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Surface Chemical Modification of Poly(dimethylsiloxane) for the Enhanced Adhesion and Proliferation of Mesenchymal Stem Cells

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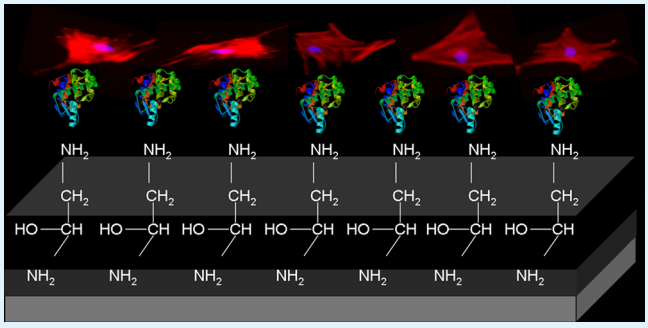
Supporting Information

ABSTRACT: The surface chemistry of materials has an interactive influence on cell behavior. The optimal adhesion of mammalian cells is critical in determining the cell viability and proliferation on substrate surfaces. Because of the inherent high hydrophobicity of a poly(dimethylsiloxane) (PDMS) surface, cell culture on these surfaces is unfavorable, causing cells to eventually dislodge from the surface. Although physically adsorbed matrix proteins can promote initial cell adhesion, this effect is usually short-lived. Here, (3-aminopropyl)triethoxy silane (APTES) and cross-linker glutaraldehyde (GA) chemistry was employed to immobilize either fibronectin (FN) or collagen type 1 (C1) on PDMS. The efficiency of these surfaces to support the adhesion and viability of mesenchymal stem cells (MSCs) was analyzed. The hydrophobicity of the native PDMS decreased significantly with the mentioned surface functionalization. The adhesion of MSCs was mostly favorable on chemically modified PDMS surfaces with APTES + GA + protein. Additionally, the spreading area of MSCs was significantly higher on APTES + GA + C1 surfaces than on other unmodified/modified PDMS surfaces with C1 adsorption. However, there were no significant differences in the MSC spreading area on the unmodified/modified PDMS surfaces with FN adsorption. Furthermore, there was a significant increase in cell proliferation on the PDMS surface with APTES + GA + protein functionalization as compared to the PDMS surface with protein adsorption only. Therefore, the covalent surface chemical modification of PDMS with APTES + GA + protein could offer a more biocompatible platform for the enhanced adhesion and proliferation of MSCs. Similar strategies can be applied for other substrates and cell lines by appropriate combinations of self-assembly monolayers (SAMs) and extracellular matrix proteins.



KEYWORDS: *(3-aminopropyl)triethoxysilane (APTES), glutaraldehyde, mesenchymal stem cells, poly(dimethylsiloxane) (PDMS), surface functionalization*

1. INTRODUCTION

For the past decade, poly(dimethylsiloxane) (PDMS) has received much attention as a biomaterial for the fabrication of biochips for multiple biological applications including the investigation of cellular communications,[1,2] mechanobiology[3−6] and development,[5−7] and cell isolation.[8] PDMS has been shown to possess numerous properties that offer advantages over many other available biomaterials such as silicon, bioglass, and other polymers. For instance, an available master allows PDMS to be molded easily into submicrometer designs as defined by the master.[1−8] Furthermore, the elastomeric properties of PDMS can be easily tuned by the base/curing agents ratio to cover a wide range of physiologically relevant elastic modulus[9] for mechanobiological studies as compared to other materials used for similar purposes, such as polyacrylamide gels,[10,11] poly(ethylene glycol),[12] and hyaluronan.[13] The surface roughness of the PDMS can also be easily adjusted with different curing temperatures.[14] These inherent properties of PDMS allow the study of physical effects on cell behavior, especially in the field of stem-cell differentiation. Adding to these advantages are their optical transparency, gas permeability, nontoxicity, and cost effectiveness, which could make PDMS a preferred material for cell-based platforms used in biomedical devices and fundamental studies.

However, the surface compatibility of PDMS for cell culture is often discouraging.[15−18] One of the major drawbacks lies in the high surface hydrophobicity of PDMS, which limits the affinity of mammalian cells to effectively adhere to the native

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PDMS surface.[15−18] Because surface wettability is one of the crucial factors that influences cell adhesion on the substrate,[19,20] previous research has focused extensively on the modification of the PDMS surface to reduce its hydrophobicity.[21−23] Although the surface hydrophobicity of PDMS can be reduced by surface treatment with plasma,[21] hydrophobic recovery was observed after plasma treatment.[22,23] Another approach to overcome this drawback is protein adsorption to the PDMS substrate, which has been one of the widely used techniques to facilitate cell adhesion because of the intrinsic biocompatibility with proteins and molecular recognition properties.[1,4−7] However, the maintenance of cell adhesion on these surfaces was transitory whereby cells were either detached after reaching confluence or aggregated to form loosely bound cell clumps.[15]

The adhesion and aggregation of proteins on the material surface during protein adsorption relies on the interaction between the charged domains on the protein and the material surface.[24] These interaction forces (e.g., electrostatic, van der Waals, and hydrophobic) are usually weak[25] and thus highly susceptible to protein leaching into the medium, reducing the biocompatibility of the protein-coated PDMS for cell culture. Efforts have been made to induce strong and stable covalent linkages of the protein to the material surfaces.[26−28] Among the covalent-binding strategies, material surfaces chemically modified with amino-silane (e.g., (3-aminopropyl)triethoxy silane (APTES)) and homobifunctional aldehyde (e.g., glutaraldehyde (GA)) chemistry have shown efficiency in immobilizing proteins and antibodies.[26−28] APTES and GA activation function as molecular spacers to minimize the direct and weak interactions of proteins with the PDMS surface and to overcome the steric hindrance from the vicinity of the support, which is essential for stronger protein attachment.[29] Such chemistry in achieving the stable covalent attachment of several proteins has been reported in applications such as immunoassays. However, the use of this chemically modified PDMS surface to stabilize cell adhesion and support cell proliferation has yet to be well investigated. Therefore, it is of interest to extend its use to study the interaction of stable extracellular matrix (ECM) protein-activated surfaces with adherent cells. In this regard, the efficiency of these APTES ± GA-modified PDMS surfaces in immobilizing common ECM proteins such as collagen type 1 (C1) or fibronectin (FN) as well as the biocompatibility of these chemically modified surfaces for the adhesion and proliferation of mammalian cells remains unknown. Consequently, we foresee a need to investigate the applicability of PDMS with a well-defined surface chemistry for cell culture applications, and we hypothesize that stable attachment of ECM proteins through covalent bonding can facilitate stronger cell adhesion and subsequently influence cell behavior.

