Endothelialization of Microporous YIGSR/PEG-Modified Polyurethaneurea

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ABSTRACT

Bioactive polyurethaneurea modified with polyethylene glycol (PEG) and the endothelial cell-adhesive peptide YIGSR was synthesized and fabricated into microporous scaffolds. This material has shown appropriate mechanical properties for vascular graft applications, resists platelet adhesion, and promotes endothelialization. In the current study, microporous scaffolds were formed by a gas-foaming and salt-leaching method. The scaffolds showed highly interconnected open pores throughout the matrices, with porosity of approximately 78% and pore sizes of 20–200 μm. The peptide modified scaffolds showed superior mechanical properties over peptide-free scaffolds (tensile strength, 1.4 ± 0.03 versus 0.19 ± 0.01 MPa; $p < 0.01$). Bovine aortic endothelial cells were seeded on the scaffolds, and cell attachment, proliferation, extracellular matrix production, and migration were investigated. Histological and scanning electron microscopy analysis showed that few cells adhered on peptide-free scaffolds, whereas confluent endothelial cell monolayers formed along the pores in peptide-modified scaffolds. DNA content, hydroxyproline production, and cell migration were also significantly greater in peptide-modified scaffolds.

INTRODUCTION

Enhanced endothelialization and minimized compliance mismatch are essential for improving clinical patency of small-diameter vascular grafts.[1] Microporous structures can induce rapid transmural and anastomotic tissue ingrowth from surrounding tissue into the graft, thus resulting in enhanced endothelialization that improves thromboresistance and maintains blood fluidity.[2–4] The improved elasticity induced by interconnected open micropores can also reduce compliance mismatching, decreasing intimal hyperplasia at the anastomotic site.[3,5]

Microporous scaffolds have been fabricated previously from polymeric materials, using various techniques such as solvent casting/salt leaching,[6] gas foaming,[7] gas foaming/salt leaching,[8] super critical point CO_2,[9] excimer laser ablation,[3,10] phase separation,[11] and freeze-drying/salt leaching.[12] Each method has its own advantages and disadvantages depending on the polymer system. For vascular graft applications, the pore structure should be open and interconnected in order to induce cell migration through the scaffolds. The pore size also needs to be precisely controlled; large enough to guide cell ingrowth yet small enough to prevent excessive blood leakage. Higher porosity will improve tissue incorporation into the scaffolds but decrease mechanical properties. Thus, porosity should also be controlled to obtain appropriate strength and compliance.[2,3,10] One advantage to gas-foaming and salt-leaching methods is the ability to simultaneously control both pore size and porosity by changing parameters such as salt size, and amount of loading salts, and concentration of acid during processing. This method has been shown to minimize the formation of closed pores and surface skin layers.[8]

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In addition to microporous structural design, the chemical properties of the biomaterial are also important to enhancing endothelialization. The modification of biomaterials with radiofrequency glow discharge plasma,[13] growth factors,[4] or cell-adhesive peptides sequences[14,15] can control the rate of tissue formation. However, the modification of biomaterials should promote endothelial cell proliferation or migration without changing the microporous structure (porosity) and biocompatibility (nonthrombogenicity). In previous work,[16] we developed a dually modified polyurethaneurea (PUU) by incorporating endothelial cell-adhesive YIGSR peptide sequences as chain extenders and nonthrombogenic polyethylene glycol (PEG) as a soft segment (PUUYIGSR-PEG) in the polymer backbone. Virtually no platelet adhesion was observed on PUUYIGSR-PEG, whereas endothelial cell adhesion, spreading, and migration were robust. Bulk modification might be particularly useful for fabrication of porous scaffolds with cell-adhesive characteristics throughout the pore structure.

In this study, we have fabricated a bioactive microporous scaffold using a gas-foaming and salt-leaching method. The effects of bioactive peptide sequences and microporous structure on endothelialization have been explored.

