Surface modification of poly(L-lactic acid) with biomolecules to promote endothelialization[a)]

Yun Xia,[b)] Freddy Boey, and Subbu. S. Venkatraman
School of Materials Science and Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore

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Rapid endothelialization is important for biodegradable blood-contacting devices not only to prevent thrombosis but also to prevent degradation debris from entering the bloodstream and causing further complications. Here the authors report a three-step surface modification method, by which biomolecules, such as gelatin and chitosan, are covalently immobilized on the surface of plasma-treated poly(L-lactic acid) (PLLA) via —COOH groups introduced by acrylic acid grafting polymerization. Surface characterization techniques, including x-ray photoelectron spectroscopy, contact angle measurement, and colorimetric methods for surface density of functional groups, proved the feasibility and stability of this surface modification method. Surface wettability was increased by biomolecules immobilization. The —COOH surface density was measured to be 4.17 ± 0.15 μmol/cm^2, the and amount of gelatin immobilized was 4.8 μg/cm^2. Human umbilical vein endothelial cell was used during *in vitro* study at seeding density of 10^4 cells/cm^2. PLLA-gAA-gelatin surface was found to enhance cell adhesion, spreading, focal adhesion formation, and proliferation significantly. Chitosan-modified PLLA shows marginally improvement in cell adhesion and proliferation. Endothelialization was achieved within 7 days on both modified PLLA surfaces. In conclusion, this work demonstrates the feasibility of the surface modification method, and its ability to promote complete endothelialization for cardiovascular applications. [DOI: 10.1116/1.3467508]

I. INTRODUCTION

Lactic and glycolic acid polymers and their copolymers are degraded by hydrolysis in physiological environment into nontoxic products. They are capable of offering a broad range of mechanical and degradation properties by varying their molecular weights and copolymerization ratios. Hence they have been investigated widely in the application of orthopedic replacements,[1] cardiovascular prostheses,[2–5] sutures,[6] and drug-delivery systems.[7] However, their applications *in vivo* are sometimes limited by their surface characteristics, which govern their interaction with biological tissues. For instance, poly(L-lactic acid) (PLLA) has been evaluated as a candidate material for vascular stent applications based on its mechanical properties.[8,9] Low endothelial cell affinity of PLLA becomes a hindrance to its medical application as part of a blood-contacting device because rapid endothelialization is crucial for a cardiovascular stent not only to prevent thrombosis and restenosis but also to prevent degradation debris from entering the bloodstream and causing further complications.[10,11] The rationale for surface modification of biomaterials becomes clear: to retain the key physical properties of biomaterials while modifying only the surface to influence the bio-interaction.

Our group has demonstrated enhanced endothelialization rate *in vitro* by several physical surface modification methods, such as salt-leaching[12] and lithography[13] to create nanosized surface features. Zhu *et al.*[14–17] used aminolysis to introduce primary amine groups on the surface of PLA, PLGA, and PCL, followed by introduction of gelatin, chitosan, and collagens through glutaraldehyde or layer-by-layer assembly. However, aminolysis is a harsh wet-chemical method and difficult to control. Plasma surface treatment causes changes to a limited depth. In addition, plasma treatment with tunable conditions provides a controllable, flexible and uniform surface modification even with complicated surface conformation. Plasma gases, such as oxygen, argon, and ammonia, have been utilized for surface modification.[18,19] However, single plasma surface modification has limited capacity to generate a conductive surface for proper cell-material interaction. One important benefit from plasma treatment is enabling the introduction of functional groups on a polymeric surface. These functional groups could be utilized to immobilize bioactive macromolecules. For example, Kang's group have modified PMMA, PET, and PU using oxygen plasma treatment followed by acrylic acid graft polymerization and immobilization of collagen and heparin.[20–22] However, these modifications have been generally confined to nondegradable synthetic polymers. Clearly, there is a need to develop a mild and effective surface modification method for the purpose of promoting endothelialization on biodegradable polymer surfaces.

In this study, we reported a three-step surface modification of PLLA with biomolecules such as gelatin and chitosan. First, PLLA surface was activated by argon (Ar) plasma

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[b)]Electronic mail: xiayun_marie@ntu.edu.sg