Mesenchymal stem cells (MSC) are a promising cell source for regenerative medicine because of their multipotency and ready availability from mesenchymal origins. Specifically, in this study, for the first time to the best of our knowledge we investigated the effect of the modified PDMS surfaces by APTES ± GA cross-linking chemistry with the attachment of either FN or C1 on MSC culture. APTES was first bound onto the plasma-activated PDMS surface followed by activation with GA. ECM proteins (C1 or FN) were bound to the substrate surface modified either through APTES or APTES + GA routes (Figure 1). Surface characterization of the chemically modified PDMS surface was performed, and its biocompatibility with the mesenchymal stem cells (MSCs) was then assessed with

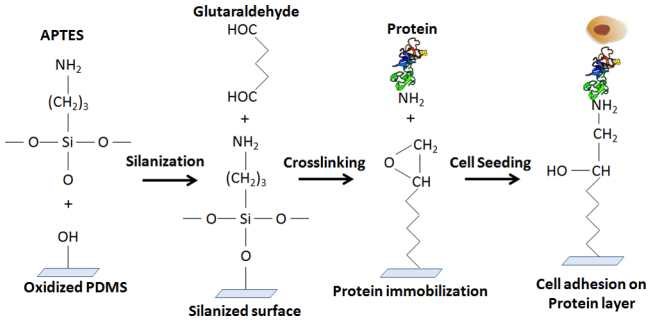

Figure 1. Schematic diagram of PDMS surface modification.

appropriate analytical techniques. We demonstrated that this approach to the chemical modification of PDMS surfaces offers an improved biocompatible platform that enhanced cell adhesion and proliferation, which are critical for the later stages of physiological cell development.

2. EXPERIMENTAL SECTION

Surface Modification of PDMS. PDMS substrates were prepared by mixing 10 parts of silicone elastomer base to 1 part of curing agent (SYLGARD, Dow Corning, USA) followed by casting onto a flat polystyrene dish or well plate. The PDMS was then degassed in a vacuum oven for 30 min to remove all of the air bubbles and cured at 70 °C for 90 min. The cured PDMS substrates were split into three groups, and their surfaces were treated as follows. Two groups of the PDMS substrates (APTES + protein and APTES + GA + protein) were subjected to oxygen plasma for 3 min in the plasma cleaner (Harrick Plasma-PDC 32G) followed by immersing the PDMS substrates in 10% APTES (Sigma-Aldrich, Singapore) at 50 °C for 2 h. The APTES solution was removed, and the samples were washed twice in nuclease-free water. One of the groups (APTES + GA) was further immersed in a 2.5% GA (Sigma-Aldrich, Singapore) solution at room temperature for 1 h. GA was then removed, and the samples were washed twice in nuclease-free water. All three groups (protein, APTES + protein, and APTES + GA + protein) were then immersed in either 0.1 mg/mL of a fibronectin (FN) (Life Technologies, Singapore) or collagen type 1 (Col1) solution (Life Technologies, Singapore) and stored at 4 °C overnight. Finally, the protein solution was removed, and the samples were washed twice with nuclease-free water. The PDMS substrates were sterilized under UV light for 60 min prior to cell culture experiments. Tissue culture plates (TCP) (Nunc, Singapore) were used as a control.

Analysis of Elemental Composition. X-ray photoelectron spectroscopy (XPS) was performed to analyze the elemental composition of the samples. XPS spectra were taken in normal emission at 9 to 10 mbar within 10 min. All C 1s peaks corresponding to hydrocarbons were calibrated to a binding energy of 284.8 eV to correct for the energy shift caused by charging. The spectra were analyzed using the Casa XPS software. A 50 eV pass energy using empirical Wagner sensitivity factors was set to obtain the required region scans on the surface.

Characterization of Surface's Hydrophilic Properties. Water contact-angle measurements were determined with a theta optical tensiometer (Attension, Finland, Europe). Briefly, 5 μL droplets of Milli-Q water (resistivity >10 MΩ) was brought into contact with the surface of the PDMS substrates, and the contact angles were measured with the static sessile drop tangent method in the Drop Shape Analysis software, where the angle formed between the substrate surface and the tangent to the drop surface was determined. At least three points of contact were measured for each sample.

Surface Protein Quantification. A micro-BCA Protein Assay Kit (Thermo Scientific, Singapore) was used to determine the amount of protein that was retained on the different PDMS substrates. Following the overnight incubation in the 0.1 mg/mL protein solution, the protein solution was discarded. The PDMS surfaces were then

incubated with 0.05% Tween 20 (Sigma-Aldrich, Singapore) for 30 min on a shaker followed by washing twice with nuclease-free water to remove loosely bound or nonadherent protein molecules. The adhered protein on the PDMS surfaces was then determined according to the stated protocol in the Micro-BCA Protein Assay Kit, where the absorbance of specimens were measured at 562 nm with Multiskan Spectrum microplate reader (Thermo Scientific, Singapore). The results were then expressed as a percentage ratio of the surface-bound protein concentration to the initial protein seeding concentration to reflect the percentage of protein retention on the PDMS surfaces.