MATERIALS AND METHODS

Fabrication of microporous scaffolds

PEG-containing polyurethane urea (PUUPPD-PEG) was synthesized by reacting 4,4′-methylene di(p-phenyl isocyanate) with poly(tetramethylene oxide)–PEG mixture (85:15 molar ratio) and extending with p-phenylenediamine. A material also containing the YIGSR peptide (PUUYIGSR-PEG) was synthesized similarly except it was extended with a combination of GGGYIGSRGGGK and PPD as previously described.[16]

Materials were characterized via ^{1}H nuclear magnetic resonance (NMR), using a 400-MHz NMR spectrometer (Advance 400; Bruker BioSpin, Rheinstetten, Germany), in N,N-dimethylformamide-d_7 (DMF-d_7; Sigma-Aldrich, Milwaukee, WI). The peaks were assigned and characterized, and successful incorporation of the peptide sequence was confirmed as described in a previous study.[16] Fourier transform infrared (FT-IR) spectra, differential scanning calorimetery (DSC), and electron spectroscopy for chemical analysis (ESCA) measurements also confirmed successful synthesis of the materials.[16] Molecular weight distributions (PUUPPD: M_n = 113,489, M_w = 153,973, polydispersity index [PDI] = 1.36; PUUPPD-PEG: M_n = 92,645, M_w = 124,072, PDI = 1.34; and PUUYIGSR-PEG: M_n = 96,675, M_w = 116,103, PDI = 1.20) were obtained by gas-phase chromatography (GPC) with ultraviolet (UV) and evaporative light scattering detectors (Polymer Laboratories, Amherst, MA).

Scaffolds were fabricated by gas foaming and salt leaching via incorporation of sodium bicarbonate. PUUPPD-PEG and PUUYIGSR-PEG were separately dissolved in anhydrous N,N-dimethylformamide (DMF; Sigma-Aldrich) at 10% (w/v) concentration, and DMF was partially evaporated at 60°C under vacuum. Sieved sodium bicarbonate salts (Sigma, St. Louis, MO; particle size of 100–200 μm, weight fraction = 90 wt%) were added into the viscous polymer solutions at room temperature and mixed well. The paste of polymer and salts was placed in a Teflon mold (75 × 25 × 1 mm) and dried for 24 h at room temperature. The polymer–salt films were immersed in a 50% citric acid solution (Sigma) for 30 min to induce gas foaming and then in deionized water for 3 days to leach remaining salts. Deionized water was changed every day, and samples were freeze-dried for 48 h: Scaffolds were cut into disk shapes (diameter, 7 mm; thickness, 1 mm; weight, 10–20 mg) for further study.

Characterization of scaffolds

The porosity of scaffold was determined with a mercury intrusion porosimeter (Autoscan-500; Quantachrome Instruments, Boynton Beach, FL). Scaffolds were loaded into the intrusion chamber for measurement. Mercury was intruded into samples at 500 lb/in^2, and the intruded mercury volume and pressure were recorded. Porosity was determined from the total intruded volume per unit mass.[6]

The microstructure of the scaffold was investigated by scanning electron microscopy (SEM). Samples were sputter coated with gold for 30 s at 100 mA with a sputter coater (Pelco Model 3 sputter coater 91000; Ted Pella, Redding, CA). The surface and cross-section of scaffolds were observed by SEM (JSM-5300 [JEOL, Tokyo, Japan]; operated at 30 kV).

Uniaxial mechanical testing was performed with a Universal materials testing machine (model 5565; Instron, Norwood, MA) at a cross-head speed of 25 mm/min with a 50-N load cell. Test specimens were prepared according to ASTM D-638-VI. Sample thickness was measured with a digital caliper (Mitutoyo, America, Aurora, IL).

Cell maintenance

Bovine aortic endothelial cells (BAECs; Cambrex Bio Science, San Diego, CA), passage 2–5, were used for this study. Dulbecco's modified Eagle's medium (DMEM; Sigma) was prepared with 10% fetal bovine serum (FBS; Cambrex Bio Science, Walkersville, MD), 2 mM L-glutamine, penicillin (1 unit/mL), and streptomycin (100 mg/L) (GPS; Sigma). Endothelial basal medium (EBM; Sigma) was prepared with 10% endothelial medium supplement (Sigma), which contained fetal bovine serum, basic fibroblast growth factor, heparin, epithermal growth

factor, and hydrocortisone. BAECs were maintained on a mixture of EBM and DMEM (25:75 volume ratio) at 37°C in a 5% CO_2 environment.

