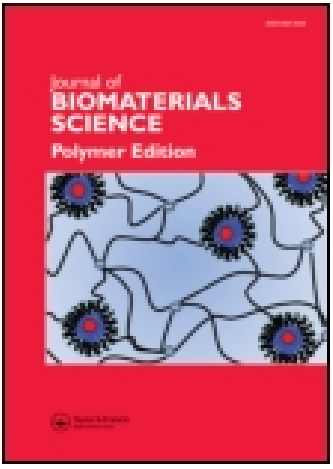

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Development of a YIGSR-peptide-modified polyurethaneurea to enhance endothelialization

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Abstract—Polyurethanes have been investigated for use as vascular grafts due to their excellent mechanical properties and relatively good biocompatibility. However, poor retention of endothelial cells and thrombogenicity *in vivo* remain problematic for vascular graft applications. The peptide YIGSR has been shown to increase endothelial cell adhesion but not attachment of platelets, suggesting its possible utility for vascular graft applications. In this study, a bioactive polyurethaneurea has been synthesized by incorporating GGGYIGSRGGGK peptide sequences into the polymer backbone. Successful incorporation of the peptides was confirmed by NMR, contact angle measurement and ESCA. Uniform distribution of peptides on the surface was observed using a fluorescent probe capable of reacting with tyrosine residues on the peptides. Hard segment domains were visualized using tapping mode AFM. Endothelial cell adhesion, spreading, proliferation, migration and extra-cellular matrix production were improved on bioactive polyurethaneurea compared to control polyurethaneurea. Competitive inhibition of endothelial cell attachment and spreading by soluble YIGSR peptides indicated that cell adhesion and spreading were specifically mediated by YIGSR-sensitive cell adhesion receptor, not just by changed surface properties. There was no significant difference in the number of adherent platelets. Therefore, this bioactive polyurethaneurea may improve vascular graft endothelialization without increasing thrombogenicity.

Key words: Polyurethaneurea; endothelialization; YIGSR; endothelial cells; vascular graft.

INTRODUCTION

Vascular diseases, especially atherosclerosis, are responsible for the majority of deaths in the United States [1]. A common procedure used to direct blood flow around occluded segments is bypass grafting. In coronary artery bypass grafting (CABG), occluded coronary arteries can be replaced with autologous tissue such as saphenous veins. However, many patients do not have suitable donor tissue

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due to peripheral vascular disease or prior surgery [2–4]. Synthetic materials have been developed for blood vessel substitutes but are not suitable for small diameter applications such as CABG. ePTFE (expanded polytetrafluoroethylene), Dacron (polyethylene teraphthalate) and microporous polyurethanes have been successfully used as large diameter (>6 mm) vascular substitutes [1, 5, 6]. However, when used in small diameter applications, they have not been satisfactory due to thrombosis and intimal hyperplasia [2, 6–8].

Polyurethane block copolymers have been widely used for biomedical applications due to their excellent mechanical properties and relatively good biocompatibility. These applications include vascular prostheses [9, 10], artificial hearts [2, 11], catheters [12] and artificial tracheae [13]. However, like other synthetic materials, they are generally thrombogenic on exposure to blood. Several surface modification strategies have been evaluated to improve blood compatibility such as introduction of ionic groups [14], heparin with hydrophilic PEO spacers [15] and thrombomodulin [16]. Nevertheless, these modifications have not been enough to completely solve the problem.

Endothelialization of synthetic grafts is a possible strategy to improve graft patency [8, 17]. However, since endothelial cells often detach on exposure physiological shear stress, the graft patency is dependent on the attachment and retention of endothelial cells on the surface. Thus, surface modifications to increase adhesion and retention of endothelial cells on artificial prostheses have been tried including plasma treatment [18, 19], adhesive peptides [20] and growth factors [21].

Adhesive peptide sequences have been extensively used for modification of biomaterials to enhance cell adhesion. Biodegradable polymers, such as block co-polymers of biotinylated poly(ethylene glycol) (PEG) with poly(lactic acid) (PLA) [22] and poly(lactic acid-co-lysine) [23–25], have been modified with RGD peptides. Endothelial cells showed enhanced spreading on these polymers. RGD-containing peptides have also been covalently grafted to carboxylated polyurethanes *via* amide bonds, resulting in improved endothelial cell attachment and spreading [26, 27]. However, thrombosis is one of the major causes of vascular graft failure. RGD is known to interact with platelet integrin glycoprotein IIb/IIIa [28, 29]; so, while RGD may enhance endothelial cell adhesion, it may ultimately decrease graft performance.

The laminin-derived peptide YIGSR has also been used to modify biomaterials to promote endothelialization. Enhanced attachment, spreading, proliferation and migration of endothelial cells on vascular graft materials are essential to obtain successful endothelialization. Massia and Hubbell modified glycophase glass, PET and PTFE with immobilized YIGSR peptide and found that attachment and spreading of human umbilical vein endothelial cells (HUVECs) on substrates modified with YIGSR increased [30]. Hubbell *et al.* also reported that HUVECs on YIGSR immobilized surface showed resistance to detachment at 20 dyne/cm^2 shear stress for 8 h [31]. Proliferation and spreading of bovine aortic endothelial cells (BAECs) also increased on glass modified with YIGSR [32]. In addition,

the combination of YIGSR peptide and fibroblast growth factor has been shown to support an increase in proliferation and motility of BAEC [33]. Importantly, YIGSR immobilized on glass or PEG-modified PET supported enhanced adhesion and spreading of HUVECs but did not support platelet attachment [34].

The surface concentration of adhesive peptides greatly affects cellular functions on bioactive materials. For example, maximal cell migration is generally obtained at intermediate surface ligand concentrations [35, 36]. In addition, Mann and West reported that migration and proliferation of smooth muscle cells were lower at higher surface concentration of RGD peptides [38]. Cell morphology [30], attachment and extra-cellular matrix production [37] have been shown to vary with surface peptide concentration for a number of different cell types. In a preliminary study, we investigated the responses of endothelial cells on YIGSR-modified surfaces at various surface concentrations of the peptide [39]. YIGSR peptides were covalently grafted to aminophase glass surfaces. Strength of adhesion was increased with increasing YIGSR concentration, but proliferation was decreased at high concentrations. Cell migration and extra-cellular matrix production were increased at low peptide concentrations but decreased at high concentrations. Platelet adhesion on YIGSR modified-surfaces were less than on the aminophase glass surfaces. Therefore, we can identify optimal peptide concentrations to enhance endothelialization without increasing thrombosis and use this information in the design of bioactive polyurethaneureas.

The goal of this research is to develop a bioactive peptide-modified polyurethaneurea to enhance endothelialization for small diameter vascular graft applications. A bioactive YIGSR-peptide-modified polyurethaneurea was developed by incorporating GGGYIGSRGGGK peptide sequences into the polymer backbone. The bioactive polyurethaneurea was characterized and the enhancement of endothelialization was evaluated.

