Differences in PDMS Modification Affect Laminin Deposition and Smooth Muscle Cell Response

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INTRODUCTION

When culturing cells on flexible surfaces, it is important to consider extracellular matrix treatments that will remain on the surface under mechanical strain. Here we investigate differences in laminin surfaces that were deposited on oxidized polydimethylsiloxane (PDMS) (plasma only) versus oxidized PDMS treated with aminopropyltrimethoxysilane (silane-linked). We use specular X-ray reflectivity (SXR), transmission electron microscopy (TEM), and immunofluorescence to probe the quantity and uniformity of the laminin on the treated PDMS surfaces. We also compare vascular smooth muscle cell (SMC) proliferation on laminin under equibiaxial strain between plasma only and silane-linked treatments.

EXPERIMENTAL

PDMS Oxidation via Plasma Treatment. PDMS surfaces were made with Sylgard 184 elastomer base and curing agent (Dow Corning, MI) [1]. All PDMS surface treatments were initiated with oxygen plasma activation for 30 s at 40 W to create a hydrophilic surface and prevent protein adsorption via hydrophobic interactions.

Surface Treatments.

A. Silane in Ethanol (Silane-linked). Silane has commonly been employed in the literature for the attachment of proteins to silicone surfaces [2,3]. Similar to these protocols, a solution of 1 % by volume aminopropyltromethoxysilane (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 silane solution to promote hydrolysis. The samples were reacted at 25 ^{o}C for 10 min and then 70 ºC for 10 min. They were then cooled to 25 ^{o}C, washed 1X with 70 % ethanol and 3X with distilled water. Substrates were incubated with mouse laminin (Invitrogen, CA) at 10 μg/mL in distilled water for 12 h. Surfaces were again washed with distilled and deionized water to remove loosely bound protein.

B. Physisorption of Proteins (Plasma only). Laminin was physically adsorbed onto plasma treated PDMS substrates via incubation at 10 μg/mL in distilled water for 12 h and washed as above.

Specular X-ray reflectivity (SXR). Samples for SXR were made and measurements performed according to previously published procedures [4].

Transmission Electron Microscopy (TEM). The substrate was coated with Pt/C by vacuum evaporation at an angle of $\approx 14°$. At this angle, surface features were shadowed 4X longer than their height. The sample was then coated with additional carbon at normal incidence. These evaporated films were then detached using a polyacrylic (PAA) acid technique. An aqueous solution of PAA (25 % mass fraction) was deposited onto the surface and allowed to dry overnight at 55 °C. Afterwards the dry glassy PAA was removed, detaching the thin films from the substrate. The resulting piece of PAA was placed on the surface of distilled water. After dissolution of the PAA, the detached films were retrieved onto copper grids and examined by TEM (EM400T, Phillips) at 120 kV bright-field using an objective aperture and slight underfocus.

Immunofluorescence. First, the laminin surfaces were blocked with 1 % by mass bovine serum albumin (Sigma-Aldrich, MO) in Dulbecco's 1X phosphate buffered saline (PBS) for 20 min. Surfaces were then labeled with primary antibody rabbit anti-laminin (1:1000, Sigma-Aldrich, MO) for 2 h at room temperature. After washing three times with PBS, substrates were incubated for 2 h with Alexa Fluor 488 goat anti-rabbit IgG (1:100, Invitrogen, Carlsbad, CA) as the secondary antibody. The fluorescence intensity from negative controls was negligible.

Cell Culture. Rat aortic smooth muscle cells (SMCs, A10) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in 5 % CO_2 at 37 ºC. SMCs were cultured in Dulbecco's Modified Eagle Media (DMEM) with high glucose (Invitrogen, CA) supplemented with 10 % fetal bovine serum (FBS, Invitrogen, CA), 2 mmol/L l-glutamine, 1 mmol/L nonessential amino acids, and 50 μg/mL each of penicillin and streptomycin, according to published protocols [5]. Cell proliferation was measured using hemocytometry on 18 samples. This data is presented along with the standard uncertainty (±) involved in the measurement.

