Figure

Optimization of Cellular Response on Flexible Surfaces Using Chemically Bound Proteins

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Abstract

Many methods exist in the literature to modify surfaces with extracellular matrix (ECM) proteins prior to cell attachment. However, there are few studies that systematically compare surface characterization and cell response results among different modification methods. In this work, we compare cell response and physical characterization results from fibronectin and laminin attached to polydimethylsiloxane (PDMS) elastomer surfaces by physical adsorption, chemisorption, and covalent attachment to determine the best method to modify a deformable surface. We evaluate modification methods based on completeness and uniformity of coverage, surface roughness, and hydrophilicity of attached ECM protein. Smooth muscle cell adhesion, proliferation, morphology and phenotype were also evaluated. We found that chemisorption methods resulted in higher amounts of protein attachment than physical adsorption and covalent bonding, although results among the chemisorption methods varied. All chemisorption methods were superior to covalent bonding methods, and most were superior to physisorption. The various chemisorption methods gave similar results for cell adhesion, area, aspect ratio and phenotype. One particular chemisorption method, silane modification at 70 °C, gave overall superior results in protein attachment and cell proliferation.

Keywords: cell adhesion, cell proliferation, fibronectin, laminin, siloxane, smooth muscle cells.

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Abstract

Many methods exist in the literature to modify surfaces with extracellular matrix (ECM) proteins prior to cell attachment. However, there are few studies that systematically compare surface characterization and cell response results among different modification methods. In this work, we compare cell response and physical characterization results from fibronectin and laminin attached to polydimethylsiloxane (PDMS) elastomer surfaces by physical adsorption, chemisorption, and covalent attachment to determine the best method to modify a deformable surface. We evaluate modification methods based on completeness and uniformity of coverage, surface roughness, and hydrophilicity of attached ECM protein. Smooth muscle cell adhesion, proliferation, morphology and phenotype were also evaluated. We found that chemisorption methods resulted in higher amounts of protein attachment than physical adsorption and covalent bonding, although results among the chemisorption methods varied. All chemisorption methods were superior to covalent bonding methods, and most were superior to physisorption. The various chemisorption methods gave similar results for cell adhesion, area, aspect ratio and phenotype. One particular chemisorption method, silane modification at 70 °C, gave overall superior results in protein attachment and cell proliferation.

Introduction

Chemical properties of surfaces can have a profound influence on cellular response, whether that effect is due directly to surface chemistry or mediated by adsorbed proteins. Curran et al. demonstrated that mesenchymal stem cells show increased viability and osteogenic differentiation on surfaces modified with amine terminated silanes as opposed to chondrocytic differentiation on carboxylic acid terminated silanes (1). Lewandowska et al. (2) concluded that the amount of fibronectin adsorption was independent of the type of silane functionality. However, spreading and differentiation responses varied, suggesting that the both fibronectin and cell surface receptor conformation varied with surface treatment. Siperko et al. (3) showed that the surface chemistry of self-assembled monolayers of various alkanethiols influenced the conformation of adsorbed fibronectin; thus exposing different integrin binding sites that influenced the degree of myoblast proliferation and differentiation, with enhanced differentiation occurring on -OH and -CH_3 terminated surfaces (4). Siperko et al. (3) used surfaces modified with collagen and RGD (arginine-glycine-aspartic acid) to demonstrate that osteoblastic differentiation depended on surface chemistry more strongly than surface roughness.

Adsorption of extracellular matrix (ECM) protein is a well accepted and widely-used method of surface modification on all types of substrates, even devices such as micro-fluidic bioreactors (5). Protein modification improves surface biofunctionality while maintaining substrate properties, especially for elastomers that are employed for investigating mechanotransduction such as polydimethylsiloxane (PDMS) (6-8). In this work, we evaluate protein attachment protocols to PDMS from several categories. We discriminate among various categories of methods in the following manner: those approaches that rely primarily on Van der Waals

interactions between the proteins and the surface will be referred to as "physisorption", methods that rely on stronger electrostatic or hydrogen bonding interaction will be called "chemisorption," and those methods that result in permanent chemical bonds between the protein and surface will be termed "covalently bonded."

Physically adsorbing proteins to a surface is not a robust approach to surface modification. Proteins physisorbed to oxidized PDMS surfaces are known to desorb in static culture over time. Cunningham et al. investigated the efficiency and long-term stability of physisorbed fibronectin on PDMS substrates (9). They showed that fibronectin initially adsorbed at a density of (310 ± 20) ng/cm^2, but more than 90 % of the protein desorbed after 7 d in static culture, leaving only (10 to 20) ng/cm^2 fibronectin on the PMDS surface. Primary smooth muscle cells on the physisorbed fibronectin / PDMS surfaces showed poor attachment and spreading compared to the polystyrene control.

A variety of organosilanes have been regularly employed for chemisorption of ECM proteins such as fibronectin and laminin to glass or PDMS surfaces. Aminosilanes, in particular, form silanols in aqueous solution that then covalently bond to surface hydroxyl groups and leave a terminal amino group for attachment to biopolymers via chemisorption (3). Other methods of protein attachment by chemisorption have also been utilized. Glass and silicone surfaces have been chemically modified using layer-by-layer self assembly of polyelectrolytes (10) or graft copolymerization of acrylic acid (11). Due to reaction simplicity and stability, surface modification via silanization is most commonly utilized. Covalent attachment of the proteins can also be achieved in a variety of ways. Volcker et al. (11) used 1-ethyl-3-(3-

dimethylaminopropyl)-carbodiimide (EDC) to activate any carboxylic acid group generated by the plasma treatment to covalently bind with the proteins. Covalent reaction of glutaraldehyde to the primary amine on the silane provides a end-group for further reaction of protein primary amines (12).

In this work, PDMS surfaces modified with physisorbed, chemisorbed, and covalently bonded proteins were prepared. Protein attachment methods were evaluated with respect to their ability to yield a uniform, high density layer of protein and to support cell attachment and growth. Both fibronectin and laminin were explored as candidate proteins for surface modification since these proteins present substantially different binding domains. All surfaces prepared in this study were characterized for protein presence and distribution by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and immunochemical staining. Surface roughness and surface energy were characterized using optical confocal microscopy and contact angle measurements.

A10 Smooth muscle cells (SMC) were cultured on the protein-modified surfaces in this study to understand how changes in the physical properties of the surface and in the type of adsorbed ECM protein influences cell behavior. Cytotoxic effects of the surface treatment were monitored via image-based cell counting. SMC adhesion was studied by visualization and quantification of focal adhesion complexes using immunochemical staining and fluorescent microscopy. Cell phenotype was characterized using RT-PCR and cell morphological analysis. Gene expression analysis focused on specific phenotypic markers for synthetic and contractile states. Cell morphologies were determined with confocal fluorescence microscopy using probes specific for

either the cytoplasm or cytoskeleton. Results obtained in these studies are consistent with results for other cell lines such as fibroblasts and osteoblasts. Establishing a baseline for surface physical properties and optimizing cellular response will be crucial in discerning the effect of external stimuli on cellular behavior.

Experimental

Surface Modification Methods

PDMS Oxidation via plasma treatment (Plasma): All PDMS surface treatments were initiated with oxygen plasma activation to create a hydrophilic surface and prevent protein adsorption via hydrophobic interactions. Substrates were activated via oxygen plasma treatment using an Anatech Plasma Etcher[#] for 30 s at 30 W and an air flow rate of 20 sccm. Immediately following plasma treatment, silane solutions were applied to the substrates using slightly modified literature protocols as detailed below.

Chemisorption of Proteins:

Proteins were chemisorbed to the silicone surfaces using aminopropyltrimethoxysilane (APTMS) under various reaction conditions.

Silane in Ethanol: Silane has commonly been employed for the attachment of proteins to silicone surfaces in the literature (13;14). Similar to these protocols, a solution of 1 % by

[#] Certain equipment, instruments or materials are identified in this paper in order to adequately specify the experimental details. Such identification does not imply recommendation by the National Institute of Standards and Technology nor does it imply the materials are necessarily the best available for the purpose.

volume APTMS (Sigma-Aldrich, MO) in absolute ethanol was prepared and added to culture plate wells containing silicone substrates. Then, 5 % by volume water was added to the sample wells containing the silane solution to prevent premature hydrolysis of the silane. The reaction occurred for 10 min, at which time samples were washed once with 70 % by volume aqueous ethanol and then three times with distilled and deionized water for preparation at room temperature (Silane_25C). Alternatively, samples were incubated at 70 ºC for 10 min (Silane_70C), cooled to room temperature, and washed as was indicated previously. Substrates were then incubated with human fibronectin (Biomedical Technologies, MA) or laminin at 10 μg/mL overnight. Surfaces were again washed with distilled and deionized water to remove loosely bound protein.

Silane in Acetone (Silane_Acet): After plasma treatment, PDMS substrates were incubated in a solution of acetone and water at 1:5 by volume adjusted to pH 3.5 using HCl and having an APTMS concentration of $1.2x10^{-2}$ mol/L. Similar to other protocols tested, substrates were exposed to the solution at room temperature for 10 min, washed then and incubated with either fibronectin or laminin as described previously.

