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Designing Tailored Biomaterial Surfaces to Direct Keratinocyte Morphology, Attachment, and Differentiation

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Abstract

Precisely engineering the surface chemistry of biomaterials to modulate the adsorption and functionality of biochemical signaling molecules that direct cellular functions is critical in the development of tissue engineered scaffolds. Specifically, this study describes the use of functionalized self-assembled monolayers (SAMs) as a model system to assess the effects of biomaterial surface properties on controlling fibronectin (FN) conformation and concentration as well as keratinocyte function. By systematically analyzing FN adsorption at low and saturated surface densities, we distinguished between SAM dependent effects of FN concentration and conformation on presenting cellular binding domains that direct cellular functions. Quantitative image analyses of immunostained samples showed that modulating the availability of the FN synergy site directly correlated with changes in keratinocyte attachment, spreading, and differentiation, through integrin mediated signaling mechanisms. The results of this study will be used to elucidate design features that can be incorporated into dermal equivalents and percutaneous implants to enhance the rate of reepithelialization and tissue regeneration. Furthermore, these findings indicate that SAM based model systems are a valuable tool for designing and investigating the development of scaffolds that regulate the conformation of extracellular matrix cues and cellular functions that accelerate the rate of tissue regeneration.

Introduction

Tissue engineered skin substitutes offer the potential of an “off the shelf” therapeutic treatment for skin injuries, including full-thickness burns and chronic non-healing ulcers. Over the past three decades, composites of cultured cells and biomaterials have been investigated for potential use as tissue engineered skin substitutes.^{1–3} Regeneration of skin tissues has been examined with a variety of dermal analogs including collagen-glycosaminoglycan sponges,^{4,5} collagen gels,^{6,7} hyaluronic acid derivatives,⁸ and synthetic polymers.⁹ Although these composite scaffolds have demonstrated efficacious results in clinical applications, problems with mechanical stability, prolonged healing times, scarring, and limited tissue functionality persist.¹⁰ The ultimate success of tissue engineered skin substitutes which promote rapid reepithelialization requires further development of dermal analogs that more closely mimic the wound environment and biochemically direct cellular function.^{1–3,11}

Reepithelialization is a critical process that initiates the restoration of the barrier function of skin during wound healing. Hours after injury, keratinocytes located at the margin of the

wound respond to a variety of biochemical signals and begin to migrate. This lateral migration entails sequential adhesion and release of the cells from ECM molecules that either originate from circulation or are deposited by the migrating keratinocytes themselves after the initial wounding phase.^{12,13} Keratinocyte attachment to these ECM proteins is mediated by integrin receptors present on the cell surface that exhibit specificity for particular domains in the ECM proteins.¹⁴ Upon binding to their extracellular ligands, integrins, intracellular proteins, and signaling molecules aggregate to form focal adhesion complexes that link integrins to the actin cytoskeleton.¹⁵ These complexes transmit information in a bi-directional manner between the outer and inner environment of the cell, facilitating the formation of multiple signaling complexes and directing cellular functions critical for reepithelialization of a wound site.^{16,17}

The role of ECM proteins in directing keratinocyte function has been studied extensively during the reepithelialization phase of wound healing,^{18–20} in tissue culture models,^{21,22} and on the surfaces of biomaterials.^{23–25} Specifically, the ECM protein fibronectin (FN) plays an integral role in the attachment of keratinocytes and the regeneration of an epidermal layer. Fibronectin contains an RGD binding domain that acts as a ligand for integrin receptors found on the surfaces of keratinocytes and other cells.^{26,27} Studies have demonstrated that the conformation of adsorbed FN affects the availability of these ligand domains for integrin binding. Furthermore, the presence of these domains directs specific functions of fibroblast,²⁸ osteoblast,²⁹ myoblast,³⁰ and endothelial cells.³¹ Although many research efforts have elucidated the importance of FN in directing keratinocyte function, little research has been conducted to evaluate the roles of biomaterial substrate properties, including chemistry and hydrophobicity, on FN conformation and subsequent FN mediated keratinocyte functions.

