). SAMs assembled on Au-coated 9 mm square coverslips were rinsed with ethanol, rinsed with deionized water, dried and assembled onto a Biacore chip holder (Biacore SIA and stored at 4 °C. The specific activity (4.39 × 10\textsuperscript{4} IUC/mL coating concentration at 22 °C). Surfaces were then blocked for 30 min in 1% hd-BSA to be consistent with the Ab assays and cell adhesion assays that require blocking of the remaining surface not covered by FN. hd-BSA was used to block nonspecific interactions such as antibody adsorption to the surfaces ensuring detachment forces.\textsuperscript{29,33} SAMs were coated with a range of FNIII\textsubscript{7-10} concentrations (0–20 µg/mL) for 30 min and blocked in 1% hd-BSA plus 0.1% nonfat dry milk for 30 min to prevent nonspecific adhesion to the substrate. NIH3T3 cells were labeled with 2 µg/mL calcine-AM and seeded at 200 cells/mm\textsuperscript{2} in 2 mM dextrose-DPBS into reassembled chamber slides for 30 min at 22 °C. The 30 min time point was chosen to investigate initial adhesion and reduce any confounding effects of cell spreading or cellular matrix secretion. The initial fluorescence intensity was measured to quantify the number of adherent cells prior to application of centrifugal force. After the wells were filled with medium and sealed with transparent adhesive tape, substrates were inverted and spun at a fixed speed in a centrifuge (Beckman Allegra 6, GH 3.8 rotor) to apply a centrifugal force corresponding to 22.4g. After centrifugation, media were exchanged and fluorescence intensity was read to determine remaining adherent cells. For each well, adherent cell fraction was calculated as the ratio of postspin to prespin fluorescence readings.

**Statistical Analysis.** Nonlinear regression analysis was used to fit experimental data to the appropriate model for radiolabeling, Ab affinity, and cell adhesion assays using SigmaPlot 5.0 (SPSS, Chicago, IL). Results for all experiments were analyzed by one-way ANOVA using SYSTAT 8.0 (SPSS). If treatments were determined to be significant, pairwise comparisons were performed using Tukey’s post hoc test with a 95% confidence level considered significant.

**Results**

**Model Surfaces and Protein.** SAMs of alkane thiols on Au were used to present a wide range of well-defined surface chemistries to examine the effects of surface chemistry on FNIII\textsubscript{7-10} adsorption. Long-chain functionally terminated alkane thiols (HS-CH\textsubscript{2}\textsubscript{n}-X, n ≥ 10) adsorb from solution onto Au to form stable, well-packed and ordered monolayers.\textsuperscript{34–36} The functional end groups (X) examined in this study were CH\textsubscript{3} (hydrophobic), OH (neutral hydrophilic), NH\textsubscript{2} (positively charged at physiological pH), and COOH (negatively charged at physiological pH). These SAM surfaces have been characterized by contact angle measurements as shown in Table 1 and as by X-ray photoelectron spectroscopy (XPS).\textsuperscript{29}

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\textsuperscript{31} Cutler, S. M.; Garcia, A. J. Biomaterials 2003, 24, 1759–1770.

FNIII7-10 is a 39 kDa recombinant fragment of FN that encompasses the 7th through 10th type III repeats of the human pFN molecule.37 FNIII7 spans the central cell binding domain which includes the PHSRN synergy site in the 9th type III repeat of FN and the RGD binding motif in the 10th type III repeat. These motifs are required for binding of integrin \( \alpha_5\beta_3 \), a cellular receptor specific for FN.38,39 The full pFN molecule is a large glycoprotein (440-480 kDa) that contains several functional domains other than cell binding, including sites for collagen and heparin binding as well as self-assembly.40 We chose to use this recombinant fragment of FN to model full pFN in order to eliminate possible confounding effects from regions outside the central cell binding domain. Recombinant FNIII7-10 was expressed and purified to high yields (Figure 1). Antibody and cell adhesion assays demonstrated equivalent functional activity between FNIII7-10 and pFN (data not shown).