Silane in Acid (Silane_Acid): A 1 mmol/L solution of acetic acid in anhydrous ethanol containing 1 % by volume APTMS was added to the PDMS substrate. 5 % by volume water was directly added to the solution in contact with the membrane and allowed to react for 10 min at room temperature and 10 min at 70 ºC. After the solution cooled to room temperature, the APTMS solution was removed and the membranes were washed and incubated with ECM protein as previously described. Experiments were also carried out similar to the literature

protocol (3) in which the silane solution was removed prior to heating. These reactions produced lower protein binding and non-uniform surfaces and are therefore not considered when mentioning acid treatment of the surfaces.

Covalent Protein Attachment

Silane then Glutaraldehyde (Silane_Glut): Following silanization at room temperature (Silane_25C), substrates were further treated with glutaraldehyde (Sigma-Aldrich, MO) (12). APTMS surfaces were washed and treated with 1 % by volume glutaraldehyde in 3.3 mmol/L KOH for 2 h. Samples were washed repeatedly with 3.3 mmol/L KOH and water and then incubated with the desired protein for 24 h.

EDC Coupling (EDC): EDC (Sigma-Aldrich, MO) was used to enhance the amount of bound protein via covalent coupling to carboxylic groups exposed at the PDMS surface (11). PDMS substrates were plasma treated and then incubated with 0.03 mol/L NaH_2PO_4 buffer at pH 4.8 containing 0.5 mg/mL EDC for 30 min at room temperature. Surfaces were then incubated with fibronectin or laminin at 10 μg/ml overnight.

Physisorption of Proteins (ABS): Following common procedures, proteins were physically adsorbed onto plasma treated PDMS substrates via incubation with 10 μg/mL fibronectin or laminin for 18 hours.

Surface Characterization

Scanning Electron Microscopy (SEM): Surface damage following oxygen plasma treatment was monitored using a Hitachi S-4700-II Field Emission SEM at 20 keV and 15 μA.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR): Attenuated total internal reflection spectroscopy was performed on a Nicolet Magna 550 FT-IR spectrometer. A background of 512 co-added scans was taken using a blank germanium prism. PDMS samples were cut to the dimensions of the prism and placed on both sides to increase the signal-to-noise ratio. 512 co-added scans were collected and ratioed against the background.

Optical Confocal Microscopy: Surface measurements and profiles are obtained using a NanoFocus μSurf confocal microscope with a rotating Nipkow disk, a xenon light source, and a charge coupled device (CCD) detector. The Nipkow disk contains approximately 120,000 pinholes. A 20x, 0.6 numerical aperture (NA) yielded a field of view of 800 μm x 800 μm with an x and y pixel resolution of 1.56 μm. The measurement z-range is set by moving the piezo driven z-stage to the lower and upper bounds of the specimen. The z resolution is 2 nm. Topography is determined by imaging at consecutive height levels and re reconstructing a single 3D image. The roughness of the surface is reported as *Ra* (15), the average roughness, for the complete 3D surface. Mathematically, the *Ra* is evaluated as follows:

$$R_a = \iint_a |Z(x,y)| dxdy$$

The standard uncertainty of roughness measurements was determined by the standard deviation of three independent measurements on three different samples prepared under identical conditions.

Contact Angle: Advancing contact angle of water on treated PDMS surfaces was measured at 25 °C via drop-shape analysis on a DSA 10 Mr2 (Krüss, Germany). The standard uncertainty of contact angle measurements was determined by the standard deviation of three independent measurements on three different samples prepared under identical conditions.

Protein Quantification and Uniformity via Immunochemistry: Surfaces modified with either fibronectin or laminin were labeled with the primary antibodies anti-human fibronectin (1:1000, Sigma) or anti-laminin (1:1000, Sigma), respectively, for 2 h at room temperature. After washing, substrates were stained with FITC (fluorescein isothiocyanate)labeled goat anti-rabbit IgG (1:100, Sigma) as the secondary antibody. Negative controls were performed with the antibodies on plasma treated PDMS. The fluorescence generated was used as a background and subtracted from the fluorescence on the protein treated surfaces. The relative amount of protein on the PDMS substrate was measured using a Molecular Devices SpectraMax M5 plate reader at an excitation wavelength of 494 nm and an emission wavelength of 520 nm. Measurement uncertainty is expressed as the standard deviation of six readings per sample and three samples. Protein uniformity was determined via imaging using a Zeiss LSM510 Confocal Microscope (LSCM) equipped with an argon ion laser for excitation at 488 nm and a (505 to 530) nm bandpass filter for emission. Images were collected using an air coupled, 20x, 0.4 NA long working distance objective. Protein modified substrates were imaged at the edge to establish a zero-intensity baseline in order to profile the image intensity and compare various substrates.

Cell Culture and Assays:

Cell Culture: Rat aortic smooth muscle cells (SMCs, A10) were purchased from ATCC (Manassas, VA) and maintained in 5 % CO_2 at 37 ºC. SMCs were cultured in DMEM with high glucose (Invitrogen) supplemented with 10 % fetal bovine serum (FBS, Invitrogen), 2 mmol/L l-glutamine, 1 mmol/L nonessential amino acids, and 50 μg/mL each of penicillin and streptomycin, according to published protocols (16).

Cell Adhesion and Proliferation: Cells were seeded at a density of 10,000 cells/well in a 48-well assay plate containing the protein modified PDMS substrates. After 4 h in culture, samples were visually checked to ensure cellular attachment and all nonattached cells were removed from the well by gentle washing twice with phosphate buffered saline. These cells were counted via hemacytometry and used to calculate the number of cells that did not adhere to the surface. As cells in TCPS (tissue culture polystyrene) control wells reached confluency, after approximately 4 d in culture, they were detached from the substrates using trypsin/EDTA and counted for total number of cells. Cell counts for adhesion and proliferation were performed in triplicate and are expressed using the average and standard deviation.

Cell Staining and Imaging: SMCs in culture for 4 d were fluorescently labeled for imaging of the cytoskeleton and the focal adhesion complexes (FAC). SMCs were fixed in 3.7 % formaldehyde for 5 min and stabilized in 0.5% Triton in cytoskeleton buffer (10 mmol/L PIPES buffer, 50 mmol/L NaCl, 150 mmol/L sucrose, 3 mmol/L $MgCl_2$, pH 6.8) for 5 min. Cells were blocked for nonspecific adsorption for 1 h with 1 % bovine serum albumin (BSA) in phosphate buffered saline and incubated with primary antibody against vinculin (antivinculin, Sigma) for 1 h. SMCs were also fluorescently stained for 1 h using FITC labeled secondary antibody to

highlight the FACs and Texas Red phalloidin to visualize the actin filaments for 1 h. SMCs were imaged on the LSCM using the argon ion laser at 488 nm for excitation and a (505 to 530) nm bandpass filter for emission of the focal adhesion complexes and the He/Ne laser at 546 nm for excitation with a long pass filter for emissions of the actin filaments. Collected images were analyzed for cell area and aspect ratio using ImagePro 6.0 (MediaCybernetics, Bethesda, MD). Uncertainty in the measurement is determined from analyzing multiple cells per image, in a minimum of 3 images per sample, and three samples per modification method.

Real Time Polymerase Chain Reaction (RT-PCR): Gene expression profiles for the SMCs on the various protein modified surfaces were analyzed using RT-PCR after 7 d in culture. RNA was extracted from cell lysates using the RNeasy Kit from Qiagen (Qiagen, Valencia, CA) according to the manufacturer's specification. Primers for RT-PCR experiments were selected from the literature and are as follows: 18S(17): sense 5'-cgacgacccattcgaacgtct-3' and antisense 5'-gctattggagctggaattaccg-3'; collagen I(18): sense 5'-gagcggagagtactggatcg-3' and antisense 5'-tactcgaacgggaatccatc-3'; smooth muscle (SM) α-actin(19): sense 5'-gatcaccatcgggaatgaacgc-3' and antisense 5'-cttagaagcatttgcggtggac-3'; and SM-myosin heavy chain (20): sense 5'-aagcagctcaagaggcag-3' and antisense 5'-aaggaacaaatgaagcctcgtt-3' (MWG Biotech, Inc., High Point, NC).

RT-PCR was performed using the QuantiTect SYBR Green RT-PCR Kit and protocol (Qiagen, Valencia, CA) on the iCycler (Bio-Rad, Hercules, CA). The protocol utilizes the following steps: Reverse Transcription: 30 min at 50 °C; Activation step: 15 min at 95 °C; 3-Step cycling: denaturation for 30 s at 95 °C, annealing for 2 min at 57 °C, and extension for 2 min at 72 °C for

45 cycles. A melt curve, 50 °C to 95 °C in 1 °C increments, was subsequently generated to analyze the product purity. The copy number for each gene was obtained by extrapolating to a standard gene curve of known concentration and copy number to yield quantitative data. 18S was included as an internal control since its mRNA levels do not change significantly with the choice of substrate. Samples were analyzed in triplicate and represented as the average gene copy number ± the standard deviation.