Understanding how to engineer biomaterial surfaces with the appropriate properties to present tailored biochemical signaling cues to keratinocytes, will ultimately lead to the design of dermal scaffolds that promote rapid reepithelialization and improve the performance of tissue engineered skin substitutes. Self-assembled monolayers (SAMs) of alkanethiols on gold substrates offer an excellent model system to evaluate the effect of biomaterial surface properties on protein conformation and subsequent cellular interactions, due to their ease of fabrication and ability to present homogenous surface chemistries.^{32,33} Various surface chemistries can be created by modifying the terminal functional group of alkanethiol molecules without altering other surface variables, such as roughness or topology. Previous studies have demonstrated that both the hydrophobicity and charge of a SAM surface directly effect protein conformation and concentration and modulate cellular signaling and subsequent cellular functions.^{28,29,32,34–37}

The goal of this study was to quantitatively evaluate the effect of FN concentration and conformation on keratinocyte attachment, morphology, and differentiation. Using SAMs as model biomaterial surfaces, we analyzed FN adsorption and conformation at both low and saturated FN surface densities, as a function of different surface chemistries. Low FN surface density experiments were conducted to compare the effects of each surface chemistry on FN conformation and subsequent cellular functions, since each surface had the same amount of protein adsorbed. Saturated FN surface density experiments were conducted to analyze the effects of surface chemistry on FN conformation and concentration as well as their roles on keratinocyte functions. The availability of cellular binding sites was evaluated and correlated with keratinocyte attachment, morphology, differentiation, as well as focal adhesion (FA) formation. Comparative analyses of keratinocyte function on FN coated SAMs suggest that NH₂ and CH₃ terminated surfaces at saturated FN densities increase binding domain availability which correlates directly with increased control of keratinocyte

attachment, morphology, and decreased differentiation through integrin mediated signaling mechanisms.

Materials and Methods

Preparation of Self-Assembled Monolayers (SAMs)

Gold surfaces on glass substrates were obtained commercially from Evaporated Metal Films (Ithaca, NY). For monolayer formation, slides were cleaned and immersed in 1 mM alkanethiol solutions in absolute ethanol of dodecanethiol (CH₃ surface, Alfa Aesar, Ward Hill, MA), 11-mercaptoundecanoic acid (COOH surface, Aldrich, Milwaukee, WI), 11-mercapto-1-undecanol (OH surface, Aldrich), and 11-amino-1-undecanethiol, hydrochloride (NH₂ surface, Dojindo Laboratories, Kumamoto, Japan) for 18 hours. After a packed monolayer formed, the slides were removed from solution, rinsed with ethanol, and dried with nitrogen following protocols previously described.³⁴⁻³⁸ New films were prepared immediately prior to each characterization and cellular experiment.

Characterization of SAMs

To measure the contact angle for each SAM surface, sessile drop measurements were obtained using a Rame-Hart model 100-00 Goniometer (Netcong, NJ) with a protractor mounted in the eyepiece on 1 µl droplets of deionized water that were applied to the surface. A minimum of four measurements were taken on each surface and the results were averaged. These measurements were repeated for each surface.

To determine the thickness of the SAM layers on the surface of each substrate, ellipsometry measurements were obtained with a Rudolph Series 439 manual null ellipsometer (Denville, NJ). Film thickness values were determined using regression algorithms with constant values of 1.457 for the index of refraction of the film, 3.50 for the substrate absorption, and varying the index of refraction of the substrate from 0.15 – 0.30 in 0.05 increments. For each SAM surface 5 measurements were made.

Culture of Neonatal Human Keratinocytes

Human keratinocytes were isolated from neonatal foreskins using an enzymatic treatment with a dispase (Gibco, Gaithersburg, MD) solution and were propagated on a feeder layer of 3T3-J2 mouse fibroblasts (generously donated by Dr. Stelios Andreadis, State University of New York at Buffalo, Buffalo, NY) following methods described previously using keratinocyte medium (KCM) consisting of a 3:1 mixture of DMEM (high glucose) (Life Technologies, Inc., Gaithersburg, MD) and Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 10⁻¹⁰ M cholera toxin (Vibrio Cholerae, Type Inaba 569 B; Calbiochem, La Jolla, CA), 0.4 µg/ml hydrocortisone (Calbiochem), 0.13 U/ml insulin (Sigma Chemical), 1.4*10⁻⁴ M adenine (Sigma), 1% penicillin/streptomycin (Invitrogen), 5 µg/ml transferrin (Calbiochem), 2*10⁻⁹ M triiodo-L-thyronine (Sigma), and 0.01 µg/ml epidermal growth factor (BD Bioscience, San Jose, CA). After 5 days of culture in 10% CO₂ at 37°C, cells were detached using 0.05% Trypsin-EDTA (Invitrogen) and rinsed with serum free and epidermal growth factor free KCM(-S-GF). For all cellular experiments, keratinocytes were seeded in KCM(-S-GF) and passage 2–4 keratinocytes were used for all experiments.

