Adhesion, proliferation, and gene expression profile of human umbilical vein endothelial cells cultured on bilayered polyelectrolyte coatings composed of glycosaminoglycans[a)]

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This study characterized human umbilical vein endothelial cell (HUVEC) adhesion, proliferation, and gene expression on bilayered polyelectrolyte coatings composed of an outermost layer of glycosaminoglycans (hyaluronan, heparin, or chondroitin sulfate), with an underlying layer of poly-L-lysine or chitosan. The proportion of cells that adhered to the various polyelectrolyte coatings after 1 and 2 h incubations was quantified by the WST-8 assay. Interchanging poly-L-lysine with chitosan resulted in significant differences in cellular adhesion to the outermost glycosaminoglycan layer after 1 h, but these differences became insignificant after 2 h. The proliferation of HUVEC on the various bilayered polyelectrolyte coatings over 10 days was characterized using the WST-8 assay. Regardless of whether the underlying layer was poly-L-lysine or chitosan, HUVEC proliferation on the hyaluronan outermost layer was significantly less than on heparin or chondroitin sulfate. Additionally, it was observed that there was more proliferation with poly-L-lysine as the underlying layer, compared to chitosan. Subsequently, real-time polymerase chain reaction was used to analyze the expression of seven genes related to adhesion, migration, and endothelial function (VWF, VEGFR, VEGFA, endoglin, integrin-α5, ICAM1, and ICAM2) by HUVEC cultured on the various bilayered polyelectrolyte coatings for 3 days. With poly-L-lysine as the underlying layer, biologically significant differences (greater than twofold) in the expression of VWF, VEGFR, VEGFA, endoglin, and ICAM1 were observed among the three glycosaminoglycans. With chitosan as the underlying layer, all three glycosaminoglycans displayed biologically significant differences in the expression of VWF and VEGFR compared to the chitosan control. CT-HA displayed the highest level of expression of VWF, whereas expression levels of VEGFR were almost similar among the three glycosaminoglycans. © *2010 American Vacuum Society.* [DOI: 10.1116/1.3483218]

I. INTRODUCTION

In recent years, there has been growing interest in utilizing multilayered polyelectrolyte films to immobilize biologically active proteins and drug-loaded nanoparticles on the surface of biomaterial implants.[1,2] These are fabricated through layer-by-layer deposition of high molecular weight polymers with opposing charges.[3,4] A particularly promising area for the therapeutic application of multilayered polyelectrolyte films is in the coating of drug-eluting coronary stents[5] as an attempt to prevent restenosis after percutaneous transluminal angioplasty.[6,7] For this purpose, it is essential that the stent coating must not only be biocompatible but should also be a surface that is conducive for the adhesion of endothelial cells so as to facilitate healing of the blood vessel and prevent restenosis and thrombosis.

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TABLE I. Gene markers examined by quantitative RT-qPCR.

	Gene marker	Putative function	References
Markers of endothelial functionality			
	VEGFA	A key cytokine/growth factor involved in the regulation of angiogenesis	Ylä-Herttuala *et al.*[a]
	VEGFR	VEGF receptor	Smadja *et al.*[b]
	Von Willebrand Factor (VWF)	Platelet adhesion to wound sites: binding to factor VIII	de Meyer *et al.*[c]
	Endoglin (CD105)	Endothelial cell proliferation, angiogenesis, and vascular remodeling	Fonsatti *et al.*[d]
Adhesion and migration markers	Integrin $\alpha 5$ subunit	Focal adhesion points	Liu *et al.*[e]
	ICAM1 (CD54)	Cell migration	Van de Stolpe *et al.*[f]
	ICAM2	Expressed at the endothelial junctions, also mediates angiogenesis	Melero *et al.*[g]

[a]Reference 63.
[b]Reference 67.
[c]Reference 60.
[d]Reference 68.
[e]Reference 71.
[f]Reference 64.
[g]Reference 72.

Nevertheless, the interactions of endothelial cells with various polyelectrolytes are currently not well-characterized with respect to their biocompatibility and ability to serve as a substratum for cellular adhesion. Of particular interest are the various glycosaminoglycans (negatively charged polyelectrolytes) together with poly-L-lysine and chitosan (positively charged electrolytes). The glycosaminoglycans are a class of high-molecular polysaccharide molecules with many interesting biological properties. For example, heparin is known to inhibit thrombosis[8] and chondroitin sulfate has been demonstrated to promote wound healing,[9,10] while hyaluronan is known to be a mediator of both wound-healing and inflammation.[11] Such biologically relevant properties would certainly be useful for incorporation within the coatings of coronary drug-eluting stents. Indeed, there have been numerous studies on the formulation of stent coatings with various combinations of heparin, hyaluronan, and chondroitin sulfate,[12–16] together with chitosan[17] and poly-L-lysine.[18]

Hence, in this study, we fabricated bilayered polyelectrolyte coatings of various combinations of negatively charged glycosaminoglycans overlaid on either positively charged poly-L-lysine or chitosan on tissue culture polystyrene (TCPS) surface. The TCPS surface, being negatively charged, is unable to bind directly to the negatively charged glycosaminioglycans, but must instead be bound first to either positively charged poly-L-lysine or chitosan prior to being overlaid with the various glycosaminoglycans. The presence of the various polyelectrolytes bound to the TCPS surface was confirmed by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, as well as atomic force microscopy (AFM). Subsequently, the study characterized HUVEC adhesion and proliferation on the various polyelectrolyte coatings with the WST-8 assay. In addition, expression profiles of seven gene markers (VWF, VEGFR, VEGFA, Endoglin, Integrin-$\alpha 5$, ICAM1, and ICAM2) related to adhesion, migration, and endothelial functions were analyzed by quantitative real-time polymerase chain reaction (RT-qPCR). The putative functions of these seven gene markers are listed in Table I.