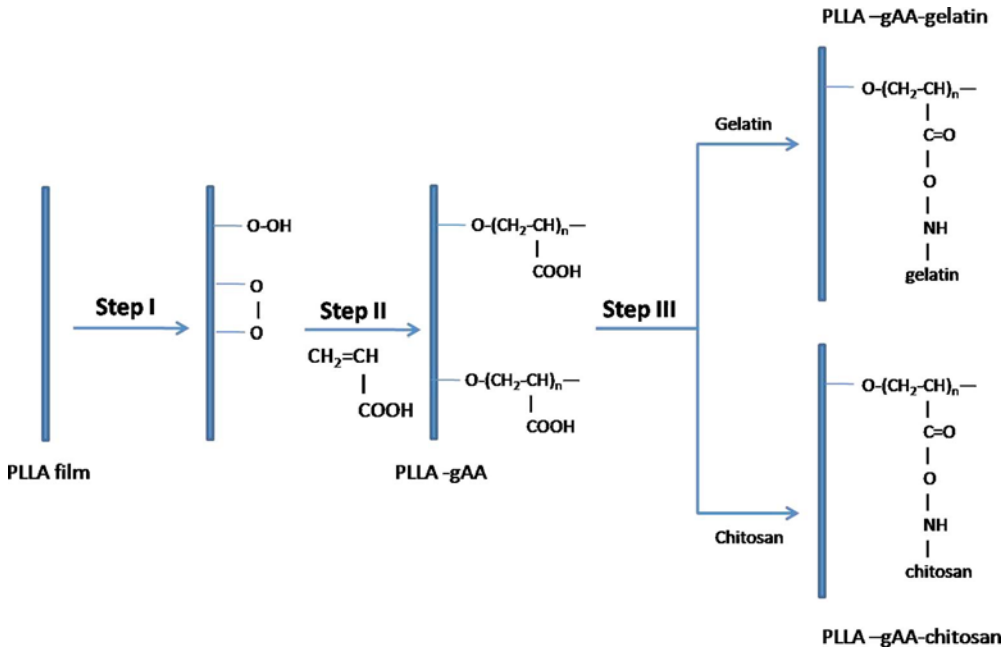

FIG. 1. (Color online) Three-step biomolecule immobilization protocol. Step I is argon-plasma treatment of PLLA. Step II is AA graft polymerization on the activated PLLA film. Step III is covalent coupling of amine groups on biomolecule with carboxylic groups on PLLA-gAA in WSC solution.

treatment. Second, carboxylic groups were introduced by acrylic acid (AA) graft polymerization on the plasma-activated PLLA surface. Finally, the biomolecules were immobilized by covalent coupling between carboxylic groups and amine groups found in biomolecules. Surface characterization techniques, including x-ray photoelectron spectroscopy (XPS), contact angle measurement, and surface density quantification of function groups, were employed to evaluate the modification method. Human umbilical vein endothelial cells (HUVECs) grown on modified PLLA surfaces showed enhanced cell adhesion, spreading, focal adhesion formation, and proliferation. Complete endothelialization was observed on both modified PLLA surface, but not on bare PLLA.

II. EXPERIMENT

A. Materials

PLLA was purchased from Purac (Singapore). Dichloromethane (DCM), 2-(*N*-Morpholino) ethanesulfonic acid (MES, low moisture content, >99%), gelatin (Porcine skin, Type A), paraformaldehyde (PFA, 95%), acrylic acid (AA, anhydrous, 99%), *N*-hydroxysuccinimide (NHS), chitosan oligosaccharide lactate (M_n=5000), sodium metabisulfite (ACS reagent, 99%), potassium persulfate (ACS reagent, 99%), rhodamine 6G, and glutaraldehyde (25%, Grade I) were purchased from Sigma-Aldrich. 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and coomassie plus (Bradford) protein assay were purchased from Thermo Scientific.

Cryopreserved Clonetics® HUVECs 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. Phosphate buffer saline (PBS) was from Gibco. WST-8 cell counting reagent kit was from Dojindo, Japan. Triton X-100 (ultrapure grade) was from USB Biochemicals. Mouse antihuman paxillin and DAPI were purchased from Molecular Probes. Secondary antibody goat antimouse IgG FITC conjugate was purchased from Sigma-Aldrich. Fluorescence mounting medium and goat serum were purchased from DAKO, Denmark.

B. Surface modification

The three-step surface modification protocol is illustrated in Fig. 1. Three steps are involved: (1) argon-plasma treatment, (2) AA graft polymerization, and (3) biomolecules immobilization.

The argon-plasma surface treatment was carried out in a March PX-500 cleaning system. The plasma power was set at 150 w at 13.56 mHz. The PLLA film was placed between the two parallel plate electrodes and subjected to the glow discharge for 120 s at an Ar pressure of 0.4 Torr. Surface activated PLLA films were then exposed in air for 30 min to allow the formation of surface peroxides and hydroperoxides, and then were immersed in a solution of AA in MilliQ water with redox agents (sodium metabisulfite and potassium persulfate) for 5 h at room temperature. The AA-grafted PLLA, termed as PLLA-gAA, was rinsed with a 0.1 wt % Triton X-100 aqueous solution in an ultrasonic cleaner for 15 min and two subsequent rinses by MilliQ water. The biomolecules immobilization was carried out in water soluble carbodiimide (WSC) which was MES buffer solution (*p*H =6.5) in the presence of EDC and NHS. The PLLA-gAA films were kept in the WSC solution at room temperature for

2 h to preactivate the carboxylic groups introduced by AA polymerization. Then, gelatin or chitosan was added to initiate the covalent coupling reaction between carboxylic groups on PLLA-gAA and amine groups found in biomolecules. The reaction was carried out for 12 h at room temperature. After immobilization, PLLA-gAA-gelatin and PLLA-gAA-chitosan were rinsed with 0.1% Triton X-100 aqueous solution and subsequently with MilliQ water.

C. Surface characterization

1. X-ray photoelectron spectroscopy

The surface composition of various PLLA films was analyzed by XPS (Kratos AXIS Ultra) with monochromatic Al $K\alpha$ (1486.71 eV) x-ray radiation (15 kV and 10 mA). 160 eV pass energy was used for survey scans, whereas 40 eV was used for the high-resolution scans.

2. Contact angle measurement

The advancing contact angle was measured by a dynamic contact angle system (FTÅ200) with the FTÅ μ-tip® needles (ID=5 μm, OD=1 mm). The pump was programed at 0.1 μl/s to achieve a relatively low expansion or contraction volume in a pseudoequilibrium condition. The spreading of the drops was monitored, from the moment they detached from the needle, by a video recorder connected to a charge-coupled device camera and a microscope. Images of growing drop were then recorded by the computer with FTÅ32 software at a rate of a picture every 2 s. An advancing contact angle was defined as the one measured when the sessile drop had the maximum volume allowable for the liquid-solid interfacial area.