Mesenchymal Stem Cell Culture. Mesenchymal stem cells (MSCs) were generated from porcine bone marrow aspirates under institutional guidelines and routinely cultured. Briefly, bone marrow was aspirated from the porcine iliac crest under aseptic conditions and washed with 1× PBS (First Base, Singapore). The bone marrow stromal cells were then resuspended in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Singapore) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Singapore), a penicillin (100 U/ml)/streptomycin(100 μg/mL) mixture (Life Technologies, Singapore), and 2 mM Glutamax (Life Technologies, Singapore) before seeding into a culture flask for culture in a humidified atmosphere of 95% air and 5% CO_2. Nonadherent cells were removed by washing after 72 h, and the adhered mesenchymal stem cells were further expanded upon reaching confluence. This protocol was established to obtain MSCs that demonstrated good purity and stability while maintaining the original cellular morphology and differentiation capability in their earlier passages.[30−32] MSCs of passages 2−4 were used in the experiments for this study.

Cell Proliferation Assay. PrestoBlue cell viability reagent (Life Technologies, Singapore) was used to assay the proliferation activity of MSCs on different modified PDMS surfaces for 2 weeks. Briefly, MSCs were seeded at a density of 1.5×10^3 cells per well (1.9 cm^2) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed twice per week. On third, seventh, and 14th days, the culture medium was removed, and the cells were washed twice with 1× PBS before they were incubated with DMEM containing 10% PrestoBlue reagent for 1 h at 37 °C in a humidified, 5% CO_2 atmosphere. DMEM containing 10% PrestoBlue reagent was incubated in the wells with no cells and served as the blank control. The absorbance of the reduced PrestoBlue reagent was read at 570 (excitation) and 600 nm (emission) with a Multiskan Spectrum microplate reader (Thermo Scientific, Singapore). Because the number of viable cells correlated with the reduction level of the PrestoBlue dye, the absorbance readings were converted and expressed as the percentage reduction of the PrestoBlue reagent according to the manufacturer's protocol.

Characterization of Cell Adhesion. For the analysis of cell-adhesion capability on different modified PDMS surfaces, MSCs were seeded at a density of 1.0×10^4 cells per well (1.9 cm^2) and incubated in a humidified atmosphere of 95% air and 5% CO_2 for 90 min. The wells were then rinsed twice with 1× PBS to remove the nonadherent cells, and the adherent cells were frozen at −80 °C. The frozen cells were then thawed and lysed by the addition of a cell lysis buffer containing 1× CyQUANT GR Dye from the CyQUANT Cell Proliferation Assay Kit (Life Technologies, Singapore) for 5 min. Fluorescence was then measured directly with an Infinite M200 series plate reader (Tecan Asia, Singapore) with excitation at 485 nm and emission detection at 535 nm. The readings were then expressed as a fold difference relative to the fluorescence intensity of the TCP (control). Because the DNA content is constant within the cell, this method uses the DNA content (reflected by fluorescence intensity) that was obtained by cell lysis to correlate to the number of cells that remained adhered on the surface. This method has proved to be a sensitive, accurate, and simple deterministic means for correlating DNA content to cell density[33,34] and thus provides an accurate quantification of the cell adhesion under conditions of different surface chemistries.

Cell adhesion area was determined as follows. MSCs were seeded onto 6-well plates at a seeding density of 1.0×10^4 per well (9.6 cm^2) and incubated in a humidified atmosphere of 95% air and 5% CO_2 for 4 h. The adhered cells were then fixed with 10% formalin (Sigma-Aldrich, Singapore) overnight, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Singapore), blocked with 1% BSA (Sigma-Aldrich, Singapore), and incubated with TRITC-conjugated Phalloidin (Millipore, Singapore) for 60 min and DAPI (Millipore, Singapore) for 5 min. The images were viewed under an IX71 inverted fluorescence microscope (Olympus, Singapore), and the area of individual cell adherence was analyzed by Image-Pro Plus (Media Cybernetics, Rockville, MD).

Statistical Analysis. The paired Student's t test was adopted for determining the statistical significance between groups using Minitab 16 Statistical Software (Minitab Inc., State College, PA). All experiments were performed in quadruplicate. A p value of <0.05 was considered to be statistically significant.

3. RESULTS

X-ray Photoelectron Spectroscopy (XPS). Surface elemental composition was obtained from XPS characterization. From Figure 2, it can be seen that the relative loading of

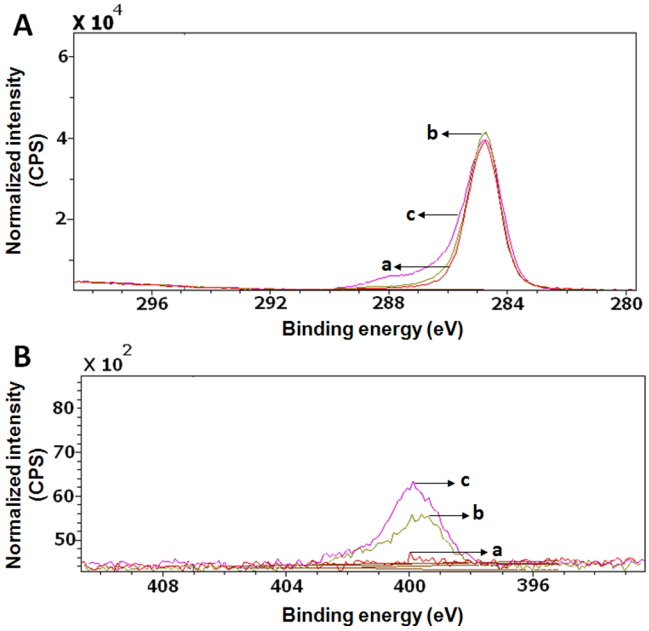

Figure 2. XPS analysis of elemental composition. (A) C 1s (carbon) and (B) N 1s (nitrogen) region XPS scans on (a) native PDMS, (b) PDMS + APTES, and (c) PDMS + APTES + GA samples.

carbon (C 1s) increased slightly in APTES and subsequently in APTES + GA-modified samples as compared to the plain PDMS. Similarly, a higher loading of nitrogen (N 1s) was seen in both the APTES and APTES + GA-modified samples because of the presence of the amine group (−NH2) of the organosilane.