II. MATERIALS AND METHODS

A. Cell lines, culture media, and reagents

HUVECs were purchased from Lonza Inc. (Walkersville, MD). Unless otherwise stated, all reagents and chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO), all culture media and supplements were purchased from Lonza Inc. (Walkersville, MD), while all labware consumables were purchased from Techno Plastic Products Inc. (Trasadingen, Switzerland).

B. HUVEC culture

HUVECs were shipped in cryovials under dry ice and were stored immediately in liquid nitrogen upon arrival. Subsequently, the cells were thawed and cultured within T-75 flasks at a density of 2.0×10^3 cells/cm^2. Upon reaching confluence, the cells were detached with 0.25% (w/v) bovine trypsin with 1 mM EDTA (Gibo-BRL Inc., Franklin Lakes, NJ) and replated on new T-75 flasks at a passage split ratio of 1:3. The culture medium utilized was endothelial growth media (EGM, Cat No. CC-3121; Lonza Inc., Walkersville, MD) supplemented with 4% (v/v) fetal bovine serum and 0.4% (v/v) bovine brain extract (Cat No. CC-4133; Lonza Inc., Walkersville, MD). The primary explanted HUVEC cells were at P0 upon arrival and were expanded up to P7 or P8 prior to being utilized for experiments.

C. Preparation of polyelectrolyte coatings for endothelial adhesion and proliferation studies

Chitosan (water soluble grade, 85% deacetylated, M.W. ≈25 KDa, Cat No. 9012-76-4, Sinopharm Co Ltd., Shanghai, China) and poly-L-lysine (hydrobromide salt, M.W. ≈20–30 KDa, Cat No. 81333, Fluka Chemie AG., Buchs, Switzerland) were constituted in de-ionized water at a concentration of 1 mg/ml, and subsequently coated onto 12-well culture dishes (≈4.8 cm^2 per well, 0.2 ml per well) by incubation for 24 h at 37 °C. Subsequently, the chitosan and poly-L-lysine coated culture dishes were rinsed three times with phosphate buffered saline (PBS) prior to the addition of 0.2 ml of 1 mg/ml hyaluronan (Healon-5™, M.W. ≈5000 KDa, Cat No. 10–2030–15, Advanced Medical Optics Inc., Uppsala, Sweden), heparin (Cat No. H4784, Sigma-Aldrich Inc.), or chondroitin sulfate (Cat No. 27042, Sigma-Aldrich Inc.). After incubation for a further 24h at 37 °C, the culture dishes were rinsed again thrice prior to being utilized for the endothelial adhesion or protein adsorption study. Altogether, there were eight experimental groups, as follows: chitosan (CT), chitosan-hyaluronan (CT-HA), chitosan-heparin (CT-HP), chitosan-chondroitin sulfate (CT-CS), poly-L-lysine (PLL), poly-L-lysine-hyaluronan (PLL-HA), poly-L-lysine-heparin (PLL-HP) and poly-L-lysine-chondroitin sulfate (PLL-CS).

D. ATR-FTIR characterization of polyelectrolyte coatings

The preparation of bilayered polyelectrolyte coatings for the purpose of ATR-FTIR was similar to what was described previously. However, rinsing was carried out in de-ionized water instead of PBS (due to the presence of various salts in PBS that might confound the ATR-FITR data). After rinsing, the samples were left to air-dry for 24 h at room temperature, prior to ATR-FITR analysis. Altogether, there were a total of 6 experimental groups including two controls for the ATR-FITR analysis. These were PLL-HA, PLL-HP, PLL-CS, CT-HA, CT-HP, and CT-CS; PLL and CT were controls. In addition, 1 mg/ml solutions of HA, HP, and CS were added directly on TCPS surfaces and left to air-dry for 24 h at room temperature. These three samples were not rinsed, since they were used as standards for later characterizations. ATR-FITR analysis was carried out with the Spectrum 200 FTIR spectrometer (Perkin Elmer Inc., Waltham, MA). All spectra were collected at a resolution of 4 cm^{-1} and for 16 scans. TCPS was scanned as background. Data at frequencies of 4000–800 cm^{-1} were collected. Results from each data set were expressed as absorbance versus wave number. Unrinsed HA, HP, and CS were scanned as standards for characterizations of the six experimental groups (excluding the two controls).

E. AFM characterization of polyelectrolyte coatings

The preparation of bilayered polyelectrolyte coatings for the purpose of AFM was similar to what was described previously. However, rinsing was carried out in de-ionized water and the samples were subsequently dried with nitrogen gas. A commercial AFM instrument (Dimension 3100 with Nanoscope IIIa controller, Veeco Instruments Inc., Fremont, CA) equipped with a scanner (90×90 μm^2) was employed. The tapping mode in air was performed to observe the various bilayered polyelectrolyte coatings on TCPS. Supersharp silicon cantilevers with the normal resonance frequency of 330 kHz and spring constants of 42 Nm^{-1} (SSS-NCH, Nanosensors) were used. All images were captured with a scan rate at 1–2 Hz and 512×512 pixel resolution.

F. HUVEC adhesion on polyelectrolyte coatings

HUVEC cells were seeded at a density of 5000 cells/cm^2 on 12-well tissue culture dishes (≈4.8 cm^2 per well) for all experimental groups. The culture media utilized was Endothelial Growth Medium (Lonza Inc.) supplemented with 4% (v/v) fetal bovine serum and 0.4% (v/v) bovine brain extract. The cells were allowed to attach for either 1 or 2 h within a humidified 5% CO_2 incubator set at 37 °C. Subsequently, the unattached cells were rinsed away with PBS, and the density of adherent cells was quantified with the cell counting kit (CCK-8) purchased from Dojindo Molecular Laboratories Inc. (Kumamoto, Japan), according to the manufacturer's protocol. Adherent cells were incubated for 2 h with 10% (v/v) WST-8 reagent[19] supplemented in culture media (250 μl per well of 12-well plate) prior to absorbance readings at 450 nm with a microplate reader. Standard curves were established by measuring absorbance at 450 nm for HUVEC cells at varying densities. The density of adherent cells was calculated accordingly by reading off from the standard curves. The percentage of adherent cells was calculated according to the formula $N_{\text{adherent}}/N_{\text{seeding}}\times100\%$, where N_{adherent} is the density of adherent cells after 1 or 2 h, and N_{seeding} is the initial cell seeding density (i.e., 5000 cells/cm^2).

