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Surface Modification of Smooth Poly(L-lactic acid) Films for Gelatin Immobilization

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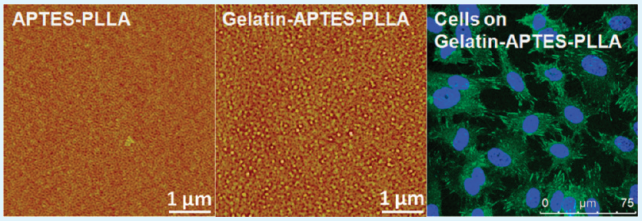
Supporting Information

ABSTRACT: Poly(L-lactic acid) (PLLA) is widely used in drug delivery and medical implants. Surface modification of PLLA with functional groups to immobilize gelatin or other extracellular matrix proteins is commonly used to improve its cellular affinity. In this work, we use the oxygen plasma to treat PLLA film followed by modification with organosilanes with different functional groups, such as amine, epoxy, and aldehyde groups. Gelatin is then immobilized on the modified PLLA film, which is confirmed by water contact angle measurement, atomic force microscopy (AFM), and laser scanning confocal microscopy (LSCM). Among the used organosilanes, aminosilane is the best one for modification of PLLA used for immobilization of gelatin with the highest efficiency. Moreover, the cellular affinity of gelatin-immobilized PLLA is studied through the evaluation of cell proliferation and focal adhesion using the human umbilical vein endothelial cells (HUVECs). Our experimental results show that the gelatin immobilized on aminosilane- and aldehyde-silane-modified PLLA improves the cellular affinity of HUVECs, whereas that immobilized on epoxy-silane-modified PLLA does not show significant improvement on the cell proliferation.



KEYWORDS: *oxygen plasma, organosilane, poly(L-lactic acid), gelatin, surface modification, human umbilical vein endothelial cells*

1. INTRODUCTION

Poly(L-lactic acid) (PLLA) is one kind of biodegradable, compostable, and recyclable polymers with good biocompatibility and eco-friendly characteristics.[1−3] It has been widely utilized in the fields of drug delivery, surgical implants, and sutures.[2,4] However, the hydrophobicity of PLLA usually results in low cell affinity and affects the cell adhesion onto its surface.[4] Therefore, it is necessary to modify PLLA with hydrophilic and biocompatible components, such as gelatin, chitosan, RGD peptide, and other extracellular matrix (ECM) proteins, to improve its biomedical applications. As PLLA is chemically inert because of the lack of reactive side-chain groups, it is a challenge to modify PLLA surface with biocompatible functional groups.

Many surface-modification methods have been reported to improve the cytocompatibility of PLLA, which include polymerization grafting,[5,6] ozone oxidization,[7,8] plasma modification,[9−12] entrapment,[13,14] hydrolysis,[15−17] aminolysis,[5,16] surface coating,[18,19] layer-by-layer self-assembly,[7,20,21] etc. Although surface coating of PLLA using ECM proteins or RGD peptide is a simple and convenient method,[2,18,19] it is time-consuming.[2] Alternatively, the entrapment of biomacromolecules, such as chitosan,[13] gelatin,[22] and poly(L-lysine),[23] on to a swollen PLLA surface is feasible after exposure of the PLLA surface into the solvent/nonsolvent mixture. However, most of the good solvent for PLLA is not biocompatible. In addition, the alkaline hydrolysis treatment is a simple and permanent way to create reactive functional groups, such as carboxyl and hydroxyl on PLLA surface by cleavage of ester bonds.[1,2,4,13,15] Importantly, these carboxyl groups can be used to covalently immobilize ECM proteins and other bioactive molecules with the help of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) reaction to improve the cell affinity of PLLA.[13,22] However, alkaline hydrolysis changes the surface roughness considerably, which can affect the cell spreading and growth.[2,24−28] As one of commonly used methods, photografting has been extensively used to tailor the surface property of PLLA through the permanent alteration of surface chemistry.[29] On the other hand, the monomer migration into the bulk film and the degradation of PLLA induced by the high power UV irradiation are often observed.[1] Although plasma treatment has been successfully used as an efficient method to modify PLLA without changing the bulk properties,[1,2,10,11] it is difficult to maintain the treated PLLA surface unchanged for a long time because of the surface rearrangement.[1,9]