Kinetics of Protein Adsorption. Kinetics of FNIII7-10 adsorption onto SAMs were determined by SPR. Kinetic profiles of fragment adsorption revealed an initial rapid association rate that then slowed with time and appeared to approach saturation (Figure 2). The saturating values varied among the surfaces, following the trend NH\(_2\) > CH\(_3\) > COOH = OH. On the CH\(_3\) surface, the response overshoots at approximately 50% saturation and then climbs to a plateau suggesting that the protein adsorbs quickly and then goes through significant reorientation or conformational change. This overshoot observation is consistent with previous work.41-43

The adsorption process was analyzed using a mass action model with two states of the adsorbed protein: (1) reversibly adsorbed state and (2) irreversibly adsorbed state.24,44,45 In the model, the protein first associates with the surface in a reversibly adsorbed state (Figure 3). A fraction of adsorbed molecules then undergo a structural/ conformational change to an irreversibly adsorbed state. This model yields the following governing equations:

\[
dY_1/dt = (kc - sY_1)(A_1 - fa_1Y_1 - fba_1Y_2) - rY_1 \quad (1)
\]

\[
dY_2/dt = sY_1A_1(1 - fa_1Y_1 - fba_1Y_2) \quad (2)
\]

\[Y_T = Y_1 + Y_2 \quad (3)\]

where \( Y_T \) is the total surface density of FNIII7-10 on the surface (ng/cm\(^2\)), \( Y_1 \) represents the surface density of molecules adsorbed in state 1 (ng/cm\(^2\)), and \( Y_2 \) represents the surface density of molecules adsorbed in state 2 (ng/cm\(^2\)). \( c \) is the concentration of molecules in solution (ng/ml), \( k \) represents the initial association rate of the protein (cm\(^{-1}\)/s\(^{-1}\)), \( s \) represents the rate of conformational change from state 1 to 2 (cm\(^{-2}\)/s\(^{-1}\)), and \( r \) represents the reversible rate of molecules in state 1 desorbed from the surface (s\(^{-1}\)). \( A_1 \) is the total surface area (0.75 mm\(^2\)), \( a_1 \) represents the area occupied by one molecule in state 1 (cm\(^{-1}\)/molecule \( \times 10^{15} \)), \( b \) represents the ratio of the area occupied by a molecule in state 2 with respect to the area occupied by a molecule in state 1, and \( f \) is Avogadro’s number divided by the molecular weight of the fragment (molecules/ng). The resulting SPR data is related to the total surface density of FNIII7-10 such that it is the sum of the surface density of molecules in both states (eq 3).

Because the governing differential equations are nonlinear and an explicit solution is not available, the equations were solved numerically in conjunction with nonlinear regression analysis to yield kinetic parameters for each data curve. The regression results for \( Y_T \), \( Y_1 \), and \( Y_2 \) on the four surface chemistries are shown in Figure 2, and the resulting kinetic parameters are listed in Table 2. The regression analysis yielded a \( r^2 \) value for each curve fit such that \( r > 0.005 \), and pairwise comparisons of model parameters yielded the following statistically significant results:

- k: CH\(_3\) > COOH = OH, NH\(_2\) > OH
- s: OH > COOH
- b: CH\(_3\) > COOH = NH\(_2\) = OH
- r: CH\(_3\) > COOH = NH\(_2\) = OH

The modeling implemented here is based on the assumption that the protein adsorbs without mass transport limitations due to the flow of the protein through the flow cell. A calculation for mass transport limitation (MTL) of the system can be made using eq 4, where \( D \) is the diffusion coefficient (cm\(^2\)/s), \( F \) is the flow rate (l/min), \( h \) and \( b \) are the dimensions of the flow cell (mm), and \( l_1 \) and \( l_2 \) are the detection points in the flow cell (mm).

\[
MTL = \frac{L_r}{L_m + L_r} + \frac{L_m}{c_m} = \frac{D^2F}{h^2bl_2} \quad (4)
\]

\[c_m = 1.47 \frac{1 - (l_1/l_2)^{2/3}}{1 - (l_1/l_2)^{3/2}} \quad (5)
\]

\[L_r = kA_1 \quad (6)
\]

MTL varies from 0 when the system is not mass transport limited to 1 when the system is absolutely mass transport limited.
For the $k$ values estimated in our analysis, the MTL index varies between 0.47 (OH) and 0.77 (CH$_3$). This range of MTL values indicates that the experiment is in the transitional regime from reaction rate limited to mass transport limited. Therefore, the fitted values for $k$ underestimate the true association rate. However, these effective parameters still reveal surface-dependent differences.

**Quasi-Equilibrium FNIII$_{1-10}$ Adsorption and Structure.** FNIII$_{1-10}$ structural changes upon adsorption were evaluated as the adsorption process approached quasi-equilibrium. First, adsorbed FNIII$_{1-10}$ surface density was quantified as a function of coating concentration using radiolabeled protein. For each surface, adsorbed FNIII$_{1-10}$ density increased linearly until saturation values were approached at high concentrations (Figure 4).