G. HUVEC proliferation on polyelectrolyte coatings

HUVEC were seeded on the various polyelectrolyte coatings (CT, CT-HA, CT-HP, CT-CS, PLL, PLL-HA, PLL-HP, and PLL-CS) at a fixed seeding density of 2500 cells/cm^2 on 12-well tissue culture dishes (≈4.8 cm^2 per well) and incubated for a period of 10 days within a humidified 5% CO_2 incubator set at 37 °C. The culture medium utilized was endothelial growth medium (Lonza Inc.) supplemented with 0.4% (v/v) bovine brain extract. Phase-contrast microscopy images of adherent HUVEC on the various polyelectrolyte coatings were captured on day 1. On days 1, 5, and 10, the density of adherent cells was quantified with the cell counting kit (CCK-8) purchased from Dojindo Molecular Laboratories Inc. (Kumamoto, Japan). Adherent cells were incubated for 2 h with 10% (v/v) WST-8 reagent[19] supplemented in culture media (250 μl per well of 12-well plate) prior to absorbance readings at 450 nm with a microplate reader. Densities of adherent cells were calculated from standard curves.

TABLE II. Primer sequences of gene markers examined by quantitative RT-qPCR.

	Gene		Primer sequences
Markers of endothelial function	VEGFA	F	CGAGGCAGCTTGAGTTAA
		R	CTGTATCAGTCTTTCCTGGTG
	VEGFR	F	GGGAAAGCATCGAAGTCTC
		R	CTCTGCGGATAGTGAGGTT
	VWF	F	ACCACTCCTTCTCCATTGTC
		R	CCCCATGCTTCAGTTTCA
	Endoglin	F	GCCAGCATTGTCTCACTT
		R	GGCACACTTTGTCTGGAT
Adhesion and migration markers	Integrin-α5 subunit	F	ACCCTGCTCATCCAGAAT
		R	GAGAAGTTGAGAGCGATGTG
	ICAM1	F	CCCATGAAACCGAACACA
		R	GGCATATGTCTTCCACTCTG
	ICAM2	F	CTTGGTCTCAAACATCTCCC
		R	CAAAGTGGGTTGCAGTGT
Housekeeping gene	RPL27	F	ATCGCCAAGAGATCAAAGATAA
		R	TCTGAAGACATCCTTATTGACG

H. Cell culture for RT-qPCR analysis

HUVEC were seeded onto the various polyelectrolyte coatings (CT, CT-HA, CT-HP, CT-CS, PLL, PLL-HA, PLL-HP, and PLL-CS) at a fixed seeding density of 2500 cells/cm^2 in 12-well culture plates ($\approx$4.8 cm^2 per well) and cultured for 72 h in a humidified 5% CO_2 incubator set at 37 °C. The culture medium utilized was endothelial growth medium (Lonza Inc.) supplemented with 0.4% (v/v) bovine brain extract. Subsequently, the cells were washed in phosphate buffered saline (PBS), trypsinized, and collected by centrifugation and subsequently flash-frozen in liquid nitrogen in 1.5 ml microcentrifuge tubes (Eppendorf GmbH, Hamburg, Germany). Cell counts within each experimental group ranged from 50 000 to 200 000 cells.

I. RNA isolation, reverse transcription, and quantitative real-time PCR

RNeasy Plus Micro Kit (Qiagen Inc., Valencia, CA) was used to extract total RNA from the HUVECs, according to the recommended protocol provided by the manufacturer. The RNA collected was kept on ice and immediately used for cDNA synthesis. Amplified RNA was reverse-transcribed using the SuperScript ViLo cDNA synthesis kit (Invitrogen Inc., Carlsbad, CA). The reaction mixture was topped up with DEPC-treated water to a total volume of 20 μl. The solution was incubated at 25 °C for 10 min, followed by 42 °C for 120 min, and finally at 85 °C for 5 min. The synthesized cDNA was then further diluted to be used for real-time PCR analysis. Each real-time PCR reaction included 1 μl of diluted cDNA solution, 8.16 μl of DEPC-treated water, 0.8 μl of forward and reverse primer mix (10 μM), 0.04 μl of ROX reference dye, and 10 μl of SYBR GreenER real-time PCR mix (Invitrogen Inc., Carlsbad, CA). Reactions were carried out in triplicates in an Applied BioSystems 7500 real time PCR system (Applied BioSystems Inc., Foster City, CA) under standard cycling conditions. The endogenous control gene RPL27 (60S ribosomal protein L27) reactions were run within every single reaction plate in parallel with other reactions. The primer sequences of the genes examined in this study are listed in Table II. The gene expression data for all bilayered polyelectrolyte coatings that utilized CT as the underlying layer (CT-HA, CT-HP, and CT-CS) were normalized against CT as the reference sample. Likewise, the gene expression data for all bilayered polyelectrolyte coatings that utilized PLL as the underlying layer (PLL-HA, PLL-HP, and PLL-CS) were normalized against PLL.