3. Surface density of carboxylic groups

The amount of carboxylic groups introduced by AA polymerization on PLLA was determined by the rhodamine-carboxyl interaction method. PLLA-gAA film was dissolved into an equal volume of benzene-dye reagent and the absorbance of the solution at 530 nm was measured using a spectrophotometer (Pharmaspec UV-1700 Shimadzu). The absorbance of bare PLLA was measured as background control. A standard curve was established using known concentrations of AA dissolved in the dye reagent as described above. The concentration of carboxylic groups in the solution was calculated from the standard calibration curve.

4. Gelatin surface density and stability

The surface density of gelatin was measured by the Bradford method according to Kang *et al.*[21] Coomassie dye binds protein in an acidic medium, and an immediate shift in absorption maximum occurs from 465 to 595 nm with a concomitant color change from brown to blue. In this study, gelatin solutions of known concentration were added to the dye solution. The gelatin-dye solutions were kept for 10 min and centrifuged at 15000 rpm for 20 min. In this process, protein-coomassie complexes were precipitated. The absorbance of supernatants at 465 nm was used for the standard calibration (supplementary data[23]). Incubating PLLA-gAA-gelatin with dye solution, protein coomassie complexes were formed on PLLA surface. The absorbance of supernatant dye solution was measured at 465 nm; the amount of gelatin immobilized on the PLLA surface was calculated based on standard calibration.

The stability of covalent immobilization of gelatin on PLLA was compared with gelatin coated onto PLLA (termed PLLA-gelatin), where the coating was done by immersing plasma-treated PLLA into 4% gelatin solution in 4% acetic acid for 12 h. Both PLLA-gAA-gelatin and PLLA-gelatin were immersed in PBS at 37 °C for 7 days. Gelatin coated on PLLA surface might dissolve into PBS due to hydrolysis or unstable absorption. The amount of residual gelatin was measured and compared with the initial one.

D. *In vitro* study

HUVECs were cultured in T25 or T75 flasks using BBE supplemented EGM in an incubator with 95% air/5% CO_2 at 37 °C. The EGM was changed every 2 days. Cells were harvested by trypsinization by 0.025% Trypsin-EDTA upon 90% confluency. Passages 4–6 were used.

1. Cell adhesion and proliferation

Unmodified and modified PLLA films were sterilized by immersing into 70% ethanol for 30 min, and then were rinsed with de-ionized water three times followed by PBS three times. PLLA scaffolds were then placed on the bottom of each cell culture plate well for cell seeding at density of 10^4 cells/cm^2. Cells were allowed to attach for 12 h. The unattached cells were rinsed by PBS. The attached cells were counted by WST-8 cell counting kit. Cell proliferation was monitored by WST-8 on days 1, 3, 5, and 7. Cell number at each time-point was calculated according to standard curve established 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). A standard curve was established by measuring absorbance of 450 nm at different cell density (supplementary data[23]). Cell doubling time (DT) of an exponential proliferation was calculated according to

$$\mathrm{DT} = \log(2)/\beta, \tag{1}$$

where β is the slop of proliferation curve plotted in logarithmic scale and log( ) is the natural logarithm.[24]

2. Cell imaging

Focal adhesion formation was observed by immunofluorescent imaging. After 12 h 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 10% goat serum in PBS for 30 min at room temperature. Paxillin was immunolabeled by mouse antihuman paxillin, 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 micro-

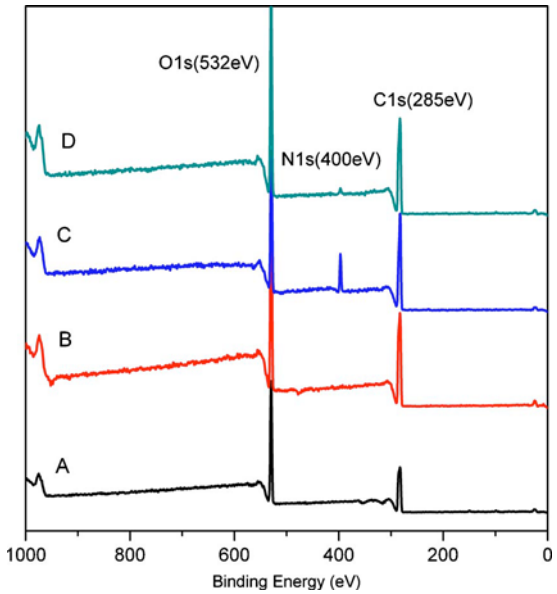

FIG. 2. (Color online) XPS survey spectra of (a) PLLA, (b) PLLA-gAA, (c) PLLA-gAA-gelatin, and (d) PLLA-gAA-chitosan.

scope (CLSM). Cell spreading area was quantified by IMAGE PRO PLUS software.

Endothelialization after 7 day incubation was observed by scanning electron microscopy (SEM) imaging. Cells grown on various PLLA substrates were fixed by 2.5% gluteraldehyde for 30 min and dehydrated by graded ethanol solutions. After gold coating, cells were observed by SEM (JEOL, JSM-6360).

E. Statistical analysis

Five specimens ($n=5$) were used in cell adhesion and proliferation study; three specimens ($n=3$) were used in other studies. The results were presented as the mean ± SD. At least 40 cells of each experimental group were analyzed for cell spreading area calculation. Each box encompasses 25–75 percentiles, with extending-lines covering the 95th and 5th percentiles, the middle line in the box representing the median (50th percentile), and the shorter line representing the mean values. Values outside the 95th and 5th percentiles were treated as outliers and were represented by dots. Surface-dependent cellular responses were analyzed using Students' *t*-test. A p value of less than 0.05 ($p<0.05$) was used to infer statistical significance of differences.