MATERIALS AND METHODS

Synthesis of polyurethaneurea (PUUPPD)

Pre-polymer was synthesized by reacting methylene di(*p*-phenyl isocyanate) (MDI; Aldrich, Milwaukee, WI, USA) with poly(tetramethylene oxide) (PTMO; Aldrich) and then extended with *p*-phenylene diamine (PPD; Acros, Fairlawn, NJ, USA) [40]. A 10% (w/v) solution of MDI (8 mmol, MW 250) in 20 ml anhydrous *N*,*N*-dimethylformamide (DMF; Aldrich) was prepared in a 100 ml three-neck round flask and stirred at room temperature. A 10% (w/v) solution of PTMO (4 mmol, MW 1000) in 40 ml anhydrous DMF was added and the mixture was heated to 75°C and held there for 2 h under argon gas. The reactor was cooled to room temperature before PPD (4 mmol, MW 108) in 4 ml anhydrous DMF was added as a chain extender. The polymer solution was then incubated at 45°C for 2 h under argon gas. The polymer solution was cooled to room temperature, precipitated in methanol and dried under vacuum.

Synthesis of bioactive YIGSR-modified polyurethaneurea (PUUYIGSR)

Pre-polymer was synthesized by reacting MDI with PTMO as described above and extended with a combination of GGGYIGSRGGGK peptide (Sigma-Genosys, Woodlands, TX, USA) and PPD. A 10% (w/v) solution of MDI (2.4 mmol) in 6 ml anhydrous DMF was prepared in 100 ml three-neck round flask and stirred at room temperature. A 10% (w/v) solution of PTMO (1.2 mmol) in 12 ml anhydrous DMF was added and the mixture was heated to 75°C and held there for 2 h under argon gas. The reactor was cooled to room temperature before GGGYIGSRGGGK peptide (0.11 mmol) in 10 ml anhydrous DMF and PPD (1.1 mmol) in 10 ml anhydrous DMF were added as chain extenders. The polymer mixture was incubated at 45°C for 2 h under argon gas. The polymer solution was cooled to room temperature, precipitated in methanol and dried under vacuum.

Polymer characterization

PUUPPD and PUUYIGSR were characterized by ^{1}H-NMR using a 400 MHz NMR spectrometer (Advance 400, Bruker, Rheinstetten, Germany) in *N*,*N*-dimethyl-formamide-d_7 (DMF-d_7; Aldrich). Molecular weight distributions were obtained by GPC with UV and evaporative light-scattering detectors (Polymer Laboratories, Amherst, MA, USA). Samples for GPC analysis were dissolved in HPLC-grade DMF at a concentration of 1 mg/ml and run at 70°C through PLgel 5 μm Mixed-C columns (Polymer Laboratories) at a flow rate of 1 ml/min. Calibration was performed using polystyrene standards (PS; Polymer Laboratories), ranging in molecular mass from 5 to 96.4 kDa. DSC thermagrams were obtained using a TA Instruments DSC 2920. Samples were cooled below −60°C and heated to 300°C at 10°C/min. Helium was used for low-temperature scans and nitrogen was employed for high-temperature scans. Fourier transform infrared (FT-IR) spectroscopy was performed using a Nicolet 500 spectrometer. The thin films of polyurethaneurea were prepared by mixing samples with KBr and pressed into pellets under vacuum. Sixteen scans were taken of each sample at a resolution of 4 cm^{-1}.

Preparation and characterization of polyurethaneurea films

Polymers were dissolved in tetrahydrofuran (THF; 0.3 wt%) and sterilized using 0.2 μm pore size PTFE syringe filters (Whatman, Clifton, NJ, USA). Polymer films were prepared on glass coverslips (18 mm; Fisher Scientific, Pittsburgh, PA, USA) by solvent casting at room temperature. Polymer films were held under vacuum for 48 h to ensure removal of the solvent.

The equilibrium contact angles of DI water on PUUPPD and PUUYIGSR films were measured using a contact angle goniometer (CAM-Micro). Six measurements were taken to calculate average contact angles on the surface of each film.

ESCA analysis was performed using a Physical Electronics Model 5700 XPS instrument. Photo-emissions were produced through the use of a X-ray source

(1486.6 eV) operated in the fixed retard ratio mode at a pass energy of 23.5 eV. Spectra were acquired over a 10–45° take-off angle range. Charge neutralization was accomplished via bombardment with a low-energy beam.

To evaluate the distribution of YIGSR peptide on the surface of the polyurethaneurea films, films were reacted with the fluorescent probe 7-chloro-4-nitrobenzo-2-ox-1,3-diazole (NBD-Cl; Molecular Probes, Eugene, OR, USA). This fluorescent compound reacts with tyrosine residues on the peptides in the polymer. 250 mM NBD-Cl was prepared in DMSO. Polyurethaneurea films were placed in a well of a 12-well tissue culture plate, 2 ml potassium phosphate buffer solution (pH 8.0) was added and then 80 μl NBD-Cl solution was added. Samples were incubated at 37°C for 2 h. Unreacted NBD-Cl was rinsed with buffer solution over one week. The surfaces were observed using a Nikon E600 fluorescence microscope equipped with a Sony DXC-950P CCD camera. A Nikon multiband DAPI-FITIC-Texas red (excitation wavelengths: 385–415, 485–505, 555–585 nm, emission wavelengths: 450–470, 510–540, 590–650 nm) filter was used.

To investigate the distribution of hard and soft segment on the surfaces, the surfaces of polyurethaneurea films were imaged by AFM (NanoScope IIIa, Digital Instruments, Santa Barbara, CA, USA). A 125 μm etched silicon cantilever was used. The radius of curvature of a silicon tip was 5–10 nm and the resonance frequency was 300 kHz. Phase and height images were obtained using tapping mode under ambient conditions from $r_{sp} = 0.92$ to 0.80 (r_{sp} = set point amplitude/free amplitude of oscillation). The data obtained were analyzed with Nanoscope IIIa controller and software.

Uniaxial mechanical testing was performed using an Instron model 5565 at a cross head speed of 25 mm/min with a 5 kN load cell. Polymers were dissolved in DMF (Aldrich) at 10 wt% and sterilized using 0.2 μm pore size PTFE syringe filters (Whatman). Polymer films were prepared in Teflon molds by solvent casting at 60°C under vacuum for 48 h. Test specimens were prepared according to ASTMD-638-VI. The tensile strength was calculated from the maximum load at break and the initial cross sectional area of the specimen. Sample thickness was measured using a digital caliper (Mitutoyo, Hauppauge, NY, USA).

Cell maintenance

Bovine aortic endothelial cells (BAEC; Clonetics, San Diego, CA, USA), passage 2–5, were used for this study. Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO, USA) was prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD, USA), 2 mM L-glutamine, 1 unit/ml penicillin and 100 mg/l streptomycin (GPS; Sigma). Endothelial basal medium (EBM; Sigma) was prepared with 10% endothelial medium supplement (Sigma) containing 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) at 37°C in a 5% CO_2 environment.

Viability of endothelial cells on polyurethaneurea films

To evaluate viability of endothelial cells growing on polymer films, 8-well FlexiPerms (Sigma) were attached to polyurethaneurea films to create culture wells on each surface. BAEC suspensions were prepared and seeded at a concentration of 1.7×10^4 cells/cm^2 on the films. Calcein AM and ethidium homodimer-1 (Live-Dead Assay Kit; Molecular Probes) were used to determine endothelial cell viability after 24 or 72 h in culture. Calcein AM is converted to a green fluorescent product within live cells due to enzymatic activity, while ethidium homodimer-1, a red fluorescent compound, accumulates in dead cells due to increased membrane permeability.