Conformational/structural changes were analyzed by ELISA with a panel of monoclonal Ab's. The use of a hyperbola (eq 5) to obtain estimates of saturation density ($FN_{ads}$) and half-maximal adsorption ($[FN]_{50}$). FNIII$_{1-10}$ saturation density followed the trend $\text{NH}_2 > \text{CH}_3 > \text{COOH} = \text{OH}$. This trend in saturation density agrees well with our kinetic observations.

$$FN_{ads} = FN_{sat}\frac{[FN]}{([FN] + [FN]_{50})}$$  

Table 2. Model Parameters Based on Fit to SPR Results (Mean ± Standard Error)

<table>
<thead>
<tr>
<th></th>
<th>$k$ (cm$^{-1}$s$^{-1}$) mean (error)</th>
<th>$s$ (cm$^{-2}$s$^{-1}$) mean (error)</th>
<th>$a_1 \times 10^{15}$ (cm$^2$/molecule) mean (error)</th>
<th>$b (a_2/a_1)$ mean (error)</th>
<th>$r$ (s$^{-1}$) mean (error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$</td>
<td>0.19 (0.014)</td>
<td>7.0 (1.0)</td>
<td>0.33 (0.026)</td>
<td>150 (5.0)</td>
<td>0.60 (0.036)</td>
</tr>
<tr>
<td>OH</td>
<td>0.049 (0.0030)</td>
<td>13 (1.3)</td>
<td>6.8 (0.32)</td>
<td>13 (0.64)</td>
<td>0.31 (0.018)</td>
</tr>
<tr>
<td>NH$_2$</td>
<td>0.14 (0.013)</td>
<td>7.3 (0.73)</td>
<td>2.4 (0.22)</td>
<td>28 (3.1)</td>
<td>0.32 (0.044)</td>
</tr>
<tr>
<td>COOH</td>
<td>0.080 (0.0041)</td>
<td>4.4 (0.48)</td>
<td>3.1 (0.23)</td>
<td>38 (3.4)</td>
<td>0.34 (0.019)</td>
</tr>
<tr>
<td>$p$</td>
<td>0.00096</td>
<td>0.041</td>
<td>0.000003</td>
<td>$5.6 \times 10^{-8}$</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Figure 2. Representative kinetic model curve fits to SPR results: (a) CH$_3$, (b) OH, (c) NH$_2$, and (d) COOH. $Y_1$ is the amount of protein in state 1, $Y_2$ is the amount of protein in state 2, and $Y_T$ is the total amount of FNIII$_{1-10}$ on the surface.

Figure 3. Protein adsorption model. $k$ represents the initial association rate of the protein, $s$ represents the rate of conformational change, $a_1$ represents the area occupied by the molecules in state 1, $a_2$ represents the area occupied by the molecules in state 2, and $r$ represents the reversible rate of molecules leaving the surface from state 1.

Figure 4. Adsorbed surface density of FNIII$_{1-10}$ on SAMs as a function of coating concentration.
monoclonal antibodies as probes for structural or conformational changes in adsorbed proteins is well documented. Adsorption of FNIII-10 onto synthetic surfaces is a relatively nonspecific process in which molecules are expected to be present in different orientations with respect to the surface. Only a portion of the adsorbed molecules are likely to display any particular domain in a position that is accessible to Ab binding. For molecules in which a particular binding domain is exposed, the average conformation of this domain is influenced by the chemical properties of the underlying surface. Three monoclonal Ab's directed against sites within the central integrin binding domain of FN were used to detect changes in conformation for both FNIII-10 and pFN. A sigmoidal binding curve (eq 6) was fit to Ab binding profiles of binding (ABbound) versus adsorbed density (FNads) where ABbkgd is the background Ab binding, ABsat is the saturation level of Ab binding, AB50 is the half-maximal Ab binding, and b is the slope at the inflection point.

\[
AB_{\text{bound}} = AB_{\text{bkgd}} + \frac{AB_{\text{sat}}}{1 + \exp\left(-\frac{FN_{\text{ads}} - AB_{\text{50}}}{b}\right)}
\]  

(6)

Shifts in the Ab binding profiles (Figure 5), characterized by AB50, reflect changes in Ab binding affinity for the protein adsorbed to the different surfaces. These changes in Ab binding affinity reflect adsorption-induced changes in protein conformation/structure and/or the prevention of Ab binding due to adsorbed orientation with respect to the surface. A right shift in the profile corresponds to a lower affinity of the Ab for that particular protein conformation because higher FNIII-10 densities are necessary to reach comparable amounts of Ab binding. Therefore, the AB50 parameter corresponds to the inverse of the Ab affinity. Similar shifts were detected for FNIII-10 and pFN (data not shown) using HFN7.1 and FNI-11, indicating that FNIII-10 is a useful model for the cell binding domain of pFN. Pairwise comparison analysis of HFN7.1 AB50 revealed statistically significant differences among surface chemistries (p < 0.05) such that HFN7.1 affinity followed the order OH > NH2 > COOH > CH3 (Table 3). Similarly, analysis of FNI-11 and mAb1937 AB50 demonstrated changes in Ab binding affinity in the order OH = COOH > NH2 > CH3.