Equibiaxial Strain Experiments. Forty-five thousand SMCs were seeded onto the laminin modified Bioflex cell culture wells and incubated for 12 h prior to straining. The wells were strained for 1 h at 5 % peak equibiaxial strain and 0.5 Hz followed by static culture for 23 h. This cycle was done twice, followed by continuous strain for 48 h. This cycle was repeated for 4 d, at which time cells were detached and counted.

RESULTS AND DISCUSSION

Laminin free control surfaces of as cured PDMS (no plasma), plasma treated PDMS, and plasma treated PDMS with silane were imaged using TEM. These control surfaces were generally smooth and featureless (results not shown). TEM micrographs of plasma only laminin treated surfaces are qualitatively different than the control surfaces. Figure 1 displays TEM micrographs at two different magnifications (A & B), where the laminin is manifested as irregularly shaped, light islands. (The arrows show the direction of shadowing.) The islands range in height from about 200 nm to around 10 nm. The average thickness of laminin patches is around (10 to 30) nm, although there is an underlying roughness that cannot be measured in these images. For comparison, confocal fluorescence microscope images of laminin immunofluorescence are displayed (C and D). The fluorescence signal is manifested by lighter gray areas while black indicates regions of no signal. The confocal images corroborate the TEM images that indicate the laminin deposited is in patchy domains on the surface.

Figure 2 displays the TEM (A and B) and fluorescence (C and D) images of laminin on the silane-linked treatment. Here several larger islands of laminin (Figure 2A.) are seen, but the laminin heterogeneity exists on a scale much smaller than in the previous figure. Even at a higher magnification, the laminin surface appears to be much smoother (Figure 2B). The image in Figure 2C reveals more uniform layer of laminin, as seen by the constant gray background of the image. This is also seen in fluorescence images of a larger area (Figures 2D). The characterization results demonstrate that, the silane-linked laminin is spread more uniformly, down to the scale of 100 nm.

The TEM and confocal fluorescence results together reveal differences in how the laminin is deposited onto the PDMS surface. Laminin has three short arms and one long arm. Two short arms are about 34 nm in length and the third is about 48 nm [6]. The long arm is about 85 nm in length and consists of three chains oriented in a triple coiled-coil structure. Globular domains within the molecule dictate its thickness, and domain at the end of the triple coiled-coil long arm is 3 nm thick [7]. Therefore, the SXR results, with laminin thicknesses from (2.0 to 4.4) nm, suggest that the laminin is laying flat on the surface, given that this technique probes uniform films. The plasma only and silane-linked laminin surfaces have around 45 % and 52 % coverage, respectively. The amount of coverage makes sense given that the four-arm protein has been shown to associate into oligomers and higher order polymers that are unable to densely pack [8]. It has been shown that laminin will self-associate through the short arm amine moieties on the arm ends. Polymerized laminin has a 35 nm strut length [9]. Although here we cannot determine precisely whether the laminin is self-associated on the PDMS surface, we can

hypothesize that there is a strong driving force for the laminin to associate with the amine group on the silane. This discourages strong self-association and globule formation. The plasma only surface consisting primarily of hydroxyl groups does not promote as much laminin surface association as does the amine functionality. Consequently, there are more globules of self-associated laminin on the surface. The agglomerated laminin seen here is similar to that visualized by atomic force microscopy on hydrophobic surfaces [10].

We hypothesized that the silane treated surfaces would be able to better retain laminin and SMCs cells than the plasma treated surfaces under mechanical deformation due to the strong polar interaction of the laminin with the silane primary amine. Laminin robustness results demonstrate that, after 4 d of continuous equibiaxial stretching at 5 % and 0.5 Hz, the plasma only treatment has a greater total loss of laminin than for the silane-linked treatment (results not shown). To test the effect of the surface treatments on SMC proliferation under strain, cells were seeded and attached for 12 h. There were no differences in the number of cells attached after 5 h. The PDMS substrates were strained at 0.5 Hz, 5 % strain for 1 hr followed by 23 h static culture for 2 d, then 2 d of continuous strain. Cell proliferation on laminin for plasma only and silane-linked substrates shows statistically more SMCs on the silane-linked surface (92000 $\pm$ 19000) than on the plasma only surface (48000 $\pm$ 25000).