Surface Wettability. The surface wettability of the native PDMS, tissue culture plate (TCP), and the chemically modified and unmodified PDMS with protein coating was evaluated by the measurement of the water droplet contact angle on the substrate (Figure 3). The wettability of the native PDMS was within the hydrophobic region (114.41 ± 3.14°). Although protein adsorption on the unmodified PDMS surface resulted in a slight decrease of the contact angle, the surface wettability still remained in the hydrophobic regions (~ 100°). However, chemical modification of the PDMS surface by APTES and/or GA with protein adsorption could further reduce the contact angle to a hydrophilic region (<70°), for which the collagen

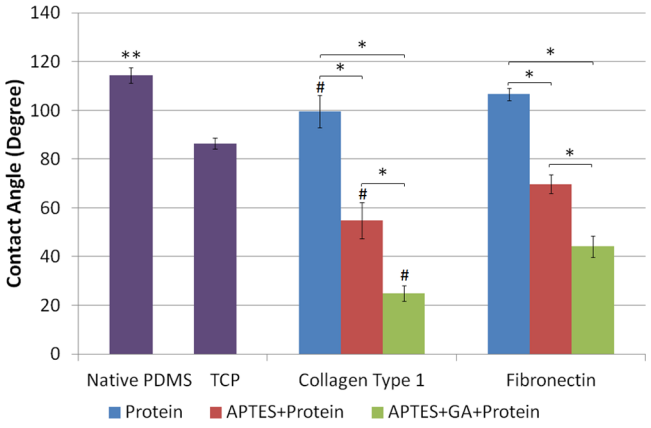

Figure 3. Average contact angle on TCP and different PDMS surfaces. The data are shown as the mean ± SD. *p value of <0.05 between two groups. **p value of <0.05 as compared to the rest of the groups. #p value of <0.05, as compared to fibronectin coated on the similar PDMS-modified surfaces.

type 1 adsorption showed significantly greater effect than the fibronectin adsorption.

Protein Adsorption. A microBCA assay was used to evaluate the extent of protein adsorption on the modified/unmodified PDMS surfaces (Figure 4). The assay revealed that

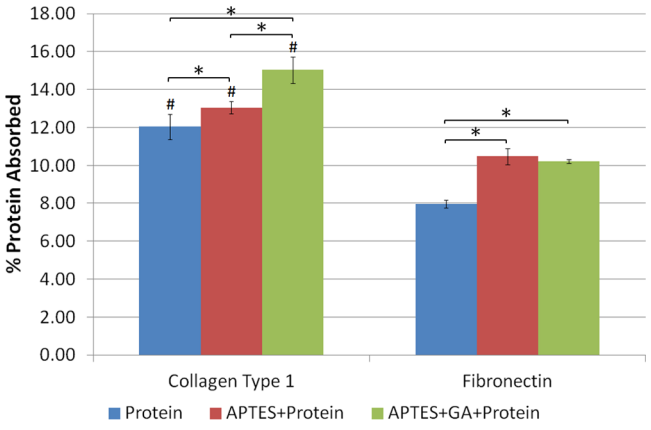

Figure 4. Amount of protein (collagen type 1 or fibronectin protein) retained on the modified/unmodified PDMS surfaces. The data are shown as the mean ± SD. *p value of <0.05 between two groups. #p value of <0.05, as compared to fibronectin coated on the similar PDMS-modified surfaces.

the chemical modifications indeed enhanced the adsorption for both proteins. However, the attachment of the C1 protein was significantly higher as compared to FN attachment on similar PDMS surfaces. The adsorption of FN, however, was enhanced in the APTES + protein group. The data also revealed a significant difference in the attachment of either protein on similar PDMS surfaces.

Cell Adhesion. To assess the influence of the chemically modified/unmodified PDMS surfaces on the adhesion of MSCs, the amount of adherent cells were determined by a CyQUANT Cell Proliferation Assay Kit after 90 min of the initial cell seeding. The adherent cell density was then expressed as a fold difference relative to that of the TCP group. Cell adhesion on PDMS modified with APTES + GA + protein was significantly higher than the rest of the groups (Figure 5). Furthermore, FN adsorption on any PDMS surface was shown to provide better cell adhesion as compared to C1 adsorption on similar PDMS surfaces (Figure 5).

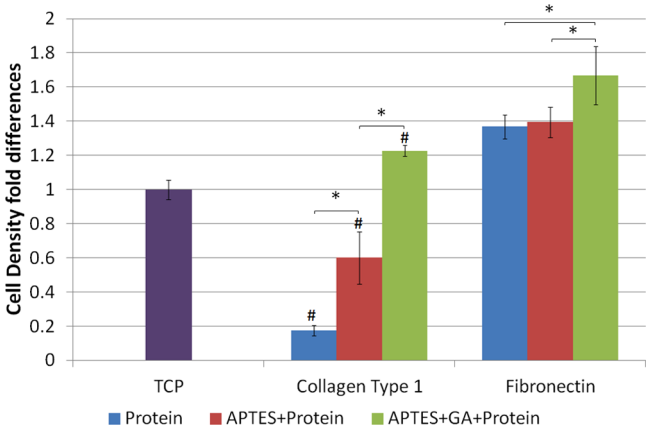

Figure 5. Adherent cell density after 90 min of initial MSC seeding on modified/unmodified PDMS surfaces. The cell density is presented as the fold difference relative to the cell density on TCP. The data are shown as the mean ± SD. *p value of <0.05 between two groups. #p value of <0.05, as compared to fibronectin coated on the similar PDMS-modified surfaces.