FN tertiary structure consists of functional β-sheet folded globular domains connected by a flexible linker (Figure 6). HFN7.1 binds to the flexible linker between the 9th and 10th type III repeats. The statistical trend for HFN7.1 binding suggests that the flexible linker undergoes significant structural changes upon adsorption of FNIII-10 with the OH SAM having the least amount of structural changes and CH3 having the largest changes. The statistical trend for FNI-11 suggests that the 9th type III repeat undergoes structural changes on the CH3 surface in comparison to the other three surfaces. Similarly, the loss in mAb1937 affinity for FNIII-10 adsorbed onto CH3 indicates considerable changes in the structure of the 8th type III repeat.

Cell Adhesion Centrifugation Assay. The activity of adsorbed FNIII-10 was investigated using a centrifugation adhesion assay that applies controlled, reproducible forces to adherent, fluorescently labeled cells. A sigmoidal adhesion curve was fit to the resulting adherent cell fraction (f) versus adsorbed fragment density (FNads) where f_{bkgd} is the background level of adhesion, f_{sat} is the maximum adhesion fraction, ADH50 is the half-maximal adhesion, and g is the slope at the inflection point.

\[
f = f_{\text{bkgd}} + \frac{f_{\text{sat}}}{1 + \exp\left(-\frac{FN_{\text{ads}} + ADH_{\text{50}}}{g}\right)}
\]  

(7)

Figure 5. Ab binding affinity curves for HFN7.1, FNI-11, and mAb1937 Abs. The concentration of FNIII-10 has been normalized by the surface density obtained by radiolabeling. Changes in Ab affinity, which reflect differences in protein conformation, are demonstrated as shifts in the normalized curves.

Table 3. Antibody-Based Assay for FN Conformation

<table>
<thead>
<tr>
<th></th>
<th>HFN7.1 AB50 (error)</th>
<th>FNI-11 AB50 (error)</th>
<th>mAb1937 AB50 (error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH3</td>
<td>160 (14)</td>
<td>74 (4.1)</td>
<td>59 (5.6)</td>
</tr>
<tr>
<td>OH</td>
<td>31 (2.2)</td>
<td>15 (1.2)</td>
<td>5.2 (0.79)</td>
</tr>
<tr>
<td>NH2</td>
<td>62 (8.9)</td>
<td>22 (2.4)</td>
<td>4.2 (0.76)</td>
</tr>
<tr>
<td>COOH</td>
<td>92 (7.6)</td>
<td>35 (2.7)</td>
<td>8.2 (1.0)</td>
</tr>
<tr>
<td>p</td>
<td>0.0013</td>
<td>0.0000029</td>
<td>0.00013</td>
</tr>
</tbody>
</table>

* AB50 represents the surface density of FNIII-10 required to reach 50% of the total Ab binding. This parameter is inversely proportional to Ab affinity (mean ± standard error).

\(50\)
Changes in strength of cell adhesion to the various surfaces are shown in Figure 7 as shifts in the adhesion curve characterized by half-maximal adhesion (ADH50). A shift in the curve to the right represents a decrease in adhesion strength since more protein is required on the surface for the adhesion of the cells to the substrate; therefore, adhesion strength is inversely related to ADH50. Pairwise comparison testing of ADH50 among the SAMs revealed statistical differences that demonstrate changes in adhesion strength following the trend OH = NH2 > COOH > CH3 (p = 0.00005).

**Structure/Function Relationships.** To provide further insights into FNIII7–10 adsorption, we examined correlations between the structural (AB50) and functional (ADH50) parameters. ADH50 correlated well with AB50 for HFN7.1 (linear, R^2 = 0.98) and FNI-11 (linear, R^2 = 0.99). This relationship indicates that structural alterations or adsorbed orientation effects in the 9th and 10th type III repeats of FN significantly modulate protein activity. In contrast, FNIII7–10 adhesive activity correlated poorly with mAb1937 AB50. This low correlation is expected because the epitope for this Ab lies outside the critical integrin binding region of FNIII7–10. Finally, we examined the ability of surface hydrophobicity to predict adhesive activity. Contrary to previous reports,24–28 surface hydrophobicity was a poor indicator of functional activity. These results indicate that surface hydrophobicity is not the primary parameter controlling adsorbed protein activity and that other surface properties, including charge, influence adsorbed protein function.