Seeding and culture of endothelial cells in scaffolds

Scaffold disks were immersed in 70% ethanol overnight before use. The scaffolds were then soaked in sterile phosphate-buffered saline (PBS) for 30 min three times, and in cell culture medium for 15 min. For cell seeding the scaffolds were placed in six-well tissue culture plates, and excess medium was removed. Forty microliters of cell suspension at 1×10^6 cells/mL was added slowly onto the top of the scaffold and allowed to permeate through the matrix. Each scaffold was turned over, and an additional 40 μL of cell suspension was added to the opposite surface. Six-well plates containing cell-seeded scaffolds were maintained at 37°C in a 5% CO_2 environment for cell attachment. After 90 min, cell-seeded scaffolds were placed in 96-well plates, culture medium was added, and constructs were incubated at 37°C in a 5% CO_2 environment. After 3 days, each cell-seeded scaffold was placed in a well of a 24-well plate, washed three times with sterile PBS, and fixed with 2.5% glutaraldehyde (Sigma) overnight at 4°C.

For evaluation by SEM, the cell-seeded scaffolds were dehydrated in a graded series of ethanol–water solutions, and dried with tetramethylsilane in an ice bath (TMS; Electron Microscopy Sciences, Fort Washington, PA). Samples were sputter coated and the morphology of cells in each scaffold was observed by SEM as described above.

For histological analysis, cell-seeded scaffolds were sectioned to 30 μm at −30°C, using a cryostat (HM505E; MICROM International, Walldorf, Germany). Sections were stained with Mayer's hematoxylin and observed by light microscopy (Axiovert 135; Zeiss, Thornwood, NY).

Migration of cells through scaffold matrix

Scaffolds were prepared as described above and placed in six-well plates, and 40 μL of cell suspension at 1×10^6 cells/mL was added onto the top of each scaffold. The plates were then incubated at 37°C in a 5% CO_2 environment for 90 min to allow cell attachment. Each scaffold was placed into a Transwell cell culture insert (Becton Dickinson, Franklin Lakes, NJ) (6.4 mm in diameter, 8-μm pore size polyester [PET] membrane) in 24-well companion plates (Corning Life Sciences, Acton, MA). Medium was added to each insert and well, and the scaffolds were then incubated at 37°C in a 5% CO_2 environment. After 7 days of culture, each scaffold was removed, from its insert well, and then each insert well was transferred in a 24-well companion plate. The membrane of the insert well was washed with sterile PBS, and adherent cells on the top and bottom of the insert membrane were removed by trypsinization and counted with a Coulter counter (Multisizer 3; Beckman Colulter, Fullerton, CA).

DNA and hydroxyproline measurement in scaffold matrix

Sterile scaffolds were prepared as described above. The scaffolds were placed in six-well plates, and 40 μL of cell suspension at 1×10^6 cells/mL was added to the top and the bottom of the scaffold, respectively. The cell-seeded scaffolds in the six-well plates were placed at 37°C in a 5% CO_2 environment for cell attachment for 90 min and then transferred to 96-well plates. Additional medium was added, and the cell-seeded scaffolds were incubated at 37°C in a 5% CO_2 environment. After 3 days of culture, cell-seeded scaffolds were washed with sterile PBS and cut into four pieces. Samples were digested with 0.1 N NaOH overnight at 37°C. The digested samples were neutralized with 0.1 N HCl. The digested samples and calf thymus DNA standards were diluted with TE buffer (200 mM Tris-HCl, 20 mM EDTA, pH 7.5) and combined with Hoechst 33258 (Molecular Probes, Eugene, OR), a fluorescent DNA binding dye. DNA content was determined by measuring fluorescence with a fluorometer (VersaFluor; Bio-Rad, Hercules, CA) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm, and compared with thymus DNA standards (0 to 100 ng/mL).

For hydroxyproline measurement, neutralized samples and hydroxyproline standards were combined with 4 N NaOH in a Wheaton cryovial (Wheaton Science Products, Millville, NJ) and autoclaved for 20 min at 247°F to hydrolyze collagen. Samples and hydroxyproline standards (in deionized water, 0–100 ng) were cooled to room temperature and neutralized with 4 N HCl. They were then oxidized with chloramine-T (MP Biomedicals, Irvine, CA) and developed with p-dimethylbenzaldehyde (MP Biomedicals). The production of hydroxyproline was determined by spectrophotometry at 550 nm.[17]

Statistical analysis

Data were compared with two-tailed, unpaired t tests. p Values less than 0.05 were considered to be significant. Data are presented as means ± standard deviation.

