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Two ply tubular scaffolds comprised of proteins/poliglecaprone/polycaprolactone fibers

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Abstract Electrospun bi-layer tubular hybrid scaffolds composed of poliglecaprone (PGC), polycaprolactone (PCL), elastin (E), and gelatin (G) were prepared and thereafter crosslinked by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). Scanning electron microscopic (SEM) images revealed a highly porous micro-structure comprising randomly distributed non-woven fibers with the majority of fibers in submicron diameters. The EDC-crosslinking yielded an average crosslinking degree of 40%. Uni-axial tensile test of hydrated scaffolds in both longitudinal and circumferential directions revealed tensile properties, comparable to those of native arteries. The graft (PGC:PCL = 1:3) did not demonstrate significant difference before and after EDC-crosslinking in tensile strength or % strain in either longitudinal or circumferential directions. However, crosslinking increased the Young's modulus of the graft along the longitudinal direction (from 5.84 to 8.67 MPa). On the contrary, the graft (3:1) demonstrated a significant decrease in maximum strain in both directions. Cyto-assay using human umbilical vein endothelial cells (HUVECs) showed excellent cell viability.

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1 Introduction

Research in vascular tissue engineering has focused on the development of a tubular scaffold that possesses comparable biological and mechanical properties as those of native blood vessels [1–5]. Recently, electrospinning has garnered special attention for its flexibility in producing ultra-fine non-woven fibers on micro/nano scales, which mimics the protein fibers in native extracellular matrix (ECM). So far, numerous attempts have been made to construct scaffolds by electrospinning natural proteins and/or synthetic polymers [6–9], including collagen or gelatin, polyglycolide (PGA), polylactide (PLA), poly(caprolactone) (PCL) and their copolymers [10–19]. The composite scaffolds composed of natural protein together with synthetic polymer can provide better cell-scaffold responses as well as biomechanical properties compared to pure synthetic and protein scaffolds. Collagen-blended poly(L-lactic acid)-*co*-poly(ε-caprolactone) scaffold with different mass ratios were fabricated by electrospinning [20]. These highly porous scaffolds feature a centralized distribution of fiber diameter, 60% of which ranges from 100 to 200 nm, and a porosity of roughly 70%. Tensile testing demonstrated an average tensile modulus of 26 MPa, a tensile strength of 1.54 MPa with a failure strain of 66% [21]. Subsequent biocompatibility evaluation using human umbilical vein endothelial cells (HUVECs) illustrated that the presence of collagen in the scaffolds enhanced cell attachment, viability and morphology. Another research group further attempted to

construct a porous scaffold via co-electrospinning of poly(lactide-*co*-glycolide) (PLGA), gelatin, and elastin [22]. This scaffold was composed of fibers with an average diameter of 380 nm and demonstrated a versatility of tensile modulus, ranging from 12 to 43 MPa, depending on the mass ratio of individual chemical components with the maximum strain reaching approximately 60%. H9c2 rat cardiac myoblasts and rat bone marrow stromal cells (BMSCs) seeded separately on the scaffolds showed satisfactory adhesion to the surface and penetration of BMSCs into the bulk of the scaffold.

Previously, we have reported a tubular scaffold, featuring a spatially layered structure, by sequential and co-electrospinning of gelatin, elastin and Maxon® blends, and its in vitro degradation-profile in phosphate buffered saline (PBS) [23, 24]. It was observed that the proteins dissolved and/or disintegrated from the scaffold after being aged in PBS for over 10 days and that the scaffold lost its mechanical integrity within 20 days. This demands, a post-fabrication crosslinking to retain the proteins in order to prolong the bioactivity and mechanical integrity of the scaffold. For electrospun fibers containing proteins, a variety of chemicals can be used as the crosslinking agents, including glutaraldehyde, formaldehyde and glyoxal [25–28]. Particularly, glutaraldehyde has been used to crosslink electrospun fibers containing collagen for its low cost, fast reaction and high degree of crosslinking [6, 26]. However, glutaraldehyde may leave cytotoxic residues in the scaffold after crosslinking [29]. Therefore, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) has drawn special attention in that it does not integrate into the crosslinked products ("zero length" crosslinking).