III. RESULTS

A. Surface characterization

1. Surface chemical composition

The surface chemical composition of the modified PLLA films was analyzed by XPS. Element survey spectra are plotted in Fig. 2. The measured oxygen-to-carbon (O/C) ratio and nitrogen-to-carbon (N/C) ratio are tabulated in Table I. The measured O/C ratio of bare PLLA surface was 0.59, which was in a good agreement to its theoretical value of 0.67.[25,26] The O/C ratio was remained similar after AA graft polymerization at 0.52, as poly(acrylic acid) (PAA) has the same theoretical O/C ratio of 0.67. No nitrogen peak was detected on PLLA, plasma-treated PLLA, and PLLA-gAA. The appearance of nitrogen was found only after immobilization of gelatin or chitosan on PLLA-gAA, indicating the successful immobilization of those biomolecules on PLLA surface. The N/C ratio is directly associated with the surface density of the immobilized gelatin or chitosan on PLLA substrate. From Table I, we noted that both PLLA-gAA-gelatin and PLLA-gAA-chitosan showed N/C ratios much smaller than their respective theoretic values. As the sampling depth of XPS in polymer matrix usually is about 7 nm, it could be concluded that the thickness of the immobilized gelatin and chitosan layers do not exceed 6–7 nm.[26]

The C 1*s* spectra were resolved into four characteristic peaks at 289.0, 288.1, 286.5, and 285 eV indicated the functional groups of O—C═O, N—C═O, C—O (C—N)), and (C—C) (C—H)), respectively (supplementary data[23]). The percentage of individual peak contribution is listed in Table I. As expected from the monomer structure, pristine PLLA gives a C 1*s* core spectrum consisting of three peaks of comparable area, which was consistent with results from other individual research groups.[25,26] After acrylic acid graft polymerization on PLLA, the C—O peak was slightly dropped and C—C was increased accordingly as expected from differences in monomer structure between acrylic acid and lactic acid. The O—C═O peak was attributed to the carboxylic side groups of PAA. It was noted that C—O peak took up 29.2% contribution in PLLA-gAA C 1*s* spectrum, which was not expected from acrylic acid monomer structure. That implied that the thickness of grafted acrylic acid

TABLE I. Atomic ratios with corresponding theoretic values indicated in parentheses and the percentage of functional groups on various surfaces by deconvoluted high-resolution C 1*s* spectra of (A) PLLA, (B) PLLA-gAA, (C) PLLA-gAA-gelatin, and (D) PLLA-gAA-chitosan.

	Atomic ratio		Contribution of C 1*s* components for surface modified PLLA			
Surface	O/C	N/C	C—C or C—H	C—O or C—N	N—C═O	O—C═O
A	0.59 (0.67)	0 (0)	38.5	31.0	···	30.5
B	0.52 (0.67)	0 (0)	40.4	29.2	···	30.4
C	0.35 (0.38)	0.12 (0.31)	33.8	26.1	24.4	15.7
D	0.47 (0.67)	0.03 (0.17)	38.7	36.4	···	24.9

TABLE II. Advancing contact angle of unmodified and modified PLLA films. $^{*}p<0.05$, referring to statistically significant difference in comparison with PLLA by Students' *t*-test.

Samples	Advancing angle
PLLA	82.3 ± 2.7
Plasma-treated PLLA*	35.5 ± 5.0
PLLA-gAA-gelatin*	52.8 ± 3.8
PLLA-gAA-chitosan*	30.7 ± 2.6

does not exceed 6–7 nm as well. With the immobilization of gelatin onto the surfaces, the carboxyl peak (O—C═O) decreased significantly and the amide peak (N—C═O) increased to 24.4% accordingly. The increment in amide peak (N—C═O) was mainly attributed to the contribution of amide bond found in the amino acid backbone of gelatin.[27] On PLLA-gAA-chitosan, a significant increase of C—O (C—N) peak coupled with a decrease in O—C═O peak was observed compared with the other PLLA substrates, which was expected from the structure of D-glucoamine unit found in chitosan.[25]

2. Surface wettability

The advancing contact angles (associated predominantly with surface wettability) of PLLA samples are shown in Table II. Unmodified PLLA is hydrophobic, having a measured advancing contact angle of 82.3°. After gelatin and chitosan immobilization, the contact angle was decreased to 52.8° and 30.7°, respectively. The better wettability of PLLA-gAA-chitosan was attributed to the presence of primary amide group (—NH_2) in each unit of D-glucosamine, which is positively charged in water. In contrast, gelatin contains a large amount of glycine (Gly) and proline (Pro) which are hydrophilic amino acids with neutral nonpolar side chains. Therefore, gelatin-modified PLLA exhibited a moderate wettability. The result suggests that biomolecules immobilization improved the PLLA surface wettability.

3. Surface density of carboxylic groups on PLLA-gAA

Carboxylic group concentration standard curve, shown in Fig. 3, was established by measuring the absorbance of an array of dye solutions mixing with known AA concentrations. The surface density of carboxylic group of PLLA-gAA was calculated to be 4.17 ± 0.15 μmol/cm^2, and was comparable with published results. Kang *et al.*[21] reported that their concentration of carboxylic acid density on AA-grafted PMMA (treated by oxygen plasma for 30 s) was 0.47–9.48 μmol/cm^2, depending on the pressure of the plasma chamber during the discharge treatment.

