Published in final edited form as:

J Biomed Mater Res B Appl Biomater. 2018 January ; 106(1): 278–290. doi:10.1002/jbm.b.33853.

Development of an electrospun biomimetic polyurea scaffold suitable for vascular grafting

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Abstract

The optimization of biomechanical and biochemical properties of a vascular graft to render properties relevant to physiological environments is a major challenge today. These critical properties of a vascular graft not only regulate its stability and integrity, but also control invasion of cells for scaffold remodeling permitting its integration with native tissue. In this work, we have synthesized a biomimetic scaffold by electrospinning a blend of a polyurea, poly(serinol hexamethylene urea) (PSHU), and, a polyester, poly-ε-caprolactone (PCL). Mechanical properties of the scaffold were varied by varying polymer blending ratio and electrospinning flow rate. Mechanical characterization revealed that scaffolds with lower PSHU content relative to PCL content resulted in elasticity close to native mammalian arteries. We also found that increasing electrospinning flow rates also increased the elasticity of the matrix. Optimization of elasticity generated scaffolds that enabled vascular smooth muscle cells (SMCs) to adhere, grow and maintain a SMC phenotype. The 30/70 scaffold also underwent slower degradation than scaffolds with higher PSHU content, thereby, providing the best option for *in vivo* remodeling. Further, Gly-Arg-Gly-Asp-Ser (RGD) covalently conjugated to the polyurea backbone in 30/70 scaffold resulted in significantly increased clotting times. Reducing surface thrombogenicity by the conjugation of RGD is critical to avoiding intimal hyperplasia. Hence, biomechanical and biochemical properties of a vascular graft can be balanced by optimizing synthesis parameters and constituent components. For these reasons, the optimized RGD-conjugated 30/70 scaffold electrospun at 2.5 or 5 mL/h has great potential as a suitable material for vascular grafting applications.

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Notes

The authors declare no competing financial interests.

Keywords

Vascular graft; elastic modulus; SMC; degradation; RGD; hemocompatibility

INTRODUCTION

One of the major challenges in the tissue engineering of small caliber (diameter < 6mm) vascular grafts is achieving an optimal balance between its biomechanical and biologic properties. There is evidence that the biologic properties of a vascular graft including biocompatibility and hemocompatibility strongly influence the biomechanical properties of the graft and vice versa.^{1,2} It is also well known that there is a linear correlation between vascular graft compliance and one-month patency-rate, i.e. whether the graft remains patent *in vivo* after implantation.³ Commonly used synthetic materials for artificial vascular grafts such as extended-polytetrafluoroethylene (e-PTFE) and Dacron generally do not promote remodeling or change in graft compliance after implantation due to improper microporous structure and/or poor vascular cell infiltration.⁴ Grafts made of synthetic materials exhibit a decrease in patency over time due to unfavorable inflammatory reactions, thrombosis due to low blood flow, improper endothelialization due to high shear, and, subsequent intimal hyperplasia (IH) and calcification.^{3–5} Synthetic grafts exhibit long term lower patency rates even with luminal modification techniques such as heparin-bonding⁶ and *in vitro* endothelialization.⁷

Synthetic vascular grafts generally do not allow easy modification of their luminal surfaces or inhibit undesired inflammatory responses by themselves. Hence, these grafts do not provide ideal long-term hemocompatibility. However, using robust techniques, anti-thrombotic biomolecules such as collagen, chitosan, heparin, thrombomodulin, Arg-Gly-Asp, and albumin, can be immobilized non-covalently on the luminal surface of the graft.^{8–13} Studies have validated the role of such biomolecules in promoting transanastomotic and blood-borne migration and attachment of endothelial cells (ECs) on the luminal surface of the vascular graft.^{14–16} With rapid endothelialization, the endothelial cells form a barrier that prevents the flux of fluid and protein into the grafts. Endothelial cells act as an anti-thrombogenic surface since they inhibit platelet adhesion, fibrinolysis and clotting.¹⁷ Endothelial cells also serve as a source of molecules that act to minimize the migration of the SMCs into the luminal side of the graft, thereby, reducing IH.^{18–20} Independent of the migratory effects of these biomolecules, they prevent the attachment of platelets to the luminal surface of the graft promoting sustained hemocompatibility.^{12,21} Hence, the immobilization of specific biomolecules can impart improved hemocompatibility to tissue-engineered vascular grafts (TEVGs). However, vascular grafts exhibiting both optimal compliance and hemocompatibility characteristics have not been well documented.

Previously, we synthesized a versatile polyurea, poly(serinol hexamethylene urea) (PSHU) and demonstrated that it can be electrospun into scaffolds suitable for guiding neuron and neuron-like cells in peripheral nerve regeneration.²² The polyurea was covalently conjugated with the Gly-Arg-Gly-Asp-Ser (herein; simply RGD) peptide in its backbone, which improved the survival, migration and attachment of neuron-like cells. Found in numerous

cell adhesion proteins, the Arg-Gly-Asp tripeptide motif, mediates cell attachment.²³ RGD sequences have also been shown to promote endothelialization of the vascular graft after implantation.¹⁰ RGD also imparts excellent hemocompatibility to the tissue engineered vascular graft since it prevents the attachment of platelets to the graft surface.¹⁰ However, PSHU is a mechanically weak polymer owing to its lower molecular weight (~9.7kDa) making it unsuitable for electrospinning as a standalone polymer. Electrospun poly-ε-caprolactone (PCL) matrices, on the other hand, have been shown to be mechanically strong yet compliant, and, used as slow degrading scaffolds capable of supporting cell attachment, growth and proliferation in various tissue engineering applications.^{24–26} However, by itself, the PCL scaffold is relatively inert and provides limited scope for biochemical modifications. Hence, PCL has been blended with other materials to improve its functionality.^{13,27}