The aim of the present study was to design a bi-layer scaffold that mimic the 'intima' and 'media' of small diameter vessels in order to engineer a functional smooth muscle and endothelium, respectively. For this purpose, the outer layer was designed to confer the overall mechanical strength of the scaffold while the inner layer was primarily to promote endothelial regeneration. The mechanical and morphological evaluations of the bi-layer scaffold, produced by sequential electrospinning of blended solution of PGC, PCL, bovine elastin, and porcine gelatin were carried out before and after EDC-crosslinking. PGC is a copolymer of glycolide (75%) and caprolactone (25%) and is used as absorbable sutures. PGC loses it tensile strength within 3 weeks and is completely absorbed roughly within 3 months, but, PCL is able to retain its mechanical integrity for a longer period and will not complete its degradation within 6 months. Therefore, more durable PCL is introduced to prolong the mechanical and structural integrity of the scaffold. This study reports the electrospinning of poliglecaprone based scaffolds, for the first time.

2 Materials and methods

2.1 Materials

PGC was obtained in the form of the surgical suture under the trade name Monocryl® (Advanced Inventory Management, Mokena, IL) and PCL was purchased from Absorbable Polymers, Birmingham, AL. Porcine gelatin B (Sigma-Aldrich, St Louis, MO) and soluble elastin (ES12) from bovine neck ligament (Elastin Products Co., Inc., Owensville, MO) were used as natural proteins.

2.2 Fabrication of the bi-layer tubular scaffold

A mixture of PGC and PCL were dissolved and homogenized in 1,1,1,3,3,3-hexafluoro-2-proanol (HFP) (Sigma-Aldrich, St Louis, MO) along with gelatin and elastin. The weight ratio of individual components in each layer is given in Table 1. The polymer solution for each layer was loaded into a 3 ml syringe capped with a 27 G needle, which was mounted on a motorized and programmable syringe pump. Then, the solutions were sequentially spun onto a stainless steel mandrel (4 mm diameter) rotating at 400 rpm, which was distanced at 30 cm from the needle tip. An electric voltage of 30 kV (M826, Gamma High-Voltage Research, Ormond Beach, FL) and a feeding rate of 3 ml/h were applied. The bi-layer scaffold fabricated by electrospinning of 3 ml of PGC/PCL/elastin/gelatin solution and 1 ml of PGC/PCL/elastin solution yielded a 20 mm long tubular graft with an overall wall thickness of approximately 0.8 mm. To ensure the uniform distribution of the fibers throughout the scaffold length, the collector was traversed at a speed of 15 mm/min.

2.3 EDC crosslinking of bi-layer scaffolds

EDC (Pierce Biotechnology, Inc., Rockford, IL) was dissolved in pure ethanol to achieve a concentration of 200 mM and the crosslinked according to the procedure adapted from Barnes et al. [18]. The tubular scaffolds were cut open along the axis and sliced into rectangular specimens ($n = 4$) measuring 12 × 3 mm. Each specimen was crosslinked for 18 h with 6 ml of EDC solution. Thereafter, the specimen was introduced into 0.1 M of sodium phosphate (Fischer Scientific, Inc.) for 2 h for hydrolysis of

Table 1 Chemical composition of each layer of bi-layer hybrid scaffolds

PGC:PCL	Inner layer chemistry	Outer layer chemistry
Graft (1:3)	(PGC:PCL):e:G = (1:3):2:1	(PGC:PCL):e = (1:3):1
Graft (3:1)	(PGC:PCL):e:G = (3:1):2:1	(PGC:PCL):e = (3:1):1

residual *O*-isoacylurea intermediates. Subsequently, all specimens were rinsed in PBS for 2 h to eliminate residual sodium phosphate. Non-crosslinked specimens of identical dimension were hydrated in PBS for 24 h prior to mechanical testing.