Fibronectin Adsorption on SAMs Surfaces

To create individual wells for cellular assays, 9 mm inner diameter adhesive silicone isolators (Grace BioLabs, Bend, OR) were affixed to SAM surfaces or tissue culture polystyrene cover slips (positive controls) (TCPS, Thermanox, Nunc, Rochester, NY). Fibronectin (BD Biosciences, San Jose, CA) was then passively adsorbed at low surface

density or saturated surface density for 1 hour at room temperature. Based on the results of previous studies 40 ng/cm² of FN for each surface was used as the low surface density. This concentration was achieved by using 10 µg/ml of FN for the OH surface, 2 µg/ml of FN for the CH₃, COOH, and NH₂ surfaces, and 1 µg/ml of FN for the TCPS surface.³⁰⁻³⁸ Saturated surface densities were achieved using 25 µg/ml of FN, based on our ellipsometry data as well as previously reported values for SAM surfaces.³³⁻³⁸ This concentration produced surfaces with FN surface densities of approximately 110 ng/cm², 360 ng/cm², 280 ng/cm², 410 ng/cm², and 400 ng/cm² for OH, CH₃, COOH, NH₂, and TCPS surfaces, respectively.³⁰⁻³⁸ After FN adsorption, each well was blocked for non-specific binding using 1% heat denatured bovine serum albumin (BSA, Sigma) in dPBS (Hyclone).

Determining Saturation Density of Adsorbed Fibronectin by Ellipsometry

The thickness of the adsorbed FN on the SAM surfaces was quantified using ellipsometry methods previously described.³³ Self-assembled monolayer substrates were prepared and individual wells were created on each surface. Stock solutions of 0, 5, 25, and 100 µg/ml of FN were added to each well in dPBS for 1 hour or 4 hours at room temperature. Each well was measured three times and average film thickness was recorded and compared with the initial value, determined during characterization of the untreated surfaces, to give the thickness of adsorbed protein on each SAM surface. This analysis was performed on each surface in triplicate and averages with standard deviations were reported.

SAM Mediated Changes in Fibronectin Conformation

To measure SAM dependent changes in adsorbed FN conformation and the availability of the central cellular binding domain on FN, a monoclonal antibody directed towards this domain (HFN 7.1, Developmental Studies Hybridoma Bank, Iowa City, IA) was used.³⁹ Low surface density and saturated surface density FN treated SAM surfaces as well as TCPS surfaces were blocked using 1% heat denatured BSA (in dPBS) then incubated with HFN 7.1 for 1 hour in 10% CO₂ at 37°C. Each surface was then rinsed in blocking buffer (0.05% Tween-20 (Sigma) and 0.25% BSA in dPBS) and incubated with 546 Alexa Fluor conjugated goat anti-mouse IgG (1:200 in blocking buffer, Molecular Probes, Eugene, OR) for 1 hour in 10% CO₂ at 37°C. Slides were then rinsed with dPBS, and images were captured using an RT Color Spot camera (Spot Diagnostics, Sterling Heights, MI). Image J Analysis software (downloaded from <http://rsb.info.nih.gov/ij/>) was used to determine the relative amount of cellular binding sites on each FN treated SAM and control surface. The total area of fluorescent pixels was calculated, normalized against the total area of each well examined, and the values are reported as HFN 7.1 percent positive area. This assay was performed four times for each surface at each surface density. Results are reported as averages and standard deviations.