Based on these observations, we hypothesized that RGD-conjugated PSHU (PSHU/RGD) blended with PCL will serve as an excellent material for a TEVG because it possesses synthetic polypeptide-like bonds and RGD groups mimicking the structure and function of the native extracellular matrix components as well as reducing the thrombogenicity of the graft long-term and exhibiting optimal elasticity. PSHU was blended with PCL ($M_w=80\text{kDa}$) and electrospun into scaffolds. The elasticity of the scaffold was controlled by varying electrospinning blend ratio (100/0, 70/30, 50/50, 30/70 and 0/100) and flow rates (1, 2.5, and 5mL/h). To determine the bulk properties, we performed structural characterization of the scaffold using scanning electron microscopy, mechanical characterization of the scaffold using tensile testing, and biochemical characterization of the scaffold using Fourier transform infrared spectroscopy (FTIR). The biocompatibility of the scaffolds were evaluated with bovine pulmonary artery SMCs and the effect of scaffold stiffness on SMC proliferation was also studied. The degradation rates of the different scaffolds were also determined in an oxidative degradation study. After optimization of the elasticity of the electrospun scaffolds, we covalently conjugated Gly-Arg-Gly-Asp-Ser (GRGDS; herein simply RGD) to the PSHU backbone to improve hemocompatibility of the electrospun scaffold. To study the hemocompatibility, clotting times, including activated partial thromboplastin time, unactivated partial thromboplastin time and thrombin time, were determined after treating the grafts with freshly collected bovine plasma. Our results demonstrate that (1) the blending the two polymers in the electrospun scaffold provides a balance between the biomechanical and the biologic properties, and, (2) the RGD-conjugated polyurea backbone imparts superior hemocompatibility making the optimized scaffold very suitable for vascular grafting.

EXPERIMENTAL SECTION

Materials

N-BOC serinol, urea, hexamethylene diisocyanate, anhydrous N,N-dimethylformamide (DMF), poly-ε-caprolactone (80,000g/mol), n-hydroxysuccinamide (NHS), anhydrous lithium-N,N-dimethylformamide (Li-DMF), hydrogen peroxide, cobalt chloride, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC-HCL) and 3.5kDa cellulose dialysis tube were purchased from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic

acid, methylene chloride, trifluoroethanol, diethyl ether and 1,1,1,3,3,3 hexafluoro-2-propanol (HFP) were purchased from Alfa Aesar (Wardhill, MA). RGD was purchased from Biomatik Inc. (Wilmington, DE, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Invitrogen (Grand Island, NY). Bovine pulmonary artery smooth muscle cells were provided by Dr. Kurt Stenmark. The hemocompatibility study kits were kindly provided by Dr. Jorge DiPaola (Department of Pediatrics-Hematology, University of Colorado Denver, Anschutz Medical Campus). Rabbit antibody for α -smooth muscle-actin (clone 1A4) and calponin (clone CALP-1) were purchased from Sigma Chemical, St. Louis, MO. Rabbit antibody for smooth muscle heavy chain myosin kindly provided by Dr. R. S. Adelstein (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD).

Equipment

For electrospinning, the syringe pump was purchased from Fisher Scientific (Pittsburgh, PA) and high-voltage power supply was purchased from Gamma High Voltage Research (Ormand Beach, FL). SEM was performed on a JEOL low vacuum scanning electron microscope. The blood coagulation studies were performed using an automatic coagulation analyzer and the glass transition temperature of the polymer was determined using a differential scanning calorimeter from Thermo Scientific Inc.

Synthesis of PSHU & PSHU/RGD

PSHU was synthesized as previously described.²² Briefly, urea and N-BOC serinol were dissolved in DMF at room temperature. Then, the reaction mixture was stirred continuously for 7 days at 90°C after hexamethylene diisocyanate was added drop-wise. After washing in diethyl ether and nanopure water thrice, the resultant product was lyophilized yielding the pale yellow PSHU. 1 g of PSHU was weighed out in a round bottom flask and 15 mL of methylene chloride was added. Then, the reaction mixture constant was stirred for 45 min at room temperature after 15mL of trifluoroacetic acid was added drop-wise. Then, after removing trifluoroacetic acid by rotoevaporation, the final deprotected PSHU (dPSHU) was obtained after washing in diethyl ether and nanopure water thrice. Then, RGD was activated with EDC-HCL and NHS in DMF under constant stirring at room temperature for 2 hours. After 2 hours, dPSHU, dissolved in DMF, was added drop-wise into the reaction mixture and the reaction was constantly stirred at room temperature for 1 day. Then, after washing in diethyl ether, the polymer was dialyzed in a 3.5kDa cellulose dialysis tube in nanopure water for 3 days, and, then, lyophilized.

Characterization of PSHU

The molecular weights of the unconjugated polymer samples were evaluated using gel permeation chromatography. The samples were dissolved in Li-DMF at 1 mg/mL concentration. These solutions were then injected through the columns of the gel permeation chromatograph to determine the size of the permeated through the columns. Each injection was repeated 3 times to get the average molecular weights of the unconjugated polymer samples.

Thermal behavior of the unconjugated polymer was analyzed using a differential scanning calorimeter. Approximately, 10 mg of the samples were placed in aluminum pans under a nitrogen atmosphere, heated to 150 °C, cooled to -10 °C, and then heated to 200 °C. The thermograms were recorded at a rate of 10 °C/min. The thermogram shown in the next section refers to the final heating. Glass transition temperature (T_g) was calculated using the software provided by Thermo Scientific, Inc.