To evaluate cytotoxicity of any leachable products, PUUPPD and PUUYIGSR films were placed in glass vials and dried under vacuum for 48 h. Each film had a 6-cm^2 surface area. According to USP extraction ratio of synthetic polymer films with thickness less than 0.5 mm [41, 42], 1 ml of HEPES-buffered saline (HBS, 10 mM, pH 7.4) solution was added to each vial. Samples were incubated at 37°C. After 30 and 60 days of extraction, endothelial cells were seeded into 24-well tissue culture plate at 1.5×10^4 cells/cm^2.24 h after cell seeding, the extract solutions were sterilized using a 0.2 μm pore size syringe filter and added to the culture medium at 10, 15, or 25% (v/v). After 24 h of incubation with the extracts, cell viability was evaluated by Calcein AM/ethidium homodimer staining as described above.

Adhesion and spreading of endothelial cells on polyurethaneurea films

To evaluate adhesion of endothelial cells, BAECs were seeded at a concentration of 1.7×10^4 cells/cm^2 in 8-well FlexiPerms attached to polyurethaneurea films. Cells were cultured at 37°C in a 5% CO_2 environment. After 4 or 24 h of incubation, non-adherent cells were removed by rinsing and adherent cells were removed with trypsin and counted using a Coulter Counter (Multisizer 3, Beckman Coulter, Miami, FL, USA). To evaluate endothelial cell spreading, after 4 or 24 h of incubation, non-adherent cells were removed by rinsing and fresh medium was added. Cells were observed by phase contrast microscopy (Axiovert 135, Zeiss, Thornwood, NY, USA) and digital image processing (Scion Image, Frederick, MD, USA) was used to determine cell areas.

Competitive inhibition of attachment and spreading of endothelial cells was also examined using soluble YIGSR peptides to ensure that increased BAEC adhesion and spreading was due to bio-specific interactions with the YIGSR peptides that were incorporated into the polymer structure. Cells were seeded at a concentration of 1.7×10^4 cells/cm^2 in 8-well FlexiPerms attached to polyurethaneurea films and incubated with soluble YIGSR peptide (Sigma) at 0.01, 0.1 and 1 mM in EBM/DMEM (25 : 75) at 37°C in a 5% CO_2 environment. After 4 h incubation, attachment and spreading of cells were evaluated as described above.

Proliferation of endothelial cells on polyurethaneurea films

To evaluate endothelial cell proliferation, 8-well FlexiPerms were attached to polyurethaneurea films. Cells were seeded at a concentration of 1.7×10^4 cells/cm^2. After 48 h incubation at 37°C in a 5% CO_2 environment, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was employed. The PCNA antibody stains cells in the S-phase of mitosis. Cells were washed with PBS, fixed with 10% formalin (Stephens Scientific, Riverdale, NJ, USA) for 10 min, permeabilized with methanol (Sigma) for 2 min and incubated in 3% H_2O_2 (Fisher Scientific) for 5 min. Cells were incubated with mouse anti-human PCNA IgG (DAKO, Carpinteria, CA, USA) for 1 h, rinsed with PBS and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (DAKO) for 40 min. Antibodies were diluted 1 : 100 in PBS containing 3% FBS and incubations were performed at room temperature in a humidified chamber. After rinsing with PBS, cells were treated with aminoethylcarbazole chromagen (DAKO) for 10 min. Counter-staining was performed with Mayer's hematoxylin (DAKO). Using this procedure, proliferating cells appear red and non-proliferating cells appear blue. Two wells were used as controls; one well with no primary (anti-PCNA IgG) antibody and one well with neither primary nor secondary (HRP-conjugated anti-mouse IgG) antibody.

Migration of endothelial cells on polyurethaneurea films

To assess migration, a fence-style assay was utilized. BAECs were seeded at a concentration of 4×10^4 cells/cm^2 in 8-well FlexiPerms attached to polyurethaneurea films. When cells reached confluence, the original boundaries were recorded and the FlexiPerm strips were removed. After 24 h of incubation at 37°C in a 5% CO_2 environment, the cells that had migrated over the original boundary were counted using a phase-contrast microscope (Zeiss Axiovert 135).

Endothelialization at an anastomotic site was also simulated in an *in vitro* system. PUUPPD and PUUYIGSR were cast on glass and then 8-well FlexiPerms were attached to the surfaces. Collagen I (Sigma) was dissolved at 2.5 mg/ml in 3% glacial acetic acid. Surfaces coated with collagen I were prepared by adding 50 μl of the collagen I solution into each well and drying under vacuum. After washing the surfaces in PBS three times, endothelial cells were seeded onto the surfaces at 3.4×10^4 cells/cm^2. After 24 h the FlexiPerms were removed, the boundaries were marked and fresh medium was added. After 48 h incubation at 37°C in a 5% CO_2 environment, the number of cells that had migrated across the boundary simulating an anastomotic site, from the collagen-I-coated surface to the polyurethaneurea surface, were counted under a phase-contrast microscope as described above.

Extra-cellular matrix production by endothelial cells

To evaluate extra-cellular matrix (ECM) production, 8-well FlexiPerms were attached to the polyurethaneurea films. BAEC suspensions were prepared with

5 μg/ml ascorbic acid added to the culture medium and seeded at a concentration of 1.7×10^4 cells/cm^2. Four of the wells on each film were cultured with 1 μCi/ml [^{3}H]glycine added to the medium to measure the ECM production via incorporation of the radioactive amino acid into newly synthesized ECM proteins. The other four wells were cultured in the absence of [^{3}H]glycine and used for cell counting. After 48 h of incubation at 37°C in a 5% CO_2 environment, cells cultured in the absence of [^{3}H]glycine were trypsinized and counted on a Coulter Counter. Cells in the remaining wells were rinsed with PBS and DI water and then lysed in 25 mM ammonium hydroxide for 30 min. The remaining ECM was washed with 70% ethanol and air dried. In order to digest glycoprotein, elastin and collagen, the ECM was sequentially exposed to 200 μg/ml trypsin for 4 h, 58 U/ml elastase for 4 h and 76 U/ml collagenase for 8 h at 37°C. Finally, the wells were incubated in 1 M NaOH for 1 h at room temperature to remove any remaining proteins. All enzyme solutions were prepared in TEC buffer (25 mM Tris-HCl, 5 mM calcium chloride, pH 8). ECM production was determined by the amount of radioactivity in the glycoprotein (trypsin-sensitive), elastin (elastase-sensitive) and collagen (collagenase-sensitive) fractions using scintillation counting (Minaxiβ Tri-Carb 4000, Packard Instrument, Meridien, CT, USA). NaOH fractions were also evaluated by scintillation counting to ensure that the ECM had been completely digested.

Effect of peptide incorporation on platelet adhesion

PUUPPD and PUUYIGSR films were cast on glass coverslips (18 mm; Fisher Scientific) as described above. A solution of 2.5 mg/ml collagen I (Sigma) solution was prepared in 3% glacial acetic acid. Glass coverslips were incubated with the collagen I solution for 45 min in a humidified environment at room temperature to provide a highly thrombogenic reference material. Blood was obtained from a healthy volunteer with 10 U/ml heparin (Sigma). 10 μM mepacrine (Sigma) was added in order to fluorescently label platelets. Collagen I (positive control), PUUPPD and PUUYIGSR film surfaces were incubated with mepacrine-labeled whole blood at 37°C for 20 min and then rinsed with PBS. The number of adherent platelets per field of view (200×) was determined using a fluorescent microscope (Zeiss Axiovert 135).