Results and Discussion

Silicone surfaces were pretreated by oxygen plasma etching to increase their hydrophilicity and expose –OH functional groups to silane for bonding prior to attachment of proteins. Increasing both the exposure time and the plasma power increases the oxygen content and subsequent cellular attachment on the surface, but it may also induce surface damage (21). The SEM images in Figure 1 show that the integrity of the PDMS surface appears to be maintained at (a) 30 s oxygen plasma treatment at 35 W but damaged at (b) 60 s as evidenced by surface cracks. Hence, all samples were plasma treated at 35 W for 30 s to increase the surface hydrophilicity while maintaining surface integrity. Figure 1 (c) shows a comparison of the FT-IR spectra of the as-received and plasma treated PDMS. The characteristic peaks appearing from the PDMS substrate are due to symmetric and asymmetric $-CH_3$ stretching from the $\equiv Si\text{-}CH_3$ group at 2870 and 2970 cm^{-1}, respectively. Changes in the PDMS substrate upon plasma treatment are evident in the IR spectra by an increase in the –OH stretching in the range of (3100 to 3600) cm^{-1}.

Proteins were attached to the PDMS surface in a variety of ways, which are described under "Methods." Briefly, proteins were physisorbed to the PDMS using a single protocol (ABS

protocol). Chemisorption of the proteins was performed in several ways, each using surface silanization with APTMS; published silanization protocols were slightly modified to optimize the protein adsorption and cellular response. Silanization prior to chemisorption was performed under the following conditions: in a solution of acetone and water at pH 3.5 and room temperature (Silane_acet protocol), in ethanol and water at 70 ºC (Silane_70C protocol) (13), in ethanol and water at room temperature (Silane_25C protocol), in ethanol, water, and acetic acid at 120 ºC (Silane_acid protocol) (3). Proteins were covalently attached to PDMS in one of two ways. In one approach, substrates were treated with glutaraldehyde following silane modification in ethanol and water at 25 °C, (Silane_glut protocol) (12). Covalent attachment was also performed by treating the PDMS substrate with EDC (EDC protocol) to activate any carbonyl functionalities on the plasma treated PDMS. Prepared surfaces were exposed to protein solution for 24 h to ensure reaction completion. Experiments with various concentrations of fibronectin (FN), (4 to 20) μg/mL, indicated that no change in surface protein density or cellular adhesion was observed beyond 1 d.

The reaction progress and protein attachment was monitored using FTIR-ATR. Spectra of fibronectin on PDMS for ABS, Silane_70C, and EDC bonded are shown in Figure 2 and compared to unmodified PDMS. The presence of the protein is assigned by characteristic peaks corresponding to the amide I and amide II peaks at 1660 cm^{-1} and 1550 cm^{-1}, respectively. It is evident from this figure that peaks in the unmodified PDMS can overlap the 1660 cm^{-1} amide I peak, while the peak at 1550 cm^{-1} remains mostly unobscured. The peak at 1550 cm^{-1} is the largest for the Silane_70C, implying that the high temperature silane modified surfaces have the most FN. This qualitatively agrees with the immunochemical results in Figures 3 and 4. The

amide II peaks from the EDC and ABS treated proteins are not resolved, most likely because the PDMS peaks obscure them.

Protein-modified surfaces were immunochemically stained to measure a relative amount of surface-bound protein on the surface and to determine surface uniformity. Figure 3 shows results of relative abundance determinations. These were obtained by using a plate reader to measure fluorescence intensities from stained, protein-functionalized substrates in 96-well plates. The surface area analyzed in the plate reader is 1 mm x 1 mm. Significant differences in the data were assessed using a pooled variance t-test at 5% significance level. Using 95 % confidence for the t-test, significant differences in amount of protein on the surface are expressed in the groupings below. The amount of surface protein detected following the protocols in the same grouping was statistically indistinguishable:

$$FN: \begin{matrix} Silane\_25C \\ Silane\_70C \end{matrix} > \begin{matrix} Silane\_Acid \\ Silane\_Acet \\ Silan\_Glut \\ EDC \end{matrix}, \qquad ABS > \begin{matrix} Silane\_Acid \\ Silane\_Acetone \end{matrix}$$

where the amount of ABS is indistinguishable from Silane_25C and Silane_70C.

$$LAM: \begin{matrix} Silane\_Acid \\ Silane\_Acet \end{matrix} > \begin{matrix} ABS \\ Silane\_25C \\ EDC \end{matrix}, \qquad \begin{matrix} Silane\_\_70C \\ Silane\_Glut \end{matrix} > \begin{matrix} ABS \\ Silane\_25C \end{matrix}$$

When comparing FN and LAM surfaces, all of the modification procedures yielded significant differences in amount of protein except for the EDC and Silane_70 treated samples. While the FN and LAM surface did not show all the same trends, the amount of surface-attached protein using the Silane_70C protocol (averaged over FN and LAM samples) was significantly higher

than the rest of the methods, whose averages were not distinguishable. This was also qualitatively seen in the ATR-FTIR results.

Figure 4 shows confocal fluorescence microscopy images of several surfaces; these were obtained to determine the uniformity of the proteins on the surface on a more local scale than the plate reader. Line intensity profiles of the FN coated surfaces were measured to highlight changes in the fluorescence intensity, i.e. protein surface coverage. These results highlight the fact that the silanization protocol was an important factor in obtaining uniform surface coverage. For qualitative comparison, physisorption of FN onto the PDMS substrate (Figure 4 (a)) displayed weak fluorescence intensity, indicating a low protein density, even though the coverage was qualitatively uniform throughout the surface. Figures 4 (b) (70 ^{o}C) and (c) (25 ^{o}C) show the proteins on the surface after modification with APTMS at 70 ºC and 25 ºC, respectively. Overall not only was FN coverage larger using the Silane_70C protocol (232 $\pm$ 25) than the other protocols, ABS (62 $\pm$ 37), Silane_25C (69 $\pm$ 47), Silane_Acid (72 $\pm$ 49), and Silane_Glut (89 $\pm$ 45), but the absolute uniformity ($\pm$ 25) was also the best among the protocols tested. The results from the fluorescence plate reader and confocal fluorescence microscope thus indicate that the Silane_70C protocol provides the highest amount and uniformity of FN, especially compared to the covalent bonding methods (EDC, Silane_Glut). Most of the differences in the results from the plate reader for LAM were not statistically significant. One conclusion is that, when considering both plate reader and confocal results, physical adsorption (ABS method) results in nominally less protein coverage when compared to the other methods. In addition, the overall amount of LAM on the surface for the Silane_70C is comparable to the FN results.

Surface roughness was determined using optical confocal microscopy. Values obtained for the average surface roughness *(R_a)* are displayed in Table 1. At 95% confidence, Silane_Glut is the roughest surface, as shown below:

$$Silane\_Glut > Silane\_Acid > \begin{matrix} Silane\_70C & EDC \\ Silane\_25C & ABS \\ Silane\_Acet & PDMS \end{matrix}$$

The average roughness of Silane_Glut treatment is statistically larger than the surface roughness resulting from the rest of the methods. The larger average roughness of the Silane_acid treatment is distinguishable at 95% confidence from all the other methods except Silane_70C from which it is marginally distinguishable. The average roughness is not statistically distinguishable between the Silane_Acet, Silane_70C, Silane_25C, EDC, ABS, and unmodified PDMS protocols. These determinations were made using optical interferometry with an (800 μm x 800 μm) field of view. Modification via glutaraldehyde produced the most non-uniform surfaces, whereas physisorption and most cases of chemisorption resulted in much more uniform surfaces on the nanoscale.

Results for contact angle on FN surfaces are shown in Table 2. Student-Newman-Keuls multiple comparison test demonstrates that, at 95% confidence, Silane_Glut is distinguished as having the lowest contact angle and therefore is the most hydrophilic, whereas PDMS is the most hydrophobic, as shown below:

$$PDMS > Plasma > Silane\_Acid > \begin{matrix} Silane\_70 \\ ABS \end{matrix} > \begin{matrix} Silane\_25 \\ EDC \end{matrix} > Silane\_Glut$$

Physical characterization of these surfaces demonstrates that various methods can be used for successful attachment of proteins to biologically inert surfaces. However, the method by which the proteins attach to the surface, via physical or chemical adsorption or covalent attachment, plays an important role in surface roughness, hydrophilicity, and protein coverage and density. To assess the role of these surface properties on cellular adhesion, proliferation, and differentiation and to ultimately determine the optimal method for attaching proteins to surfaces, cell studies were performed. Smooth muscle cells were chosen as a model cell system for the biological characterization of the protein modified surfaces. SMCs display a single phenotypic shift, under specific biochemical culture conditions, from the highly proliferative synthetic state to the near-quiescent contractile state (22). The phenotypic shift can be followed specifically by a change in the gene expression profiles but is also accompanied by a shift from a rounded to an elongated morphology (23).