4. Surface density and stability of immobilized gelatin

In order to evaluate the efficiency of covalent immobilization, surface density of gelatin of PLLA-gAA-gelatin was measured and compared with that of PLLA-gelatin in which gelatin was physically adsorbed on PLLA. As shown in Fig. 4, gelatin surface density on PLLA-gAA-gelatin was

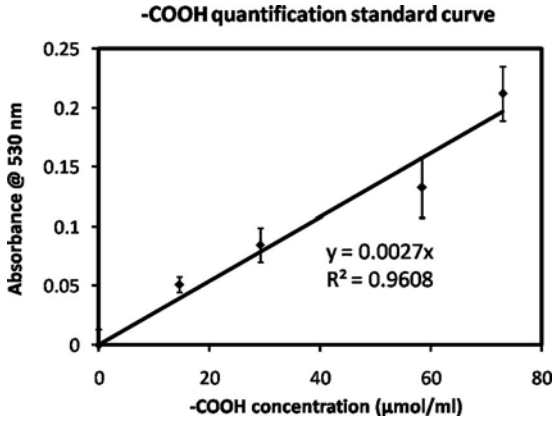

FIG. 3. Carboxylic group concentration standard curve measured by rhodamine-carboxyl interaction method.

4.8 μg/cm^2, which was significantly higher than that of PLLA-gelatin (1.5 μg/cm^2), implying the advantage of covalent immobilization over physical coating. The stability of immobilized gelatin was investigated by immersing PLLA-gAA-gelatin and PLLA-gelatin in PBS over 7 days. The surface density of gelatin on both surfaces was measured before and after immersion. Indicated in Fig. 4, the gelatin surface density on either PLLA-gelatin or PLLA-gAA-gelatin did not show significant loss after 7 day immersion in PBS (N.S. refers to $p>0.05$). However, in the physiological environment, it is likely that the physically adsorbed gelatin might be less stable, especially in the presence of enzymes and at different *p*H values.

B. *In vitro* study

1. Cell adhesion

Cell adhesion is the first event of cell-biomaterial interaction after adherent cells are plated on substrate, which is mediated by cell membrane receptors (mainly from the integrin superfamily). Upon cell adhesion, focal adhesion formation take place to provide cell anchorage points on substrate, which normally contain a multitude of cytosketletal and sig-

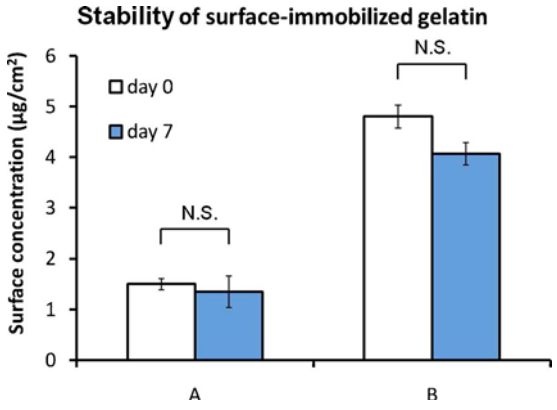

FIG. 4. (Color online) Surface density and stability of gelatin immobilized on PLLA, comparing physical coating of (a) PLLA-gelatin with covalent coupling of (b) PLLA-gAA-gelatin. N.S. refers to no significant difference.

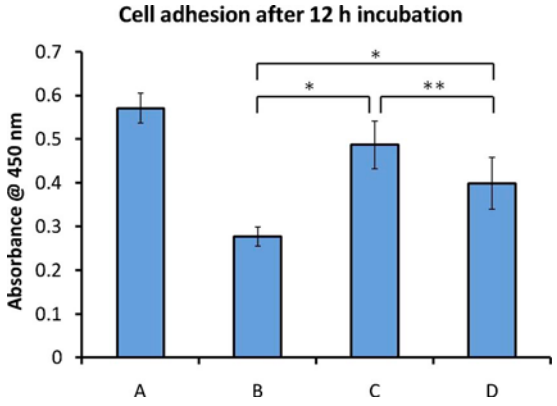

FIG. 5. (Color online) Cell adhesion after 12 h incubation at 37 °C in humidified air with 5% CO_2. Cell seeding density 10^4 cells/cm^2. Significance was tested by the Students' t-test. $^*p<0.05$, referring to statistically significant difference compared to PLLA. $^{**}p<0.05$, referring to statistically significant difference compared to PLLA-gAA-gelatin.

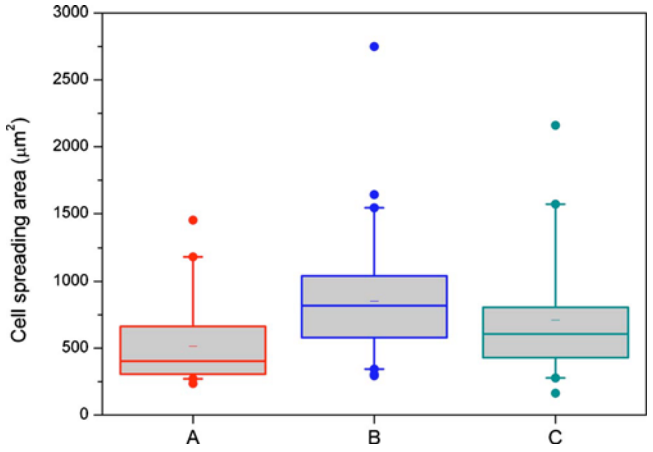

FIG. 6. (Color online) Cell spreading area observed on (a) PLLA, (b) PLLA-gAA-gelatin, and (c) PLLA-gAA-chitosan at 12 h postseeding. High resolution immunofluorescent images of individual cells were taken by Leica TCS SP5 CLSM and then analyzed by IMAGE PRO PLUS software. At least 40 cells were measured for each sample group.

nal transduction molecules that specifically accumulate at these sites.[28] One of the most representative protein markers is paxillin, which serves as a platform for the recruitment of numerous regulatory and structural proteins that together control the dynamic changes in cell adhesion, cytoskeletal reorganization, and gene expression.[29] Depending on the physical and chemical properties of substrates, cells exhibit different adhesion behavior. After 12 h incubation, unattached cells were rinsed off. The remaining adhered cells were evaluated for cell-adhesion behavior, in term of quantity of adhered cells, cell spreading area, and focal adhesion formation.