It is well-known that organosilanes can react with hydroxyl groups on many substrates to form stable self-assembled monolayers (SAMs).[30−38] As oxygen plasma treatment can introduce hydroxyl groups on PLLA surface,[9] it should be feasible to introduce functional groups on oxygen plasma-treated PLLA film through the reaction of organosilanes and

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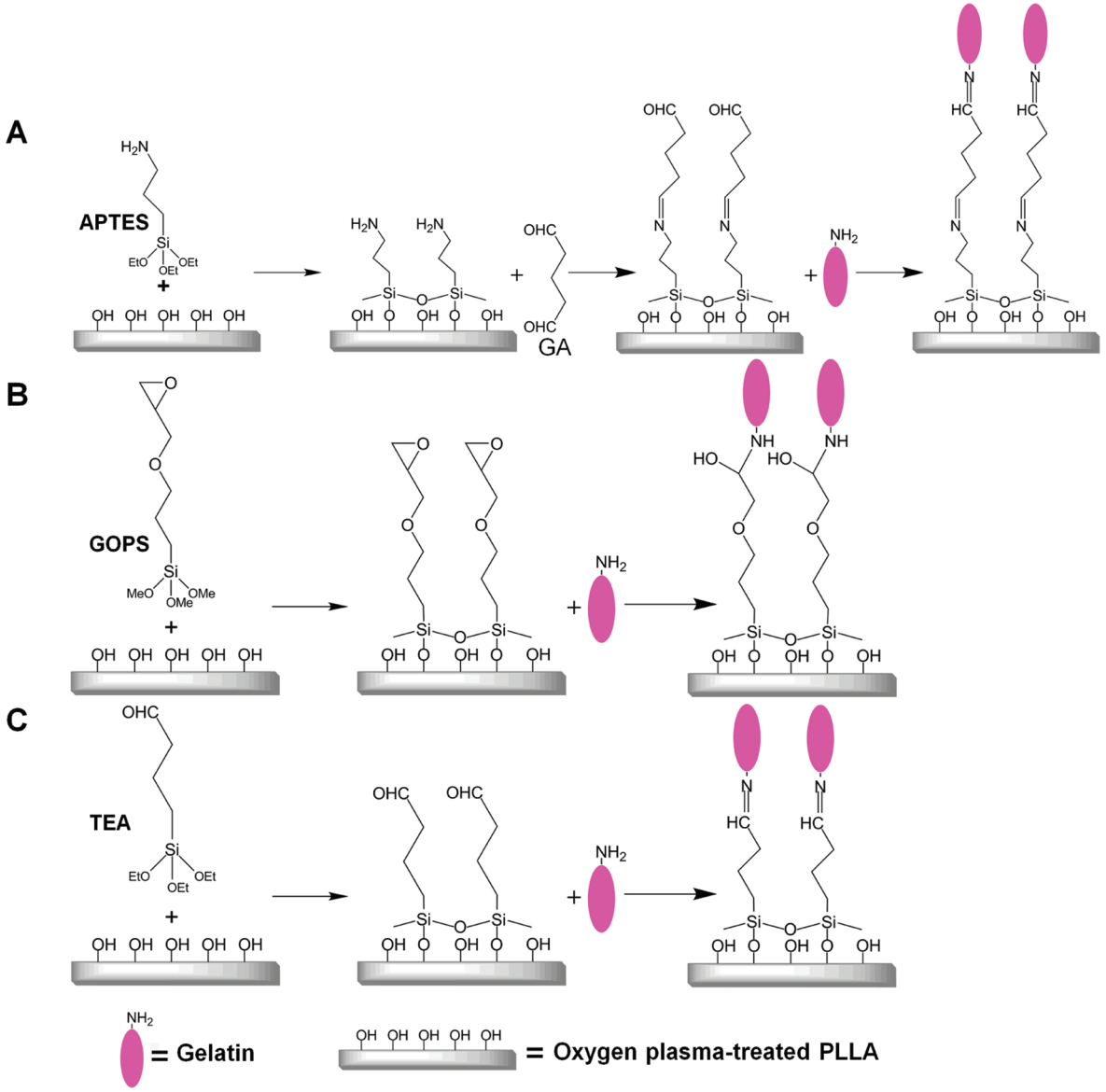
Scheme 1. Schematic Illustration of Surface Modification of Oxygen Plasma-Treated PLLA Films with (A) Aminosilane (APTES),[a] (B) Epoxy-Silane (GOPS), and (C) Aldehyde-Silane (TEA), Used for Gelatin Immobilization



[a]Glutaraldehyde (GA) is used as linker to covalently immobilize gelatin on APTES-modified PLLA film.

hydroxyl groups. Since the surface roughness of biomaterial-coated substrate could significantly affect the cell behaviors especially at the nanometer scale,[4,25,39] keeping the nanoscale topography of the modified surface is also important.