Electrospinning of PSHU blended with PCL

To electrospin the polymer, PSHU was blended with PCL in different ratios (70/30, 50/50, 30/70, 0/100) at 8% concentration in HFP. The blended solutions were electrospun on flat stationary aluminum sheet or an 4mm aluminum rod rotating at speed of 600 rpm set to a voltage of 15 kV. The PSHU-PCL (30/70) was electrospun at different electrospun flow rates, 1, 2.5, 5 mL/h. Flat samples were used in biochemical, biomechanical, structural characterizations, biocompatibility and degradation studies. Tubular samples were used for the hemocompatibility studies.

Structural characterization using SEM

PSHU-PCL (70/30, 50/50, 30/70, 0/100) scaffolds electrospun at 1 mL/h and PSHU-PCL (30/70) samples electrospun at 2.5 and 5 mL/h were dehydrated in a lyophilizer for 48 h. The samples were sputter-coated with a thin layer of gold across the sample surface for 30s at an operating current of 40 mA in a vacuum chamber. The sample was imaged under an operating voltage of 5 kV. The pore sizes of the PSHU-PCL (30/70) electrospun at 1, 2.5, 5 mL/h were calculated from the SEM images using ImageJ software.

Biomechanical testing

PSHU-PCL (70/30, 50/50, 30/70, 0/100) scaffolds electrospun at 1 mL/h and PSHU-PCL (30/70) samples electrospun at 2.5 and 5 mL/h were placed in 1X PBS for 24 h to maintain hydration. Uniaxial tensile testing with a 5 kN load cell was performed using a 5 kN MTS Insight load frame material testing system. Uniaxial tensile testing was performed either at room temperature in dry conditions or in an environmental chamber with deionized water circulated at 37°C, with the grips pulled apart at a 5 mm/min strain rate to 200% strain or to failure, whichever occurred earlier. The elastic modulus was calculated in the initial linear region. The effect of variation of blending ratio and the flow rates on the elastic modulus of the electrospun scaffolds was studied.

Cell culture studies

PSHU-PCL blends (100/0, 70/30, 50/50, 30/70 and 0/100) were electrospun at 1 mL/h and in the ratio 30/70 at 1, 2.5 and 5 mL/h directly onto circular glass cover slips. Then these samples were placed in a 24-well plate and treated with high glucose DMEM substituted with 10% bovine calf serum for 24 h to maintain hydration. After this, the samples with the varying blend ratios were seeded with bovine pulmonary artery SMCs at a concentration of approximately 4×10^4 cells in 300 μ L of media. A plain polystyrene plate was used as control. On days of 2, 4 and 6, the media was carefully removed and fresh media was added to the culture. The cells were allowed to proliferate on these scaffolds for 7 days and, on

days of 1, 3, 5 and 7, they were trypsinized from the scaffolds and counted using a hematocrit counter. The cell counts were normalized with respect to initial plating density. The cell numbers were used to study the effect of the stiffness of the matrix on cell proliferation varied by the blending ratio as well as the flow rate.

Immunostaining of cells cultured on electrospun scaffolds

For immunostaining, bovine pulmonary artery SMCs (approximately 4×10^4 cells in 300 μ L of media) were plated onto 30/70 scaffold electrospun both in 4mm aluminum rod as well as circular glass cover slips. After 3 days, cells were fixed in 1:1 Acetone-Methanol and immunolabeling was performed using antibodies against the following smooth muscle variants of contractile/cytoskeleton proteins: α -smooth muscle-actin (α -SM-actin), calponin (both at 1:100 dilution); affinity-purified rabbit polyclonal antibodies against smooth muscle variants of myosin heavy chains (SM-Myosin)²⁸ and used at 1:2000 dilution (these antibodies react strongly with SM-1 and SM-2 isoforms of myosin heavy chains and, at the dilution used (1:2000), do not recognize non muscle myosin heavy chain isoforms).

Scaffold degradation study

The scaffold degradation study was performed as described previously.²⁹ Briefly, the treatments were conducted at 37°C on unstrained scaffolds, PSHU-PCL (70/30, 50/50, 30/70, 0/100) electrospun at 1 mL/h and PSHU-PCL (30/70) electrospun at 1, 2.5 & 5 mL/h, in an oxidative solution that simulates the effect of the *in vivo* environment at an enhanced rate. A 20% hydrogen peroxide in 0.1 M cobalt chloride oxidative solution was used to treat electrospun scaffolds for up to 28 days. Solutions were changed every 3 days to relatively maintain a constant concentration of radicals. Scaffolds were removed every 7 days to examine chemical and physical degradation. Specimens were rinsed thrice in nanopure water and dehydrated in a lyophilizer. The weights of the samples were measured before the beginning of the treatment with the oxidative solution and after lyophilization post-treatment. Three samples were used to calculate the average change in the weights. Based on the weight change the rate of degradation of the scaffolds was also determined by fitting the data points to straight line using Microsoft Excel®.

Hemocompatibility study

Blood was collected from a cow in citrate tubes and plasma was extracted by centrifuging the blood at 5000 rpm and 4°C. The plasma was then frozen in 1.5 mL centrifuge tubes and stored in a -80°C freezer for at least 48 h. The hemocompatibility study was performed as described previously.¹² Tubular scaffolds of PSHU-PCL (30/70), PSHU/RGD-PCL (30/70) and PCL were cut into smaller cylinders of approximately 3 mm height. These samples were placed in 1.5 mL centrifuge tubes. Then, 500 μ L of bovine plasma was thawed and was put into each of the sample tubes. The plasma was allowed to incubate for 1 h at 37°C. Untreated plasma was used as control. The full bovine plasma activated partial thromboplastin time (APTT), unactivated partial thromboplastin time (UPTT) and thrombin time (TT) of each sample were measured on an automatic coagulation analyzer. The data were averaged from measurements on three specimens.

