Characterization of Laminin on Silanized Polydimethylsiloxane

Joy P. Dunkers, Lisa M. Pakstis, Hae-Jeong Lee, Marvi A. Matos, and Marcus T. Cicerone

Polymers Division, National Institute of Standards and Technology, Gaithersburg, MD 20899

INTRODUCTION

There are many *in vivo* processes that rely on mechanical stimulation to elicit the appropriate tissue response. For example, bone responds to bearing weight by mineralizing (increasing strength) and demineralizes (decreases strength) when not loaded. Cartilage, muscle, tendons and ligaments have analogous responses when studied under load/no load conditions. Therefore, there is considerable interest in the translation of mechanical stimuli to cellular response.

Integrins are a group of transmembrane glycoproteins that provide a linkage between extracellular matrix (ECM) proteins and the cell and are one potential path by which mechanical signals are transmitted. It has been shown that the interaction between ECM proteins and integrins influences cell response [1]. Therefore, the characterization of these protein, especially under load, is of great interest. Previous work has shown that physically adsorbed proteins quickly desorb [2]. The focus here is on creating a robust ECM surface treatment that will withstand mechanical loading.

In this work, we characterize a protein surface on a flexible polydimethylsiloxane substrate (PDMS) capable of undergoing strain. We compare the results from two different surface treatments: physically adsorbed laminin on PDMS and laminin deposited on silane treated PDMS. Immunofluorescence and x-ray reflectivity were used to measure the amount of laminin from each surface treatment. Vascular smooth muscle cell attachment and proliferation were compared between the methods. Additional work will concentrate on evaluating the robustness of surface treatments under strain.

EXPERIMENTAL

PDMS Oxidation via Plasma Treatment (Plasma). PDMS surfaces were made with Sylgard 184 elastomer base and curing agent (Dow Corning, MI). 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 Treatment: Silane in Ethanol. Silane has commonly been employed in the literature for the attachment of proteins to silicone surfaces [3,4]. Similar to these protocols, a solution of 1 % by volume aminopropyltromethoxysilane (APTMS) (Sigma-Aldrich, MO) [5] 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 room temperature. The samples were then incubated at 70 ºC for 10 min (Silane_70C), cooled to room temperature, and washed 1X with 70 % ethanol and 3X with distilled water. Substrates were then incubated with mouse laminin (Invitrogen, CA) at 10 μg/mL overnight. Surfaces were again washed with distilled and deionized water to remove loosely bound protein.

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 h.

Specular X-ray Reflectivity. Three types of samples were made: laminin on PDMS, laminin on plasma-treated PDMS, and plasma treated PDMS with APTMS and laminin. For all samples, a 5 vol % solution of Sylgard 184 elastomer in heptane was spun onto a silicon wafer. The wafer was heat treated at 70 ^{o}C for 45 min in air. For two methods, the wafers were subjected to oxygen plasma treatment and then treated with laminin as previously explained. All samples were then washed with 100 % ethanol and dried with nitrogen gas. The wafers were incubated overnight in 10 μg/ml laminin (Sigma, St. Louis, MO). The surfaces were then washed with water to remove unbound protein, dried with nitrogen, and stored for 24 h at ambient conditions before analysis.

The SXR measurements were accomplished using a modified high-resolution X-ray diffractometer in a θ–2θ configuration at the specular conditions with the incident angle equal to the detector angle. A finely focused Cu K_α X-ray source with a wavelength, λ, of 1.54 Å was conditioned with a four-bounce Ge (220) monochromator and focused onto the film of interest. A three-bounce Ge (220) channel cut crystal was also used to direct the reflected X-rays into the detector. The sample and detector goniometer have an active servo feedback system to provide an angular reproducibility of ± 0.0001º [6]. Typically, the specular reflected intensity was collected as a function of incident angle between 0.1º to 1.0º. The detailed SXR measurements and data analysis were performed in a manner reported elsewhere [7]. The sampling area ranges from 100 mm^2 to 140 mm^2.

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 [8].

RESULTS AND DISCUSSION

We evaluate the two modification methods based on completeness and uniformity of coverage of attached laminin. Smooth muscle cell adhesion and proliferation were also evaluated on these surfaces.

As seen in Figure 1, results from three immunochemistry relative fluorescence intensity experiments and pooled variance t-tests have shown qualitatively that the amount of laminin deposited using APTMS on plasma treated PDMS (Silane Treated) is significantly higher than for laminin only on plasma-treated PDMS (Adsorbed). This is presumably due to the polar or hydrogen bonding interactions the silane has with the laminin compared to the more hydrophobic plasma treated PDMS. Analogous work using fibronectin shows the same trends.