Cell Spreading

To quantify the effect of FN treated SAM surfaces on keratinocyte morphology, cell spreading was measured using fluorescein-5-maleimide, as previously described.⁴⁰ Keratinocytes were seeded at a density of 5,000 cells/well in KCM(-S-GF) and allowed to attach for 3 hours in 10% CO₂ at 37°C. After attachment, the wells were rinsed using PBSABC (EMD Chemicals, Gibbstown, NJ), fixed using 16% formaldehyde (Ted Pella, Inc., Redding, CA), and then permeated using 0.1% Triton X-100 (Sigma). Fluorescein-5-maleimide (Molecular Probes) at 0.6 mM was added to the cells for 1 hour at room temperature. After 1 hour, cells were rinsed with dPBS and cell nuclei were stained with a 0.06 mM solution of Hoechst nuclear reagent (Molecular Probes) for 5 minutes at 37°C. All images of stained cells were thresholded using the same image analysis protocol to clearly define cellular areas. Each cell was traced and its area was calculated with Image J software. For each surface condition, 15 cells were analyzed.

Analysis of Keratinocyte Attachment

Keratinocytes were seeded at a density of 5,000 cells/well in KCM(-S-GF) and allowed to attach for 3 hours in 10% CO₂ at 37°C on each FN treated surface. The wells were then rinsed using PBSABC and the cell nuclei were stained Hoechst nuclear reagent. Images were captured to determine the number of attached cells in a defined region. Assuming the cells were homogenously dispersed in each well, this value was used to extrapolate the total number of attached cells per well and normalized to the initial number of cells seeded. For each surface condition examined, 3 separate wells were imaged and each experimental surface condition was assayed in duplicate. Values are reported as percent keratinocyte attachment.

Analysis of Keratinocyte Differentiation

The percentage of involucrin positive keratinocytes was detected by immunofluorescence staining using methods previously described.⁴¹ Involucrin is expressed by keratinocytes that have committed to terminal differentiation.^{42,43} Keratinocytes were allowed to attach on FN treated surfaces for 3 hours in KCM(-S-GF) in 10% CO₂ at 37°C. Following incubation, the wells were rinsed with PBSABC, fixed with 4% formaldehyde, and then permeabilized with 0.5% Triton X-100 in dPBS. The cells were treated with a monoclonal mouse anti-human involucrin antibody (Clone SY5, Sigma) (1:50 dilution in blocking buffer; 0.05% Tween 20 and 0.25% BSA in dPBS) for 1 hour at room temperature. Cells were then rinsed with blocking buffer and incubated with an Alexa Fluor 546 conjugated goat anti-mouse secondary antibody (1:200 dilution in blocking buffer) for 45 minutes in 10% CO₂ at 37°C. Immunofluorescence images were converted to binary images by thresholding to 50% of maximum intensity (127 pixels/256 pixels). Thresholded cells that exhibited a fluorescent area greater than 100 μm^2 were marked involucrin positive. The total cell number was also evaluated for each image and the percentage of involucrin positive cells for each image was reported. For each surface condition, 4 samples were evaluated and each experimental surface condition was assayed in duplicate.

Fluorescence Quantification of Focal Adhesion Formation

To further evaluate keratinocyte attachment on precisely tailored SAM surfaces, quantitative immunolocalization studies were used to evaluate the expression of the FA protein vinculin within the cells. Keratinocytes were seeded on FN treated surfaces for 3 hours in 10% CO₂ at 37°C. Each of the cell seeded surfaces was then rinsed with PBSABC, treated for 10 minutes with a solution to fix and permeabilize the cells (4% formaldehyde, 0.2% Triton X-100 (Sigma) in PBS), and then treated for 10 minutes with a blocking solution (1% BSA in dPBS) to minimize nonspecific binding. The cells were then incubated with mouse anti-human vinculin primary antibody (Clone HVIN-1, Sigma) (1:100 dilution in blocking solution) for 45 minutes in 10% CO₂ at 37°C. Following primary incubation, the surfaces were rinsed in 1% BSA and incubated with Alexa Fluor 546 conjugated goat anti-mouse secondary antibody (1:100 in blocking solution) for 30 minutes in 10% CO₂ at 37°C. Images were all exposed to the same fluorescence intensity to allow for quantification of FAs. Using Image J analysis software, the area of each individual FA was measured and the total area of FAs was summed for each individual cell. This value was then normalized to the area of the corresponding cell to calculate the area density of focal adhesions for each cell. For each surface condition, 10 cells were analyzed.