RESULTS

Characterization of scaffolds

PUUPPD-PEG and PUUYIGSR-PEG scaffolds were successfully fabricated by gas foaming and salt leaching. The porosity of the scaffolds was characterized by mercury intrusion porosimetry and SEM. Both PU-UPPD-PEG and PUUYIGSR-PEG scaffolds showed
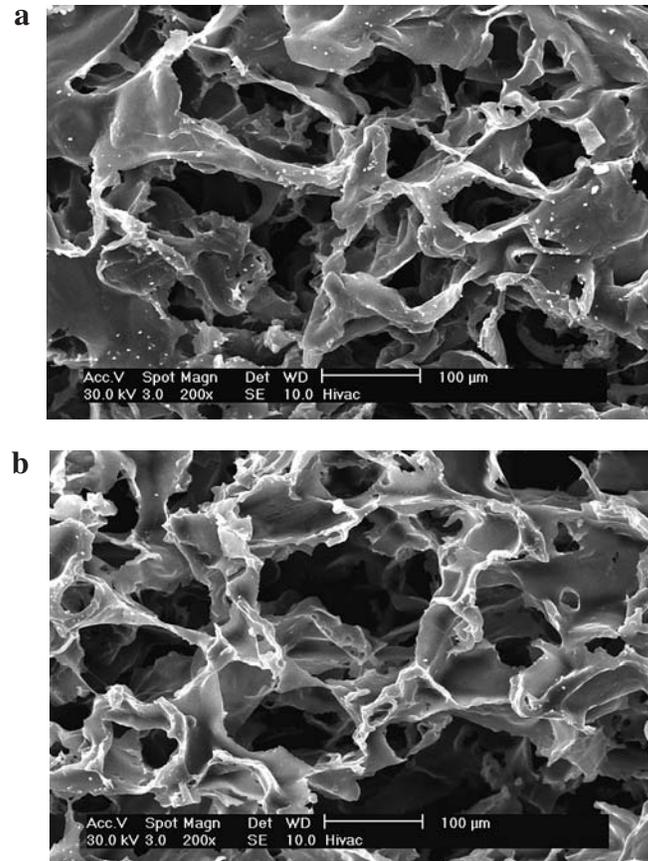
FIG. 1. Scanning electron micrographs of surface and cross-section of scaffold matrices. (**a**) Surface of PUUPPD-PEG; (**b**) cross-section of PUUPPD-PEG; (**c**) surface of PUUYIGSR-PEG; (**d**) cross-section of PUUYIGSR-PEG.

highly interconnected pore structures throughout the matrices, with porosities of approximately 78% (PUUPPD-PEG, 78 ± 3.6%; PUUYIGSR-PEG, 77 ± 8.4%) and pore sizes of 20–200 μm. The pores were highly open on both surface and cross-section views of the scaffolds (Fig. 1).

Uniaxial mechanical testing was performed to determine mechanical properties of the scaffolds, as shown in Table 1. PUUYIGSR-PEG scaffolds showed greater tensile strength and elongation at failure compared with PUUPPD-PEG (tensile strength: 1.4 ± 0.03 versus 0.19 ± 0.01 MPa, $p < 0.01$; elongation: 796 ± 122 versus 129 ± 2.08%, $p < 0.02$). There was no significant difference in elastic modulus (PUUYIGSR-PEG; 0.33 ± 0.1 MPa; PUUPPD-PEG, 0.21 ± 0.01 MPa).

Endothelial cell culture in scaffolds

Endothelial cells were seeded at the top and bottom of scaffolds and cultured for 3 days. Cell attachment and morphology in the scaffolds were visualized by SEM, as shown in Fig. 2. On PUUPPD-PEG scaffolds, few cells were found to adhere through the scaffolds. Individual cells were distributed randomly on the scaffolds, and cell colonies were not found. However, confluent endothelial cell attachment and spreading were found throughout the PUUYIGSR-PEG scaffold matrix. Endothelial cells created monolayers along the interconnected pore network in the scaffold. This was also consistent with the histological analysis (Fig. 3).