The MSCs were further examined for their spreading area on the different chemically altered PDMS surfaces 4 h after the initial cell seeding by TRITC-conjugated phalloidin staining. The stained cells were viewed with an inverted fluorescent microscope. The spreading area of each single cell was measured by the Image-Pro Plus software. For the PDMS surfaces with collagen adsorption, cell adhesion on APTES + GA + protein resulted in a significantly higher spread area compared to the other two modified/unmodified PDMS surfaces (Figures 6 and 7). However, for PDMS surfaces with

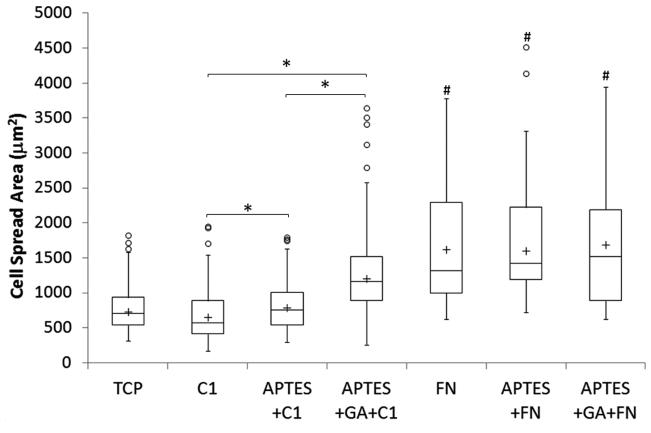

Figure 6. Mean and median cell-spread area on modified or unmodified PDMS surfaces displayed as Box and Whiskers plots (n > 30). The mean data are represented by +. *p value of <0.05 between two groups. #p value of <0.05, as compared to collagen type 1 coated on the similar PDMS-modified surfaces.

FN adsorption, there was no significant difference in the spread area of the cells on all three modified/unmodified PDMS surfaces (Figures 6 and 7), whereas in general the cells spread much wider compared to the adhered MSCs on TCP surfaces.

Cell Proliferation. The proliferation of MSCs on different chemically modified/unmodified PDMS surfaces was evaluated

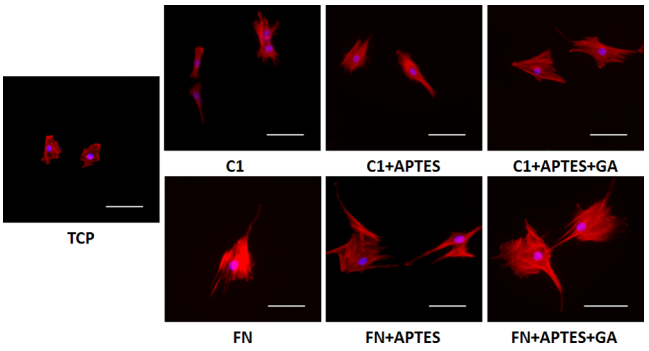

Figure 7. Cell-spread area of MSCs on modified/unmodified PDMS surfaces. The actin cytoskeleton of MSCs was stained red, and the cell nucleus was stained blue. Scale bar, 50 μm.

with PrestoBlue Cell Viability Reagent for a duration of 14 days. On the modified PDMS surfaces with C1 adsorption, MSCs remained metabolically active and continued to proliferate, whereas MSCs showed a higher proliferation activity on the APTES + GA + C1 PDMS surface (Figure 8A). However, MSCs that adhered to the unmodified PDMS

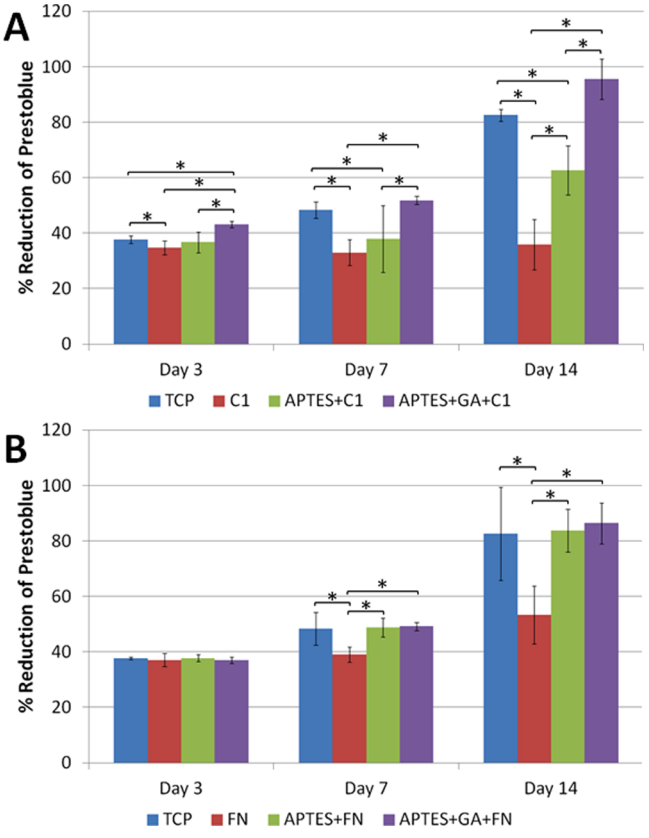

Figure 8. PrestoBlue assay for the proliferation of MSCs cultured on PDMS-modified/unmodified surfaces with (A) collagen type 1 or (B) fibronectin coating at 3, 7, and 14 days after seeding. *p value of <0.05 between two groups.

surfaces with C1 adsorption remained metabolically inactive (Figure 8A). For the modified/unmodified PDMS with FN adsorption, there were no significant differences in PrestoBlue dye reduction on day 3, but the subsequent data revealed significant differences in their proliferation activities on days 7 and 14, when the MSCs exhibited enhanced proliferation activities on the modified PDMS surfaces as compared to the unmodified surfaces (Figure 8B).

4. DISCUSSION

In recent years, the exploration of cell-surface interactions has been gaining importance, especially in the area of biomaterial research for therapeutics, diagnostics, and regenerative medicine. Investigating the effects of surface chemical modifications on cellular behavior is critical to assess the optimal conditions for cell adhesion, expansion, and proliferation. This requires a thorough understanding of surface functionalities and cell-surface interactions that beneficially influence cell anchorage on the chemically modified substrates.