Statistic analysis

Data were compared with two-tailed, unpaired t-tests. P-values less than 0.05 were considered to be significant. All error bars in this paper represent standard deviations.

RESULTS

Synthesis and characterization of polyurethaneurea

The NMR spectra of PUUPPD and PUUYIGSR were obtained and the characteristic proton peaks of tyrosine (6.5–7.0 ppm) from the GGGYIGSRGGGK sequence indicated the successful incorporation of the peptide sequence into the PUUYIGSR polymer. To ensure that the peptides were not just physically entangled or mixed in polymer matrix, the polymer was washed and filtered several times with methanol prior to NMR measurement. The peaks of pre-polymer, PUUPPD and PUUYIGSR were also assigned and characterized. Then the number of the protons was calculated from the intensity. The intensity was compared to theoretical values. The reactivity of the peptide into the polymer was nearly 100%. ESCA and contact angle measurements also supported incorporation of the peptide sequences. The peptide concentration of the polymer matrix was approx. 56 μmol/g, determined from NMR.

The number-average molecular weight (M_n), the weight-average molecular weight (M_w) and the polydispersity index (PDI) were determined by GPC using polystyrene standards. The PUUPPD and PUUYIGSR polymers had similar molecular weights (PUUPPD: $M_n = 40\,001$, $M_w = 53\,307$, PDI $= 1.33$ and PUUYIGSR: $M_n = 43\,054$, $M_w = 54\,531$, PDI $= 1.27$).

The surface atomic concentration was determined using ESCA with various take-off angles (PUUPPD: C = 83.6%, N = 1.1%, O = 15.3% at 10° and C = 86.7%, N = 0.8%, O = 12.5% at 45°, PUUYIGSR: C = 70.6%, N = 3.4%, O = 26.0% at 10° and C = 77.2%, N = 1.3%, O = 21.4% at 45°). Nitrogen (N) and oxygen (O) concentrations are related to the urea linkages of the hard segments and amide linkages of the peptide sequences. The higher level of nitrogen and oxygen detected on the PUUYIGSR surface indicates that the GGGYIGSRGGGK peptide sequences were successfully incorporated into the polymer backbone and present at the surface of the material.

There was no significant difference in the FT-IR spectra between PUUPPD and PUUYIGSR. For both PUUPPD and PUUYIGSR, the hydrogen-bonded urea carbonyl peak occurred at 1640 cm^{-1}. The urethane carbonyl peaks appeared at 1720 cm^{-1} for hydrogen bond and at 1740 cm^{-1} for free bond. The CH stretch peaks of the soft segment, PTMG, appeared at 2850 cm^{-1} and 2940 cm^{-1} and the hydrogen-bonded NH peak also appeared at 3310 cm^{-1} [40, 43].

The thermal behaviors of PUUPPD and PUUYIGSR were examined using DSC. The glass transition temperature (T_g) was determined to be −30°C for PUUPPD and −45°C for PUUYIGSR. The melting point (T_m) was approximately 260°C for both polymers. Thus, the introduction of peptides into the polymer backbone lowered the T_g of the soft segment, but did not affect the T_m of the hard segment.

Characterization of polyurethaneurea film

The polymer films were fabricated using a solvent-casting method on glass coverslips. The bulk polymer characterization shows that GGGYIGSRGGGK peptide sequences were successfully incorporated into the polymer at a ratio of approx. 56 μmol/g polymer. Based on this, the surface peptide concentration of PUUYIGSR is approx. 0.2 nmol/cm^2. This concentration was calculated using the polymer density (1.25 g/cm^3) and the thickness of the monolayer (20 nm). The thickness of the monolayer was obtained based on hard segment length [44–46]. Water contact angle measurements showed that the contact angle on PUUYIGSR ($68.8 \pm 1.17^\circ$) was approximately about 10° lower than the contact angle on PUUPPD ($80.16 \pm 1.72^\circ$), indicating that the surface of PUUYIGSR is more hydrophilic and polar due to the incorporated peptides.

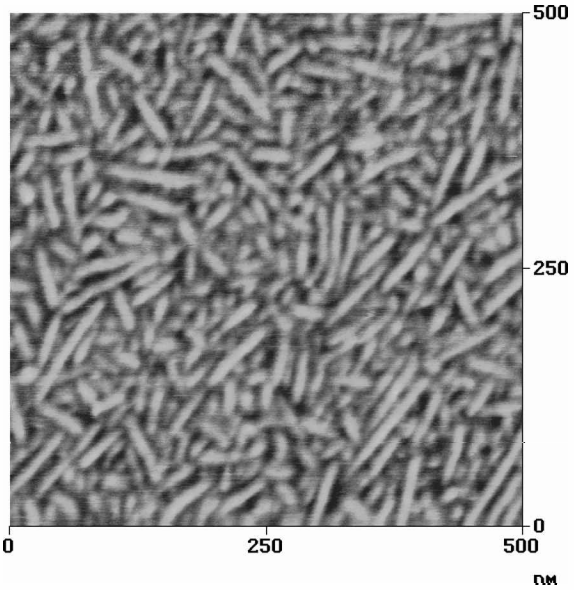
Distribution of peptides and hard segment domains

In order to study the peptide distribution on the surface of bioactive polyurethaneurea films, a fluorescent dye, NBD-Cl, capable of reacting with the tyrosine residues under aqueous conditions [47], was employed. When reacted with NBD-Cl, PUUPPD showed no fluorescence; however, PUUYIGSR showed bright orange fluorescence evenly distributed over its entire surface area. This result suggests that peptide is uniformly distributed on the surface of the bioactive PUUYIGSR polymer.

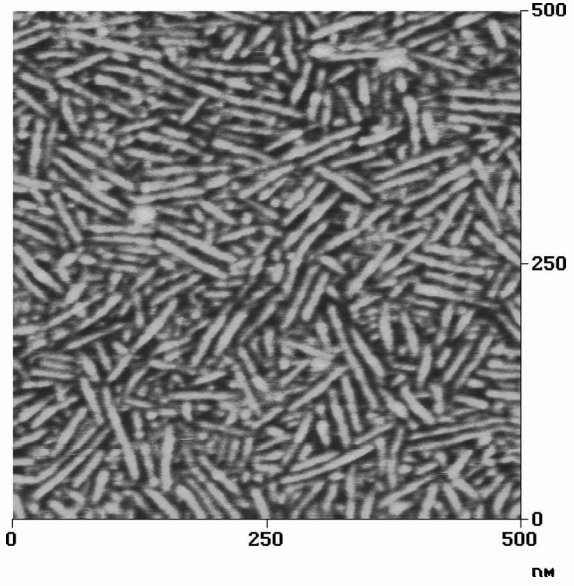
The hard segment distribution on the surface was also examined using AFM. Figure 1 shows AFM tapping mode images from the surface of polyurethaneurea films at $r_{sp} = 0.88$. The r_{sp} value is the ratio of set point amplitude and free amplitude of oscillation. At $r_{sp} = 0.88$, the very top layer of the surface is being detected [46]. The hard segment domains were exposed to the surface and appeared bright. The cylindrical and spherical hard segment domains were distributed in the soft segment matrix very randomly on the PUUPPD surface. However, on PUUYIGSR, most hard segment domains were cylindrical shape and arranged parallel to the plane of the surfaces. Even at low tapping force, $r_{sp} = 0.80$, the patterns of hard segment distribution in PUUPPD and PUUYIGSR were very similar to $r_{sp} = 0.88$.