J. Statistical analysis of data

The results from the cell proliferation assay were expressed as mean $\pm$ standard deviations ($n=3$ for all data sets). Statistical differences between data sets were assessed by the Student's t-test, with a p-value less than 0.05 being considered significantly different. For the RT-qPCR analysis, a more than twofold change in gene expression in relation to the reference sample was considered to be biologically significant.

III. RESULTS

A. ATR-FITR

As seen in Fig. 1, both PLL-HA and CT-HA show two characteristic peaks at 1616 and 1413 cm^{-1} due to asymmetric and symmetric stretching of the planar carboxyl groups on HA respectively, as well as another characteristic peak at 1046 cm^{-1} related to the stretching of the carbohydrate group on HA.[20,21] For PLL-HP and CT-HP (Fig. 2), a characteristic peak at 1418 cm^{-1} related to a weaker stretching of carboxylate groups on HP is observed.[22,23] Additional characteristic peaks observed at 1245 and 1028 cm^{-1} are due to

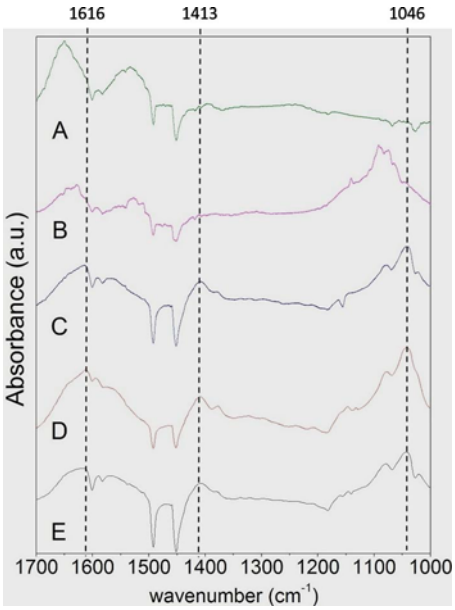

FIG. 1. (Color online) ATR-FTIR characterization curves for (a) PLL, (b) CT, (c) HA, (d) PLL-HA, and (e) CT-HA coatings on TCPS.

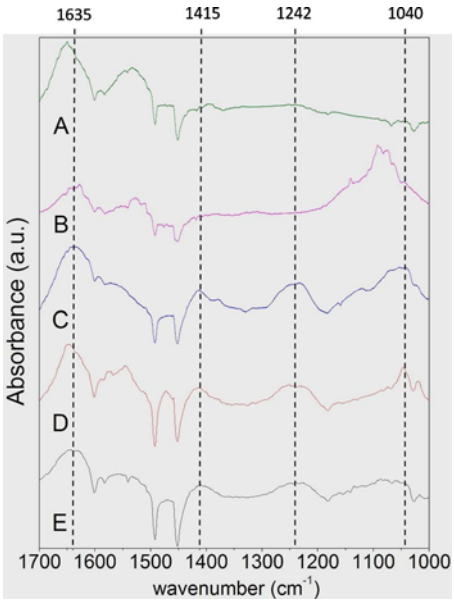

FIG. 3. (Color online) ATR-FTIR characterization curves for (a) PLL, (b) CT, (c) CS, (d) PLL-CS, and (e) CT-CS coatings on TCPS.

the SO_3 groups of HP.[22,23] For PLL-CS and CT-CS (Fig. 3), distinctive peaks at 1635 cm^{-1} (amide I band) and 1242 cm^{-1} (amide III band) characteristic of CS are observed.[24] Additional characteristic peaks at 1415 and 1040 cm^{-1} are related to the stretching of saccharide alkyl and alkoxyl groups on CS.[25] The ATR-FITR data thus positively confirmed the presence of bound glycosaminoglycan molecules on the TCPS surface.

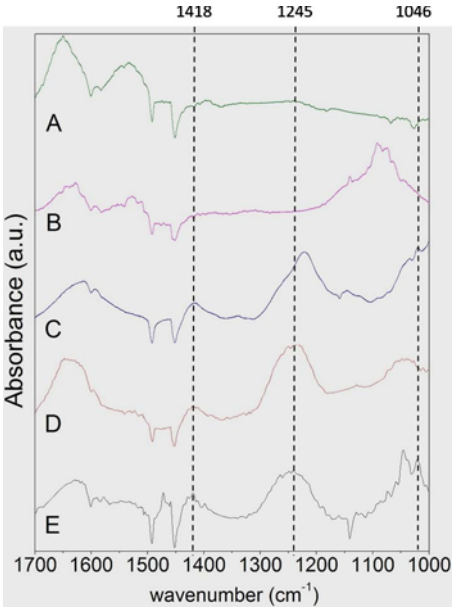

FIG. 2. (Color online) ATR-FTIR characterization curves for (a) PLL, (b) CT, (c) HP, (d) PLL-HP, and (e) CT-HP coatings on TCPS.

B. AFM

As seen in Fig. 4, AFM confirms the deposition of the various polyelectrolytes on the TCPS (tissue culture polystyrene) surface. Although there may not be complete surface coverage, and the glycosaminoglycans may be concentrated within isolated nanosized droplets for some samples [i.e., Figs. 4(e), 4(f), and 4(h)], the surface coverage is relatively homogenous as a whole within a fixed 5×5 μm^2 area (Fig. 4). This is much smaller than the typical dimensions of an endothelial cell, so it can be assumed that the cells make contact with a relatively homogenous surface for all coated samples.