The number of adhered cell was assessed by WST-8 cell counting kit. According to its standard curve (supplementary data[23]), the absorbance of WST-8 reagent solution, which had been incubated with attached cells for 4 h, was linearly proportional to the cell number. The absorbance measured from different PLLA surfaces was plotted in Fig. 5. The TCPS, which is a cell-adhesion promoting surface, was treated as a reference. Compared with PLLA, PLLA-gAA-gelatin, and PLLA-gAA-chitosan enhanced cell adhesion ($^*p<0.05$). Moreover, PLLA-gAA-gelatin was found to be superior than PLLA-gAA-chitosan ($^{**}p<0.05$). Figure 5 suggested that cell adhesion was surface dependent. Biomolecule-modified PLLA improved cell adhesion.

Cell-adhesion behavior was further evaluated by cell spreading area, which measured the extent of cell spreading on a substrate. After 12 h of incubation, cells seeded on PLLA-gAA-gelatin exhibited the average spreading areas of 851 μm^2 and 711 μm^2 on PLLA-gAA-chitosan, as indicated in Fig. 6. While the spreading area found on PLLA was 515 μm^2, which was significant smaller than that of both modified PLLA substrate (Students' t-test, $p<0.05$). That suggested biomolecule-immobilized PLLA substrates promoted cell spreading.

Focal adhesion formation is another criterion to evaluate the quality of cell adhesion. After 12 h incubation, cells were fixed and paxillin was immuno labeled. Images captured by CLSM on different PLLA substrates were shown in Fig. 7. A limited number of focal adhesions (pointed by arrows) were found in cells grown on PLLA. However, the number of focal adhesion was increased in cells on both biomolecule-immobilized PLLA substrates. Especially cells on PLLA-gAA-gelatin shown in Fig. 7(c), the abundant and well-organized focal adhesions observed throughout the cell suggested the matured and strong cell adhesion on PLLA-gAA-gelatin.

Conclusively, surface modification by gelatin and chitosan immobilization on PLLA not only enhanced the number of cells adhered on substrates, but also improved cell spreading and focal adhesion formation.

2. Cell proliferation and endothelialization

The attached cells were allowed to proliferate in BBE-supplemented EGM for 7 days. Figure 8 shows the cell proliferation profile on various PLLA substrates as a function of culture duration. The doubling time observed on each substrate is presented in Table III. Cell proliferation profile on TCPS was taken as a reference. Cells grown on TCPS and PLLA-gAA-gelatin proliferated exponentially with doubling

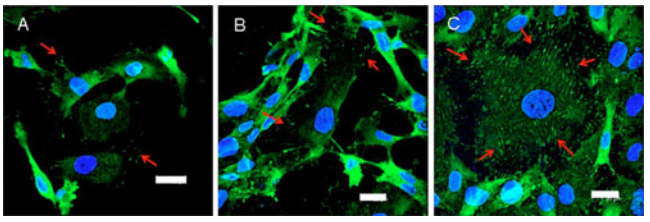

FIG. 7. (Color online) Focal adhesion formation, as denoted by arrows, exhibits surface dependence. Cells were seeded on (a) PLLA, (b) PLLA-gAA-chitosan, and (c) PLLA-gAA-gelatin at a seeding density of 10^4 cell/cm^2. After 12 h culture, unattached cells were removed by PBS rinsing. The remaining cells were fluorescently stained to label paxillin and nuclei. The images were captured by Leica TCS SP5 CLSM. Scale bar $=20$ μm.

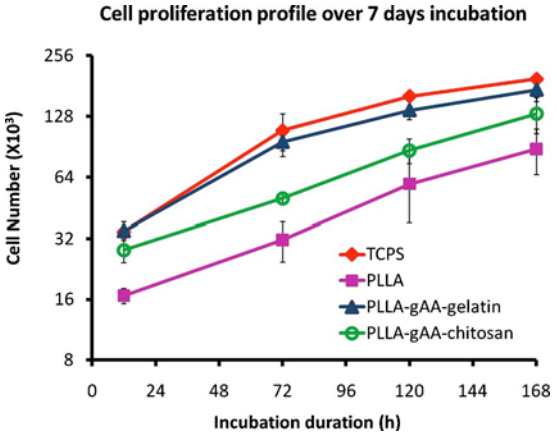

FIG. 8. (Color online) HUVEC proliferation profile cultured over 7 days at 37 °C in humidified air with 5% CO_2. Cell seeding density is 10^4 cells/cm^2.

time of about 16–18 h. Then cell proliferation slowed down after 72 h with a much longer doubling time of about 50 h. It is believed that contact inhibition took place as cell density increased to about 6×10^4 cell/cm^2 on both surfaces at 72 h. In contrast, cells grown on PLLA-gAA-chitosan and PLLA proliferated exponentially with a constant doubling time until 168 h of incubation. The doubling time on PLLA-gAA-chitosan was marginally shorter than that on PLLA. The cell proliferation study suggests that cell proliferation rate was discriminative with respect to surface chemistry. Gelatin immobilization shortened the doubling time by a factor of 2. Only marginal improvement on proliferation was found on PLLA-gAA-chitosan. After 7 day incubation, cells grown on different PLLA substrates were fixed and observed by SEM (Fig. 9). Complete endothelialization was found on PLLA-gAA-gelatin and PLLA-gAA-chitosan. HUVECs were well spread and formed monolayer of endothelium. However, endothelialization was absent on PLLA.