Cell adhesion was measured via cell counting by seeding at an initial density of 10,000 cells per well and allowing cells to attach to the surfaces for 5 h. Non-adherent cells were subsequently removed from the sample wells and counted. The compliment of this number was taken to be the number of cells adhering to the substrates; these values are displayed in Figure 5. Physisorption, chemisorption, and covalent binding via EDC produced consistently reproducible results, whereas covalent binding via glutaraldehyde occasionally resulted in SMC toxicity and/or cell detachment. Also, for the FN surfaces, the number of cells attached using the Silane_Glut modification was significantly lower compared with the remaining protocols. Results obtained with this protocol were highly dependent on the amount of surface washing before cell addition or the reaction time the glutaraldehyde solution. At 95 % confidence, the number of cells

attached using the ABS protocol was significantly lower than Silane_25 and Silane_Acid. The number of cells attached on FN surfaces among the remaining protocols was indistinguishable. For the LAM surfaces, the number of cells attached is not distinguishable among any of the protocols. Similarly, the number of cells attached when comparing FN and LAM within a protocol was not distinguished. Overall, there is little difference between protocols for the number of cells attached.

Although the number of initially attached cells could not be used to positively distinguish any protocol, cell counts for each modified substrate after 4 d in culture are significantly different, as displayed in Figure 6. For the FN and LAM treated surfaces, the Silane_Glut cell counts were significantly lower than the rest of the modifications. Comparison of the remaining protocols is shown below:

$$\begin{matrix} Silane\_25C \\ Silane\_70C \end{matrix} > \begin{matrix} Silane\_Acet \\ EDC \\ ABS \\ Plasma \end{matrix}, \qquad Silane\_Acid > \begin{matrix} EDC \\ Plasma \end{matrix}$$

Among the Silane_25C, Silane_70C, and Silane_Acid modifications, there is no significant difference in cell counts after 4 days. For the LAM treated surfaces, the Silane_70C modification yields significantly more cells than the Plasma protocol. The cell counts for the rest of the protocols are not distinguished from each other. In addition, there is no statistical difference between FN and LAM for the cell counts of a particular protocol. If the total number of cells for FN and LAM are considered, the Silane_70C protocol resulted in the most cells on the surface with Silane_25C and Silane_Acid having the second most.

Figure 7 displays confocal fluorescence images of SMCs tagged with cytoskeleton and FAC labels. We observe that cell morphology, including cell area and aspect ratio, was not significantly altered regardless of the modification method or protein on the surface. Table 3 displays area and aspect ratios for all SMCs on control or modified surfaces. Changes in aspect ratio would be an indicator of the changes in morphology that accompany a phenotypic shift from rounded cells in the synthetic state to elongated cells in the contractile state. As the measurements show, however, the aspect ratio was similar for all modifications and comparable to the control. In some instances, cells adopted a slightly elongated morphology in static culture that was independent of phenotype, and that seemed to occur only after cells reached confluency.

RT-PCR results confirm that SMCs remain in their original synthetic state. SMCs in the synthetic state produce a high amount of collagen, and as they transition to the contractile state, begin producing SM α-actin, calporin and caldesmon, and, ultimately, myosin heavy chain (MHC)(23). One expects that, as SMCs transition to the contractile state, the amount of SM α-actin produced would decrease while the amount of MHC would increase. Figure 8 shows that for all modification methods and proteins, the protein expression profiles show no significant deviation from the TCPS control, indicating that all cells remain in synthetic state. SMC behavior was measured on LAM and FN modified surfaces since the rate at which primary contractile SMCs return to the synthetic state is delayed in the presence of laminin as opposed to fibronectin (16). Although this study was not conducted with primary cell lines, investigations with LAM were done to determine whether the protein could induce changes in cell phenotype. SMC area and aspect ratio measurements and gene expression profiles indicate that the type of

protein had no effect on this transition in static culture and imply that a phenotypic shift is dependent on a mechanical or chemical stimulus not investigated in this work.

Conclusions

We have evaluated several methods of attaching protein to PDMS surfaces, including physisorption, chemisorption, and covalent bonding methods. We observe significant variation in the amount and uniformity of bound protein, as well as surface roughness among the methods tested. We observe little variation in cell adhesion, but significant variation in cell proliferation of SMCs on these surfaces. We note that exchange of LAM for FN made little or no statistical difference in any of the properties or outcomes we measured; we also saw no indication of ECM-induced phenotype shift.

In terms of protein coverage and uniformity, one of the chemisorption methods, Silane_70C, yielded results that were significantly better than the others. This same protocol also resulted in the highest cell counts after 4 d incubation in static culture. A covalent bonding method, the Silane_Glut protocol, on the other hand, exhibited the roughest surface with the highest protein non-uniformity and was the most hydrophilic. This method also resulted in poor cell adhesion at 5 h, and significant cytotoxicity at 4 d culture. The physisorption (ABS) protocol resulted in a nominally lower amount of protein coverage and poor cell proliferation when compared to the best chemisorption methods.

This work provides evidence that methods for protein adsorption to PDMS can yield widely varying results, and should be carefully evaluated before being adopted in biotechnological

applications. This work also establishes a necessary baseline for future investigations involving the introduction of mechanical stimuli to SMCs on protein modified elastomeric substrates. The Silane_70C protocol will be strongly considered for use in our future SMC work on surface-treated deformable substrates.

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References

1. Curran JM, Chen R, Hunt JA. Controlling the phenotype and function of mesenchymal stem cells in vitro by adhesion to silane-modified clean glass surfaces. Biomaterials 2005 Dec;26(34):7057-67.

2. Lewandowska K, Pergament E, Sukenik CN, Culp LA. Cell-type-specific adhesion mechanisms mediated by fibronectin adsorbed to chemically derivatized substrata. J Biomed Mater Res 1992 Oct;26(10):1343-63.

3. Siperko LM, Jacquet R, Landis WJ. Modified aminosilane substrates to evaluate osteoblast attachment, growth, and gene expression in vitro. J Biomed Mater Res A 2006 Sep 15;78(4):808-22.

4. Lan MA, Gersbach CA, Michael KE, Keselowsky BG, Garcia AJ. Myoblast proliferation and differentiation on fibronectin-coated self assembled monolayers presenting different surface chemistries. Biomaterials 2005 Aug;26(22):4523-31.

5. Figallo E, Cannizzaro C, Gerecht S, Burdick JA, Langer R, Elvassore N, et al. Micro-bioreactor array for controlling cellular microenvironments. Lab Chip 2007 Jun;7(6):710-9.

6. De Jong KL, MacLeod HC, Norton PR, Petersen NO, Jasnin MF. Atomic force microscopy compatible device for stretching cells and adsorbed proteins. Review of Scientific Instruments 2006;77(2).

7. Lahiji K, Polotsky A, Hungerford DS, Frondoza CG. Cyclic strain stimulates proliferative capacity, alpha2 and alpha5 integrin, gene marker expression by human articular chondrocytes propagated on flexible silicone membranes. In Vitro Cell Dev Biol Anim 2004 May;40(5-6):138-42.

8. Miller CE, Donlon KJ, Toia L, Wong CL, Chess PR. Cyclic strain induces proliferation of cultured embryonic heart cells. In Vitro Cell Dev Biol Anim 2000 Nov;36(10):633-9.

9. Cunningham JJ, Nikolovski J, Linderman JJ, Mooney DJ. Quantification of fibronectin adsorption to silicone-rubber cell culture substrates. Biotechniques 2002 Apr;32(4):876, 878, 880.

10. Brown XQ, Ookawa K, Wong JY. Evaluation of polydimethylsiloxane scaffolds with physiologically-relevant elastic moduli: interplay of substrate mechanics and surface chemistry effects on vascular smooth muscle cell response. Biomaterials 2005 Jun;26(16):3123-9.

11. Volcker N, Klee D, Hocker H, Langefeld S. Functionalization of silicone rubber for the covalent immobilization of fibronectin. J Mater Sci Mater Med 2001 Feb;12(2):111-9.

12. Lateef SS, Boateng S, Ahluwalia N, Hartman TJ, Russell B, Hanley L. Three-dimensional chemical structures by protein functionalized micron-sized beads bound to polylysine-coated silicone surfaces. J Biomed Mater Res A 2005 Mar 15;72(4):373-80.

13. Altankov G, Grinnell F, Groth T. Studies on the biocompatibility of materials: fibroblast reorganization of substratum-bound fibronectin on surfaces varying in wettability. J Biomed Mater Res 1996 Mar;30(3):385-91.

14. Lateef SS, Boateng S, Hartman TJ, Crot CA, Russell B, Hanley L. GRGDSP peptide-bound silicone membranes withstand mechanical flexing in vitro and display enhanced fibroblast adhesion. Biomaterials 2002 Aug;23(15):3159-68.

15. ASME B46.1-2002 Surface Texture. 2003. New York, American Society of Mechanical Engineers.
Ref Type: Generic

16. Pauly RR, Bilato C, Cheng L, Monticone RE, Crow MT. Vascular Smooth Muscle Cell Cultures. Methods in Cell Biology 1997;52:133-54.