**Discussion**

We analyzed several adsorption parameters, including adsorption kinetics, quasi-equilibrium values, and structural changes (as determined by changes in Ab binding affinity), upon adsorption to well-defined surface chemistries for a FN model fragment. These parameters were correlated to the adhesivity of the adsorbed protein in order to provide insights into adsorption–function relationships. The 30 min time point for cell adhesion to FNIII7–10 was chosen to restrict interactions to initial adhesion events, ensuring that differences in adhesion could be attributed to FN conformation and thus eliminating confounding effects. Our group has shown that detachment of cells occurs at the integrin–FN bond for these cells.31 In addition, blocking the integrin–FN interaction eliminates cell adhesion to these surfaces, confirming that our adhesion assay detects the functional activity of FN on each surface.29

Recent studies with model surfaces have proposed that surface hydrophobicity is a good predictor of adsorption parameters and that proteins adsorbed onto hydrophobic surfaces undergo greater unfolding/denaturation than on hydrophilic supports.25–28,41,50 In agreement with these studies, our kinetic results indicate faster FNIII7–10 adsorption rates for the CH3 SAM and that proteins adsorbed onto this surface undergo that largest change in protein unfolding as determined by the ratio of area per molecule of irreversibly adsorbed protein (state 2) to area per molecule of reversibly adsorbed protein (state 1). Analysis of protein conformation using antibodies as structural probes revealed drastic reductions in Ab binding affinity for epitopes in the 8th, 9th, and 10th type III repeats of FN for proteins adsorbed onto the CH3 functionality compared to other surfaces. These results suggest gross changes in FN structure throughout the entire molecule and agree well with the conclusions obtained from the kinetic analysis. Finally, FNIII7–10 adsorbed onto the CH3 SAM displayed poor cell adhesion activity. This loss in activity is in good agreement with the significant structural changes observed.

In contrast, kinetic parameters and surface hydrophobicity did not correlate well with FN activity on the OH, NH2, and COOH surfaces. Similarly, no significant differences in the structure of the 8th type III repeat were detected among these surfaces using mAb1937. On the other hand, Ab measurements with two different Ab’s revealed surface-dependent differences in the structure of the central cell binding domain of FN localized to the 9th and 10th type III repeats. These structural changes correlated well with surface-dependent differences in cell adhesive activity (Figure 8). FNIII7–10 adsorption onto the neutral hydrophilic OH SAM exhibited the highest Ab binding affinity for Ab’s localizing to the cell binding domain as well as the highest cell adhesion activity, suggesting that this functionality induces the least amount of protein unfolding or denaturation. The adsorption and functional behavior on the OH surface, as well as the

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hydrophobic CH₃, may be explained by water solvation and restructuring effects at the interface.²²,²³ This analysis indicates that surface hydrophobicity and adsorption kinetic parameters are partial predictors of adsorbed FN functional activity when comparing hydrophobic and hydrophilic supports. These determinants are likely to be effective for gross/global changes in protein structure. However, these parameters cannot discriminate among neutral, positive, and negative hydrophilic surfaces, even though adsorption onto these functionalities significantly modulates the activity of the adsorbed protein. In contrast, probes for specific structural/functional domains, such as the Ab’s used in the present study, provide robust determinants of adsorbed FN activity. Furthermore, the Ab-specific differences among SAMs indicate that local, indicated by each individual Ab, as well as global, indicated by combining the results of all three Ab’s, changes in protein structure potentiate protein activity. While the present study provides insights into adsorption-induced changes in structure and function, detailed structure–function analyses are required to elucidate mechanisms involved in surface-dependent modulation of protein activity. As an initial step to this goal, we recently implemented computational molecular modeling approaches to the adsorption of the FN fragment onto the SAMs examined in the present work.²⁴ This computational molecular model predicted adsorption free energy following the trend NH₂ > CH₃ > COOH > OH and structural changes following the trend CH₃ > COOH = NH₂ > OH, both of which are consistent with the experimental results obtained in the present study. Finally, similar structure–function analyses with other proteins are necessary to establish the broad applicability of this approach to analyze protein adsorption to synthetic supports.

Conclusions
Using model surfaces with well-defined surface properties and investigating a range of surface chemistry, we demonstrate significant surface-chemistry-dependent structural changes in FN central cell binding domains and adhesive activity. These findings indicate that adsorption-induced changes in protein structure and activity are not dominated by surface hydrophobicity alone. This study also establishes a relationship between surface-dependent changes in structural domains of FN and FN functional activity.

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