In this paper, the surface chemical modification of smooth PLLA film was achieved after the reaction of oxygen plasma-treated PLLA film with organosilanes. Various reactive functional groups, such as amine, aldehyde, and epoxy () groups were introduced on the PLLA film. These functional groups were subsequently used to immobilize gelatin, which was extensively used to improve the cytocompatibility of PLLA film.[17,21,22] AFM and water contact angle measurements were used to characterize the surface structures. Micropatterns of fluorescent labeled gelatin were fabricated on the surface-modified PLLA film by microcontact printing, which were imaged by the confocal laser scanning microscopy. Moreover, the cellular affinity of gelatin-immobilized PLLA was studied through the evaluation of cell proliferation and focal adhesion using the human umbilical vein endothelial cells (HUVECs).

2. EXPERIMENTAL SECTION

2.1. Materials. Poly(L-lactic acid) (PLLA, intrinsic viscosity (IV): 2.38, Bio Invigor) was used without further purification. Glutaraldehyde, (3-aminopropyl)triethoxy silane (APTES), (3-glycidoxypropyl)-trimethoxy silane (GOPS), and gelatin (from porcine skin, ~175°g Bloom) were purchased from Sigma-Aldrich Pte. Ltd. and used as received. Triethoxysilylbutyraldehyde (TEA, tech-90) was purchased from Gelest Inc.

Cryopreserved Clonetics human umbilical vein endothelial cells (HUVEC) in endothelial growth medium (EGM), Clonetics EGM BulletKit supplemented with 0.4% bovine brain extract (BBE), Hepes buffered saline solution (HBSS), and 0.025% Trypsin-EDTA were purchased from Lonza Walkersville Inc. (USA). Phosphate buffer saline (PBS), WST-8 cell counting reagent kit, and Triton X-100 (ultrapure grade) were purchased from Gibco (Life Technologies Corporation, USA), Dojindo Laboratories (Japan), and USB

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Biochemicals (Affymetrix Inc., USA), respectively. Monoclonal antivinculin produced in mouse (V9264) and secondary antibody goat antimouse IgG FITC conjugate were purchased from Sigma-Aldrich. DAPI and Molecular Probes Fluorescence mounting medium were purchased from DAKO, Denmark.

2.2. Fabrication of Smooth PLLA Films. Freshly cleaned glass slides (size: 15 mm ×12 mm) were immersed into 1% APTES aqueous solution for 15 min, dried by nitrogen gas, and then heated at 120 °C for 2 h.[40] The obtained APTES-modified glass slides were immersed in a 0.5% glutaraldehyde (GA) solution overnight.[30] PLLA thin films were prepared on the above modified glass slides by spin-coating 3.0 wt.% PLLA solution in dichloromethane at 3000 rpm for 30 s. The thickness of obtained PLLA film is ~650 nm.[41] The aforementioned experiment was carried out in order to ensure the strong attachment of PLLA on glass slides and avoid the peeling-off of PLLA film during the washing process.

2.3. Fabrication of Polydimethylsiloxane (PDMS) Stamps. PDMS stamps were fabricated by pouring a mixture of Sylgard 184 elastomer and curing agent with weight ratio of 10:1 on a Si master and heating at 70 °C for 12 h after degassing.

2.4. Oxygen Plasma Treatment and Organosilane Modification of PLLA films. The prepared smooth PLLA films were treated by oxygen plasma cleaner (PDC-32G-2, Harrick Plasma, Ithaca, NY) at pressure of 120 mTorr and power of 10.8 W for 1 min, which were then immediately immersed into the three kinds of silane solutions. The first oxygen plasma-treated PLLA film was immersed into 10 mL 1% APTES aqueous solution for 30 min to form APTES-PLLA, which was then immersed into 10 mL 0.5% GA solution overnight to form GA-APTES-PLLA , used for gelatin immobilization. Two other oxygen plasma-treated PLLA films were immersed into 10 mL of the mixture of GOPS:ethanol:H_2O (v:v:v = 1:10:89) or TEA:ethanol:H_2O (v:v:v = 1:98:1) for 30 min to fabricate GOPS-PLLA or TEA-PLLA, respectively.