17. Lin ZH, Fukuda N, Jin XQ, Yao EH, Ueno T, Endo M, et al. Complement 3 is involved in the synthetic phenotype and exaggerated growth of vascular smooth muscle cells from spontaneously hypertensive rats. Hypertension 2004 Jul;44(1):42-7.

18. Li J, Lin SG, Yu XY, Lin QX. [Effects of macrophage migration inhibitory factor on collagen I and III mRNA expression in vascular smooth muscle cells in vitro]. Di Yi Jun Yi Da Xue Xue Bao 2005 Jun;25(6):684-6.

19. Park F, Mattson DL, Roberts LA, Cowley AW, Jr. Evidence for the presence of smooth muscle alpha-actin within pericytes of the renal medulla. Am J Physiol 1997 Nov;273(5 Pt 2):R1742-R1748.

20. Low RB, White SL, Low ES, Neuville P, Bochaton-Piallat ML, Gabbiani G. Age dependence of smooth muscle myosin expression by cultured rat aortic smooth muscle cells. Differentiation 1999;65(3):151-9.

21. Hettlich HJ, Otterbach F, Mittermayer C, Kaufmann R, Klee D. Plasma-induced surface modifications on silicone intraocular lenses: chemical analysis and in vitro characterization. Biomaterials 1991 Jul;12(5):521-4.

22. Nerem RM, Stegemann JP. Functional Requirements for the Engineering of a Blood Vessel Substitute. In: Guilak F, Butler DL, Goldstein SA, Mooney DJ, editors. Functional Tissue Engineering. 1 ed. New York: Springer; 2003. p. 87-95.

23. Owens GK. Regulation of differentiation of vascular smooth muscle cells. Physiol Rev 1995 Jul;75(3):487-517.

Figure Captions:

Figure 1: Oxygen plasma of the silicone substrates at 35 W did not alter the surface at (a) 30 s but did induce surface damage at (b) 60 s. (c) FT-IR of the PDMS substrate, as is and after oxygen plasma treatment. The spectra shows an increase in surface oxygen due to the increase in intensity at (3700 to 3000) cm-1 from the –OH stretching vibration.

Figure 2: FT-ATR spectra showing the presence of the protein on the plasma treated PDMS surface. Traces show the amide I and amide II peaks 1660 cm^{-1} and 1550 cm^{-1}, respectively for fibronectin attached to the surface via physical adsorption, chemisorption, or covalent bonding with EDC as compared to unmodified PDMS.

Figure 3: Immunochemistry results showing relative amounts fibronectin and laminin on PDMS using various surface treatments.

Figure 4: Fluorescence imaging of fibronectin bound to the PDMS surface using immunohistochemical staining. Uniform surface coverage occurs when proteins are (a) physically adsorbed to the surface or (b) silanization occurs at 70 ºC compared to (c) silanization at room temperature. Protein coverage is shown graphically below each figure.

Figure 5: Percent cells attached from the surface treated with FN or LAM. Initial density of cells seeded were 10,000/well.

Figure 6: SMC proliferation for FN and LAM surfaces as a function of modification method.

Figure 7: In static culture, the SMCs on the modified substrates did not show any significant difference in morphology, area, aspect ratio, or the number of focal adhesions, as shown on (a) TCPS, (b) FN modification via silane at 70 ^{o}C, (c) FN modification via EDC only, (d) physically adsorbed FN, (e) FN modification via silane at 25 ^{o}C, and (f) FN modification under acidic conditions. Actin filaments are shown in red, FAC and nucleus in green.

Figure 8: PCR results indicate that the cells stay in the synthetic state as indicated by the large amount of smooth muscle actin (SMA) relative to myosin heavy chain (MHC).

Figure

Modification Protocol	R_a (nm)
Unmodified PDMS	5.7 ± 1.2
ABS	7.0 ± 1.0
Silane_Acet	5.7 ± 0.6
Silane_Acid	16.7 ± 2.5
Silane_25C	7.0 ± 1.0
Silane_70C	10.3 ± 1.2
Silane_Glut	32.3 ±12.6
EDC	8.9 ± 3.5

Table 1: Surface roughness measurements for various modified surfaces after fibronectin has been attached. R_a is the average roughness in nm and the uncertainty is represented as the standard deviation of multiple samples with measurements at three different locations per sample. Studies using laminin produced similar results.

Figure

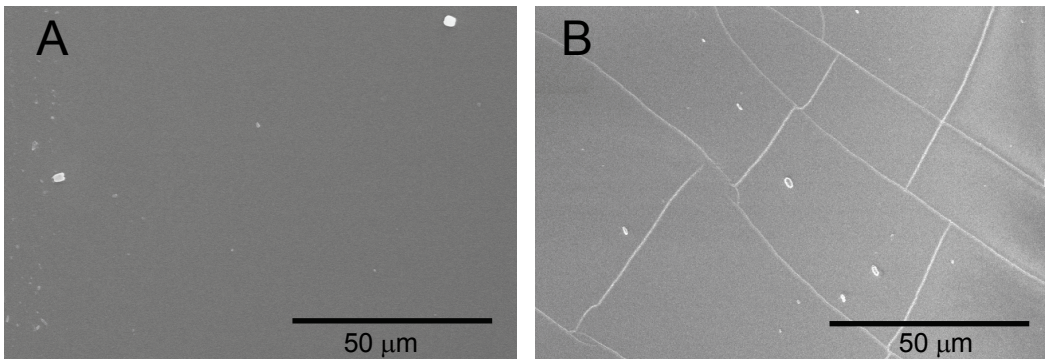
Modification Protocol	Contact Angle (degrees)
Unmodified PDMS	109.5 ± 3.1
Plasma	98.0 + 2.0
ABS	84.4 ± 1.6
Silane_Acid	93.3 ± 0.3
Silane_25C	74.0 ± 3.4
Silane_70C	82.7 ± 1.3
Silane_Glut	43.2 ± 3.5
EDC	73.2 ± 2.8

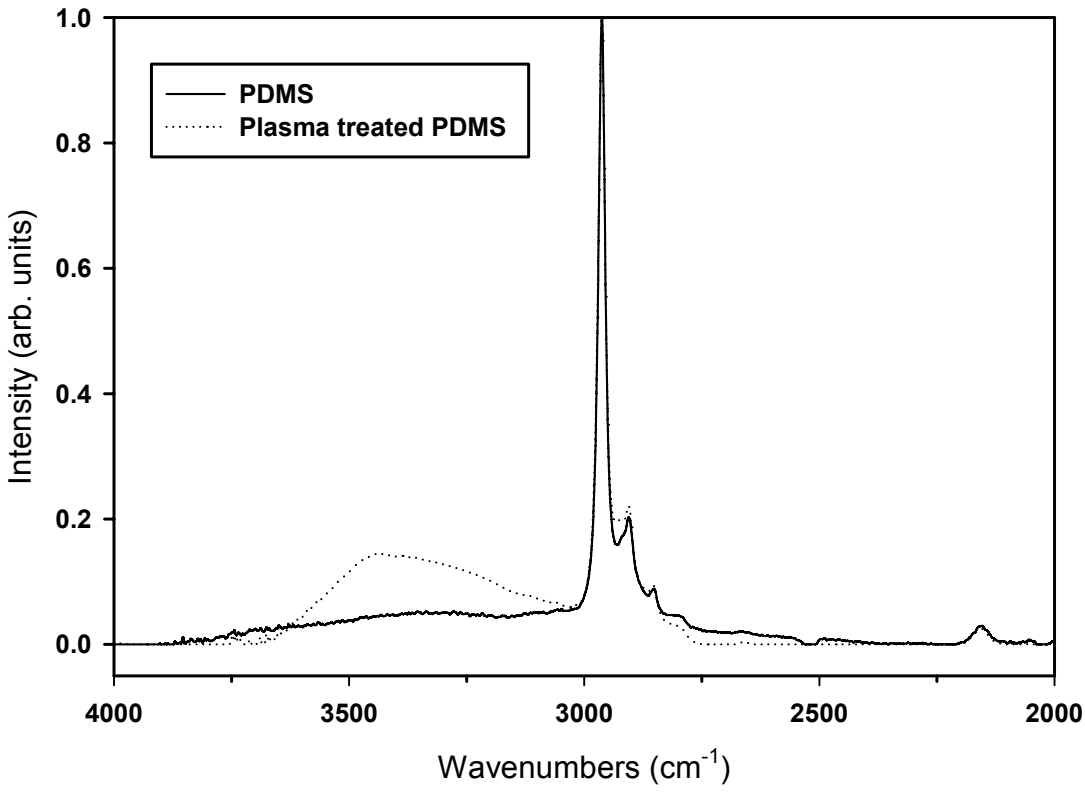
Table 2: Average contact Angle of the protein modified surfaces. Measurement uncertainty is represented by standard deviation. The average consists of measurements at three different locations per substrate for multiple samples.