Statistical analysis

The data was analyzed using ANOVA and student t-tests to determine statistical significance for which a p-value greater than 0.05 was chosen ($p > 0.05$).

RESULTS

Synthesis and characterization of PSHU conjugated with and without RGD

The determination of the structure of the PSHU polymer (Figure 1(A)) was previously described in detail in our earlier work.²² The number average molecular weight (M_n) of the PSHU polymer from gel permeation chromatography was determined to be 9,725 Da with a moderate polydispersity index of 1.29. The glass transition temperature of PSHU was determined to be 55°C.

Electrospinning polyurea blended with polyester yields highly porous scaffolds

PSHU (with and without RGD) and PCL were blended in different ratios (100/0, 70/30, 50/50, 30/70, 0/100) and electrospun into different matrices. Figure 1(B) shows sample tubular matrices electrospun with the 30/70 blending ratio at 1, 2.5 and 5 mL/h. The 30/70 scaffold was chosen for this figure since no difference in the gross morphology or structure was observed amongst the scaffolds of various blend ratios and electrospun rates. Scanning electron microscopy images of the 30/70 scaffold were used to study the effect of flow rate on the pore size of the electrospun matrices. Cylindrical sections were frozen in liquid nitrogen and cut along the longitudinal axis of the samples. The sections were imaged by scanning electron microscopy (Figure 2) and the pore size was determined using ImageJ. The average pore size of the scaffold calculated from 6 different images was found to increase with flow rate (Figure 3(A)). The fiber density was higher in the scaffolds electrospun at 1 mL/h than that in scaffolds electrospun at 2.5 and 5 mL/h. Using ImageJ, the diameters of the fibers in scaffold electrospun at 1 mL/h were measured to be statistically less than those electrospun at 2.5 and 5 mL/h (Figure 3(B)).

FTIR reveals no change in chemical structure of blended electrospun scaffolds

The FTIR spectroscopy of the scaffolds with varying blend ratio is illustrated in Figure 1(C). The areas under the different sections of the FTIR curve varied with the amount of PSHU and PCL present in the blend. With the addition of PCL to the blend, the areas under the hydroxyl ($-OH$) and primary amine ($-NH_2$) stretch peak at 3320 cm^{-1} , the carbonyl ($-C=O-$) stretch peak at 1690 cm^{-1} and the amide ($-NH-$) stretch peak at 1525 cm^{-1} were significantly reduced. Also, with the addition of PCL the hydroxyl (and primary amine) stretch and the carbonyl stretch peaks shifted to the right and the left, respectively. Furthermore, with the addition of PCL to the blend, new peaks can be seen at 2910 cm^{-1} (asymmetric $-CH_2-$ stretching) and 2845 cm^{-1} (symmetric $-CH_2-$ stretching). Essentially, the FTIR curves indicated that the blending did not significantly affect the general structure of PSHU and the polypeptide-like bonds were also retained.

Optimizing blending ratio and electrospinning flow rate produces scaffolds with biomechanical properties similar to native tissue

To determine the elastic modulus of the electrospun matrices, we performed uniaxial tensile testing. We found that the mechanical properties of the matrices are strongly related to electrospinning blend ratio and flow rate. Mechanical properties were dependent on the hydration of the scaffolds and a significant difference was observed between samples tested at room temperature in air and the samples tested in deionized water at 37°C (Figure 4). In the hydrated condition, all the scaffolds except the 70/30 exhibited high strain at break (> 125%) while in the dry condition, the scaffolds failed at values similar to those reported previously for electrospun polyurethanes (data not shown). Figure 4 summarizes the elastic modulus of the matrices with variations of the PSHU-PCL blend ratio (Figure 4(A)) and the electrospinning flow rate (Figure 4(B)) in dry and hydrated conditions. In the hydrated condition, the elastic modulus of the 70/30 scaffold was 4226.21 ± 346.36 kPa while the pure PCL scaffold (0/100) scaffold possessed an elastic modulus of 892.7 ± 120.57 kPa. It is clearly seen that the modulus of the electrospun scaffolds was dependent on the concentration of PSHU in the blend. The blends with higher concentration of PSHU possessed higher elastic modulus than the scaffolds with higher PCL content. The average molecular weight and the viscosity, listed in Table 1, increase with the increase in the PCL concentration in the blend. Also, the elastic modulus of the electrospun matrices decreases with increase in electrospinning flow rate. In the hydrated condition, the elastic modulus of the 30/70 electrospun at 1, 2.5 and 5 mL/h are 2221.37 ± 265.63 kPa, 1451.69 ± 285.04 kPa and 323.30 ± 265.81 kPa, respectively. The elastic modulus of the scaffolds electrospun at 2.5 and 5 mL/h were significantly different from those at 1 mL/h. The elastic modulus of the scaffolds tested in dry conditions at room temperature was significantly different from that at hydrated conditions at 37°C.