The high hydrophobicity of a PDMS surface and poor physical adsorption of ECM components, such as fibronectin, has commonly caused the early detachment of cells and the uneven layering and clumping of a loosely bound cell population, thereby limiting the use of PDMS in cell culture.[15−18] This could be attributed to the relative instability of the physically adsorbed surface proteins that are vulnerable to detachment. A significant loss of bioactivity through disruptions of the protein's conformation or weak attachment is bound to occur in subsequent washing or media-changing steps[35] or even during routine perfusion processes. In this study, PDMS was functionalized with APTES and GA cross-linking chemistry, which provided −NH2 and −CHO functional groups, respectively, to achieve a stable covalent bonding of C1 or FN. The C 1s region scan data from XPS revealed the presence of a higher percentage of carbon in the successive modification steps (Figure 2). This confirmed the modification of PDMS surfaces with APTES and GA because of the presence of triethyl (−CH2) and aldehyde (−CHO) groups, respectively. Similarly, the N 1s region scans revealed the presence of a higher percentage of nitrogen in the silanized surface because of the presence of the amine group (−NH2) of the APTES molecules. Subsequently, the surface characterization and biocompatibility analysis of the chemically modified PDMS surfaces were performed to investigate the feasibility of the mentioned surface chemistry for application in cell culture studies.

Cell adhesion is vital for viability and growth, and the cellular adhesion relationship with the substrate is usually influenced by the substrate surface properties, such as biomolecular/biochemical composition, wettability, charges, and chemistry.[36,37] Therefore, it was of fundamental interest to investigate the level of cell attachment and spread area on the unmodified/modified PDMS surfaces. The surface wettability of the substrates has been known to be one of the determining factors that influence cell adhesion. To relate the surface wettability of our functionalized surfaces to the influence of cell adhesion and behavior, the hydrophobicity of the unmodified/modified PDMS surfaces was assessed by the measurement of the water contact angle. Previous studies have reported that PDMS surface functionalized with APTES and/or GA could result in a more hydrophilic surface.[38,39] These reported phenomena were observed to be consistent with the water contact-angle measurements performed on the APTES ± GA modified surfaces (data not shown) in which the reduced hydrophobicity could be attributed to their hydrophilic carbonyl and amine functional groups. The physical adsorption of protein alone (without any surface chemical treatment) was able to reduce the hydrophobicity of the native PDMS, but the surfaces were still hydrophobic (>90°) (Figure 3). However,

protein attachment on APTES + GA surfaces proved to be the most effective chemical modification to render a hydrophilic surface among the three experimental groups, with C1-bound surfaces more hydrophilic than the FN-bound surfaces (Figure 3). Despite the higher wettability on collagen-bound surfaces compared to that of FN-bound surfaces, cell adhesion was reported to be weaker on C1-bound surfaces (Figure 5). These data suggested that cell adhesion to a substrate surface was determined not only by its wettability but also by the types of proteins or functional groups present on the substrate surfaces.

Cell interactions with ECM proteins are vital to many biological processes,[40,41] and in numerous studies matrix proteins such as FN and collagen have been coated on a substrate for in vitro cell-based experiments.[1,4−7] Hence, it is essential to generate the stable attachment of matrix proteins by surface chemical functionalization. Past studies have shown that chemical modification leads to a higher surface stability than physical adsorption alone,[26−28] which could probably result in an enhanced biocompatibility for cell culture. To assess the stability of proteins bound to each unmodified/modified surface, Tween 20 was used to remove loosely bound proteins. The formation of stable protein−substrate covalent linkages resulted in a higher amount of protein being strongly attached on the chemically modified PDMS (Figure 4). Meanwhile, it was observed that C1 had a greater affinity than FN on the unmodified/modified PDMS surfaces. This could be attributed to the differences in their amino acid composition and protein conformation that influence the intermolecular forces between the proteins and the substrates, which would facilitate the attachment of proteins.[42] Functionalizing APTES and GA cross-linkers on the PDMS surface offers the possibility for the preferential orientation and covalent attachment of proteins through their respective affinity for moieties that bind to the reactive functional groups.[43]

We observed that surfaces coated with C1 had an increased wettability (Figure 3) with a higher amount of absorbed proteins (Figure 4) than those coated with FN, which could be favorable conditions for cell adhesion. However, the comparison of actual MSC adhesion on FN and C1 showed an opposite trend. We compared the level of cell adhesion and cell spreading on unmodified/modified surfaces with different protein adsorption, and the findings revealed a relatively lower level of cell adhesion and spreading on all of the C1-adsorbed surfaces as compared to FN-adsorbed surfaces. These results suggested that besides surface wettability and the underlying immobilization chemistry, the particular type of biomolecules (proteins) present on the surfaces also had an effect on the level of cell adhesion and spreading. Many studies have reported that cell adhesion on a collagen-coated surface is inferior to those on FN-coated surfaces.[37−46] The presence of cell adhesion motifs in ECM proteins[37,47] and the multiple integrin receptors on cells with different ligand specificities[48] can mediate cell adhesion to different matrix proteins. Additionally, the protein conformation on the substrate surfaces could also contribute to differential affinity for cell adhesion on the different protein-coated surfaces.[49]

Arima et al[36] reported that functional groups expressed on the substrate surface had an impact on the adhesion of cells, where amino groups encouraged better adhesion of endothelial cells than hydroxyl and carboxylic groups. In our study, chemically modified surfaces with either C1 or FN protein coating improved cell adhesion (Figure 5). The effect on cell spreading for FN-coated PDMS surfaces, however, is not as notable as those on C1−coated PDMS surfaces (Figures 6 and 7). All of the above-mentioned phenomena suggested that the enhanced MSC adhesion/spreading on APTES + GA + protein surfaces could be attributed to the synergistic effects of multiple contributing factors, such as the increased wettability of the surfaces, the influence of reactive functional groups in APTES/GA on protein attachment/conformation, and the choice of matrix protein, given their preferential affinity.