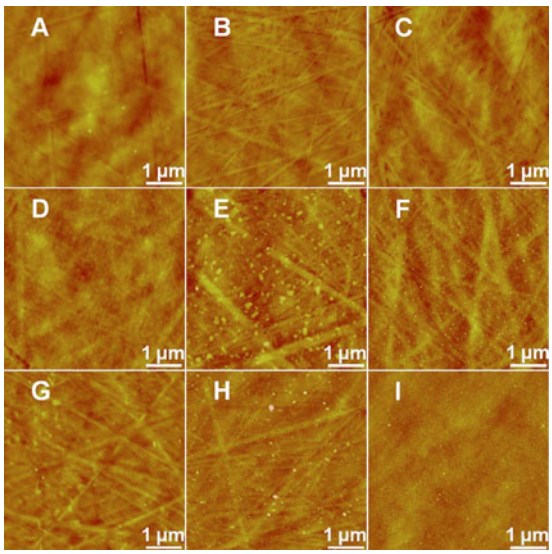

FIG. 4. (Color online) AFM images of TCPS (tissue culture polystyrene) coated with (a) blank, (b) PL, (c) CT, (d) PL-HA, (e) PL-HP, (f) PL-CS, (g) CT-HA, (h) CT-HP, and (i) CT-CS. Scale bar represents 1.0 μm.

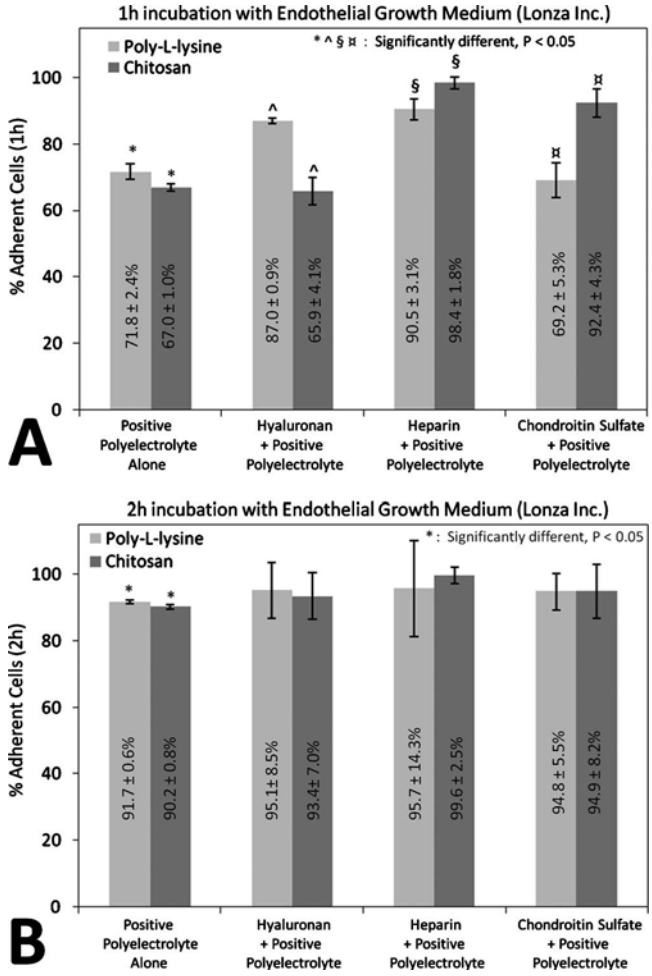

FIG. 5. Percentages of HUVEC cells that adhere onto the various polyelectrolyte coatings after incubation for 1 h (a) and 2 h (b) in commercially available endothelial growth medium, supplemented with 4% (v/v) fetal bovine serum and 0.4% (v/v) bovine brain extract.

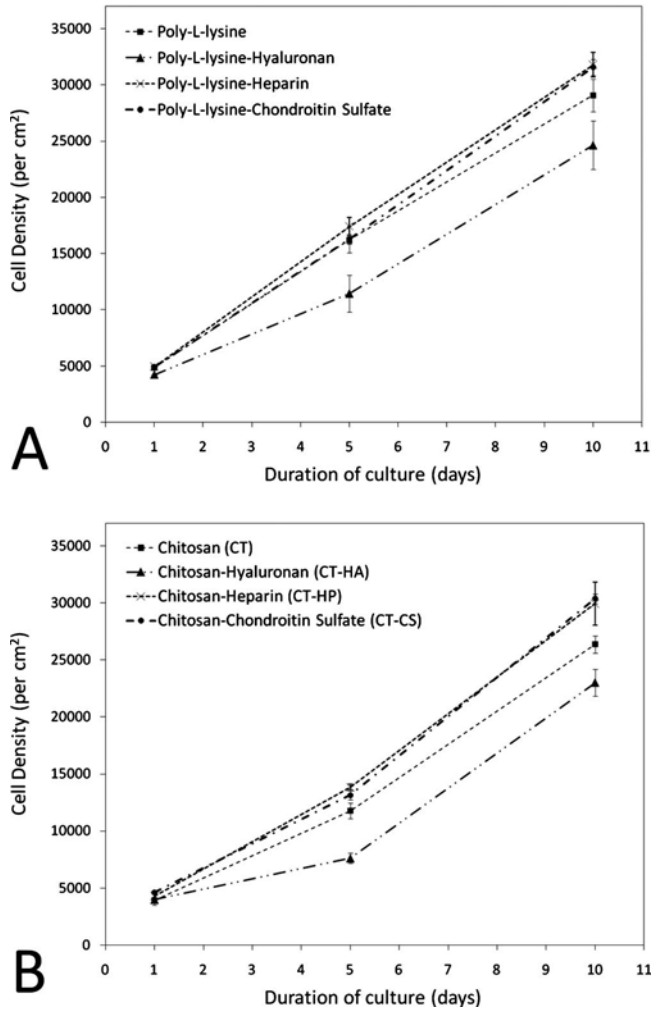

FIG. 6. HUVEC proliferation on bilayered polyelectrolyte coatings with (a) poly-L-lysine and (b) chitosan as the underlying layer.