Elastic electrospun scaffolds support SMC growth and phenotype maintenance

The electrospun scaffolds were biocompatible with the bovine pulmonary artery SMCs and allowed their attachment and growth. It was evident that none of the electrospun scaffolds imparted any toxicity to the cells. The effect of the elastic modulus on cell proliferation was studied by varying the blend ratio as well as the electrospinning flow rate, and cell counts from days 1, 3, 5 and 7 are shown in Figure 5. SMC proliferation was less on softer than on stiffer matrices. This was observed in both the cases of varying the blend ratio (Figure 5(A)) and the electrospinning flow rate (Figure 5(B)). The lowest proliferation was observed in the 30/70 scaffold at 2.5 and 5 mL/h. Furthermore, we evaluated the phenotype of SMCs plated onto 30/70 scaffold. As shown in Figure 6, SMCs maintained the expression of SM-specific markers, calponin (green fluorochrome) and SM-myosin (red fluorochrome) on the tubular 30/70 scaffold electrospun at 1 mL/h (Figure 6(A)), and, α -SM-actin (green fluorochrome) and SM-myosin (red fluorochrome) on the flat 30/70 scaffold electrospun at 2.5 mL/h (Figure 6(B)).

Degradation rate of electrospun scaffolds slows down with increase in PCL content

To study the effect of the PSHU-PCL ratio and electrospinning flow rate on the degradation rates of the electrospun scaffolds, oxidative degradation experiments were performed. The

scaffolds were treated with a 20% hydrogen peroxide in 0.1 M cobalt chloride oxidative solution at 37°C. At each time-point, the scaffolds were carefully weighed and the change of weight over the 28 days was plotted for each scaffold type (Figure 7). These curves were fit linearly using the linear-fit function in Microsoft Excel® and the goodness-of-fit (R^2) values were calculated. The rates of degradation were calculated as the coefficient of the variable. These equations and the R^2 -values are tabulated in table 2. Scaffolds with higher PSHU content (70/30) degraded more rapidly (18.8% per day) than scaffolds with higher PCL content (0/100) (1.3% per day).

Electrospun scaffolds conjugated with RGD exhibits excellent resistance to thrombosis

To evaluate the thrombogenicity of the scaffold surface, we incubated the scaffolds in bovine plasma at 37°C, and then calculated the clotting times using a blood coagulation analyzer. Activated partial thromboplastin time (APTT), unactivated partial thromboplastin time (UPTT) and thrombin time (TT) were measured (Figure 8). Untreated bovine plasma at 37°C was used as the control. The 30/70 scaffold was chosen for this study since we have previously determined that this ratio imparted biomechanical properties to the scaffold similar to host tissue. APTT for plasma treated with 30/70 scaffold (44.90 ± 3.52 s) was significantly lower than that with 30/70 scaffold conjugated with RGD (60.36 ± 3.98 s). The APTT for the plasma treated with the 0/100 scaffold (40.73 ± 4.45 s) was also significantly lower than that with 30/70 scaffold conjugated with RGD (60.36 ± 3.98 s). APTTs of the plasma treated with all the 30/70 scaffolds, with and without RGD, were significantly higher than that of the untreated control bovine plasma (30.70 ± 1.56 s). UPTTs calculated also revealed a similar trend. UPTT for plasma treated with 30/70 scaffold (64.38 ± 3.02 s) was not significantly different from that with 30/70 scaffold conjugated with RGD (65.45 ± 2.88 s). Yet, UPTTs of the plasma treated with all the 30/70 scaffolds, with and without RGD, were significantly higher than that of the untreated 0/100 scaffolds (57.42 ± 3.06 s) and the control (56.57 ± 1.29 s). The TTs of the plasma treated with any of the samples were not significantly different from one another or from the control.

DISCUSSION

The optimization of the biomechanical and the biochemical properties of the tissue engineered vascular scaffold is one of the most challenging steps in designing and developing TEVG. The biochemical properties of the scaffold include chemical composition, invasion, attachment and proliferation of vascular and inflammatory cells in the scaffold, degradation of the scaffold, and, the remodeling of the scaffold over time. The biomechanical properties of the scaffold include integrity, elasticity, stiffness and porosity. The elastic modulus of the underlying matrix controls the survival, motility, proliferation and differentiation of host cells.^{30–33} There is a strong intrinsic relationship between the biomechanical and the biochemical properties of a scaffold. The biochemical composition of the matrix components also determines the attachment of cells. These adherent cells are, then, responsible for remodeling of the degrading scaffold, and, hence, alter the biomechanical properties of the scaffold in turn. There is a constant feedback mechanism between the biomechanical and the biochemical properties of a graft helping maintain its structure and function.

In this study, the fiber in the electrospun scaffold has a polyurea component, PSHU, and, a polyester component, PCL. PSHU predominantly influences the biochemical properties while PCL mainly influences biomechanical properties. This is because the polyurea consists of polypeptide-like bonds ($-\text{CO}-\text{NH}-$), possesses lower molecular weight and degrades faster, while the polyester possesses higher molecular weight and degrades slower. PSHU allows biochemical modification while PCL is relatively inert. This biomimetic scaffold tries to capture important properties of native tissues such as RGD cell-attachment sites, polypeptide bonds in extracellular matrix proteins, porous microstructure and differential degradation rates of extracellular matrix components.