We used X-ray reflectivity to provide quantitative results for protein thickness and coverage. For the untreated PDMS, there is an ~50 Å layer of material that is about 70 % less dense than the bulk. The plasma treatment induces a densified layer ~100 Å thick and results in a decrease of film thickness of about 200 Å. Approximately 10 Å of the silanized layer appears after treatment with APTMS. By comparing the experimental protein layer density with the known protein density, one can deduce the coverage of protein. For example, if the protein layer shows 50 % density compared to the known density of the protein, this indicates a protein coverage of 50 % on the substrate. We calculate the density of laminin (850 kDa) to be 1.42 g/cm^3 [9]. If a lower limit density of 1.3 g/cm^3 is assumed, then all surface coverages are 3 % higher.

The detailed structural characteristics determined from SXR are summarized in Table I. All the protein layers have the same thickness, within experimental error. This thickness is an average thickness over the entire sampling area. Protein molecules are adsorbed most effectively on the functionalized PDMS with APTMS, following the trend from plate reader results. The PDMS layer with plasma treatment results in the second-best adsorption, while PDMS with no treatment displays the least coverage of laminin.

For the cell adhesion data, there is no difference in the number of smooth muscle cells adhered after 5 h of attachment. But after 4 d, there are significantly more cells adhered to the silane treated surface than to the plasma only surface.

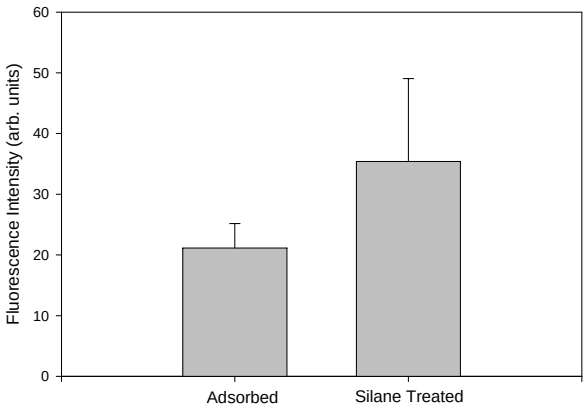

Figure 1. Immunochemistry data analyzed using pooled variance t-test show that the amount of laminin on the APTMS and plasma treated PDMS surface (SIlane treated) is significantly more than on the plasma only PDMS surface (adsorbed).

Table 1. The detailed structural characteristics determined from fits to the SXR experimental data. The relative standard uncertainties of the scattering length density (SLD), film thickness, and density are ± 0.01 × 10^{-3} Å^{-2}, ± 10 Å, and ± 0.01 g/cm^3 and respectively.

Substrate	SLD/10^{-3} (Å^{-2})	Thickness (Å)	Density (g/cm^3)	Surface coverage (%)
PDMS	0.218	30	0.479	**34**
PDMS/plasma	0.281	20	0.617	**44**
PDMS/plasma/APTMS	0.329	22	0.723	**51**

CONCLUSIONS

We have shown that treating oxidized surfaces with APTMS at elevated temperatures increases the amount of ECM protein adsorbed. The surface coverage of laminin varies from (35 to 50) %, depending upon treatment method. The number of cells attached to the substrates after 5 h was statistically identical. However, cell proliferated more on the laminin surfaces attached using APTMS than with plasma treatment only. Current work focuses on evaluating the robustness of these surface treatments under various loading conditions.

REFERENCES

1. Simmons, C. A.; Mooney, D. J. In *Functional Tissue Engineering,* Guilak, F.; Butler, D.; Goldstein, S; Mooney, D.; Eds., Springer-Verlag: New York, NY, 2004; pp 292.
2. Cunningham, J. J.; Nikolovski, J.; Linderman, J. J.; Mooney, D. J. *Biotechniques* **2002**, *32* (4), 876-+.
3. Ochsenhirt, S. E.; Kokkoli, E.; McCarthy, J. B.; Tirrell, M. *Biomaterials* **2006,** *27* (20), 3863-3874.
4. Altankov, G.; Grinnell, F.; Groth, T. *JBMR* **1996,** *30* (3), 385-391.
5. 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.
6. The data throughout the manuscript and in the figures are presented along with the standard uncertainty (±) involved in the measurement.
7. Wu, W. L.; Wallace, W. E.; Lin, E. K.; Lynn, G. W.; Glinka, C. J.; Ryan, E. T.; Ho, H. M. *J. Appl. Phys.*, **2000**, 87, 1193-1200.
8. Pauly, R. R.; Bilato, C.; Cheng, L.; Monticone, R.; Crow, M. T. Vascular Smooth Muscle Cell Cultures. In *Methods in Cell Biology*, Academic Press: 1998; pp 133-154.
9. Fischer, H.; Polilarpov, I.; Craievich, A. F. *Protein Science,* **2004**, 13, 2825-2828. Equation 2.