Results

Model Surfaces with Precisely Tailored Surface Chemistries

Self-assembled monolayer surfaces presenting nonpolar hydrophobic (CH_3), negatively charged (COOH), neutral hydrophilic (OH), and positively charged (NH_2) surfaces were produced at physiological conditions (pH 7.4). Contact angles obtained from this study were $112 \pm 1^\circ$ for CH_3 , $20 \pm 2^\circ$ for OH , $29 \pm 2^\circ$ for COOH , and $46 \pm 2^\circ$ for NH_2 and are comparable to previously reported values for the same or similar alkanethiols.^{38,44}

Determining Saturation Levels of Fibronectin

At 1 hour, ellipsometry showed that the thickness of adsorbed FN increased between the surfaces treated with 5 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$ and plateaued at a concentration of 25 $\mu\text{g/ml}$ on all SAM surfaces. No statistical differences were observed between 25 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ on any surface examined (Student's t-test $p < 0.05$) (Figure 1). When FN was adsorbed onto SAM surfaces for 4 hours, the thicknesses of the FN were comparable to those measured at 1 hr (data not included). When comparing the thicknesses of FN on each SAM surface after 25 $\mu\text{g/ml}$ was adsorbed, no statistical differences were found (One Way ANOVA, Tukey post-hoc analysis $p < 0.05$). Based on our ellipsometry data, we chose to use 25 $\mu\text{g/ml}$ as our FN concentration to saturate each SAM surface.

Analysis of SAM Directed Changes in Fibronectin Conformation

Quantitative analyses of fluorescent images indicated that at low surface density, FN treated OH surfaces exhibited a statistically greater HFN 7.1 positive area than the other surfaces evaluated (* denotes significant differences, One Way ANOVA, Tukey post-hoc analysis $p < 0.05$) (Figure 2). When surfaces were treated with saturated surface densities of FN, HFN 7.1 positive areas on CH_3 , and NH_2 functionalized substrates as well as TCPS were statistically greater than the OH and COOH functionalized surfaces (* denotes significant differences, One Way ANOVA, Tukey post-hoc analysis $p < 0.05$).

SAM Dependent Changes in Fibronectin Treated Surfaces Direct Keratinocyte Morphology

At low FN surface densities, OH terminated SAM surfaces facilitated a statistically significant increase in cell spreading relative to all other surfaces analyzed (* denotes significant differences, One Way ANOVA, Tukey post-hoc analysis $p < 0.05$) (Figure 3). In comparing keratinocyte spreading at low surface density and saturated surface densities of FN for each surface condition, a statistically significant increase was found for all surfaces except for OH terminated surfaces (Student's t-test, $p < 0.05$). When we evaluated the effects of FN treated SAM substrates at saturated surface densities, it was found that NH_2 , CH_3 , and TCPS surfaces mediated the same amount of keratinocyte spreading, and all of these surfaces promoted significantly greater keratinocyte spreading than OH and COOH functionalized surfaces (* denotes significant differences, Kruskal-Wallis One Way ANOVA on ranks, Tukey post-hoc analysis, $p < 0.05$).

SAM Dependent Changes in Fibronectin Treated Surfaces Direct Keratinocyte Attachment

The percentage of keratinocyte attachment at low FN surface density was found to be significantly higher on OH terminated SAM surfaces (25%) than other surfaces evaluated (14–18%) (*denotes significant differences, One Way ANOVA, Tukey post-hoc analysis, $p < 0.05$) (Figure 4). When comparing keratinocyte attachment at low surface density and saturated surface densities of FN for each surface condition, a statistically significant increase was found for all surfaces except for OH terminated surfaces (Student's t-test, $p < 0.05$). At saturated surface densities of FN, keratinocyte attachment values on CH_3 , NH_2 , and TCPS surfaces were comparable to each other and significantly greater than on the other

surfaces, 55% vs. 25%, respectively (* denotes significant differences, One Way ANOVA, Tukey post-hoc analysis, $p < 0.05$). These findings exhibit a trend that is consistent with the results of analyses of available cell binding epitopes for FN treated SAM surfaces.