We have designed various experiments to optimize the biomechanical and biological properties of the scaffolds by varying the polymer blending and the electrospinning flow rate. The general structure and function of the PSHU-PCL blended scaffolds were not drastically altered from that of PSHU scaffold. Electrospinning of the polymer blends by varying the electrospinning flow rate as well as the polymer blend ratio yielded a scaffold with varying pore sizes and elastic modulus. One of the most important effects of the variation of the flow rate is the variation of the pore size of the electrospun matrix. Pore size increases with flow rate and this is in good agreement with other studies.³⁴ The increase in porosity is generally due to the increase in the diameter and the density of the fibers. The pore size of the scaffold regulates the infiltration of vascular and inflammatory cells as well as matrix elasticity. The increase in electrospinning flow rate increases porosity of the matrix, and, hence, decreases the elastic modulus. Scaffolds electrospun at 1 mL/h provided highly dense matrices, which did not allow the infiltration of SMCs. Hence, we chose to electrospin the scaffold at higher flow rates to increase the pore size, though no studies were performed to confirm this. Of course, one of the major issues associated with electrospinning is heterogeneity in fiber diameter and pore size distribution.^{35,36} This can induce differences in the potential initial adherence, surface area for growth as well as the invasion of the cells into the electrospun scaffolds.

Further, two factors that are very critical in formation of fibers during electrospinning are the average molecular weight and the viscosity of the polymer blend. The lower values of average molecular weight and viscosity contribute to formation of shorter fibers and defects while their higher values produced superior electrospun fibers imparting suitable properties to the scaffold. This is a plausible reason for the high modulus seen in the blends with higher PSHU. Further, PSHU and PCL are both semi-crystalline polymers with a T_g of 54.5°C and -60°C ³⁷, respectively. Crystallinity of PCL decreases with increase in molecular weight³⁸⁻⁴¹. The glass transition temperature of PSHU is higher than room temperature. Below its T_g , PSHU behaves like a hard, brittle, glass-like polymer. On the other hand, the glass transition of PCL is below room temperature and hence it behaves like an elastomer. The lowering of T_g is observed in increase in the quantity of PCL in PCL-based polymer blends⁴¹. Above its T_g , an increase in PCL quantity in blend would increase the elasticity of the electrospun scaffold. Also, polymers with less or no branching exhibit a decrease in crystallinity with increase in molecular weight^{38,39}. The variation of crystallinity has been observed in blends of PCL with other polymers too⁴¹⁻⁴³. Hence the mechanical properties of the PSHU-PCL blend are dependent on the blend ratio. These high values of the elastic modulus in the dry conditions do not accurately model the properties of the *in vivo*

mammalian tissue since it is always in a hydrated state due to contact with interstitial fluids and blood. Hence, the mechanical testing was performed in the hydrated condition to accurately mimic the *in vivo* environments. Furthermore, the stiffness values obtained from the uniaxial tensile testing indicate that the hydrated electrospun scaffolds are highly elastic and capable of withstanding dilation under the pulsatile flow of the blood *in vivo*. From these studies, it is also evident that these electrospun scaffolds possess mechanical properties similar to those of native vessels. Thus, further experiments were performed with focus on the 30/70 scaffolds electrospun at 2.5 and 5 mL/h.

SMCs in adult blood vessels exhibit low rate of proliferation, low synthetic activity, and expression of distinctive SMC markers such as α -SM-actin, SM-myosin and other SM-specific markers, including calponin.^{44,45} SMCs in vascular grafts exposed to stiffer matrices tend to undergo dedifferentiation, and transit into a pro-proliferative myofibroblast-like phenotype, particularly at the site of suture.^{46,47} The difference in the elasticity of the native artery and the vascular graft at the site of suturing is called compliance mismatch. Abnormal proliferation of SMC due to compliance mismatch has been identified as a major cause for the formation of intimal hyperplasia that leads to the formation of occlusion.^{48–50} Hence, a reduction in the proliferation of SMC is a critical step in designing scaffold suitable for vascular grafting.

In our study, SMCs seeded on 30/70 and 0/100 scaffolds proliferated less than those seeded on polystyrene plastic plates (control) and scaffolds with higher polyurea concentration in the blend (100/0, 70/30 and 50/50). When these studies were repeated for the scaffolds electrospun at higher rates (1, 2.5, and 5 mL/h), cell proliferation decreased even more. Polystyrene plastic plates, used as control surfaces in the proliferation experiment, have an extremely high value of elasticity ($E = 3.7$ GPa)^{51,52} and are capable of attachment and growth of SMCs. The 30/70 scaffold is much more soft ($E = 2.5$ MPa) in comparison to the polystyrene surface. Hence, SMCs proliferate faster on these plastic plates and slower on the 30/70 scaffolds. We calculated the average percent change in the number of cells over 7 days (Supplemental figure 1) and we found that the polystyrene plate had the most number of cells than the 70/30, 50/50, and 30/70 scaffolds in that order. Elasticity of the 30/70 scaffold was further reduced by increasing the electrospinning flow rate from 1 mL/h to 2.5 mL/h ($E = 1.5$ MPa) and 5 mL/h ($E = 325$ kPa), respectively. This further reduced the proliferation of SMCs on these softer scaffolds. These studies helped to optimize the biomechanical properties of the electrospun scaffolds to control SMC proliferation *in vivo*, which will be further evaluated in future studies. Furthermore, SMCs plated onto 30/70 scaffolds maintained a relatively differentiated phenotype as defined by expression of several SM-specific markers (α -SM-actin, SM-myosin, calponin). This supported the idea that 30/70 scaffolds would allow SMCs to retain a phenotype most similar to the native vessel, i.e. low proliferative SMC exhibiting expression of contractile proteins *in vivo* too.