2.4 Estimation of the degree of crosslinking

The degree of crosslinking of natural proteins in the scaffold was measured by the percentage of free amino groups using 2,4,6-trinitrobenzenesulfonic acid solution (TNBS, 5% v/v in water, Research Organics, Inc., Cleveland, OH) as described elsewhere [30]. Three specimens from each type of scaffold were crosslinked by 1 ml of EDC solution for 18 h following the protocol above with uncrosslinked ones as the control. Afterward, each specimen was transferred into a solution containing 1 ml of 4% (w/v) sodium bicarbonate (Fischer Scientific, Inc.) and 1 ml of 0.5% (v/v) TNBS dissolved in deionized water and incubated for 2 h at 40°C. Then, 3 ml of 6-M HCl (Fischer Scientific, Inc.) was added into each solution, and the temperature was increased to 60°C for an additional 1.5 h. Finally, 5 ml of deionized water was used to dilute each solution. 200 μl of each solution was introduced into a reservoir of a 96-well plate with a total of 1000 μl of each specimen, giving five readings for each solution; absorbance (Abs) readings of 345 nm were recorded using BIO-TEK 96-well micro-plate reader (Instruments, Inc). The degree of crosslinking was calculated using the following formula with *c* denoting the crosslinked specimen while *nc* specimen without crosslinking.

$$cross-linking\,\% = \left(1 - \frac{\frac{Abs\,c}{mass\,c}}{\frac{Abs\,nc}{mass\,nc}}\right) \times 100$$

2.5 Morphological characterization of the crosslinked scaffolds

Square specimens measuring 5 × 5 mm were sliced from the scaffold and crosslinked using 1 ml of EDC solution for each one. Then, they were vacuum desiccated for drying and sputter-coated by gold. A scanning electron microscope (SEM) (Philips SEM 510) was employed to capture images of both the lumen and outer surfaces for each specimen at an acceleration voltage of 30 kV.

2.6 Mechanical properties of scaffolds

Both crosslinked and uncrosslinked specimens ($n = 3$) hydrated in PBS for 4 h and then subjected to a uni-axial tensile testing at a force rate of 1 N/min. The uni-axial tensile testing was conducted using both the longitudinally and circumferentially cut rectangular specimens (12 × 3 mm). The maximum stress value was taken as the tensile strength and Young's modulus of each specimen was determined from the slope of the stress–strain curve within initial 10% strain.

In addition, creep test and stress-relaxation test were used to evaluate their behavior under fatigued conditions. Prior to the creep and stress-relaxation tests, the linear viscoelastic zone for each type of scaffold was determined by a dynamic mechanical analyzer (DMA, TA Instruments Inc., DE). In creep testing, specimens with the same dimensions were held at 0.04 MPa constantly for 10 min for the strain to increase. In stress-relaxation testing, specimens was stretched at a constant extension rate to a strain of 2.5% and then held for 3 min.

2.7 In vitro cell viability with endothelial cells

MTT assay was carried out using rectangular specimens (20 mm^2) of the inner layer of the candidate graft having composition PGC:PCL:elastin:gelatin = 1:3:2:1 by direct contact of the surface with a monolayer of human umbilican vein endothelial cells (HUVECs). HUVECs were subcultured from stock culture (supplied by Lonza, Walkersville, MD) by trypsinization and seeded onto a multi-well tissue culture plate. Cells were fed with 0.5 ml EBM2 medium and incubated at 37°C in humidified 5% carbon dioxide atmosphere. When the cells attained confluent monolayer, the rectangular sheet of graft was placed gently over the cells such that the lumen surface is in contact with the cells and incubated further for 24 and 48 h separately. Following the incubation, cultures were assayed for cell viability by a colorimetric tetrazolium salt (MTT) assay using a kit obtained from American Type Culture collections, Manassas, VA as per the protocol published earlier [31]. In brief, for this assay MTT solution (20 μl, final concentrated 0.44 mg/ml) was added to each well of the microplate. Plates were shaken briefly and incubated further for 4 h at 37°C. After careful aspiration of the supernatant, 100 μl of 2-propanol was added to each well to solubilize the formazan and absorbance was measured at 590 nm against blank wells (without cells but incubated with MTT solution) using a microtiter plate reader (Biotek, USA). The relative percentage of living cells was calculated with respect to the culture cells without test material. Positive (rubber) and negative (ultra high molecular weight polyethylene) controls were also tested in parallel for comparison. All the experiments were done in triplicate.

2.8 Statistics

The statistical analysis was performed based on Student *t*-test and Tukey test ($\alpha = 0.05$) to determine differences

across the groups in the calculation of crosslinking degree and mechanical test results (JMP® 7, 2007 SAS Institute Inc.).