C. Endothelial adhesion on polyelectrolyte coatings

The percentages of adherent HUVEC on the various polyelectrolyte coatings after incubation for 1 and 2 h are shown in Figs. 5(a) and 5(b), respectively. Interchanging poly-L-lysine with chitosan resulted in significant differences ($P<0.05$) in the percentages of cells that adhered to the various outermost glycosaminoglycan layer after 1 h [Fig. 5(a)]. Nevertheless, these differences became insignificant ($P>0.05$) after 2 h [Fig. 5(b)].

D. Endothelial proliferation on polyelectrolyte coatings

HUVEC proliferation differed significantly on the various polyelectrolyte coatings (Fig. 6). Regardless of whether the underlying layer was CT or PLL, HUVEC proliferated least on HA as the outermost layer, whereas there appeared to be no significant difference in HUVEC proliferation on either HP or CS as the outermost layer. Additionally, it was observed that proliferation was generally better with PLL as the underlying layer, compared to CT, particularly during the earlier phase of cell culture. As seen in Fig. 6(b), there appeared to be a lag in HUVEC proliferation on CT-HP and CT-CS from days 1–5, which was not observed for HUVEC proliferation on PLL-HP and PLL-CS [Fig. 6(a)]. The phase-contrast microscopy images of adherent HUVEC on the various polyelectrolyte coatings after 1 day of culture are shown in Fig. 7. The cells exhibited a distinctively different morphology on the HA outermost layer, regardless of whether the underlying layer was PLL or CT. As seen in Figs. 7(b) and 7(f), the cells adopted a more rounded and less-elongated morphology and had smaller spreading area on HA, as compared to the other polyelectrolyte coatings.

E. RT-qPCR analysis

With poly-L-lysine as the underlying layer, PLL-HA exhibited significant upregulation in expression (greater than twofold) of VWF, VEGFA, and ICAM1 compared to the PLL control [Fig. 8(a)]. In the case of PLL-CS, there was also a significant upregulation (greater than twofold) of VWF though not as dramatic as was seen for PLL-HA [Fig. 8(a)]. For PLL-HP, there was observed to be significant upregula-

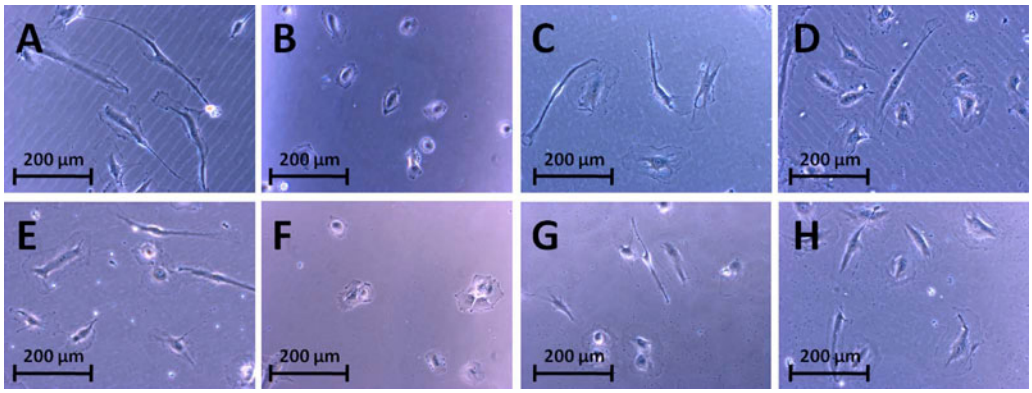

FIG. 7. (Color online) Phase-contrast microscopy images of HUVEC cultured for 24 h on (a) PL, (b) PL-HA, (c) PL-HP, (d) PL-CS, (e) CT, (f) CT-HA, (g) CT-HP, and (h) CT-CS. Scale bar represents 200 μm.

tion (greater than twofold) of both VEGFR and Endoglin compared to the PLL control. With chitosan as the underlying layer [Fig. 8(b)], biologically significant differences (greater than twofold) in the expression of VWF and VEGFR were observed for all three glycosaminoglycans compared to the chitosan control. CT-HA displayed the highest level of expression of VWF, whereas expression levels of VEGFR were almost similar among all three glycosaminoglycans.

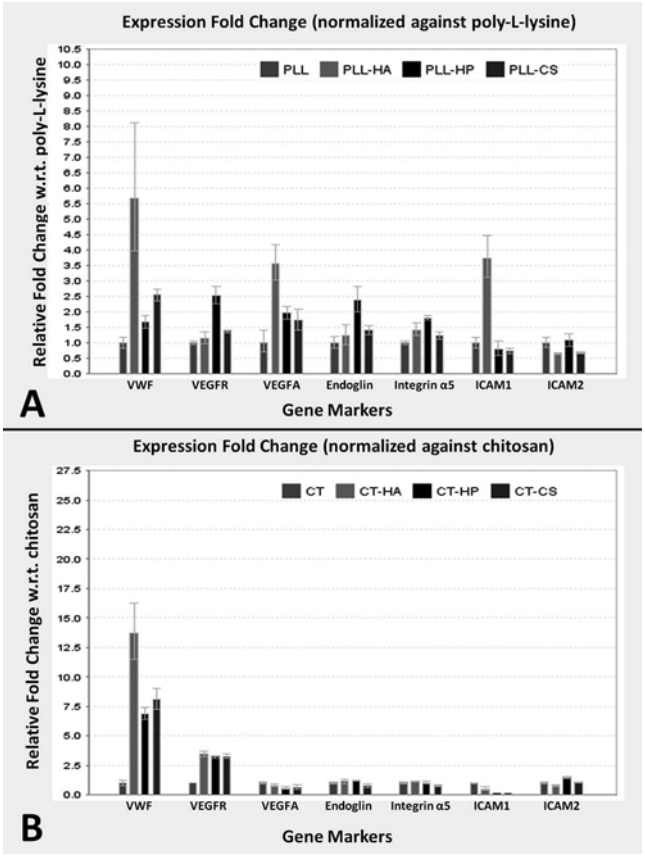

FIG. 8. RT-qPCR analysis of gene markers related to adhesion, proliferation, and endothelial functionality for HUVEC seeded on bilayered polyelectrolyte coatings with (a) poly-L-lysine and (b) chitosan as the underlying layer.