IV. DISCUSSION

The biological response to biomaterials and devices is controlled largely by their surface chemistry and topology. Hence, the rationale for surface modification of biomaterials is to retain the key physical properties of biomaterials while modifying only the surface to influence the bio-interaction. In order to improve endothelial cell-interaction with PLLA substrates and to promote complete endothelialization, a three-step surface modification was developed and evaluated in this study. With tunable conditions, plasma surface treatment provides a controllable, flexible and uniform surface modification even with complicated surface conformation. In addition, plasma surface treatment only cause changes to a limited depth, while being able to introduce active functional groups on a polymeric surface. In this study, PLLA surface was treated by Ar plasma, then followed by acrylic acid graft polymerization. By this way, carboxylic groups were incorporated on PLLA surface and enabled subsequent biomolecules immobilization through covalent coupling.

TABLE III. Doubling time (h) of various surfaces (A) TCPS, (B) PLLA, (C) PLLA-gAA-gelatin and (D) PLLA-gAA-chitosan.

	A	B	C	D
12–72 h	15.8	31.1	17.9	28.5
72–168 h	49.3	29.9	48.5	28.1

Gelatin and chitosan were selected to modify the PLLA surface in this study because of their chemical and structural similarities with type IV collagen and glycosaminoglycans (GAGs), respectively, which are the main components of basal lamina beneath the vascular endothelium. It is well known that the incorporation of gelatin can enhance biocompatibility of materials because gelatin contains cell-adhesion amino acid sequences such as RGD that can bind cell surface receptors.[30] Chitosan is also one of the common naturally occurring biomolecules that can potentially enhance endothelial cell affinity.[31] Furthermore, gelatin and chitosan are cost-effective model biomolecules. The surface modification method could be extended to extracellular matrix proteins, such as collagen IV and laminin,[32] and to cell-adhesion motifs, such as RGD (Ref. 33) and REDV.[34]

Surface characterization analyses, including XPS, contact angle measurement, and surface density of function groups, had provide strong evidences that the three-step surface modification method developed in this study was able to immobilize biomolecules onto PLLA surface, resulting in dif-

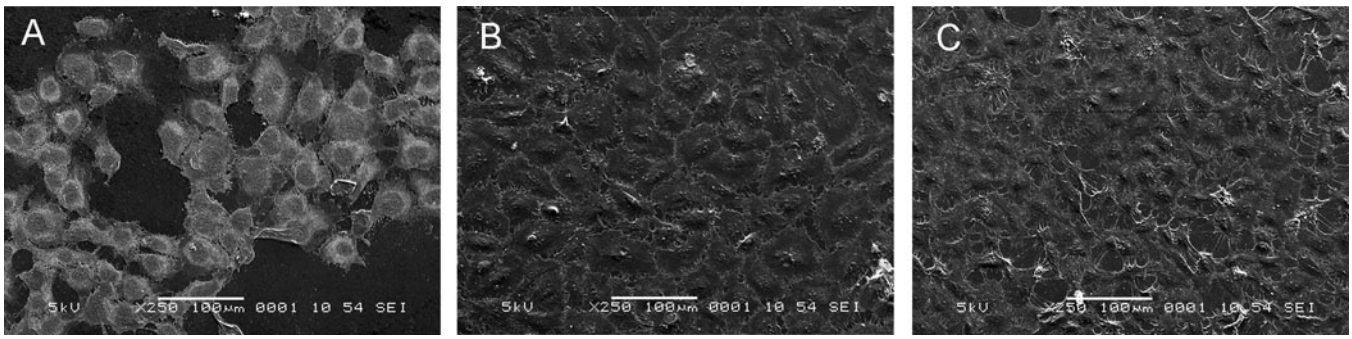

FIG. 9. Cell morphology observed on (a) PLLA, (b) PLLA-gAA-gelatin, and (c) PLLA-gAA-chitosan by SEM at day 7. Complete endothelialization was observed on both modified PLLA substrates, but not on PLLA substrate. Scale=100 μm.

ferent surface properties while preserving the bulk physical properties. After step II of AA graft polymerization, the incorporation of carboxylic groups was quantified by rhodamine-carboxyl interaction method, and the surface density was calculated to be 4.17 ± 0.15 μmol/cm^2. After step III of immobilization gelatin/chitosan, nitrogen peaks appeared in the XPS spectra of PLLA-gAA-gelatin and PLLA-gAA-chitosan, while being absent in that of PLLA and PLLA-gAA, indicating successful immobilization was achieved. The approach of covalent coupling of biomolecules on PLLA-gAA surface was proved to produce a surface with higher gelatin surface density than that by physical adsorption of gelatin. The amount of immobilized gelatin on PLLA-gAA-gelatin, as shown in Fig. 4, was twofold of that on PLLA-gelatin. Moreover, negligible loss of gelatin was observed on PLLA-gAA-gelatin, proving stability of the surface modification method. Contact angle measurement showed in Table II indicated that immobilization of gelatin and chitosan increased surface wettability, which has been shown to be one of important surface properties affecting cell-substrate adhesion.[35]