3 Results

Our previous studies on protein blended vascular scaffolds [23, 24] mandate that a more durable polymer is required to prevent the early collapse of the tubular structure. Accordingly, two tubular grafts (4 mm I.D.) were prepared from PGC, PCL and native proteins (elastin and gelatin) having a total composition of 67% by weight polymer and 33% by weight proteins in the bi-layer scaffold.

3.1 Crosslinking degree calculation

Figure 1 gives the crosslinking degree of both types of electrospun scaffolds based on the TNBS assay. The graft (1:3) yielded an average crosslinking degree of 35 ± 8.5% while the graft (3:1) an average of 44 ± 12.5%. Student *t*-test ($\alpha = 0.05$) reveals a lack of significant difference between the two groups, which can be accounted by the equal concentration of natural proteins in respective scaffolds.

3.2 Morphological characterization

SEM images of each layer in both tubular scaffolds before and after crosslinking are given in Fig. 2. In general, prior to the crosslinking treatment, each layer features a random distribution of non-woven nano/microfibers with a highly porous micro-structure. From Fig. 3, it is obvious that the majority of fibers in each layer possess a diameter below 1200 nm. In addition, the inner layer in each type of scaffold features a wider fiber-diameter distribution than the outer layer, which can be attributed to the higher elastin concentration and the lack of gelatin in the outer layer. Fiber diameters of inner layers fall in each range roughly in equal proportion while 90% and above of fibers of outer layer have diameters below 1200 nm, over 50% of which are even below 600 nm.

After crosslinking treatment, the porous structure in each layer is preserved to a great extent despite the occurrence of individual fiber swelling and fusion with adjacent fibers (Fig. 2c, d and g, h). Though the scaffolds became more densely packed with fibers [13], their fibrous structure is preserved. It should be noted that no individual fiber breakdown was observed, confirming the structural integrity remained intact throughout the crosslinking process, which is greatly due to the high total concentration of synthetic polymers (67 wt%) in the scaffold.

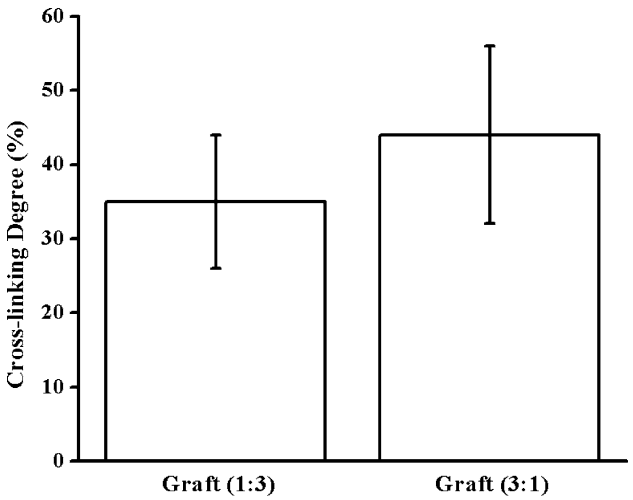

Fig. 1 Crosslinking degree (%) of PGC/PCL/Protein grafts due to EDC-crosslinking

3.3 Mechanical characterization

Table 2 gives the tensile properties of graft (1:3) and graft (3:1) both before and after crosslinking treatment under hydrated conditions. The graft (1:3) did not demonstrate significant change due to crosslinking in maximum stress or strain in either longitudinal or circumferential directions. However, EDC-crosslinking increased the Young's modulus of the graft (1:3) along the longitudinal direction from 5.84 to 8.67 MPa. On the contrary, the graft (3:1) demonstrated a significant decrease only in maximum strain in both directions with all other properties unchanged. Both grafts demonstrated anisotropy in properties with higher values for Young's modulus and maximum stress in longitudinal direction. This complicated mechanical behavior can be attributed to the combined result of both material properties, deviation occurred during the fabrication, and the crosslinking treatment, all of which conferred the anisotropic property to both grafts. Due to the fact that protein and synthetic polymer were blended in solution prior to electrospinning, each individual fiber in the scaffold is a chemically composite one, which causes uneven distribution of protein concentration on a single fiber. This heterogeneity, together with the complex structure of the scaffold, leads to the varied crosslinking degree in different regions of the scaffold, which further translated into this complicated mechanical behavior. A representative stress–strain curve for each type of scaffold (hydrated) before and after crosslinking is plotted in Figs. 4 and 5.