IV. DISCUSSION

Currently, a major technical challenge faced by coronary stents is the tendency for platelets to adhere/deposit onto the stent surface and the resultant thrombogenesis that occurs.[26,27] A possible strategy to overcome this problem would be to formulate coatings that can promote optimal attachment and growth of endothelial cells on the stent surface, thereby ensuring hematocompatibility.[28,29] For this purpose, the class of polysaccharide molecules known as glycosaminoglycans holds particular promise[12–16] because many of these molecules are known to exert a positive stimulatory effect on angiogenesis and endothelial function.

For example, hyaluronan has been widely utilized in tissue engineering applications to promote both angiogenesis and wound-healing at the same time.[30–33] Angioplasty and subsequent stent placement inevitably result in blood vessel injury, and because hyaluronan is known to enhance wound healing,[30–33] it would therefore be a suitable candidate molecule for stent coating formulation.[15,16,18] The stimulatory effect of hyaluronan on angiogenesis has been reported to be mediated by two key receptors found on endothelial cells and their progenitors: CD44 (Ref. 34) and RHAMM.[35] Heparin could be another good candidate molecule for stent coating formulation due to its antithrombogenic properties.[12,17] For instance, heparin is known to influence angiogenesis and endothelial function through binding and sequestering of various angiogenic factors, such as VEGF (Ref. 36) and FGF-2.[37] Indeed, the binding affinity of heparin for angiogenic factors has been exploited in various tissue engineering applications for the immobilization of these proteins on biomaterial implants.[38,39] Interestingly, it was also demonstrated that binding of heparin to angiogenic factors shielded them from enzymatic degradation.[40] Although its physiological effects on endothelial function and angiogenesis have not been as well-characterized, chondroitin sulfate has been shown to have desirable properties as another possible candidate molecule for stent coating formulation.[14] Chondroitin sulfate is known to be the major component of arterial walls and has

been shown to inhibit the formation of arteriosclerotic plaques within animal models,[41] as well as enhance would healing.[9,10]

Although there have already been several studies on endothelial cell interaction with various glycosaminoglycan coatings[42–44] and other multilayer polyelectolyte films,[45–47] there has not yet been a systematic comparison of the adhesion, proliferation, and gene expression profile of endothelial cells on various glycosaminoglycan-coated surfaces. Given the vast potential of glycosaminoglycan-based multilayered polyelectrolyte films in the fabrication of stent coatings[12–18] and other tissue engineering applications,[8–11] it is of great interest to determine which particular combination of glycosaminoglycans with other polyelectrolytes (i.e., chitosan, poly-L-lysine) would yield the most conducive substrata for endothelial cell adhesion, proliferation, and expression of differentiated phenotype.

In utilizing glycosaminoglycan molecules for stent coatings, alternating layer-by-layer deposition of these negatively charged polymers together with a positively charged polyelectrolyte (i.e., poly-L-lysine and chitosan) is often carried out to achieve full-surface coverage.[17,18,21,48–50] This invariably results in a relatively thick multilayered coating that tends to be soft and gel-like due to the natural physiological role of glycosaminoglycans in absorbing and retaining water,[51] as in the case of synovial fluid[52] and vitreous humor.[53] Generally, most mammalian cells, including endothelial cells, prefer a stiff rigid surface for attachment,[54,55] thus a soft gel-like substrate is most often nonconducive for cellular adhesion and subsequent growth. Indeed, apoptosis (programed cell death) has been reported to manifest in cells cultured on soft nonrigid substrates.[56] It is precisely because of this reason that there is a preference to utilize polymers that form a stiffer, less gel-like coating on stents, such as polystyrene-sulfonate/poly-allylamine hydrochloride (PSS/PAH),[46,57–59] which does not exhibit the water-retaining and absorbing properties of glycosaminoglycans.

Hence, previous data on endothelial cell interaction with multilayered polyelectrolyte films comprised of glycosaminoglycans may, in fact, be skewed or "clouded" by the biomechanical effect of low substrate rigidity of thick multilayered films. This could in turn obscure the intrinsic physiological effects of glycosaminoglycans on endothelial cell function. To overcome this inherent limitation of previously reported data, this study therefore investigated endothelial cell interaction with a relatively thin polyelectrolyte bilayer on a rigid TCPS substrate. No doubt, there may now be less complete surface coverage with a polyelectrolyte bilayer (AFM data, Fig. 4), so that the cells may also sense the underlying TCPS substrate. Nevertheless, it must be noted that because the polyelectrolyte bilayer is relatively thin, there is probably more or less consistent stiffness being provided by the underlying rigid TCPS substrate across all experimental groups. Hence, the intrinsic physiological effects of the various glycosaminoglycans on endothelial cell function can now be more clearly manifested with less influence from mechanical parameters, such as substrate rigidity. In any case, the AFM images (Fig. 4) showed that, although isolated nanosized droplets of glycosaminoglycans may be present for some samples, the surface coverage is relatively homogeneous as a whole within a fixed 5×5 μm^2 area, which is much smaller than the typical dimensions of an endothelial cell (Fig. 7). Hence, it can be assumed that the cells are sensing a relatively homogenous surface for all coated samples.