Migration of cells through scaffold matrix

To study the interconnectivity of the scaffold matrix and bioactivity of the peptide sequences on the polymer matrix, the migration of cells through the scaffolds was studied. Cells were seeded on top of scaffolds, and cell-seeded scaffolds, maintained in tissue culture plates for 90 min to ensure cell attachment on the scaffolds, were then placed into Transwell inserts. After 7 days of culture, scaffolds were removed. The insert wells were transferred to a companion plate, and cells on the top and bottom of the membrane were counted. No cells were observed at the bottom of the companion plate. As shown in Table 1, the number of cells that had migrated through the PUUYIGSR-PEG matrices (4266.7 ± 482.2) was significantly greater than for PUUPPD-PEG (1026.7 ± 266.3, $p < 0.005$).
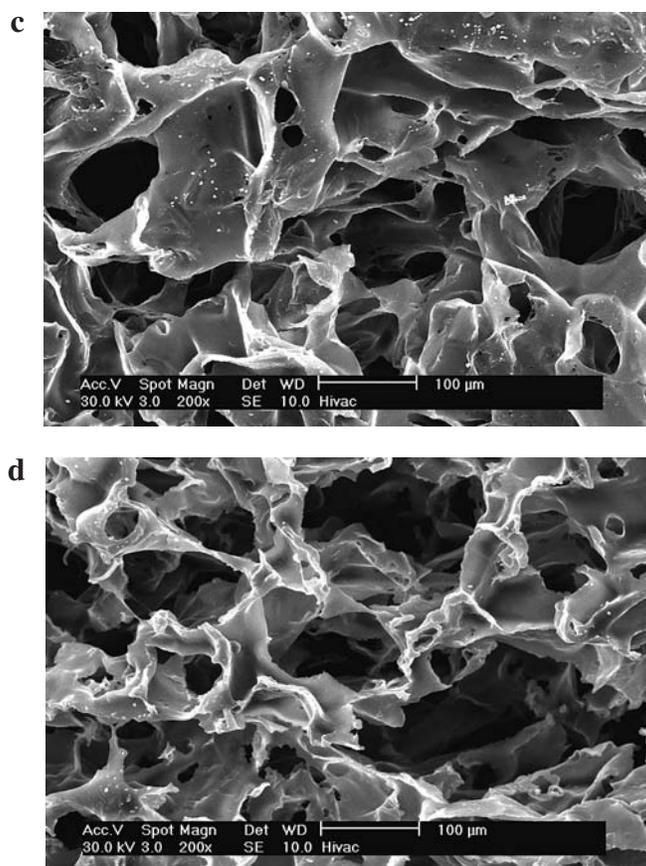
FIG. 1. (*Continued*)

DNA and hydroxyproline measurement in scaffolds

The effect of bioactive peptide sequence on endothelial cell proliferation and extracellular matrix production in the scaffolds was studied as shown in Table 1. DNA concentration in PUUYIGSR-PEG matrices (4.41 ± 0.34 ng/scaffold) was significantly greater than in PUUPPD-PEG (1.84 ± 0.26 ng/scaffold, $p < 0.001$). Hydroxyproline production, a marker of collagen synthesis, in PUUYIGSR-PEG matrices (1.092 ± 0.323 ng/ng DNA) was also significantly greater than in PUUPPD-PEG (0.069 ± 0.062 ng/ng DNA, $p < 0.05$). A higher DNA concentration in PUUYIGSR-PEG scaffold matrices indicates a greater number of cells; thus PUUYIGSR-PEG scaffold matrices were expected to have higher hydroxyproline production. However, significantly greater hydroxyproline production per cell (nanograms of hydroxyproline per nanogram of DNA) indicates that the bioactive peptide sequences might not only enhance cell adhesion but also facilitate extracellular matrix production.

TABLE 1. SUMMARY OF MECHANICAL PROPERTIES, MIGRATION, DNA, AND HYDROXYPROLINE PRODUCTION IN PUUPPD-PEG AND PUUYIGSR-PRG SCAFFOLD MATRICES