Mechanical properties of polyurethaneureas

The incorporation of the peptides into the polymer backbone did not significantly affect the tensile strength (7.3 ± 0.29 MPa for PUUPPD and 7.6 ± 0.71 MPa for PUUYIGSR). However, the elastic modulus was decreased from 3.9 ± 0.70 MPa for PUUPPD to 0.9 ± 0.07 MPa for PUUYIGSR ($P < 0.005$) and elongation was increased from $123.2 \pm 18.6\%$ for PUUPPD to $512 \pm 59.6\%$ for PUUYIGSR ($P < 0.001$).



(a)



(b)

Figure 1. AFM tapping mode phase images of the surfaces of polyurethaneurea at $r_{sp} = 0.88$. Phase images are 500 × 500 nm. (a) PUUPPD, (b) PUUYIGSR.

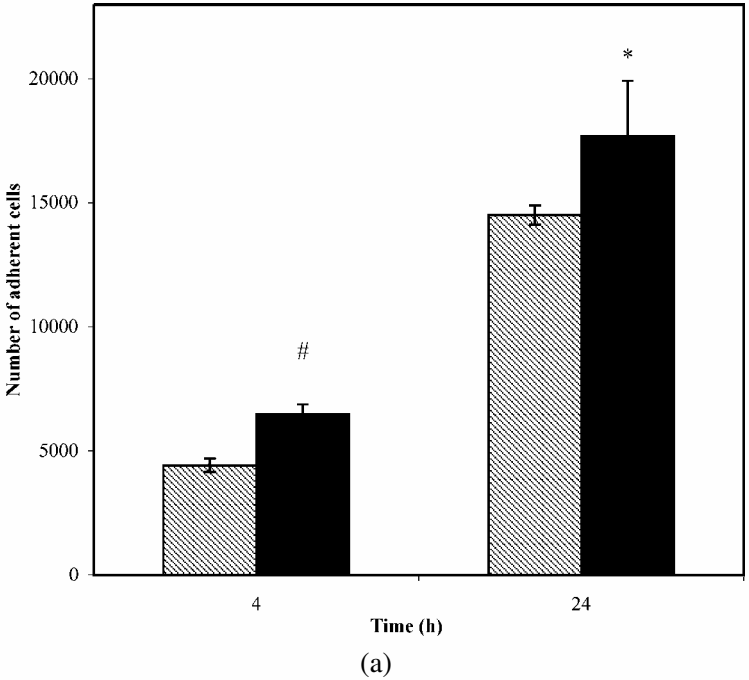
BAEC viability and cytotoxicity of polyurethaneurea films

BAECs were seeded and cultured on the polyurethaneurea films and also cultured in extract solutions from the films to evaluate cytotoxicity of any leachables. Over

95% of endothelial cells remained viable at 24 or 72 h when cultured on PUUPPD and PUUYIGSR films. In addition, to evaluate cytotoxicity of any leachables under aqueous conditions, the polyurethaneurea films were incubated in HBS for 30 or 60 days and BAECs were then cultured with the extract solutions. Over 90% of BAECs were viable over the three different extract concentrations for both PUUPPD and PUUYIGSR.

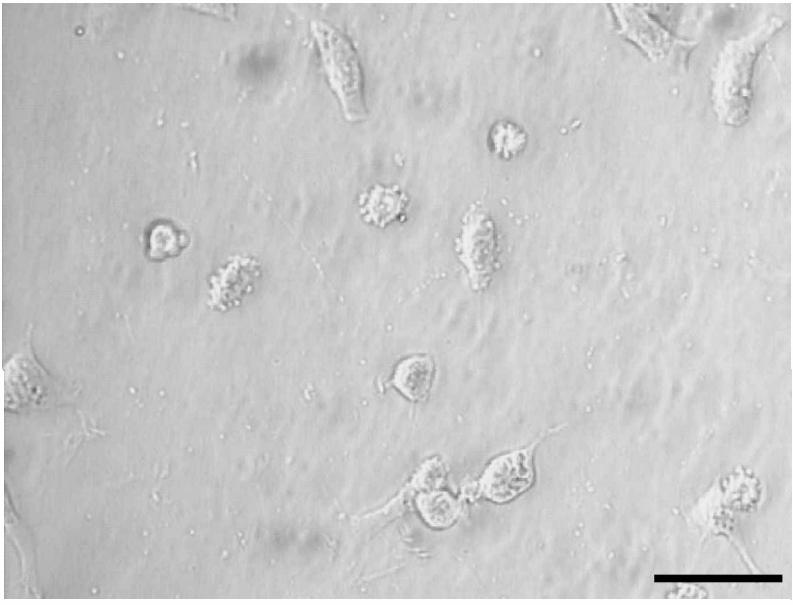
BAEC adhesion and spreading on polyurethaneurea films

We examined whether the peptides incorporated into the polymer backbone could enhance BAEC adhesion and spreading. The attachment of BAECs on polymer films is shown in Fig. 2. The number of adherent cells increased with increasing incubation time. The number of adherent cells on PUUYIGSR was significantly higher than on PUUPPD after both 4 and 24 h incubation ($P < 0.01$ for 4 h and $P < 0.05$ for 24 h). Spreading of endothelial cells on polyurethaneurea films was also investigated (Fig. 3). Cell areas and the percent of spread cells increased with increasing incubation time for both surfaces. However, BAECs on PUUYIGSR

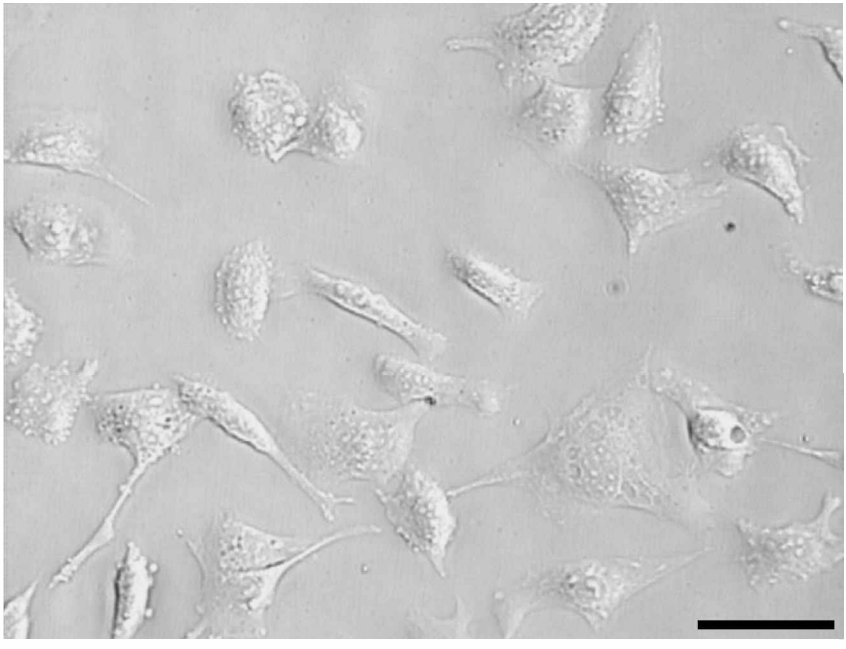


(a)

Figure 2. Attachment and phase-contrast micrographs of BAECs on polyurethaneurea films. (a) Number of adherent cells after 4 and 24 h incubation. Data represent the mean of 3 samples. $^{*}P < 0.05$, $^{\#}P < 0.01$, compared to PUUPPD. Shaded columns represent PUUPPD and filled columns PUUYIGSR. Phase-contrast micrographs of BAECs on (b) PUUPPD and (c) PUUYIGSR films after 4 h incubation. Scale bar = 50 μm.