Protein adsorption on a substrate surface, cell-substrate surface adhesion, and cell spreading were a few critical steps that preceded cell proliferation in the later stages. Besides, a study by Chen et al. showed that promoting increased cell-spread area could potentially lead to an enhanced proliferation of the cells.[50] Cell proliferation on each unmodified/modified PDMS surfaces was thus evaluated with a PrestoBlue assay and correlated with results of the cell-spread area, as discussed earlier. On C1-coated surfaces, it was shown that the spreading area and proliferation of MSCs were both significantly higher on PDMS functionalized with APTES + GA (Figures 6−8A), whereas there was no significant MSC growth observed on the unmodified PDMS surface over the 14 days (Figure 8A). However, the MSC spreading area measured on FN-coated surfaces showed little difference among the three experimental surfaces (Figures 6 and 7). However, enhanced MSC proliferation was observed on FN-coated surfaces functionalized with APTES ± GA (Figure 8B). Because the MSCs were cultured on different functionalized surfaces, these observations suggested that MSC proliferation could not be attributed only to the extent of cell spreading, which implied that other factors, such as the surface chemistry, wettability, and the protein types, could also potentially influence MSC proliferation.

The drawback of native PDMS is that it is unfavorable to cell culture. Although the physical adsorption of ECM protein might help in initial cell adhesion, early detachment of cells and cell clumping were observed during cell confluence or even before reaching confluence.[15−18] Furthermore, this proved to be disadvantageous for long-term cell based applications, such as tissue engineering. Our study demonstrated that a PDMS surface functionalized with APTES + GA + protein adsorption provided a biocompatible surface for cell-based experiments. Our data showed that chemical modification not only provided a hydrophilic surface but also showed a stable covalent linkage of proteins, which was a prior step to establish the strong anchorage of cells. Furthermore, strongly adhered MSCs could have a great potential in long-term cell-based studies. Future experiments will be directed toward demonstrating the versatility of this surface treatment for long-term culture. Because these surfaces offer a convenient platform for long-term studies on MSCs, analytical validation could be performed to evaluate the functionality characterization of MSCs on these functionalized surfaces.

5. CONCLUSIONS

PDMS surfaces functionalized with APTES ± GA and the attachment of either C1 or FN were used in this study to demonstrate the ability of modified PDMS to serve as a biocompatible substrate for long-term cell culture. Chemical functionalization was shown to reduce the hydrophobicity of the native PDMS surfaces. APTES and GA cross-linking chemistry showed stable covalent attachment of the matrix proteins on the basis of the available active surface functional groups. The notable enhancement of MSC adhesion, spread area, and proliferation were best observed on the PDMS

surfaces functionalized with APTES + GA + proteins. For C1-coated surfaces, the chemical modification improved cell adhesion, spreading, and proliferation significantly. For FN-coated surfaces, although the promotion of cell spreading is not as notable as C1 groups, the chemical modification did greatly enhanced the cell proliferation. However, in general, the MSCs demonstrated a higher affinity, in terms of cell adhesion and spreading, on FN-coated PDMS than on C1−coated PDMS. These results indicated that surface chemical modification with APTES + GA + proteins had a profound effect on cell adhesion, spread area, and proliferation in which the synergistic effects of multiple contributing factors, such as surface wettability, protein matrix, and APTES/GA functional groups, played a combined role. We reported the utility of biocompatible PDMS surfaces functionalized with APTES + GA + proteins for enhanced cell culture stability, which can be potentially used for the study of cellular physiology, especially in the area of tissue engineering and mechanobiology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Images of MSCs spreading on modified/unmodified PDMS surfaces. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Chung, S.; Sudo, R.; Mack, P. J.; Wan, C. R.; Vickeman, V.; Kamm, R. D. *Lab Chip* **2009**, *9*, 269−275.

(2) Zhang, Q.; Liu, T.; Qin, J. *Lab Chip* **2012**, *12*, 2837−2842.

(3) Park, J. Y.; Yoo, S. J.; Lee, E. J.; Lee, D. H.; Kim, J. Y.; Lee, S. H. *BioChip J.* **2010**, *4*, 230−236.

(4) Wu, M. H.; Wang, H. Y.; Liu, H. L.; Wang, S. S.; Liu, Y. T.; Chen, Y. M.; Tsai, S. W.; Lin, C. L. *Biomed. Microdevices* **2011**, *13*, 789−798.

(5) Fu, J.; Wang, Y. K.; Yang, M. T.; Desai, R. A.; Yu, X.; Liu, Z.; Chen, C. S. *Nat. Methods* **2010**, *7*, 733−736.

(6) Yim, E. K. F.; Pang, Y. T.; Leong, K. W. *Exp. Cell Res.* **2007**, *313*, 1820−1829.

(7) Kilian, K. A.; Bugarija, B.; Lahn, B. T.; Mrksich, M. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 4872−4877.

(8) Carlo, D. D.; Aghdam, N.; Lee, L. P. *Anal. Chem.* **2006**, *78*, 4925−4930.

(9) Palchesko, R. N.; Zhang, L.; Sun, Y.; Feinberg, A. W. *PLoS One* **2012**, *7*, e51499-1−e51499-13.

(10) Sunyer, R.; Jin, A. J.; Nossal, R.; Sackett, D. L. *PLoS One* **2012**, *7*, e46107-1−e46107-9.

(11) Fischer, R. S.; Myers, K. A.; Gardel, M. L.; Waterman, C. M. *Nat. Protoc.* **2012**, *7*, 2056−2066.

(12) Peyton, S. R.; Raub, C. B.; Keschrumrus, V. P.; Putnam, A. J. *Biomaterials* **2006**, *27*, 4881−4893.

(13) Rehfeldt, F.; Brown, A. E.; Raab, M.; Cai, S.; Zajac, A. L.; Zemel, A.; Discher, D. E. *Integr. Biol.* **2012**, *4*, 422−430.

(14) Ortiz, G. C. The Study of Polydimethylsiloxane (PDMS) Thin-Film Surface Roughness Properties and Its Resistance in Elevated Temperature and UV Light Resistant Applications, Proceeding of the National Conference on Undergraduate Research, Ithaca, New York, March 31−April 2, 2011; Ithaca College: New York, 2011.