After the as-prepared GA-APTES-PLLA, GOPS-PLLA, and TEA-PLLA films were immersed into 1 mg/mL gelatin solution overnight to form gelatin-immobilized PLLA films, referred to as gelatin-GA-APTES-PLLA, gelatin-GOPS-PLLA, and gelatin-TEA-PLLA, respectively, they were thoroughly washed with PBST solution (0.5% Tween-20 in PBS buffer) and then PBS solution for 3 times, respectively, to eliminate noncovalent immobilization of gelatin.

2.5. Microcontact Printing of FITC Conjugated Gelatin on the Modified PLLA Films Imaged by Confocal Laser Scanning Microscopy. 100 μL of 0.5 mg/mL FITC conjugated gelatin (FITC-gelatin) solution was dropped on a PDMS stamp with dot size of 10 μm and spacing of 10 μm. After inking for 30 min and being dried with N_2 gas, the PDMS stamp was brought into contact with the organosilanes-modified PLLA film for 30 min before it was withdrawn from the surface. After immobilization of FITC-gelatin, the sample was thoroughly washed with PBST solution and then PBS solution for 3 times, respectively. The immobilized FITC-gelatin micropatterns were imaged with a Leica TCS SP5 laser-scanning spectral confocal microscope (CLSM) (Leica, Wetzlar, Germany).

2.6. AFM Imaging. A commercial AFM instrument (Dimension 3100 with Nanoscope IIIa controller, Veeco Instruments Inc., CA) equipped with a scanner (90 × 90 μm^2) was used to image the samples in tapping mode in air. Si cantilever with the normal resonance frequency of 300 kHz and spring constants of 40 N/m (Tap300Al-G, Budget Sensors, Innovative Solutions Bulgaria Ltd., Bulgaria) was used. All images were captured at scan rate of 1−2 Hz and 512 × 512 pixel resolution.

2.7. Contact Angle Measurement. Contact angle was measured using the sessile drop technique with FTA200 Goniometer (First Ten Angstroms, Inc., USA). Milli-Q water was used for the measurement.

2.8. Cell Proliferation. Passage 2 HUVECs were cultured in T75 flasks using BBE supplemented EGM in an incubator with 95% air/5% CO_2 at 37 °C for 1 week. The EGM was changed every two days. Cells were harvested by trypsinization by 0.025% Trypsin-EDTA upon 90% confluency. All PLLA films were sterilized by immersing into 70% ethanol for 30 min, and then were rinsed with deionized water three times followed by PBS three times. PLLA substrates were then placed on the bottom of each cell culture well plate for cell seeding at density of 1 × 10^4 cells/cm^2. Cell proliferation was monitored by WST-8 on day 1, 3, 5, and 7 according to manufacturer's protocol. Briefly, HUVECs were incubated with WST-8 reagent for 4 h, and the absorbance at 450 nm was measured by microplate reader (Tecan).

2.9. Immunofluorescent Imaging. Focal adhesion formation was observed by immunofluorescent imaging. After 3-day incubation, adherent cells were fixed for 15 min with 4% PFA, permeabilized with 0.1% triton X-100 for 10 min, and subsequently incubated with 1% BSA in PBS for 30 min at room temperature. Vinculin was immunolabeled by mouse antihuman vinculin, and then visualized by goat antimouse IgG FITC conjugate. Nuclei were labeled with DAPI. Cell images were captured by Leica TCS SP5 laser-scanning spectral confocal microscope (CLSM) (Leica, Wetzlar, Germany).

2.10. Statistics. Five specimens (n = 5) were used in the cell proliferation study. The results were presented as the mean ± SD. Surface-dependent cellular responses were analyzed using ANOVA. A p value of less than 0.05 ($p < 0.05$) is taken as statistical significance of differences.

3. RESULTS AND DISCUSSION

3.1. Water Contact Angle and AFM Characterizations of PLLA Film, Organosilane-Modified PLLA Films, and Gelatin-Immobilized PLLA Films. As shown in Scheme 1, after the oxygen plasma-treated PLLA films were immersed into organosilane solutions, i.e., APTES, GOPS, or TEA solutions, the organosilanes formed self-assembled monolayers (SAM) on the hydroxyl group-terminated PLLA. The functional groups of these organosilane SAMs can be used for subsequent gelatin immobilization. On APTES-PLLA film, glutaraldehyde (GA) was used as a linker (GA-APTES-PLLA) to bind gelatin (Scheme 1A). GA consists of 2 aldehyde groups. One aldehyde group reacts with the amine group in APTES, and the other one is available for gelatin immobilization. On the GOPS-PLLA or TEA-PLLA film, gelatin can be immobilized through the reaction between aldehyde or epoxy groups on substrates and amine groups in gelatin (Scheme 1B, C).