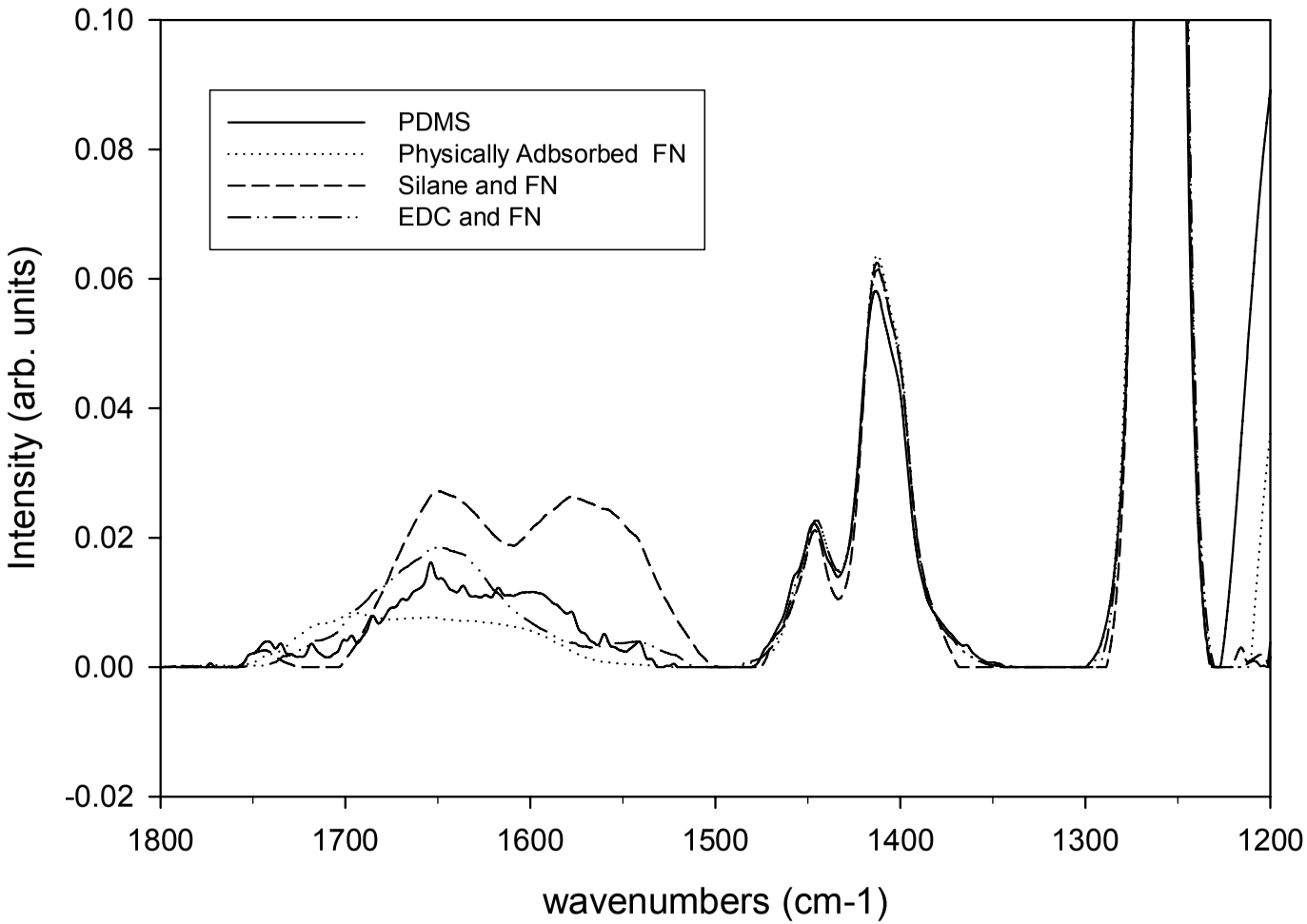
Figure

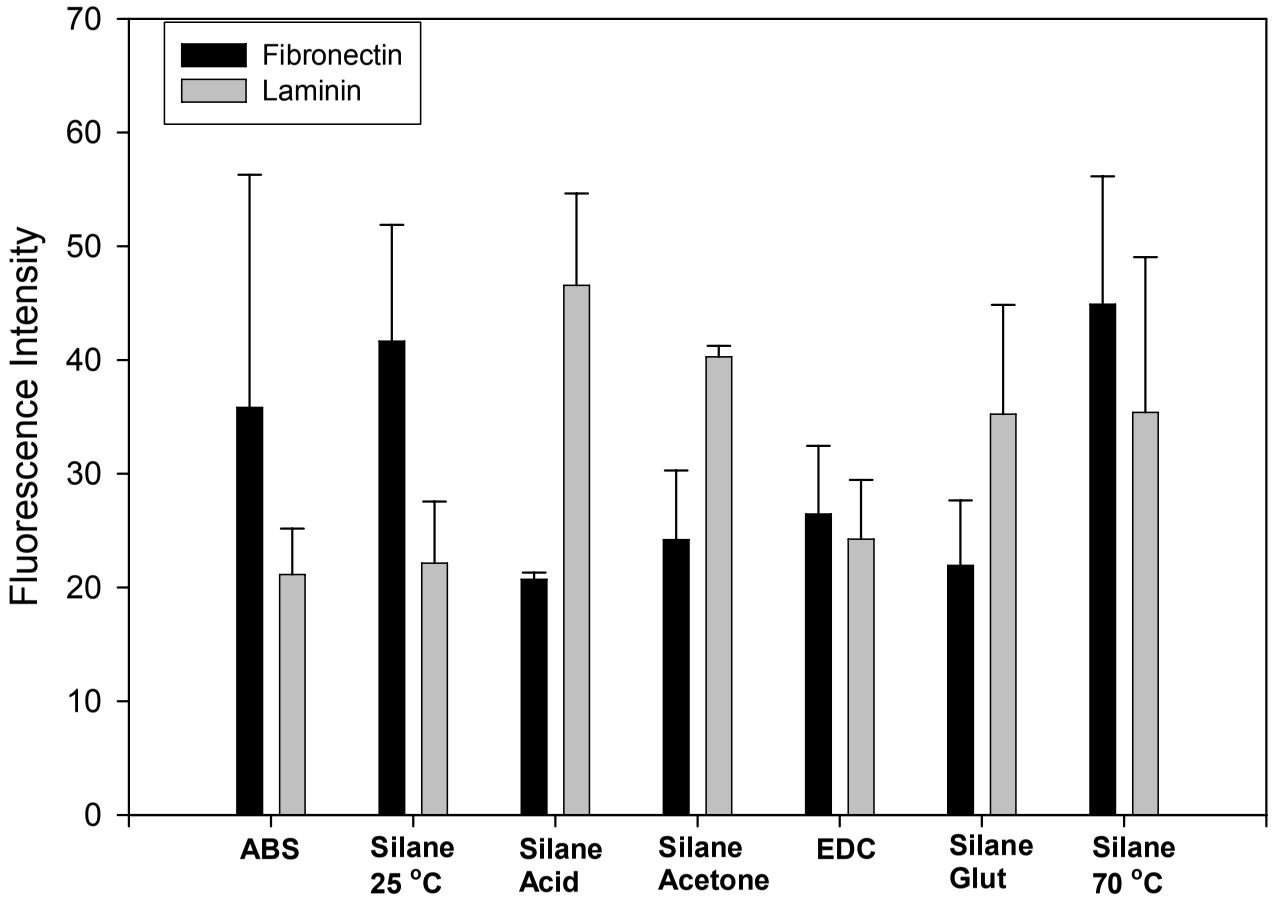
Modification Protocol	Area (μm^2)	Aspect Ratio
ABS	6628 $\pm$ 3499	3.0 $\pm$ 1.4
Silane_Acid	7100 $\pm$ 1308	1.9 $\pm$ 0.8
Silane_25C	6026 $\pm$ 2970	3.0 $\pm$ 1.1
Silane_70C	6875 + 3787	1.9 $\pm$ 1.7
TCPS	6213 $\pm$ 4325	2.7 $\pm$ 2.0