(15) Lee, J. N.; Jiang, X.; Ryan, D.; Whitesides, G. M. *Langmuir* **2004**, *20*, 11684−11691.

(16) Fuard, D.; Chevolleau, T. T.; Decossas, S.; Tracqui, P.; Schiavone, P. *Microelectron. Eng.* **2008**, *85*, 1289−1293.

(17) McDonald, J. C.; Duffy, D. C.; Anderson, J. R.; Chiu, D. T.; Wu, H.; Schueller, O. J. A.; Whitesides, G. M. *Electrophoresis* **2000**, *21*, 27−40.

(18) Duffy, D. C.; McDonald, J. C.; Schueller, J. A.; Whitesides, G. M. *Anal. Chem.* **1998**, *70*, 4974−4984.

(19) Tamada, Y.; Ikada, Y. *J. Colloid Interface Sci.* **1993**, *155*, 334−339.

(20) Lee, J. H.; Khang, G.; Lee, J. W.; Lee, H. B. *J. Colloid Interface Sci.* **1998**, *205*, 323−330.

(21) Tan, H. M. L.; Fukuda, H.; Akagi, T.; Ichiki, T. *Thin Solid Films* **2007**, *515*, 5172−5178.

(22) Bodas, D.; Khan-Malek, C. *Sens. Actuators, B* **2007**, *123*, 368−373.

(23) Hillborg, H.; Gedde, U. W. *Polymer* **1998**, *39*, 1991−1998.

(24) Yao, X.; Wu, H. X.; Wang, J.; Qu, S.; Chen, G. *Chem.—Eur. J.* **2007**, *13*, 846−853.

(25) Lodish, H.; Berk, A.; Zipursky, S. L.; Paul, M.; David, B.; James, D. *Molecular Cell Biology*, 4th ed.; WH Freeman and Company: New York, 2000.

(26) Yuan, H.; Mullett, W. M.; Pawliszyn, J. *Analyst* **2001**, *126*, 1456−1461.

(27) Halliwell, C. M.; Cass, A. E. G. *Anal. Chem.* **2001**, *73*, 2476−2483.

(28) Wang, Z. H.; Jin, G. J. *Immunol. Methods* **2004**, *285*, 237−243.

(29) Deshpande, K.; Ahamed, T.; van der Wielen, L. A.; Horst, J. H.; Jansens, P. J.; Ottens, M. *Lab Chip* **2009**, *9*, 600−605.

(30) Hui, J. H. P.; Li, L.; Teo, Y. H.; Ouyang, H. W.; Lee, E. H. *Tissue Eng.* **2005**, *11*, 904−912.

(31) Kretlow, J. D.; Jin, Y. Q.; Liu, W.; Zhang, W. J.; Hong, T. H.; Zhou, G.; Baggett, L. S.; Mikos, A. G.; Cao, Y. *BMC Cell Biol.* **2008**, *9*, 60-1−60-13.

(32) Briquet, A.; Dubois, S.; Bekaert, S.; Dolhet, M.; Beguin, Y.; Gothot, A. *Haematologica* **2010**, *95*, 47−56.

(33) Crouzier, T.; Jang, H.; Ahn, J.; Stocker, R.; Ribbeck, K. *Biomacromolecules* **2013**, *14*, 3010−3016.

(34) Lu, W.; McCallum, L.; Irvine, A. E. *J. Cell Commun. Signaling* **2009**, *3*, 147−149.

(35) Macdonald, C.; Morrow, R.; Weiss, A. S.; Bilek, M. M. *J. R. Soc., Interface* **2008**, *5*, 663−669.

(36) Arima, Y.; Iwata, H. *Biomaterials* **2007**, *28*, 3074−3082.

(37) Cooke, M. J.; Phillips, S. R.; Shah, D. S. H.; Athey, D.; Lakey, J. H.; Przyborski, S. A. *Cytotechnology* **2008**, *56*, 71−79.

(38) Lee, M.; Kim, K. H.; Park, J. G.; Lee, J. H.; Lim, H. W.; Park, M. Y.; Chang, S. I.; Lee, E. K.; Lim, D. W.; Choo, J. *BioChip J.* **2012**, *6*, 10−16.

(39) Sandison, M. E.; Cumming, S. A.; Kolchbc, W.; Pitta, A. R. *Lab Chip* **2010**, *10*, 2805−2813.

(40) Kleinman, H. K.; Philp, D.; Hoffman, M. P. *Curr. Opin. Biotechnol.* **2003**, *14*, 526−532.

(41) Adams, J. C.; Watt, F. M. *Development* **1993**, *117*, 1183−1198.

(42) Heilshorn, S. C.; Liu, J. C.; Tirrell, D. A. *Biomacromolecules* **2005**, *6*, 318−323.

(43) Wu, Y.; Buranda, T.; Metzenberg, R. L.; Sklar, L. A.; Lopez, G. P. *Bioconjugate Chem.* **2006**, *17*, 359−365.

(44) Sengel, P.; Kieny, M. *Br. J. Dermatol.* **1984**, *111*, 88−97.
(45) Chen, G.; Kawazoe, N.; Tateishi, T. *Open Biotechnol. J.* **2008**, *2*, 133−137.
(46) Junker, J. L.; Heine, U. I. *Cancer Res.* **1987**, *47*, 3802−3807.
(47) Feng, Y.; Mrksich, M. *Biochemistry* **2004**, *43*, 15811−15821.
(48) Clyman, R. I.; McDonald, K. A.; Kramer, R. H. *Circ. Res.* **1990**, *67*, 175−186.
(49) Grinnell, F.; Feld, M. K. *J. Biol. Chem.* **1982**, *257*, 4888−4893.
(50) Chen, C. S.; Mrksich, M.; Huang, S.; Whitesides, G. M.; Ingber, D. E. *Science* **1997**, *276*, 1425−1428.