SAM Dependent Changes in Fibronectin Treated Surfaces Direct Keratinocyte Differentiation

11-mercapto-1-undecanol SAM surfaces treated with low surface densities of FN exhibited a significantly lower percentage of involucrin positive cells when compared to the other surfaces, 20% vs. 32–37% (Figure 5) (* denotes significant differences, One Way ANOVA, Tukey post-hoc analysis, $p < 0.05$). At saturated FN surface densities, involucrin expression decreased significantly for all surfaces except OH terminated surfaces (Student's t-test, $p < 0.05$). Additionally, CH_3NH_2 and TCPS surfaces were shown to have the lowest levels of involucrin positive expression (~11%) (*denotes significant differences, One Way ANOVA, Tukey post-hoc analysis, $p < 0.05$).

Quantification of Focal Adhesion Binding on FN Treated SAM Surfaces

Fluorescent images of keratinocytes on OH surfaces at low FN surface density suggest these cells had a greater spreading area and appeared to express more FAs than cells on the other surfaces. At saturated FN surface densities, cells cultured on CH_3 , NH_2 , and TCPS surfaces displayed larger spreading areas and appeared to have more FAs than cells cultured on COOH and OH surfaces (Figure 6). Quantitative analyses showed that at a low FN surface density, the area density of FAs in each cell on OH terminated surfaces was statistically greater than on other surfaces (* denotes significant differences, One Way ANOVA, Tukey post-hoc analysis, $p < 0.05$). At saturated FN surface densities, all surfaces except for the OH surface exhibited an increase in area density of FAs when compared to low FN surface densities (Student's t-test, $p < 0.05$) and the area density of FAs on NH_2 , CH_3 , and TCPS surface was significantly greater than on the OH and COOH surfaces (Figure 7) (* denotes significant differences, One Way ANOVA, Tukey post-hoc analysis, $p < 0.05$). Additionally, at saturated FN surface densities, we observed that the size and amount of FAs were greater on the NH_2 , CH_3 , and TCPS surfaces.

Discussion

A critical component in the advancement of tissue engineered skin substitutes is the development of biomaterials that are tailored to include specific biochemical cues which direct cellular signaling and subsequent physiological functions. In the present study, we used SAMs as model biomaterial surfaces to examine the role of surface chemistry on mediating the conformations and concentrations of adsorbed FN, as well as on directing keratinocyte functions that guide reepithelialization of dermal equivalents. Overall, our results indicate the NH_2 and CH_3 functional groups at saturation densities facilitate the largest quantity of FN to be adsorbed, in a manner that promotes cell binding. We also showed that the availability of synergy sites directly correlates with the number of FAs and consequently integrin mediated mechanisms that modulate keratinocyte spreading, attachment, and differentiation.

The availability of the central cellular binding domain of FN, which spans the 9th and 10th type III repeats of the molecule, is known to play a major role in cellular attachment. This FN domain encompasses the RGD and PHSRN binding sites which are critical for integrin binding and subsequent cellular signaling.^{45–47} Availability of this region, and the biological activity of the protein, is highly dependent on the proper structural orientation of the protein. The results of our studies indicate structural orientation and biological activity

of FN is modulated by the surface chemistry of the substrate in a manner that is consistent with the results of previous research.³⁰

Low FN density experiments were carried out to evaluate the effects of conformation of FN since at low density the same amount of FN was adsorbed on each surface (40 ng/cm²). Our results indicate at low FN surface density, FN adsorbed on the OH terminated SAM exhibited a conformation that provided an increase in available cell binding sites, relative to the other SAMs and the control surface. When evaluating keratinocyte spreading and attachment at the low surface density, we found a direct relationship between the number of available binding sites and keratinocyte spreading and attachment and an inverse relationship for keratinocyte differentiation.