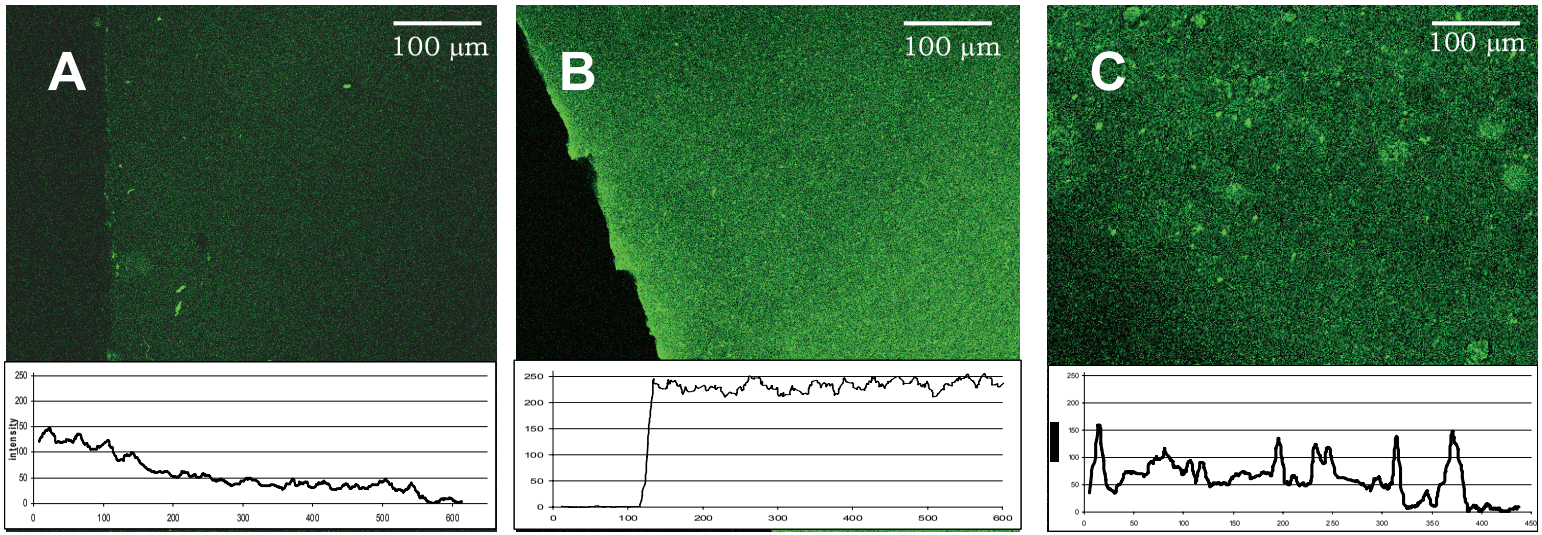
Table 3: Area (μm^2) and aspect ratio of SMC cells on surfaces with different modifications

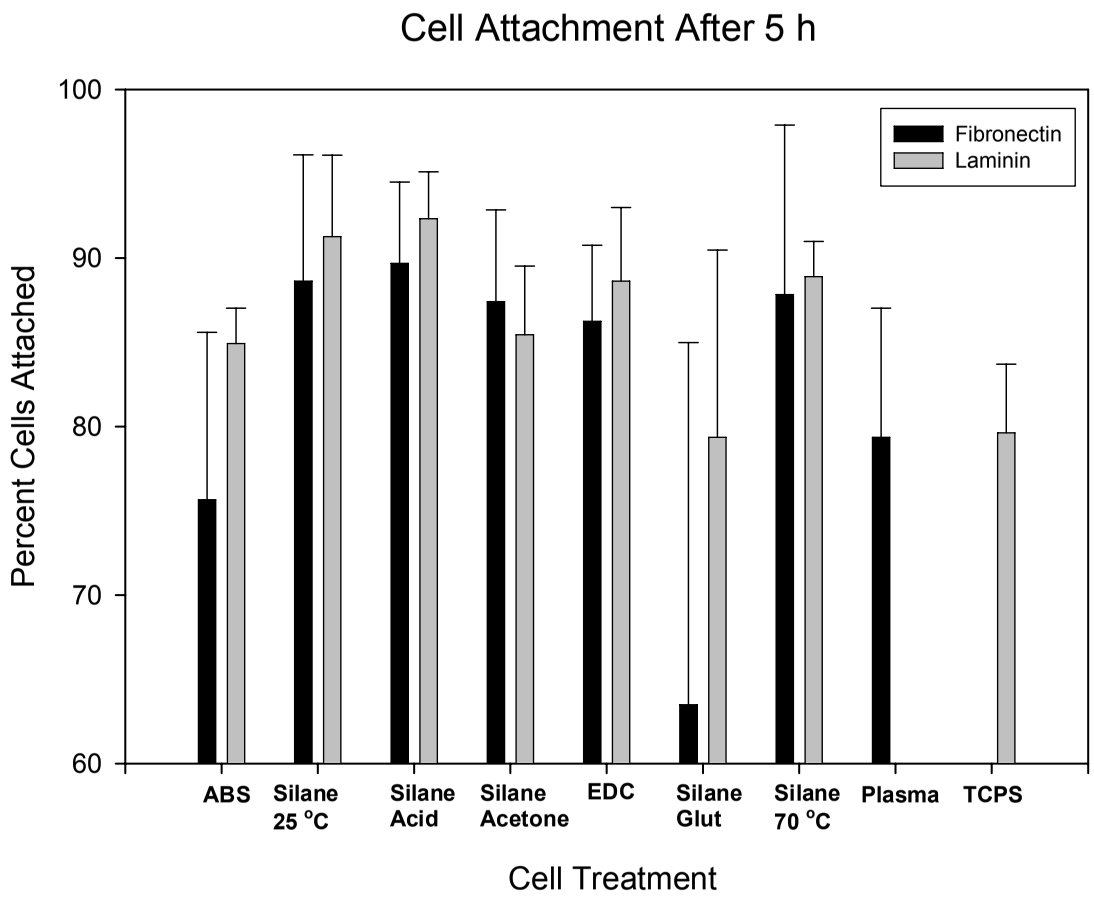

A
50 μm
B
50 μm

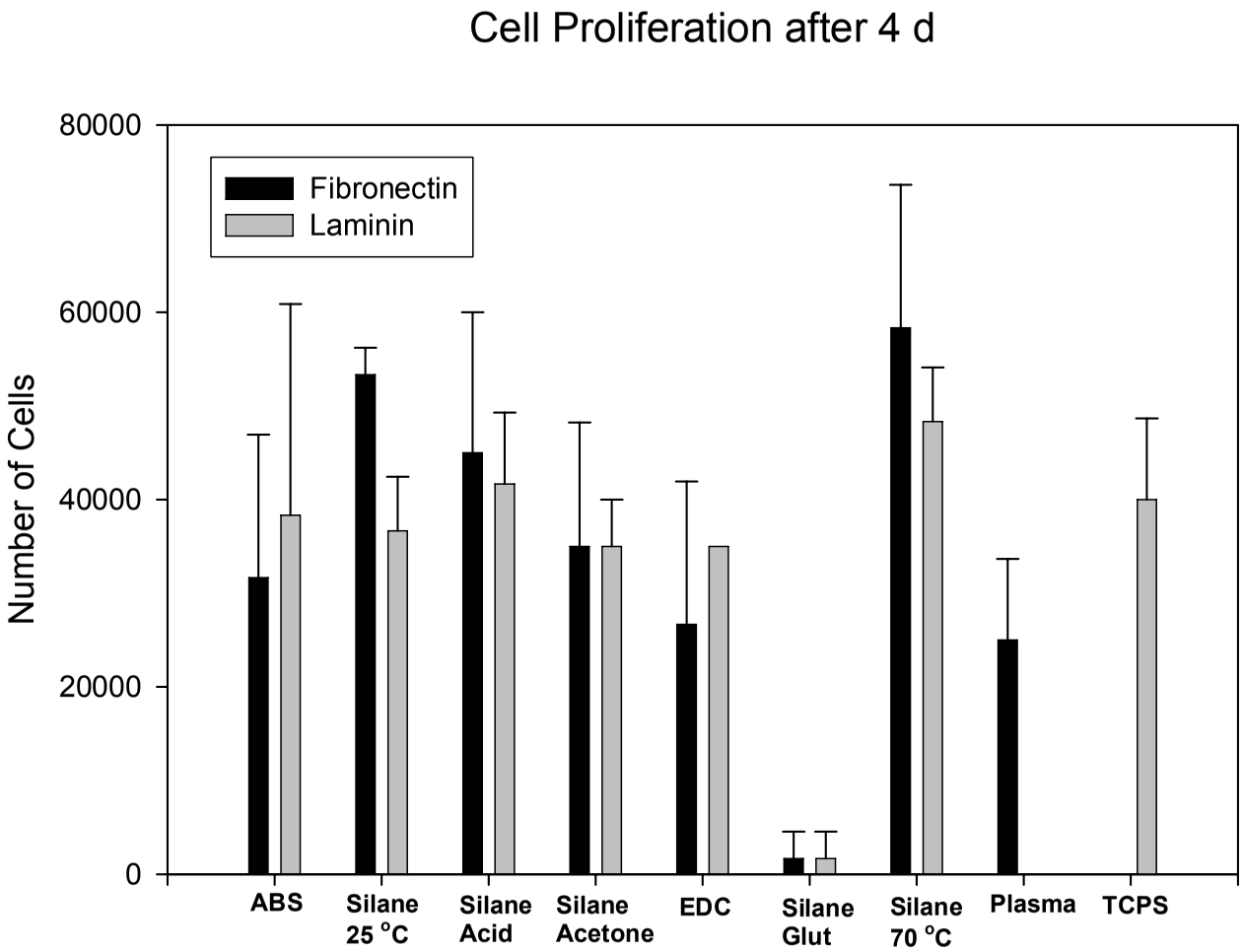

PDMS
Plasma treated PDMS
Intensity (arb. units)
1.0
0.8
0.6
0.4
0.2
0.0
4000
3500
3000
2500
2000
Wavenumbers (cm-1)