The modified PLLA substrate was further evaluated by *in vitro* study of endothelial cell interactions. Results suggested that immobilizing gelatin and chitosan on PLLA improves cell adhesion (Fig. 5), spreading (Fig. 6) and focal adhesion formation (Fig. 7) after 12 h incubation, and promote cell proliferation (Fig. 8) and complete endothelialization (Fig. 9) observed over 7 days. PLLA-gAA-gelatin improved cell adhesion by almost 50% compared with bare PLLA and shortened the doubling time by 10 h. The cell spreading and focal adhesion formation were improved significantly by gelatin-modified PLLA. At initial seeding density of 10^4 cells/cm^2, endothelialization was achieved on PLLA-gAA-gelatin within 7 days.

Two facts are needed to understand prior to the discussion of cell-biomaterials interaction. First, protein adsorption from body fluids *in vivo* or from cell culture medium *in vitro* on the biomaterial surfaces always takes place before cells arrive. Second, cell-substrate interaction is mediated by affinity binding of integrins to cell-adhesion ligands. Such ligands could be present in proteins or other biomolecules that have been spontaneously adsorbed onto the material surface, or they could be present after intentional immobilization upon the material surface.

In the case of unmodified PLLA, cell adhesive proteins such as fibronectin and vitronectin from medium serum were adsorbed on the surface. However, the stronger protein binding and unfolding of protein due to the dehydration mechanism on hydrophobic surface are probably casing conformation changes in the protein molecules leading to a decreased accessibility of specific epitopes on PLLA.[35] Consequently, only a small number of integrin was able to activated through binding with cell-adhesion domains, leading to less focal adhesions observed on PLLA than that on two modified PLLA substrates (Fig. 7).

In the case of PLLA-gAA-gelatin, immobilized gelatin containing integrin-binding domains was present on surface with a density of 4.8 μg/cm^2, providing cell-adhesion sites. Moreover, PLLA-gAA-gelatin showed a contact angle of 52.8°, which was in the wettability range favorable for cell adhesion. Studies have shown that cells effectively adhere onto polymer surfaces presenting moderate wettability with water contact angles of 40°–70°.[36,37] In contrast to hydrophobic PLLA, adsorbed serum proteins were loosely bond. The accessibility of specific domains was increased through remodeling of this adsorbed proteins.[35] The presence of both immobilized gelatin and adsorbed proteins was believed to improve cell affinity, resulting in better cell spreading and abundant focal adhesions observed on PLLA-gAA-gelatin in Fig. 7.

Unlike gelatin containing cell-adhesion domains, chitosan is considered as positively charged molecule. Hence, PLLA-gAA-chitosan appeared to be the most hydrophilic surface with contact angle of 30.7°. The enhanced cell adhesion in the first 12 h might be due to the electrostatic interaction between cell surfaces, which are usually negatively charged, with the positively charged PLLA-gAA-chitosan surface. However, proliferation rate (i.e., doubling time) was improved only marginally on PLLA-gAA-chitosan. The positive charged surface is able to attract cell during the cell-adhesion stage, but the electrostatic force between cell surface and substrate might reduce cell motility and hinder cell proliferation. Although proliferation rate of PLLA-gAA-chitosan was similar to that of PLLA, endothelialization was achieved within 7 days in this study on PLLA-gAA-chitosan but not on PLLA. It was largely attributed to the improved cell adhesion achieved at initial stage. This implied the importance of cell adhesion in studying cell-biomaterial interaction and its further biomedical applications. Other groups have reported similar observations in their study of immobilization of gelatin and chitosan to improve polymer cytocompatibility by other means.[16]

Collectively, by introducing biomolecules on PLLA surface, surface treatment not only changed the surface chemistry but also improved surface wettability. Both factors contributed to better cell affinity on modified PLLA surface which led to successful endothelialization.

V. CONCLUSIONS

A mild and easily controlled surface modification method, involving argon-plasma treatment, acrylic acid graft polymerization, and covalent immobilization of biomolecules, is reported to improve biocompatibility of biodegradable lactic/glycolic acid polymers. Surface characterization analyses via XPS, contact angle measurement, and colorimetric methods for surface density of functional groups have proven the feasibility and stability of this three-step surface modification method. Results from *in vitro* study using HUVECs have shown better cell affinity of both modified PLLA substrates in comparison with PLLA, by improving cell adhesion, spreading, and focal adhesion observed at 12 h, as well as by promoting cell proliferation and complete endothelialization observed over 7 days. Gelatin-modified PLLA was found to be superior over chitosan-modified PLLA. It has been largely

attributed to the presence of cell affinity motifs in gelatin molecules and the moderate wettability of its resultant surface. Conclusively, this work demonstrated the feasibility of the three-step surface modification method and its ability to promote complete endothelialization for cardiovascular applications.

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[23]See supplementary material at E-BJIOBN-5-003003 for (1) Fig. 1 gelatin concentration standard curve by Bradford method; (2) Fig. 2 cell number standard curve measured by WST-8 cell counting kit; (3) Fig. 3 the C1S spectra of (A) PLLA, (B) PLLA-gAA, (C) PLLA-gAA-gelatin, and (D) PLAA-gAA-chitosan resolved into four characteristic peaks at 282.2, 288.1, 286.5, and 285 eV indicated the functional groups of O-C=O, N-C+O, C-O (C-N), and C-C, respectively.
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