It is well-known that the immobilization of gelatin on PLLA can increase its hydrophilicity. To confirm the immobilization of gelatin, we conducted water contact angle measurement to investigate the wettability alternation of PLLA films before and after modification. As shown in Table 1, the unmodified PLLA

Table 1. Water Contact Angle Measurements of the PLLA Films before and after Modification (n = 10, mean ± SD)

sample	water contact angle (deg)
PLLA	77 ± 1
APTES-PLLA	71 ± 4
GOPS-PLLA	64 ± 3
TEA-PLLA	67 ± 3
Gelatin-GA-APTES-PLLA	42 ± 2
Gelatin-GOPS-PLLA	54 ± 2
Gelatin-TEA-PLLA	44 ± 2

surface gives a relatively high contact angle (77 ± 1°), which is consistent with the previous reports.[42,43] Such a hydrophobic substrate has low cell affinity and poor cell adhesion. After the modification of PLLA by organosilanes, the APTES-PLLA, GOPS-PLLA, and TEA-PLLA films give water contact angle of 71 ± 4°, 64 ± 3°, and 67 ± 3° (Table 1), respectively. It indicated that the organosilane modification increases the wettability of PLLA. After gelatin is immobilized on GA-APTES-PLLA (gelatin-GA-APTES-PLLA) and TEA-PLLA (gelatin-TEA-PLLA), the water contact angles further decrease

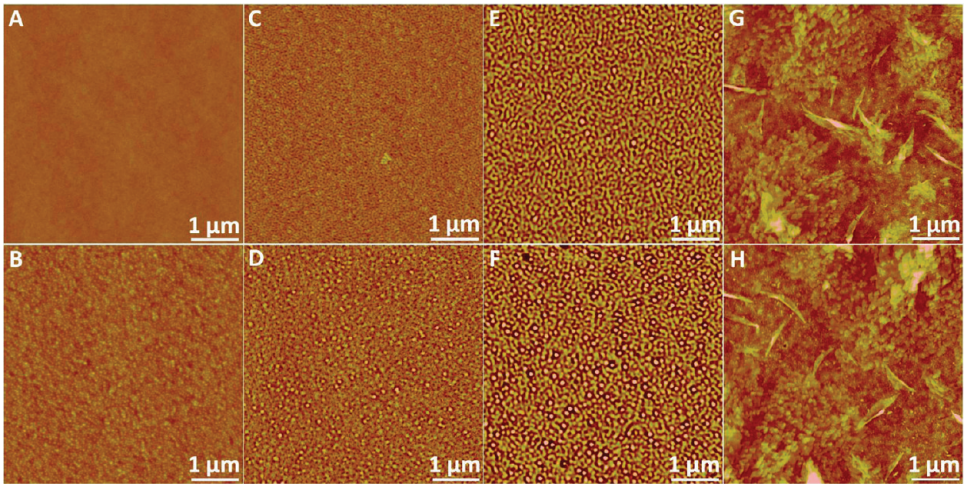

Figure 1. AFM images of PLLA films before and after surface modification. (A) PLLA film, (B) PLLA film after oxygen plasma treatment, (C) after aminosilane (APTES) modification of oxygen plasma-treated PLLA (APTES-PLLA), (D) after gelatin immobilized on APTES-PLLA with glutaraldehyde as linker (GA-APTES-PLLA), (E) after epoxy-silane (GOPS) modification of oxygen plasma-treated PLLA (GOPS-PLLA), (F) after gelatin immobilized on GOPS-PLLA, (G) after aldehyde-silane (TEA) modification of oxygen plasma-treated PLLA (TEA-PLLA), (H) after gelatin immobilized on TEA-PLLA. The Z scale is 20 nm for A–F, and 50 nm for G and H, respectively.

to 42 ± 2° and 44 ± 2° (Table 1), respectively, which are hydrophilic and comparable to the literature.[21] This wettability alteration confirmed the good immobilization of gelatin on modified PLLA. However, gelatin-immobilized GOPS-PLLA (gelatin-GOPS-PLLA) gives a higher water contact angle of 54 ± 2°. In addition, X-ray photoelectron spectroscopy (XPS) was used to confirm gelatin was successfully immobilized on GOPS-PLLA film (see Figure S1 in the Supporting Information).