3.4 In vitro cytocompatibility

The HUVECs cultured in direct contact with the scaffold surface for incubation periods of 24 and 48 h has reveled

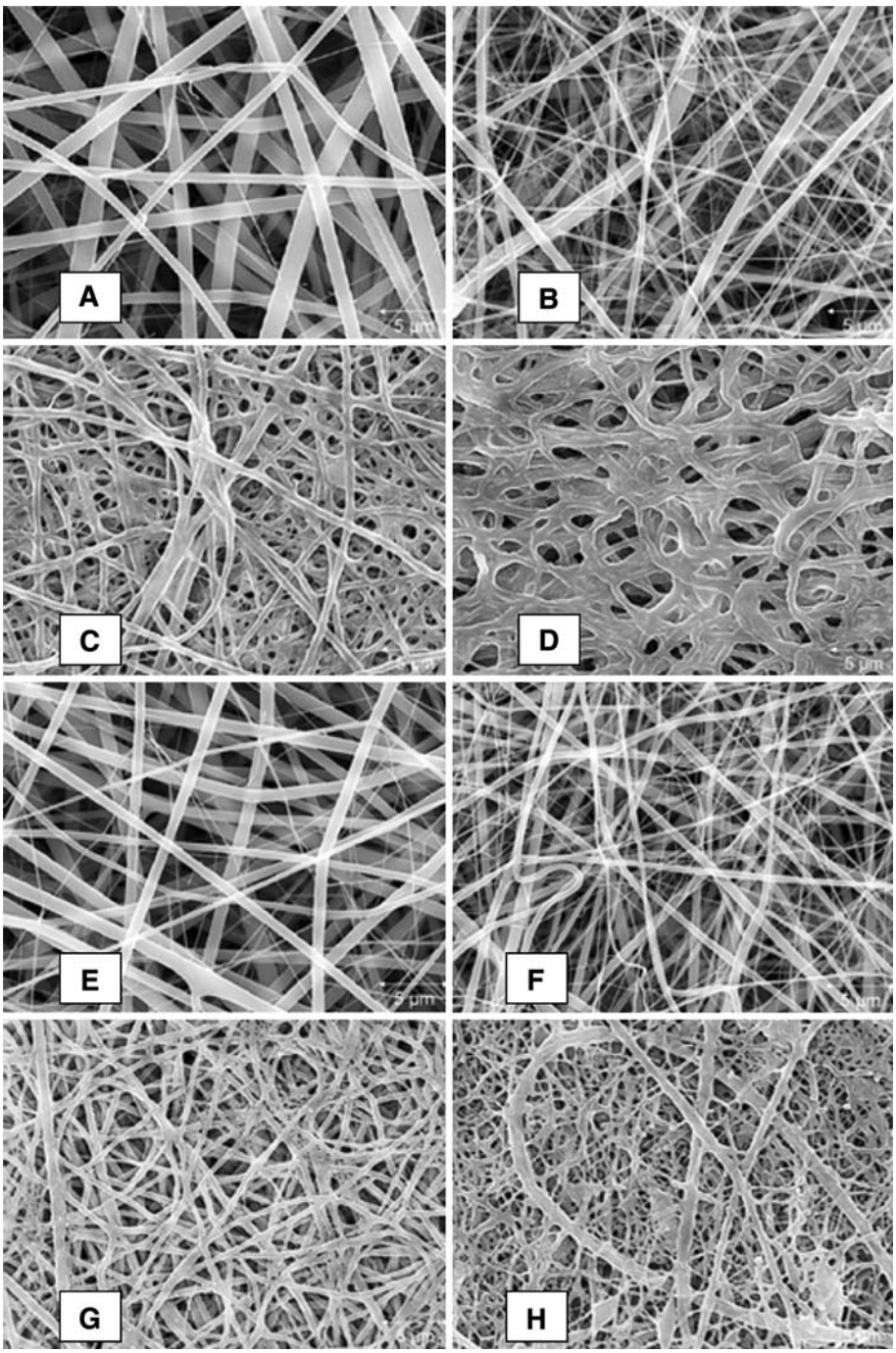

Fig. 2 SEM images (**a**–**d**) of bi-layer scaffold of graft PGC:PCL = 1:3: **a** inner layer before crosslinking; **b** outer layer before crosslinking; **c** inner layer after crosslinking; **d** outer layer after crosslinking; SEM images (**e**–**h**) of bi-layer scaffold of Graft PGC:PCL = 3:1: **e** inner layer before crosslinking; **f** outer layer before cross-linking; **g** inner layer after cross-linking; **h** outer layer after cross-linking. Both the grafts contained same amount of proteins

excellent cell-viability (Fig. 6). A positive control (rubber latex) and negative control (ultra-high molecular weight polyethylene) were also tested in parallel. No statistically significant decrease in cell growth could be observed in the presence of the scaffolds compared with negative control and is therefore considered non-cytotoxic. However, the positive control revealed severe toxicity to the HUVECs, validating the test.

4 Discussion

Both PCL and PGC are FDA approved for clinical absorbable sutures and give electrospun graft the mechanical strength and together with elastin give the necessary elasticity. Unlike hard tissue substitutes, vascular grafts must possess appropriate flexibility comparable to their native counterparts to withstand the pressure exerted by the blood