CONCLUSIONS

This study has shown that coating the laminin onto a surface with a tethered silane provides a more uniform layer and improved retention under mechanical strain than standard physical adsorption. It is hypothesized this improved laminin retention results in higher cell proliferation.

REFERENCES

1. Certain commercial materials, equipment, and software are identified in this paper in order to specify adequately the experimental and analysis procedures. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology (NIST) nor does it imply that they are necessarily the best available for the purpose. Official contribution of the National Institute of Standards and Technology; not subject to copyright in the United States.).
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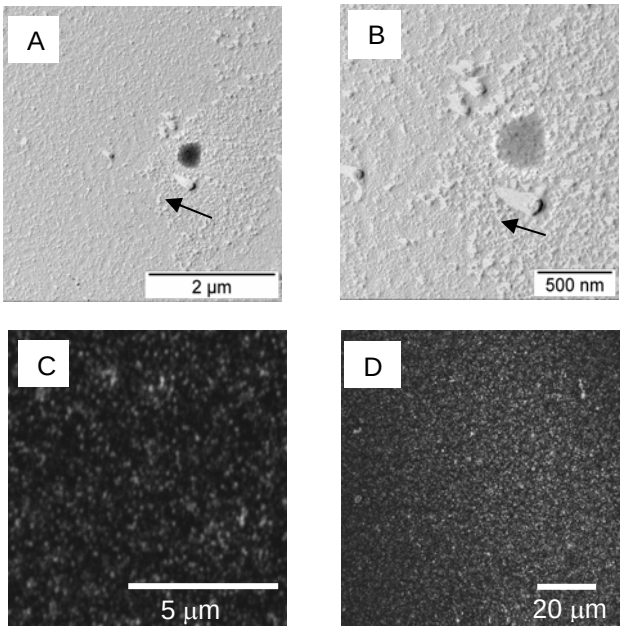

Figure 1. TEM Images of plasma only PDMS with laminin with arrow showing the direction of shadowing (A). TEM with features marked for height measurement with features 2 and 3 magnified in insets (B). High (C) and low (D) magnification immunofluorescence images. All images show heterogeneous nature of laminin deposition.

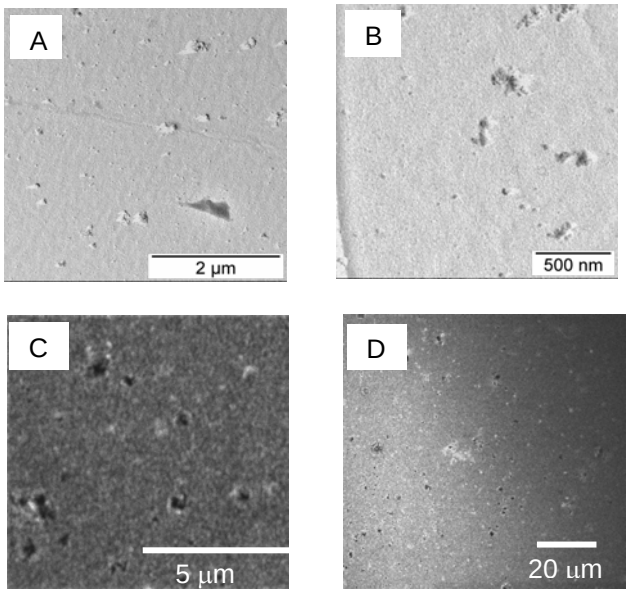

Figure 2. TEM Images of silane-linked laminin on PDMS, low (A) and high (B.) magnification. High (C) and low (D) magnification immunofluorescence images. All images show mostly uniform laminin deposition with intermittent defects such as holes or islands.