AFM, a powerful tool for surface characterization, was used to measure the PLLA film before and after surface modification. Figure 1A shows a smooth PLLA film formed on the modified glass by spin-coating. The surface roughness, root-mean-square (rms) value, of this bare PLLA film is 0.3 nm in 5 × 5 μm^2 (Table 2), which is comparable to the cleaned SiO_2 substrate

Table 2. AFM Measured Surface Roughness (in area of 5 × 5 μm^2), Root-Mean-Square (rms) Value, of PLLA Films before and after Modification

sample	rms value (nm)
PLLA film	0.3
oxygen plasma-treated PLLA	0.8
APTES-PLLA	0.9
GA-APTES-PLLA	1.0
gelatin-GA-APTES-PLLA	1.3
GOPS-PLLA	3.1
gelatin-GOPS-PLLA	3.4
TEA-PLLA	4.8
gelatin-TEA-PLLA	5.0
petri dish	2.4

(0.2 nm). After oxygen plasma treatment, the rms value of PLLA film increased to 0.77 nm, indicating that oxygen plasma-treated PLLA film (Figure 1B) is slightly rougher than the PLLA film (Figure 1A). The rms values of APTES-PLLA film (Figure 1C) before and after GA modification increased to 0.9 and 1.0 nm (Table 2), respectively, which are much smoother than a clean Petri dish with a rms value of 2.4 nm (Table 2).

The GOPS-PLLA (Figure 1E) and TEA-PLLA (Figure 1G) films are rougher than the clean Petri dish and their rms values are 3.1 and 4.8 nm (Table 2), respectively. The obvious increased rms value after modification of PLLA with GOPS and TEA can be attributed to the dissolution of PLLA film in ethanol, as ethanol was used in preparation of GOPS and TEA solutions. This was further confirmed by a control experiment, in which the oxygen plasma-treated PLLA film became much rougher after being immersed into ethanol (see Figure S2 in the Supporting Information). Note that APTES is water-soluble and no ethanol was involved in the APTES modification, resulting in the smoothest surface of APTES-PLLA film, compared to GOPS-PLLA and TEA-PLLA films. After gelatin immobilization, the rms values of gelatin-GA-APTES-PLLA (Figure 1D), gelatin-GOPS-PLLA (Figure 1F), and gelatin-TEA-PLLA (Figure 1H) films increased only slightly to 1.3, 3.14, and 5.0 nm (Table 2), respectively, indicating that the immobilization of gelatin could not dramatically change the surface roughness of these films.

3.2. Micropatterns of Gelatin on Surface-Modified PLLA Film. FITC-gelatin was patterned on GA-APTES-PLLA, GOPS-PLLA, and TEA-PLLA by microcontact printing (μCP), and then imaged using AFM and laser-scanning spectral confocal microscopy (CLSM) after washing thoroughly. As shown in Figure 2A, the height of gelatin pattern is ~2.1 nm. Figure 2B–D show the CLSM images of FITC-gelatin micropatterns immobilized on GA-APTES-PLLA, GOPS-PLLA, and TEA-PLLA, respectively. By comparing the fluorescent intensity profiles in Figure 2B–D, gelatin immobilized on GA-APTES-PLLA shows the strongest fluorescent intensity, followed by TEA-PLLA and GOPS-PLLA, indicating that the GA-APTES-PLLA has the best immobilization efficiency of gelatin. We believe this arises from the smallest rms value of GA-APTES-PLLA, compared to the other two films. The smoothest GA-APTES-PLLA film leads to the closest contact with the PDMS stamp used for micropatterning gelatin. As a result, the largest amount of gelatin can be transferred from the stamp onto GA-APTES-PLLA. However, the modification of PLLA with TEA and GOPS significantly increases the surface roughness as compared to GA-APTES-PLLA, which might result in less efficient transfer of gelatin from the PDMS stamp. The lowest fluorescent