(b)



(c)

Figure 2. (Continued).

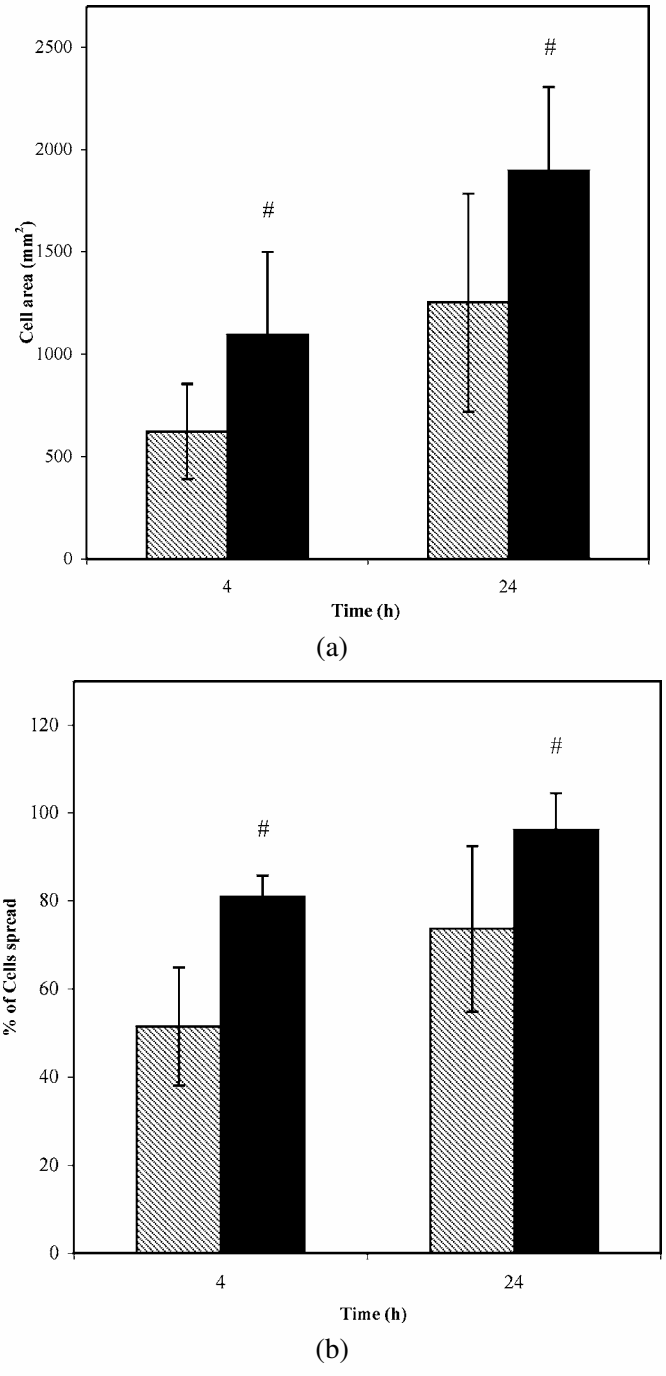


Figure 3. Spreading of BAECs on polyurethaneurea films. (a) Cell-surface area, (b) percent of cells that were spread. Data represent the mean of 30 samples for cell-surface areas and 10 samples for percent of cell spreading. $^{\#}P < 0.01$, compared to PUUPPD. Shaded columns represent PUUPPD and filled columns PUUYIGSR.

showed significantly greater cell-surface area and percent of spreading cells than on PUUPPD after both 4 and 24 h of culture ($P < 0.01$).

In order to ensure that the improved cell adhesion and spreading were due to bio-specific interactions with YIGSR peptides in the bioactive polymer, competitive inhibition of endothelial cell attachment and spreading was investigated using soluble YIGSR peptides in the culture media. As shown in Fig. 4a, the number of adherent cells on PUUYIGSR was significantly greater than on PUUPPD but the values were reduced in the presence of soluble YIGSR peptides over the entire ranges of the soluble peptide concentrations. Cell-surface area and the percent of cell spreading were also reduced over the entire range of soluble peptide concentrations (Fig. 4b and 4c). Thus, these results indicate that the improved cell adhesion and spreading were specifically mediated by YIGSR-sensitive cell-adhesion receptors.

BAEC proliferation, migration and ECM production on polyurethaneurea films

The effect of peptide incorporation on endothelial cell proliferation was examined using immunohistochemical staining with a PCNA–HRP conjugate. PCNA is present in cells in the S-phase of mitosis and indicates proliferative activity. The percent of PCNA-positive cells on PUUYIGSR was significantly greater than on PUUPPD after 48 h of culture ($78 \pm 5\%$ *vs.* $60 \pm 4\%$, $P < 0.001$).

A fence-style assay was used to assess endothelial cell migration on the polyurethaneurea films. The number of cells that had migrated over the original boundary was significantly greater on PUUYIGSR compared to that on PUUPPD (63 ± 8 *vs.* 37 ± 12, $P < 0.05$). In addition, endothelialization at an anastomotic site was simulated in an *in vitro* system. It is very important for endothelial cells to migrate across the anastomotic site, from a natural ECM environment to the synthetic polymer material, to cover the surface of implanted grafts. A fence-style assay was also used and the number of cells that had migrated over the original boundary from the collagen-I-coated surface to the polyurethaneurea surface was determined using a phase-contrast microscope. On PUUYIGSR, more cells migrated across anastomotic sites than on PUUPPD (216 ± 95 *vs.* 76 ± 29, $P < 0.02$), as shown in Fig. 5.

To evaluate extracellular matrix (ECM) production, cells were incubated in the presence of [^{3}H]glycine on the polyurethaneurea films. ECM production was determined by incorporation of [^{3}H]glycine into glycoprotein, elastin and collagen of ECM by using sequential enzyme digests. The ECM production by BAECs was significantly increased on PUUYIGSR compared to PUUPPD (0.0075 ± 0.0004 cpm/cell *vs.* 0.0055 ± 0.001 cpm/cell, $P < 0.05$).