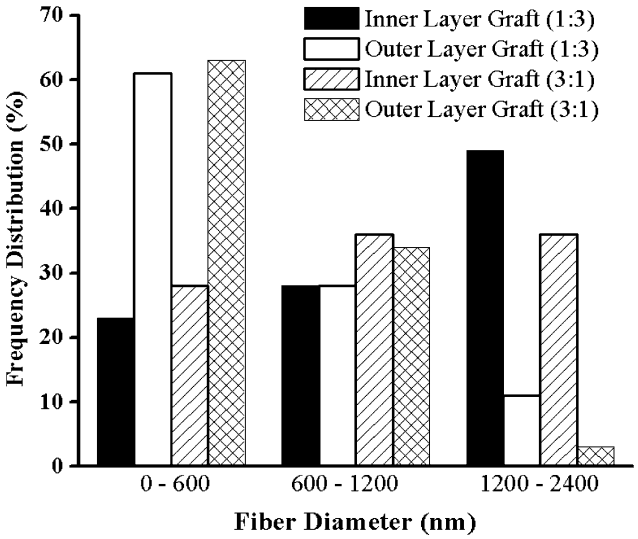

Fig. 3 Fiber diameter distribution in hybrid scaffolds

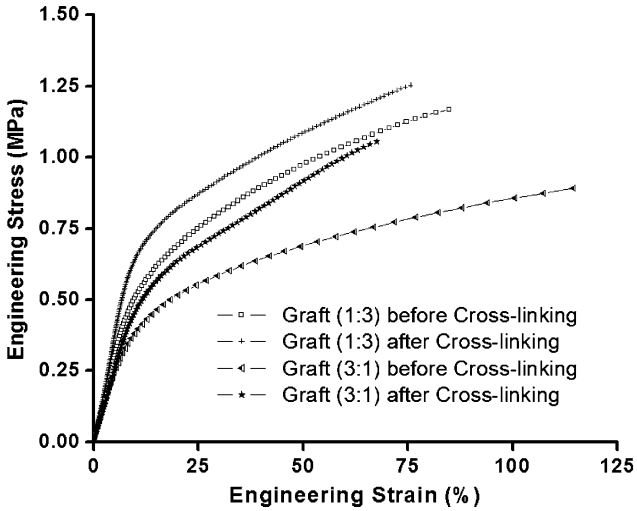

Fig. 4 Representative stress–strain curves of scaffold cut longitudinally

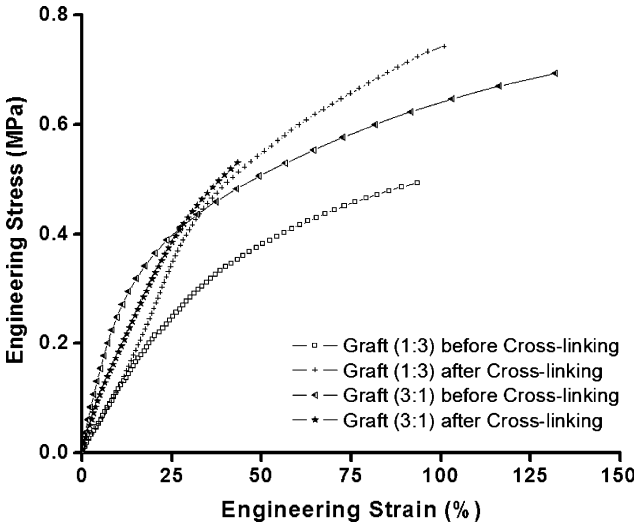

Fig. 5 Representative stress–strain curves of scaffold cut circumferentially

and to prevent mechanical failure because of mismatch of mechanical properties. For that, a vascular protein such as elastin was incorporated into the outer layer of the scaffold to enhance the elasticity of the graft. Considering that gelatin is chemically similar to collagen, another vascular protein, and capable to improve endothelial spreading and proliferation [32], this low cost natural protein (compared to collagen) is incorporated into the lumen layer of vascular scaffold. In a recent study, Lee et al. [15] have shown that composite vascular system based on PCL-collagen fibers possessed adequate biomechanical properties that resist high degree of pressurized flow over the long-term. By blending PGC and native proteins with more durable PCL, the early collapse of the composite scaffold can be prevented. The protein components, elastin and gelatin, in the present scaffold are expected to endow favorable cell-signaling features to promote cell adhesion and proliferation. For example, gelatin is chemically similar to collagen but, fibronectin, a cell adhesive protein has more affinity towards gelatin than collagen.