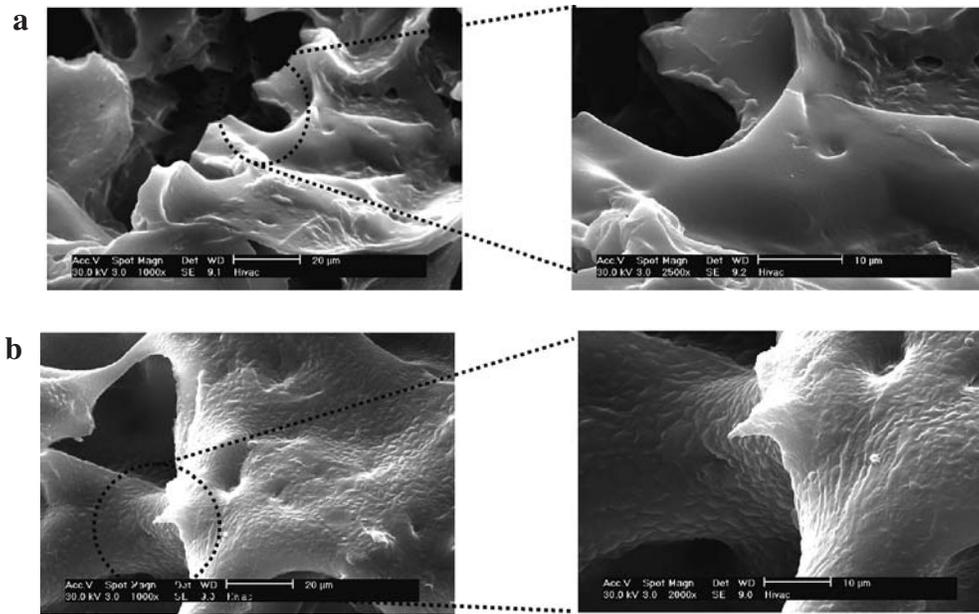|	*PUUPPD-PEG*	*PUUYIGSR-PEG*
Tensile strength (MPa)	0.19 ± 0.01	1.4 ± 0.03
Elongation at failure (%)	129 ± 2.08	796 ± 122
Number of cells migrated through scaffolds	1026.7 ± 266.3	4266.7 ± 482.2
DNA (ng)/scaffold	1.84 ± 0.26	4.41 ± 0.34
Hydroxyproline production (ng/ng DNA)	0.069 ± 0.062	1.092 ± 0.323

FIG. 2. Scanning electron micrographs of endothelial cells cultured for 3 days in (**a**) PUUPPD-PEG and (**b**) PUUYIGSR-PEG scaffold matrices.

DISCUSSION

Polyurethaneurea scaffolds fabricated by gas foaming and salt leaching showed a uniform distribution of open and interconnected pores throughout the matrix. In addition, there was no closed or nonporous skin on the external surface, as is usually found with the salt-leaching or gas-foaming method.[7] Gas foaming and salt leaching
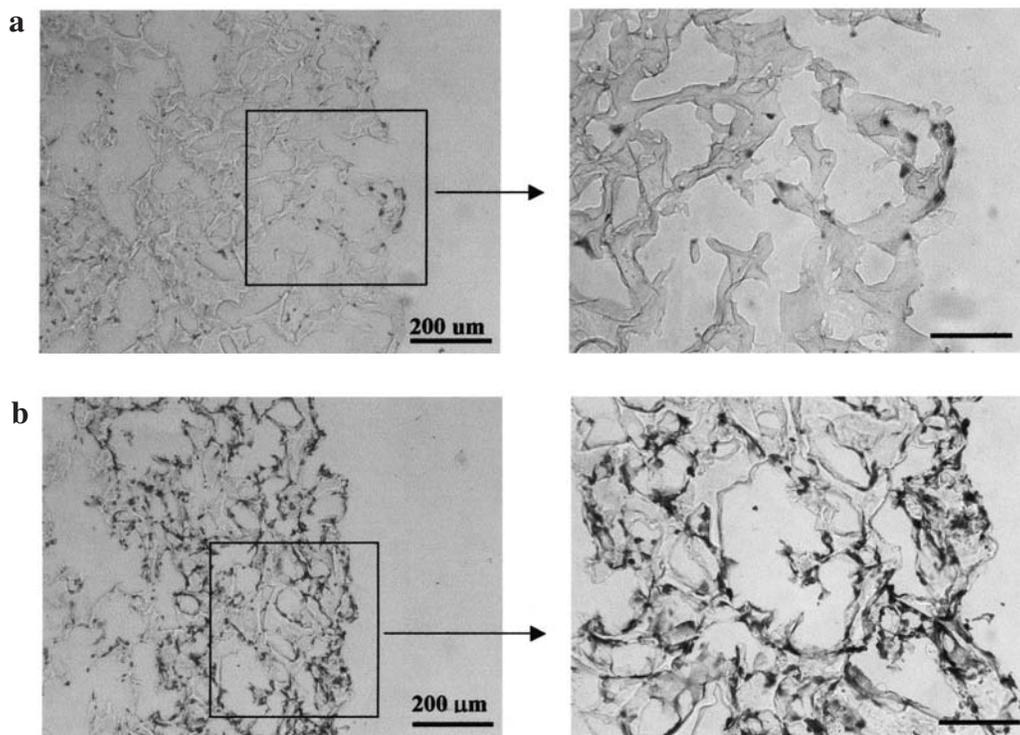