Both ATR-FITR (Figs. 1–3) and AFM (Fig. 4) data positively confirmed the presence of polyelectrolytes on the coated TCPS surface. Nevertheless, it must be noted that both the AFM and ATR-FITR data may be flawed by the rinsing procedure with de-ionized water, which could induce partial release or restructuration of the deposited material. Additionally, AFM of a dried sample surface may not accurately reflect the physiological situation *in vivo* within an aqueous environment. However, there is no other choice available but to use de-ionized water to rinse the samples followed by subsequent drying prior to AFM or ATR-FITR analysis, or else the presence of crystallized salts (i.e., if PBS was used instead for rinsing) or remaining water would be detected on the coated surfaces, which would in turn obscure the data.

Subsequently, the adhesion study (Fig. 5) showed that interchanging PLL with CT resulted in significant differences in cellular adhesion to the outermost glycosaminoglycan layer only at the 1 h time-point [Fig. 5(a)], but these differences became insignificant after 2 h [Fig. 5(b)]. By the 2 h time-point, $>90\%$ of the seeded cells have adhered to the various polyelectrolyte coatings [Fig. 5(b)]. The results thus demonstrate that within the short-time frame of 2 h, endothelial cells adhered equally well on the various polyelectrolyte coatings. The question at this point was whether endothelial cells would also proliferate equally well on the various polyelectrolyte coatings.

As seen in Figs. 6(a) and 6(b), results from the proliferation study showed obvious differences in HUVEC proliferation among the various polyelectrolyte coatings. Regardless of whether the underlying layer was poly-L-lysine or chitosan, HUVEC proliferation on the hyaluronan outermost layer was significantly lower compared to heparin and chondroitin sulfate, which were in turn not significantly different from each other. This is likely to be correlated with the unusual HUVEC morphology (more rounded and less elongated, with smaller spreading area) observed when cells were cultured on hyaluronan [Figs. 7(b) and 7(f)]. Additionally, it was observed that HUVEC proliferation was generally better with PLL as the underlying layer instead of CT, particularly during the earlier phase of cell culture from days 1–5.

An interesting point to note is that most of the observed disparity among experimental groups arises during the initial phase of culture from days 1–5. As seen in Figs. 6(a) and 6(b), the curve gradients (i.e., rate of increase in cell numbers) varied significantly between experimental groups only from days 1–5; whereas from days 5–10, the curve gradients were almost similar for all experimental groups. It is well-known that various somatic cell types, including endothelial

cells, typically exhibit a "lag" phase characterized by reduced mitotic activity during the initial period of cell culture immediately following trypsinization and reseeding onto a new substrate. This could be caused by various factors, as follows: (i) the newly seeded cells may need time to adhere and adjust to the new substrata before undergoing mitosis; (ii) extracellular matrix and surface proteins of the newly seeded cells may get digested by trypsin, and the cells may need time to resynthesize these molecules before displaying optimal proliferation rate, (iii) the newly seeded cells may need time to migrate and cluster together to restore intercellular contacts and paracrine signaling that are likely to be essential for achieving optimal proliferation rate.

Because differences in endothelial proliferation on the various polyelectrolyte coatings are most obvious during the initial phase of cell culture, this would thus imply that some combinations of polyelectrolytes are better than others in mitigating the deficiency of mitotic activity during the putative lag phase. In particular, HUVEC appears to proliferate best on PLL-HP and PLL-CS, and worst on either CT-HA or PLL-HA. However, during the later phase of cell culture from days 5–10, differences in HUVEC proliferation rates (curve gradients in Fig. 6) on the various polyelectrolyte coatings are diminished, probably because by then, the cells would have already overcome their initial deficit in paracrine signaling, intercellular contacts, and extracellular matrix synthesis.

The results of the RT-qPCR analysis showed that with poly-L-lysine as the underlying layer [Fig. 8(a)], PLL-HA exhibited higher expression of VWF, VEGFA, and ICAM1 compared to PLL-HP and PLL-CS. VWF is a secreted blood glycoprotein involved in hemostasis[60–62] and plays an important role in stopping the bleeding process during blood vessel injury. VEGFA is a key cytokine involved in the regulation of angiogenesis,[63] while ICAM1 is a gene marker for cell migration.[64] Both endothelial migration and homeostasis are key processes in the repair of damaged blood vessels. Hence, the higher expression of VWF, VEGFA, and ICAM1 on PLL-HA compared to PLL-HP and PLL-CS may somehow be correlated with its well-known role in angiogenesis and the repair of damaged blood vessels.[65,66] Additionally, it was also observed that PLL-HP displayed higher expression of endoglin and VEGFR compared to PLL-HA and PLL-CS [Fig. 8(a)]. Both VEGFR and endoglin are well-known markers of angiogenesis.[67,68] Hence the observed upregulation of these two genes on PLL-HP maybe related to the reported stimulatory effects of heparin on angiogenesis.[69,70]

With chitosan as the underlying layer [Fig. 8(b)], it was observed that the expression of VWF and VEGFR on all three glycosaminoglycans outermost coatings were significantly higher compared to chitosan alone, although there were no significant differences in the expression of VEGFR among the three glycosaminoglycan coatings on chitosan. It is likely that because chitosan does not occur naturally in mammalian biological systems, it is not a conducive substrate for mammalian endothelial cells; hence the observed extremely low expression levels of VWF and VEGFR on chitosan, both of which are key markers of differentiated endothelial cell functionality.[62,67]

Among the three glycosaminoglycans coatings on chitosan, VWF is more highly expressed on CT-HA as compared to CT-HP and CT-CS [Fig. 8(b)]. This concurs with the results obtained with poly-L-lysine as the underlying layer [Fig. 8(a)], and maybe related to the role of hyaluronan in the repair of damaged blood vessels,[65,66] as discussed earlier.

We have shown that polyelectrolyte coatings differ in their interactions with HUVEC, especially during the initial stages of the proliferative phase. Our experimental results indicate that bilayered polyelectrolyte coatings composed of either HP or CS with PLL would provide a favorable substrate for endothelial cell proliferation.

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