Saturated FN density experiments were also performed to analyze the effects of surface chemistry on conformation and concentration of FN. At saturation densities, the OH surface exhibited the same amount of binding sites as the low density of FN on the OH surface, suggesting that the surface was saturated at both densities that were analyzed. When characterizing the relative number of binding sites on FN on the CH₃, NH₂, COOH, and TCPS surfaces, an increase was found between low and saturated densities. Fibronectin treated CH₃, NH₂, and TCPS surfaces at saturation densities exhibited a greater number of binding sites than the COOH or OH surfaces. In a previous study, Keselowsky et al., reported that the density of FN on CH₃ and NH₂ surfaces was not statistically different from COOH SAM surfaces, at theoretical saturation densities.³⁸ Our data indicates a less preferential cellular binding conformation was achieved on the COOH surface relative to the CH₃ and NH₂ surfaces. This study also showed that there was a significant difference between the density of FN on OH and COOH surfaces at saturation densities. However, in our studies, there were no differences in available binding sites or functional measurements between these surfaces. Together, these observations indicate that even though a greater FN density was achieved on the COOH surface, it does not yield a preferential cellular binding conformation in comparison with the NH₂ and CH₃ surfaces. In addition, although the OH surface at the low surface density had increased cellular binding sites in comparison to the other surfaces, when the CH₃ and NH₂ surfaces received their saturation density of FN, they exhibited more binding sites. It cannot be determined however, if the increased binding sites on the CH₃ and NH₂ surfaces in comparison to the OH surfaces were due to conformation or concentration, or a combination of the two, since more FN was adsorbed at the saturated densities on the CH₃ and NH₂ surfaces.

One proposed mechanism to explain non-specific protein adsorption on well defined SAM surfaces, is the hydrophobicity, or the wettability of the surface. It has been reported that as a surface decreases in wettability (greater contact angle), an increase in non-specific protein adsorption occurs.³² Other, findings suggest that, that other mechanisms such as adsorption by charge-charge interactions as well as specific structural features of the surface, are necessary to consider for the adsorption of high molecular weight proteins, such as FN.^{48–50} Evaluating the wettability and charge of the SAMs used in this study, we found the same relative amount of FN binding site presentation on CH₃ and NH₂ surfaces, which are hydrophobic (contact angle 112°) and neutral, and hydrophilic (contact angle 46°) and positive, respectively. These results suggest that a combination of mechanisms govern non-specific FN adsorption.

The differences in FN conformation and availability of binding sites coupled with variations in cellular responses on SAMs indicated that increased binding site presentation had a direct effect on controlling cellular processes on model biomaterial substrates. To examine the role by which the surface properties contribute to directing cellular processes, we measured changes in FA formations by probing the expression of vinculin in FA complexes. Our FA

data directly correlated with the number of binding sites as well as the up and down regulation of the cellular processes we examined. Focal adhesion complexes facilitate the transmission of information between the intra- and extracellular environments through integrin based mechanisms. Although we did not specifically probe for integrin subunits in this study, the strong correlations between FA expression and changes in cellular responses to FN treated surfaces suggest the cellular functions we evaluated were governed by integrin mediated signaling mechanisms. Previous studies examining integrin expression profiles of activated keratinocytes, both in the wound environment and culture conditions, suggest that $\alpha 5 \beta 1$ is a principle integrin that interacts with the FN and transmitting information that directs cellular processes involved in reepithelialization.^{22,51,52} These observations, together with the findings in this study, suggest that FN mediated regulation of keratinocyte functions is directed through the $\alpha 5 \beta 1$ integrin signaling pathway. In future studies, we plan to develop quantitative relationships between keratinocyte functions, specific integrin signaling pathways, and the conformation of FN. These studies will yield a series of design parameters that will enhance the ability of biomaterials to control keratinocyte functionality for use as dermal equivalents. Similarly, these design parameters will be applied to the development of percutaneous devices such as catheters and prosthesis attachments, which depend upon the rapid formation of a robust cutaneous seal with the surrounding epithelial tissue to prevent infections and implant failure.

To improve the design of dermal analogs, it is essential to develop quantitative relationships characterizing keratinocyte interactions with FN moieties, presented at different surface densities and different conformations on the surfaces of biomaterials. Recently, our laboratory showed that passively adsorbing FN to collagen membranes increased keratinocyte attachment, relative to untreated collagen membranes.²³ When we treated these membranes with FN and measured relative quantity of cell binding domains, we observed a direct correlation between the number of binding sites and the efficiency of keratinocyte attachment (unpublished data). Together, these findings suggest that passively adsorbing FN to collagen surfaces and collagen dermal equivalents represents a promising, but suboptimal approach to directing keratinocyte functions on engineered biomaterials. The results of our present study suggest that strategically conjugating FN to collagen surfaces will increase the availability of the FN synergy sites and will significantly enhance keratinocyte attachment and reepithelialization of collagen based dermal equivalents.