Effect of peptide incorporation on platelet adhesion

Since some peptides, such as RGD, support adhesion of platelets [28, 29], the effect of the YIGSR peptide sequences incorporated in the polymer backbone on platelets

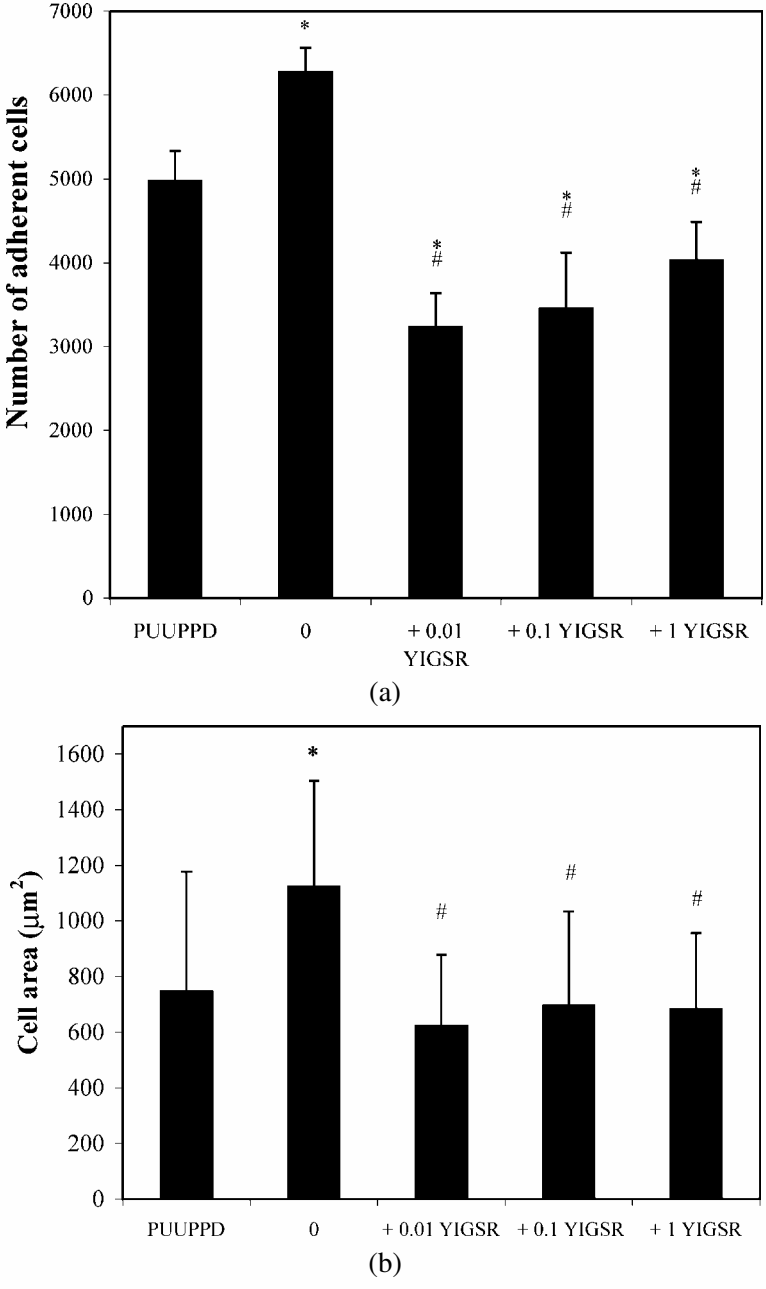


Figure 4. Competitive inhibition of attachment and spreading of BAECs by soluble YIGSR peptides. BAECs were incubated with soluble YIGSR peptides at three different concentrations (0.01, 0.1 and 1 mM). (a) Competitive inhibition of attachment. Data represent the mean of four samples. $^{*}P < 0.02$, compared to PUUPPD, $^{\#}P < 0.01$, compared to untreated PUUYIGSR (0 mM soluble peptide). (b) Cell-surface area and (c) percent of cells that were spread. Data represent the mean of 30 samples for cell-surface areas and 10 samples for percent of cell spreading. $^{*}P < 0.05$, compared to PUUPPD. $^{\#}P < 0.001$, compared to untreated PUUYIGSR (0 mM soluble peptide).

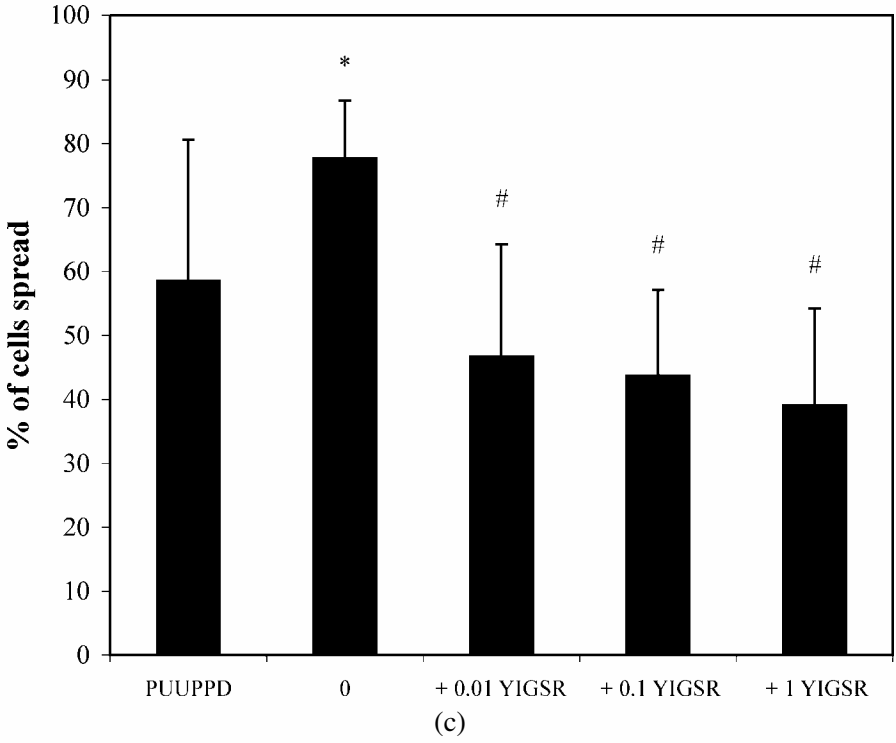


Figure 4. (Continued).

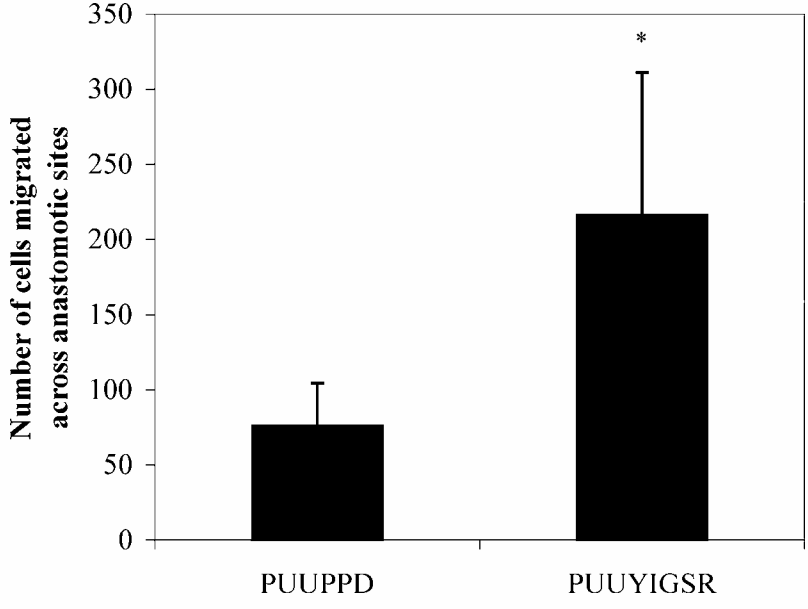


Figure 5. Migration of BAECs across simulated anastomotic sites; from collagen I surfaces to polyurethaneurea surfaces. Data represent the mean of 6 samples. $^{*}P < 0.02$, compared to PUUPPD.