In addition to the chemistry, the nanoscale morphologies of the electrospun scaffold plays a crucial role in the mechanical and biological properties and performance of the construct. Compared to fibers on micro-scale, nano-fibers provide more opportunities for ligand-binding of seeded cells. To obtain optimal mechanical properties, natural proteins are often electrospun with synthetic polymers. Previous study in our lab demonstrates that blending natural proteins

Table 2 Tensile properties of bi-layer hybrid scaffolds before and after cross-linking

Graft (PGC:PCL)	Maximum stress (MPa)		Maximum strain (%)		Young's modulus (MPa)	
	Longitudinal	Circumferential	Longitudinal	Circumferential	Longitudinal	Circumferential
Graft (1:3) Uncrosslinked	1.19 ± 0.02	0.55 ± 0.11	85.52 ± 3.49	90.97 ± 11.14	5.84 ± 0.22	1.26 ± 0.16
Graft (1:3) Crosslinked	1.30 ± 0.17	0.70 ± 0.06	100.04 ± 28.24	106.63 ± 6.15	8.67 ± 1.00	1.03 ± 0.12
Graft (3:1) Uncrosslinked	0.90 ± 0.04	0.69 ± 0.07	117.46 ± 8.23	113.21 ± 24	5.29 ± 0.90	2.63 ± 0.49
Graft (3:1) Crosslinked	0.98 ± 0.06	0.55 ± 0.06	60.72 ± 7.05	49.95 ± 7.32	4.79 ± 0.44	2.06 ± 0.12

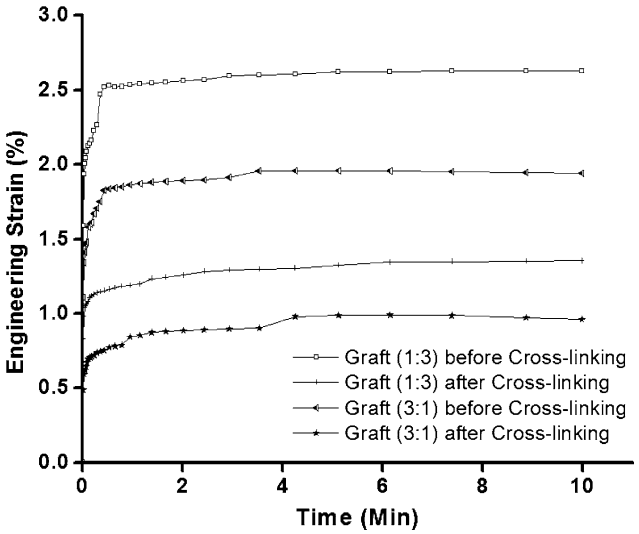

Fig. 6 Representative creep behavior curves

with synthetic polymer decreases the average diameter of individual fibers in the electrospun scaffold [23].