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Figure 1. Relationship Between Fibronectin Concentration and Thickness on SAM Surfaces
 Ellipsometry was used to determine saturation levels of adsorbed FN on CH₃, COOH, OH, and NH₂ terminated SAM surfaces. Saturation was achieved for all surfaces at 25 μg/ml. Sample values of n= 4 and data is plotted as mean ± standard deviations.

Figure 2. Analysis of Changes in Fibronectin Conformation on SAM Surfaces

To determine the relative quantity of cell binding sites of FN an antibody directed towards the RGD and PHSRN sites of was used. At low FN surface density, OH terminated surfaces promoted a significant increase in positive HFN 7.1 area and at saturated FN surface densities, CH₃, NH₂, and TCPS surfaces facilitated a significant increase in positive HFN 7.1 area. Sample values of $n = 4$ and data is plotted as mean $\pm$ standard deviations.

Figure 3. Keratinocyte Spreading Area on Fibronectin Treated SAM Surfaces

For morphological assessment of keratinocytes, cell spreading area was analyzed. At low FN surface density, OH terminated surfaces promoted a significant increase in keratinocyte spreading and at saturated FN surface densities, CH₃, NH₂, and TCPS surfaces facilitated a significant increase in cell spreading. Sample values of $n = 15$ and data is plotted as mean $\pm$ standard deviations.

Figure 4. Keratinocyte Attachment on SAM Surfaces

Cell nuclei were stained to determine attachment percentage of keratinocytes on various SAM surfaces. Results indicate that at low surface density OH terminated surfaces promoted a significant increase in percent keratinocyte attachment and at saturated surface densities there was a statistically significant increase in percent keratinocyte attachment on CH₃, NH₂, and TCPS surfaces. Sample values of $n=3$ and data is plotted as mean $\pm$ standard deviations.

Figure 5. The Effect of SAM Dependent Changes in Fibronectin on Keratinocyte Differentiation
 Keratinocytes expressing involucrin were considered differentiated and can be seen as the cells that are stained positive (open arrows), whereas keratinocytes that did not express the protein were considered to stain negative (closed arrows). Fluorescent images of keratinocytes on NH₂ surface at A) low surface density and B) at saturated surface densities. A greater percentage of cells stained positive on the low surface density (A), in comparison to the percentage of cells that stained positive on the saturated surface densities (B). At low surface density, the OH SAM has statistically lower amounts of positive involucrin expression than the other surfaces evaluated. At saturated surface densities, CH₃, NH₂, and TCPS surfaces have statistically lower amounts of positive involucrin expression than COOH or OH terminated surfaces (C). Scale bar represents 50 μ m. Sample values of n = 4 and data is plotted as mean $\pm$ standard deviations.

Figure 6. Focal Adhesion Analysis on SAM Surfaces

An anti-vinculin antibody was used to determine presence of FAs. Keratinocytes cultured on OH at low FN surface density appeared to have more FAs than on the other surfaces evaluated. Cells cultured on CH₃, NH₂, and TCPS at saturated FN surface densities exhibited larger spreading areas and appeared to have more FA than the COOH and OH surfaces. Additionally, when comparing FAs at low and saturated surface densities, CH₃, COOH, NH₂, and TCPS surfaces exhibited a difference in the size and number of FAs. Scale bar represents 25 μ m.

Figure 7. Focal Adhesion Quantification

The area density of FAs was determined using immunostaining techniques coupled with fluorescent microscopy. Keratinocytes cultured on OH at low FN surface density exhibited increased area density of FAs in comparison with the other surfaces examined. At high FN surface density, increased area density of FAs were expressed on CH₃, NH₂, and TCPS surfaces in comparison with the OH and COOH surfaces. Sample values of $n = 10$ and data is plotted as mean $\pm$ standard deviations.