When a vascular graft is implanted *in vivo*, almost immediately, proteins and cells from blood adhere to its surface.^{53,54} This is an important step initiating the formation of intimal and ultimately the occlusion of the graft. In an attempt to abrogate this response, we conjugated the RGD peptide sequence to the backbone of PSHU since it provides attachment sites for vascular cells as well as improve thromboresistance. Also, this

conjugation is advantageous since the RGD peptide is not released into the blood and thereby could provide long-term thromboresistance. Activation of plasma factors is a critical variable to study while evaluating the hemocompatibility of biomaterials. Bovine plasma has been used to study blood coagulation due to contact activation because of its similarity to human plasma, availability and cost.^{55–58} The assays used in this study measure the time for clotting based on the activation of certain plasma factors, while coming in contact with a foreign material, involved in the clot formation via intrinsic and extrinsic pathways.^{59,60} APTT measures the clotting time of recalcified citrated plasma upon addition of plasma thromboplastin as a phospholipid suspension and an activator such as kaolin.^{61,62} APTT also measures the time required to convert prothrombin into thrombin with clotting factor X or prothrombinase, and, after the formation of thrombin, it induces the formation of fibrin from fibrinogen.^{60,63} UPTT measures the time and the factors directly involved in the generation of plasma thromboplastin, thrombin and fibrin.^{60,64} TT measures the time of clot formation by measuring the time required to convert fibrinogen to fibrin, when an excess of thrombin is added to the activated plasma.⁶⁰ An increase in APTT and UPTT values is used as evidence for an increase in thromboresistance.^{12,65} Increased APTT or UPTT implies that a longer time is necessary for the activation factors to modify thromboplastin and thrombin, thereby slowing down the clot formation. We observed clotting times that were higher than times specified in general blood coagulation physiology, yet they were not artificially high (> 2.5 times, historically accepted value in laboratories) as imparted by heparin-based vascular grafts.¹² Hence, this study strongly supported the idea that the RGD conjugation to the backbone of the polyurea component of the electrospun scaffold exhibits improved thromboresistance.

The degradation rate of the scaffold influences the structural integrity of the graft as well as the rate of remodeling by host cells *in vivo*. Generally, a quick-degrading scaffold provides the host cells a chance to replace the scaffold rapidly while a slow-degrading one provides mechanical stability and support to the graft over a longer period of time. Tissue engineered scaffolds are designed with degradation rates suitable for the particular host tissue they are replacing. Vascular grafts replace arterial tissue, which is normally extremely durable and relatively compliant, and, thus, the grafts must have compatible properties both initially and over time. Degradation of the implanted vascular scaffold is very critical in maintaining the compliance of the graft *in vivo* over time. Vascular graft integration and replacement is a long-term process; hence the graft should be capable of providing good mechanical stability as well. Polyureas are known to be very sensitive to oxidative degradation and more stable to hydrolysis, since polyureas formed from the reaction of diisocyanates are insoluble in water and organic solvents [REF 5]. The dual amide structure of urea protects the polymer against hydrolysis due to the conjugation stabilization effects [REF 6]. Hence, an accelerated oxidative degradation experiment was chosen instead of hydrolytic degradation. 30/70 Scaffolds electrospun at different rates had similar degradation rates. The varied degradation rates were caused by the different degradation mechanisms of the scaffold components, which involve the degradation of PSHU by the hydrolysis of the amide and the ester bonds and the degradation of PCL by the hydrolysis of the ester group. The molecular weights of PSHU and PCL (PSHU lower; PCL higher) also played an important role in the degradation of the scaffold: the lower molecular weight polymer degrades faster than high molecular

weight polymer.^{66,67} The 30/70 scaffold degraded at rates appropriate for *in vivo* implantation because it has just enough PSHU to allow the cells to remodel the matrix while the PCL would degrade slower providing prolonged mechanical support to the scaffold. Hence, the different studies performed to evaluate and optimize the properties of the electrospun scaffolds indicate that the scaffolds with the 30/70 blending achieved biomechanical and biologic properties similar to native tissue.

CONCLUSIONS

Herein, we have demonstrated the potential of a PSHU-PCL blended polymer, conjugated with RGD, as a novel biomimetic material for vascular grafts. The properties of the electrospun scaffolds were optimized by varying the polymer blending ratio and/or by varying the electrospinning flow rate. The scaffolds with higher concentrations of PSHU in the blend had higher elastic modulus while the scaffolds with a higher PCL concentration had the lower elastic modulus. Additionally, the elastic modulus decreased with the increase in electrospinning flow rate. With a decrease in elastic modulus of the scaffold, SMC proliferation decreased and they maintained expression of SM-specific markers. We also confirmed that the PSHU and the PCL components of the scaffold degraded at different rates suggesting that the faster degrading PSHU would aid regeneration of scaffold with cells and the slower degrading PCL component would provide mechanical stability during remodeling. The RGD sequence conjugated to the backbone of the polymer reduced the thrombogenicity of the optimized scaffold by increasing the clotting times. Based on our findings, (1) the 30/70 blend electrospun at 2.5 or 5 mL/h provided elasticity and biologic properties similar to native vessels, and, (2) the conjugation of RGD to the PSHU backbone reduced the thrombogenicity of the surface significantly, thereby making this scaffold a very suitable for vascular grafting. Furthermore, the versatility of the 30/70 scaffold electrospun at 2.5 or 5 mL/h and the wide-range of possibilities in chemically modifying the PSHU backbone makes it scaffold as very suitable candidate for a range of tissue engineering applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was funded by the Colorado Research Foundation, Children's Hospital of Colorado, Aurora, CO, a University of Colorado start up grant, a NIH Program Project Grant (HL014985-39) and a NIH Axis Grant (HL114887-03). The authors express their gratitude to Mr. Eric Wartchow (Children's Hospital of Colorado, Aurora, CO) with assistance in SEM. The authors would like to thank Dr. Jeff Stansbury and Mr. Matt Barros for use of the viscometer and the differential scanning calorimeter and interpretation of the differential scanning calorimetry data. The authors thank Ms. Brittany McKeon for her help with the scaffold cryosectioning. The authors also thank Dr. Jorge DiPaola, Ms. Elizabeth Villalobos-Menuet and Ms. Linda Jacobson for their help with the hemocompatibility studies.