FIG. 3. Hematoxylin-stained endothelial cells on cross-section of (**a**) PUUPPD-PEG and (**b**) PUUYIGSR-PEG scaffolds. Endothelial cells were cultured for 3 days.

have also been shown to provide control over porosity and mechanical properties.[8] In this study, sodium bicarbonate was chosen because of its superior stability compared with ammonium bicarbonate in polyurethaneurea systems. Thus, undesirable gas formation at an early processing stage was avoided, even in the peptide-incorporated polyurethaneurea.

For vascular graft applications, appropriate strength and compliance are important.[2,3,18] Porosity decreases mechanical properties but also promotes cellular invasion. The gas-foaming and salt-leaching method results in highly interconnected open pore structures leading to improved mechanical properties of the scaffolds. PUUYIGSR-PEG showed superior mechanical properties compared with PUUPPD-PEG. Because porosity and pore size were nearly identical for both scaffolds, the difference in mechanical properties may be inherent to the material. In a previous study, we reported that bioactive peptide sequences could affect not only cellular behavior but also bulk mechanical properties.[16] Hard segment domains of polyurethaneurea serve as cross-linking sites, allowing materials to possess high elastic moduli.[2,19] The immiscibility between hard and soft segments causes phase separation that is responsible for mechanical properties.[2] Hydrogen bonding between a donor (NH) and acceptor (C = O) in urea groups plays a critical role in determining the degree of phase separation.[2] The peptide sequences also have donors and acceptors for hydrogen bonding,[20] altering hydrogen bonding and phase separation within the material. Thus, the mechanical properties of PUUYIGSR-PEG may be influenced by both molecular interactions in the polymer chain and the restriction of motility of the PEG chains by long amino acid peptide sequences and uniform distribution of micropores throughout the scaffold matrix.

One of the advantages of peptide incorporation into the polymer main chain was the ability to fabricate a bioactive scaffold easily without further modification. When modifying the surface of materials by traditional methods, such as the grafting of adhesive peptides,[21–23] it becomes difficult to gauge their bioactivity, because most grafted peptide sequences are embedded in the polymer during fabrication process. It is also challenging to modify scaffolds without changing the porous structure. The bioactivity of YIGSR peptide sequences incorporated was evaluated by assessing cell attachment, migration, and extracellular matrix production. Endothelial cells were seeded into the scaffolds and cultured under static conditions. Cells adhered and spread through the PUUYIGSR-PEG matrix but few cells adhered to the PUUPPD-PEG matrix. The biospecificity of endothelial cell adhesion on this material was evaluated in a previous study.[16] Competitive inhibition of cell attachment and spreading on the PUUYIGSR-PEG film was observed when soluble YIGSR peptide was added to the cell culture medium, indicating that adhesion and spreading were mediated by the interaction between YIGSR peptide and receptors on the cell surface. A higher level of migration was also found in the PUUYIGSR-PEG matrix compared with the PUUPPD-PEG matrix. This indicates that incorporated peptide sequences maintain their bioactivity and promote cell migration throughout the matrix. Thus, it can also be expected that this scaffold might promote transmural and anastomotic tissue ingrowth from the surrounding tissues and form a continuous monolayer on the surface of an implanted vascular graft.

Cells in the PUUYIGSR-PEG matrix showed not only higher cell densities but also higher hydroxyproline production per cell. Higher hydroxyproline production (an indication of extracellular matrix production) seems to be enhanced by incorporated peptides, and may lead to better tissue formation and maintenance under shear stress. It could also be helpful for compliance matching at anastomotic sites.

CONCLUSION

A bioactive microporous scaffold has been fabricated, using a gas-foaming and salt-leaching method. This scaffold has an interconnected open pore structure with uniform distribution throughout the matrix, which contributes to the mechanical properties of the material. The peptide sequences incorporated into the polymer main chain maintain their bioactivity, enhancing endothelialization. This bioactive scaffold is an attractive candidate for small-diameter vascular graft applications.

ACKNOWLEDGMENTS

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