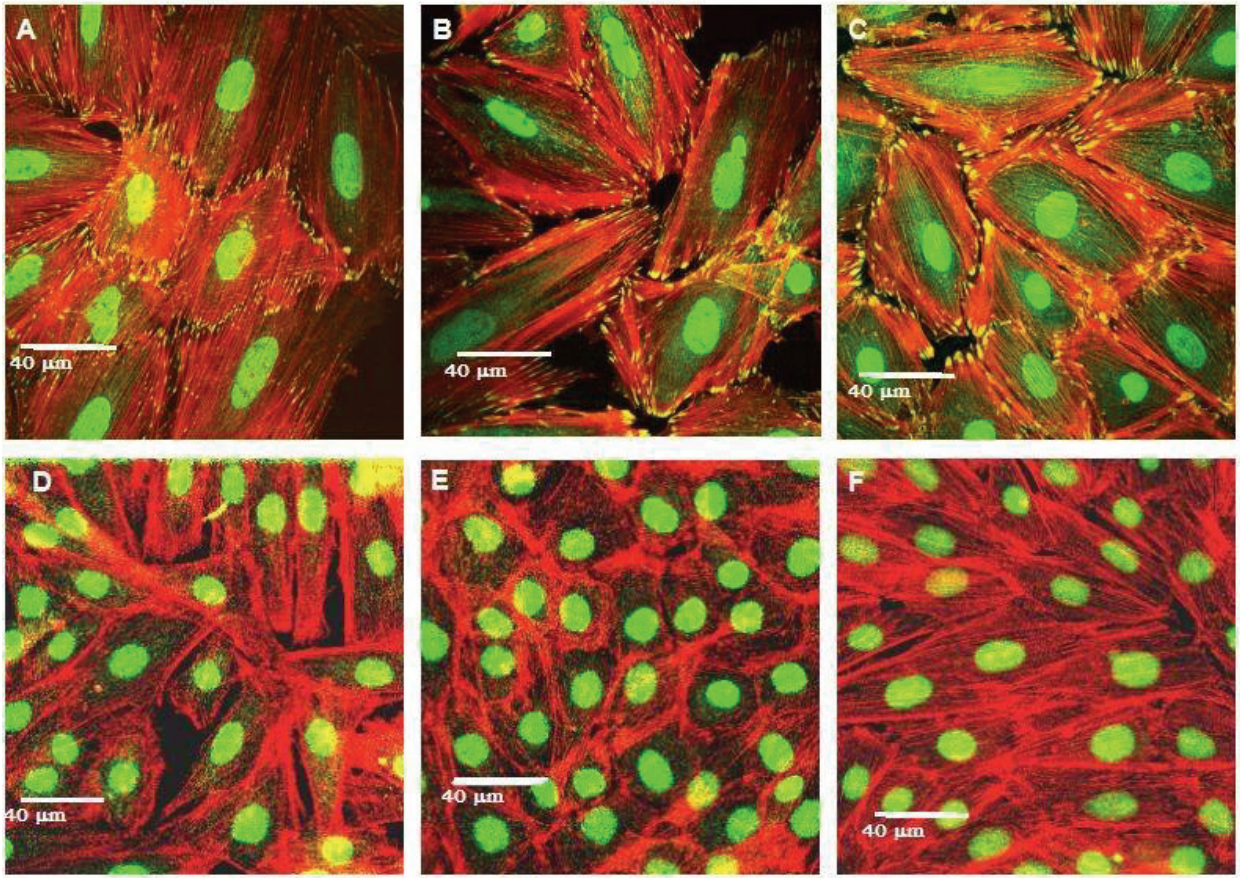

PDMS
Physically Adbsorbed FN
Silane and FN
EDC and FN
Intensity (arb. units)
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
1800
1700
1600
1500
1400
1300
1200
wavenumbers (cm-1)

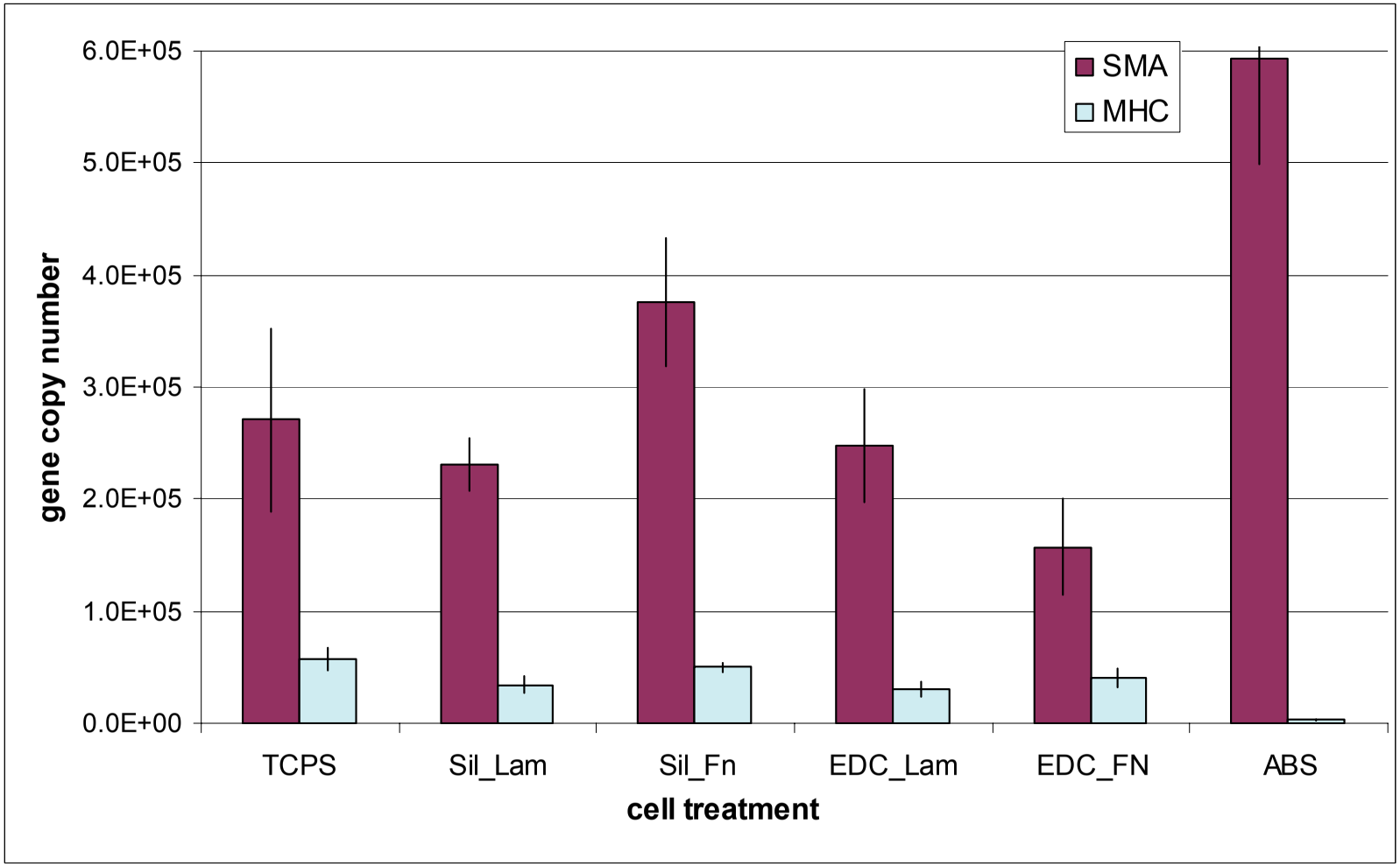

Fibronectin
Laminin
Fluorescence Intensity
70
60
50
40
30
20
10
0
ABS
Silane 25 °C
Silane Acid
Silane Acetone
EDC
Silane Glut
Silane 70 °C


A
100 µm
B
100 µm
C
100 µm
Intensity
0
50
100
150
200
250
0
100
200
300
400
500
600


Cell Attachment After 5 h
Fibronectin
Laminin
Percent Cells Attached
100
90
80
70
60
ABS
Silane 25 °C
Silane Acid
Silane Acetone
EDC
Silane Glut
Silane 70 °C
Plasma
TCPS
Cell Treatment


Cell Proliferation after 4 d
Number of Cells
80000
60000
40000
20000
0
Fibronectin
Laminin
ABS
Silane 25 °C
Silane Acid
Silane Acetone
EDC
Silane Glut
Silane 70 °C
Plasma
TCPS


A
40 µm
B
40 µm
C
40 µm
D
40 µm
E
40 µm
F
40 µm


6.0E+05
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
gene copy number
SMA
MHC
TCPS
Sil_Lam
Sil_Fn
EDC_Lam
EDC_FN
ABS
cell treatment