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Figure 1.

(A) Chemical structure of PSHU-RGD. (B) 5.5mm diameter electrospun scaffolds fabricated using PSHU-PCL (30/70) polymer blends at electrospinning flow rates of 1, 2.5 and 5 mL/h. Scale = 10 mm. (C) FTIR curves for PSHU-PCL in various blend ratios.

Figure 2. SEM of electrospun PSHU-PCL (30/70) scaffolds at different electrospinning flow rates. (A) 1 mL/h, (B) 2.5 mL/h, and, (C) 5 mL/h. Scale bar: 10 μ m.

Figure 3. PSHU-PCL (30/70) electrospun scaffold microstructure. (A) Average pore size in square microns (μm^2). (B) Average fiber diameter in microns (μm). Statistically significant difference ($p < 0.05$) with respect to * - 1 mL/h and † - 2.5 mL/h.

Figure 4.

Elastic modulus of electrospun PSHU-PCL scaffolds. (A) Variation of elastic modulus with blend ratio in the dry condition at room temperature and hydrated condition at 37°C. $p < 0.05$: * - statistically significant difference with respect to 70/30 scaffold, † - statistically significant difference with respect to 50/50 and ‡ - Statistically significant difference with respect to 30/70 scaffold. (B) Variation of elastic modulus with flow rate the dry condition at room temperature and hydrated condition at 37°C. $p < 0.05$: * - statistically significant difference with respect to 1 mL/h electrospinning flow rate in dry condition, † - statistically significant difference with respect to 1 mL/h electrospinning flow rate in hydrated condition and ‡ - Statistically significant difference with respect to 2.5 mL/h electrospinning flow rate in hydrated condition.

Figure 5.

Proliferation of SMCs on electrospun PSHU-PCL scaffolds. (A) Variation of polymer blend ratio. (B) Variation of flow rate. Note: All cell counts normalized to day 0 cell number, i.e. initial cell plating density. Polystyrene plastic plates were used as controls.

Figure 6.

The SMCs were plated onto 30/70 scaffolds electrospun as tubes at 1 mL/h (A–C) and flat surfaces at 2.5mL/h (D–F) and after 3 days, cells were fixed in 1:1 Acetone-Methanol. In the tubular scaffolds, SMCs immunostained with antibodies against calponin (green fluorochrome) (A) and smooth muscle myosin heavy chains (SM-Myosin, red fluorochrome). In the flat scaffolds, SMCs were immunostained with antibodies against α -smooth muscle actin (α -SM-Actin, green fluorochrome) (D) and smooth muscle myosin heavy chains (SM-Myosin, red fluorochrome) (E). C & F represent the green and red fluorochromes merged together in the respective conditions. In all conditions, SMC nuclei were counterstained with DAPI (blue). Note: Autofluorescence of tubular vascular graft indicated as G. Scale bar: 100 μ m.

Figure 7.

Percent weight loss over 28-day time period of electrospun scaffolds subject to oxidative degradation. The numbers in the brackets (2.5) and (5) indicate that the scaffolds were electrospun at 2.5 and 5 mL/h, respectively. The conditions without these numbers in brackets indicate that the scaffolds were electrospun at 1 mL/h.

Figure 8.

Plasma clotting times. (A) Activated partial thromboplastin time, (B) Unactivated partial thromboplastin time, (C) Thrombin time. $p < 0.05$: * - Statistically significant difference with respect to 30/70 scaffold. † - Statistically significant difference with respect to 30/70 with conjugated with RGD. ¶ - Statistically significant difference with respect to pure PCL (0/100). Untreated bovine plasma was used as control.

Table 1

Average molecular weight and viscosity of 8% PSHU-PCL electrospinning solutions

Blend ratio	Average molecular weight	Average viscosity measured using a viscometer
Units	kDa	mPa.s
100/0	9.7	86
70/30	30.65	1121
50/50	44.75	2848
30/70	58.85	2657
0/100	80	5730

Table 2

Degradation Rates of electrospun PSHU-PCL scaffolds as linear fits. Note: Numbers in the bracket indicate the electrospinning flow rate in ml/h.

Scaffold Blend Ratio	Rate Equation	R ²	Rate (% per day)
<i>Variation of blend ratio</i>			
70/30	$y = 18.756x - 12.445$	0.99	18.756
50/50	$y = 14.105x - 9.9662$	0.93	14.105
30/70	$y = 12.279x - 4.8293$	0.91	12.279
0/100	$y = 1.3159x - 0.7323$	0.98	1.3159
<i>Variation of flow rate</i>			
30/70 (1)	$y = 12.279x - 4.8293$	0.91	12.279
30/70 (2.5)	$y = 6.2744x + 5.4106$	0.95	6.2744
30/70 (5)	$y = 9.6702x - 3.7393$	0.96	9.6702

Table 3

Elastic Modulus of blood vessels

Blood Vessel	Elastic Modulus
Coronary Artery	$1.55 \pm 0.26 \text{ MPa}^{68}$
Radial Artery	$3.28 \pm 2.11 \text{ MPa}^{69}$
Common Carotid Artery	$53.33 \pm 21.47 \text{ kPa}^{70}$
Saphenous Vein	$8.5 \pm 0.5 \text{ MPa}^{71}$