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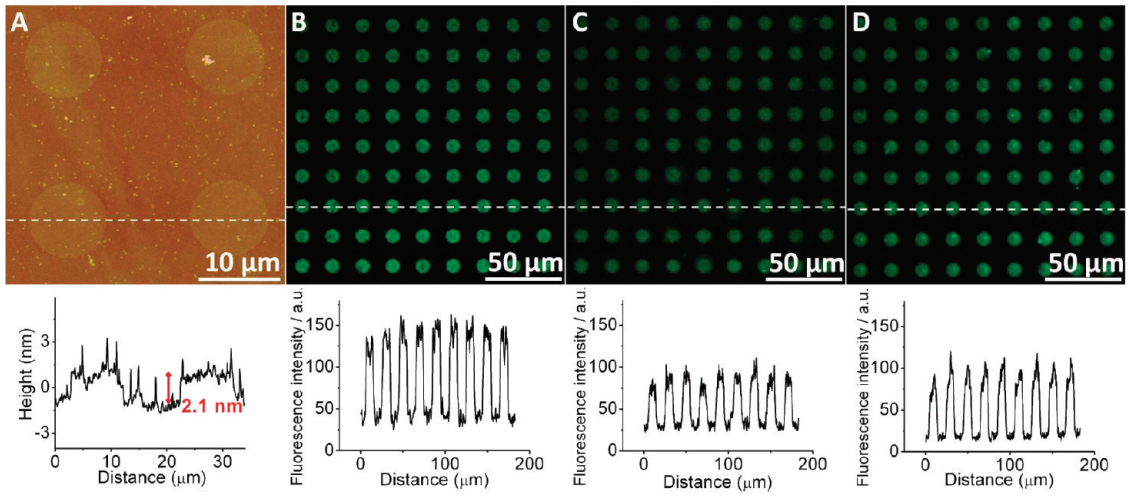

Figure 2. (A) AFM image of FITC-gelatin pattern on GA-APTES-PLLA, the Z scale is 50 nm. (B–D) CLSM images of FITC-gelatin patterns on (B) GA-APTES-PLLA, (C) GOPS-PLLA, and (D) TEA-PLLA. Bottom: Height or fluorescent intensity profiles of the dashed lines in the corresponding AFM or CLSM images.

intensity profile of gelatin-GOPS-PLLA means the lowest immobilization efficiency of gelatin on GOPS-PLLA. All these results are also consistent with the water contact angle results (Table 1). Therefore, the modification of PLLA with APTES is the best way to modify PLLA film for gelatin immobilization compared to the other two organosilanes used in this work.

3.3. Cell Proliferation and Focal Adhesion Formation. HUVECs proliferated in BBE-supplemented EGM for 7 days. The proliferation was analyzed using WST-8 cell counting kit.[12,44] Figure 3 shows the absorbance at 450 nm, which is

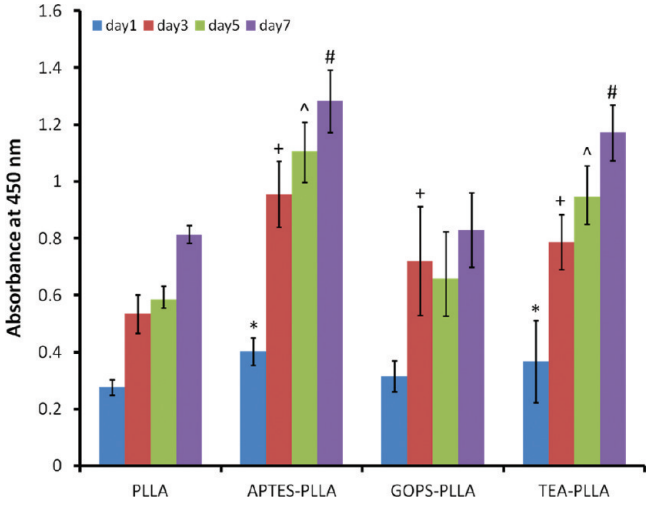

Figure 3. Proliferation of HUVECs cultured on PLLA and gelatin-immobilized PLLA films over 7 days at 37 °C in humidified air with 5% CO_2. Cell seeding density is 1×10^4 cells/cm^2. The absorbance of WST-8 cell proliferation reagent was measured at 450 nm. *$p < 0.05$, ^$p < 0.05$, +$p < 0.05$, and #$p < 0.05$ refer the significant absorbance difference with comparison of PLLA at day 1, 3, 5, and 7, respectively.

linearly proportional to the cell count measured at day 1, 3, 5, and 7 on various PLLA substrates. On APTES-PLLA and TEA-PLLA, the absorbance was significantly greater than that on PLLA after 7 days of incubation. It suggested that the gelatin immobilized on PLLA through APTES or TEA improved the cellular affinity of HUVECs. However, the gelatin immobilized on GOPS-PLLA did not give significant improvement on the cell proliferation. These results are consistent with the low density of gelatin immobilization on GOPS-PLLA as mentioned in section 3.2.