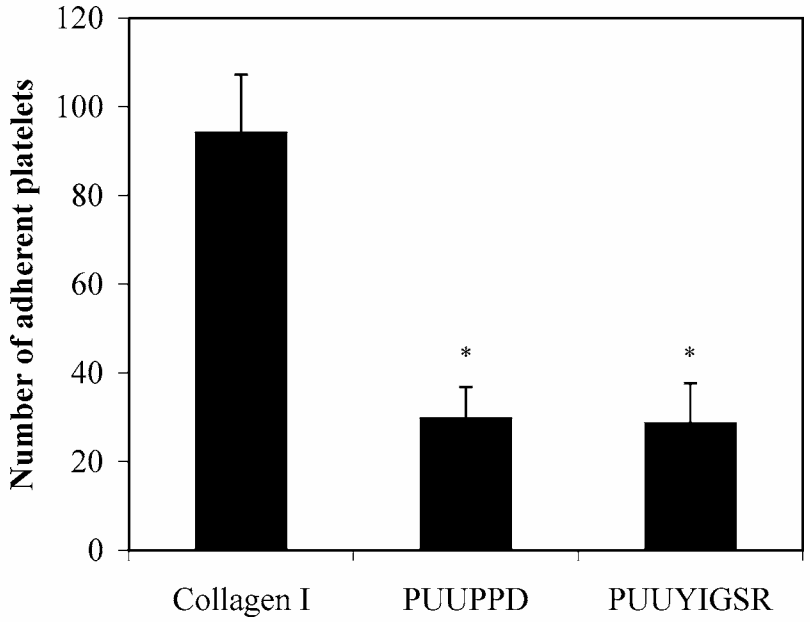


Figure 6. The effect of the YIGSR peptide in the polyurethaneurea structure on the platelet adhesion. Platelets were fluorescently labeled with mepacrine. Data represent the mean of 10 samples. $^{*}P < 0.001$, compared to collagen I.

adhesion was examined using mepacrine-labeled whole blood (Fig. 6). Platelet adhesion on PUUPPD (29.7 ± 7.1) and PUUYIGSR (28.6 ± 9.1) was dramatically lower than on collagen I (94.1 ± 13.1), the positive control. Additionally, there was no significant difference in the number of adherent platelets between PUUPPD and PUUYIGSR, indicating that the incorporation of the YIGSR adhesion peptide did not enhance thrombogenicity.

DISCUSSION

Endothelialization of polyurethaneurea grafts is a possible strategy to improve graft patency, especially for small diameter vascular grafts. The laminin-derived peptide YIGSR has been used to improve endothelial cell adhesion and spreading [31, 32, 39] and, thus, may aid in graft endothelialization. We have developed bioactive polyurethaneurea by incorporation of the peptide sequence GGGYIGSRGGGK into the polymer backbone. This peptide contains two amine groups (one at the N-terminus and one on the lysine residue) to allow incorporation into the polymer backbone. Glycine group was used as a spacer. Incorporation of peptides into the polymer structure is a very easy way to modify polyurethaneureas compared to surface modification because the peptide can be used as a chain extender during polymer synthesis. Additionally, bulk incorporation allows one to develop microporous materials that are uniformly cell adhesive.

Successful incorporation of the peptide sequences into the polymer backbone was assessed by several polymer characterization techniques. The NMR spectra, ESCA and contact angle results support the successful incorporation of the peptide sequence into the polymer backbone. Phase separation of polyurethaneurea was evaluated using differential scanning calorimetery (DSC). Previously, the introduction of a second diamine was shown to decrease phase separation [48]. However, in this study, the peptide incorporation did not significantly affect phase separation according to DSC results. In addition, interesting results were found with respect to mechanical properties. The peptide incorporation made the polymer more elastic without decreasing its tensile properties. The improved elasticity may be helpful to transmit mechanical stimuli to the seeded endothelial cells and also improve compliance matching, hopefully reducing intimal hyperplasia at the anastomotic sites [49].

The distribution of the peptides on the surfaces of the PUUYIGSR films was investigated using fluorescent probes. Previously, YIGSR was immobilized to the glass surfaces and dansyl chloride was reacted with the amino-terminus of the peptides [50]. They reported that the peptides were not uniformly distributed and bright fluorescent clusters were found on the surfaces. In this study, bright orange fluorescence was found evenly on the entire surface area of the PUUYIGSR film, but no fluorescence was observed on PUUPPD. The distribution of the hard segment domains was also examined using AFM. The morphology of the phase-separated microdomains can be visualized using tapping-mode AFM [48]. Cylindrical or spherical hard segment domains were visualized clearly on the tapping mode images. In this study, we found that the second diamine (peptides) can affect the distribution or the arrangement of hard segment domains on the surfaces: the hard segment domains of PUUYIGSR were arranged parallel to the plane of the surface compared to random distribution of those of PUUPPD.

Cell adhesion on biomaterials is mediated by cell-surface receptors and cell-adhesion proteins adsorbed to the material surfaces. Thus, modification of biomaterials with adhesive peptides has been shown to promote cell adhesion and spreading [26, 27, 51, 52]. Furthermore, the surface peptide concentration has been shown to affect cellular functions. Massia and Hubbell suggested that minimal RGD peptide spacing of 440 nm for spreading and 140 nm for contact formation were required on glass substrates. They found focal contacts of spreading human foreskin fibroblasts at 10 and 100 fmol/cm^2 [30]. Endothelial cell spreading was improved at 13 pmol/cm^2 of RGD-modified poly(lactic acid-co-lysine) [25]. Adhesion of endothelial cell was increased more than 160% at 30–40 pmol/cm^2 of RGDS-modified poly(ester-urethane)ureas compared to tissue-culture polystyrene [53]. Hubbell *et al.* found that cellular attachment was affected by peptide surface concentration, spacer length and particular peptide immobilized [34]. Therefore, the optimal surface concentrations of the peptides might be dependent on cell type, peptides and substrates.

We demonstrated that attachment, proliferation, migration and extra-cellular matrix production of endothelial cells were enhanced without increasing platelet ad-

hesion on PUUYIGSR. In addition, endothelialization at an anastomotic site was simulated in an *in vitro* system. The greater number of cells had migrated across anastomotic sites on PUUYIGSR compared to PUUPPD. To enhance endothelialization *in vivo*, it is very important for endothelial cells to migrate across anastomotic sites, from the adjacent vessel wall to the synthetic biomaterial, to cover the surfaces of implanted grafts.

CONCLUSIONS

A bioactive YIGSR-peptide-modified polyurethaneurea (PUUYIGSR) has been successfully synthesized by incorporating the peptide GGGYIGSRGGGK into the polymer backbone. Cellular responses of endothelial cells on PUUYIGSR show that the incorporated peptides maintain their bioactivity and may potentially improve vascular graft endothelialization.

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