Focal adhesion (FA) formation is another criterion to evaluate the cellular affinity and FA sites can be identified by the existence of vinculin. After 3 days of incubation, cells were fixed and vinculin was immunolabeled. Images captured by CLSM on different PLLA substrates are shown in Figure 4. A

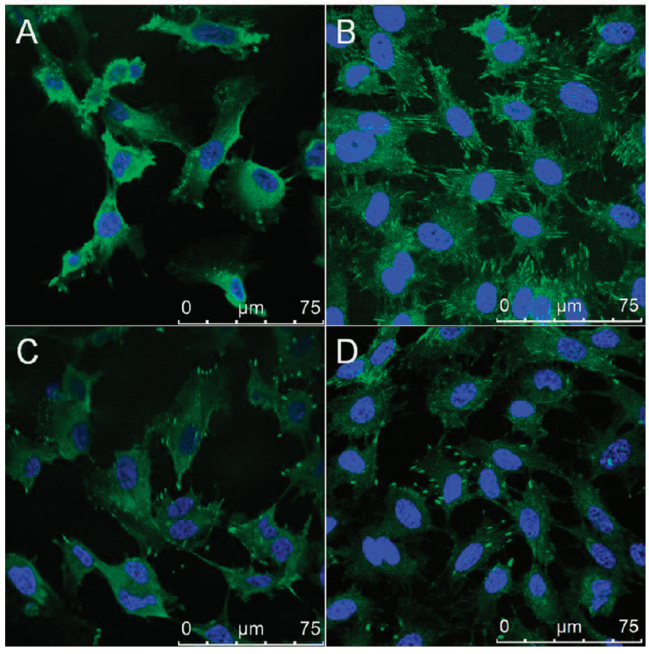

Figure 4. Focal adhesion formation was observed by vinculin immunofluorescent staining after 3-day incubation on (A) PLLA, (B) gelatin-immobilized APTES-PLLA, (C) gelatin-immobilized GOPS-PLLA, and (D) gelatin-immobilized TEA-PLLA, respectively. The seeding density was 1×10^4 cells/cm^2. The images were captured by Leica TCS SP5 CLSM.

limited number of focal adhesion was found in cells grown on PLLA (Figure 4A). However, the number of focal adhesion

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increased in cells on the three kinds of gelatin-immobilized PLLA substrates. The highest increase of FA was found on APTES-PLLA (Figure 4B), and the abundant and well-organized focal adhesion observed throughout the cell suggested the matured and strong cell adhesion. Focal adhesion sites found on GOPS-PLLA (Figure 4C) and TEA-PLLA (Figure 4D) were comparably shorter and less dense but better than that on PLLA. This observation was in a good agreement with the lower gelatin immobilization efficiency on GOPS- and TEA-modified PLLA (Figure 2). Importantly, the cell affinity further confirmed that the APTES modification of PLLA is the best method to immobilize gelatin among all organosilanes used in this work.

4. CONCLUSION

In summary, oxygen plasma-treated PLLA film was modified by organosilanes with different functional groups for immobilization of gelatin. Water contact angle measurement, AFM, and laser-scanning spectral confocal microscopy (CLSM) were used to characterize the PLLA films before and after modification. Cell proliferation and focal adhesion formation were also studied to evaluate the cellular affinity of these modified PLLA. It was found that among the organosilanes used in this work, APTES is the best one to modify PLLA film for the highest immobilization efficiency of gelatin and best cellular affinity. The results of cellular affinity indicated that the gelatin immobilized on aminosilane- and aldehyde-silane-modified PLLA improved cellular affinity of HUVECs, whereas that immobilized on epoxy-silane-modified PLLA did not give significant improvement on the cell proliferation. In conclusion, the organosilane modification of oxygen plasma-treated PLLA provides an alternative surface modification method for PLLA film to immobilize extracellular matrix proteins, which are useful to improve the cytocompatibility of PLLA films.

■ ASSOCIATED CONTENT

Supporting Information

XPS spectra of PLLA, APTES-PLLA, GOPS-PLLA, and gelatin-GOPS-PLLA. AFM image of oxygen plasma-treated PLLA after being immersed into ethanol for 15 min. 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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