A successful vascular scaffold is expected to provide adequate mechanical support for a desired length of time to restore and maintain the normal physiological functions of native blood vessels prior to the complete regeneration of native blood vessels. Therefore, satisfactory initial mechanical performances with appropriate degradation properties are critical. The PGC-PCL blend in the scaffold offers the flexibility to tailor the mechanical and degradation properties based on the initial study results. Under in vivo condition, an artery is typically in an extended mode along the axis and subject to burst pressure exerted by the pulsatile blood flow in the lumen. Thus it is critical to evaluate the scaffold's tensile properties and other mechanical performances.

Crosslinking is an effective method to enhance the structural an mechanical stability of protein scaffolds. During the crosslinking reaction, two adjacent proteins are crosslinked to form a peptide bond between amino functional groups of lysine and hydroxylysine and *O*-isoacylureas, the intermediate produced by the reaction of carboxylic functional groups of aspartic and glutamic acids with EDC [28]. The degree of crosslinking can thus be controlled by the quantity of EDC present in the system and the extent of crosslinking reaction [18, 27]. To avoid fiber swelling due to the presence of hydrophilic proteins, ethanol is preferred to water as the medium for crosslinking reaction [18]. Pieper et al. [30] reported that the porosity of the collagen scaffold was preserved during the crosslinking process in ethanol. Crosslinking agent typically causes dimensional shrinkage and confers stiffness to the treated scaffolds. However, the high percentage of synthetic polymer in the scaffold greatly retarded the dimensional shrinkage and no measurable dimensional changes were observed.

EDC-crosslinking increased the Young's modulus of the graft (1:3) along the longitudinal direction from 5.84 to 8.67 MPa, comparable to that of native arteries (tensile strength of 1–2 MPa, tensile modulus of 8–12 MPa with a strain of 80% [8, 23]). Reports showed that a pure collagen scaffold after EDC-crosslinking demonstrated overall dimensional shrinkage (12–33%) due to the absorption of moisture during the hydration process of crosslinking [30]. Pure collagen scaffold treated by 200 mM EDC (crosslinking degree of 29%) illustrated an average peak stress of 0.57 MPa, significantly lower than the dry uncrosslinked scaffold's 2.39 MPa, and an average tangential modulus of 0.92 MPa compared to the 51.45 MPa of the uncrosslinked one [18]. However, the average of strain at break increased tremendously from 9.53% before crosslinking to 128.27% after EDC treatment [18]. It is known that pure protein scaffolds lose mechanical and structural integrity upon hydration. Therefore, crosslinking is widely used to stablize them prior to the cell culture study, which is typically conducted in an aqueous environment [16, 17]. The presented PGC/PCL/proteins hybrid scaffolds illustrated superior preservation of mechanical and structural integrities throughout the crosslinking. This can be attributed to the combined complexity of the scaffold's spatial structure and chemical composition. The presence of a durable synthetic polymer, PCL, in the scaffold would retard the structural deterioration and thus would contribute to maintain the biomechanical properties under physiological conditions. Appropriate fatigue resistance (30 days of in vitro cycling under physiological loading without marked dilation) is one of the criteria to maximize the likelihood of graft success [33]. As reported by Lee et al. [15] PCL based composite vascular graft can withstand the pressurized flow conditions and provide prolonged structural support for vascular cells.

Because the native artery is constantly extended in the longitudinal direction under in vivo conditions, the tissue engineered vascular scaffold's capacity of withstanding long term fatigue determines its potency for clinic application. Therefore, the scaffold was subjected to longitudinal creep (Fig. 7) and stress-relaxation (Fig. 8) testing within its elastic limit. Neither of these two tests yielded any significant differences for the two types of scaffolds before and after crosslinking treatment, revealing that crosslinking treatment did not compromise the mechanical integrity. The scaffolds reached their steady status within a few minutes upon the start of the creep and stress-relaxation testing, making them a promising candidate for in vivo implant.

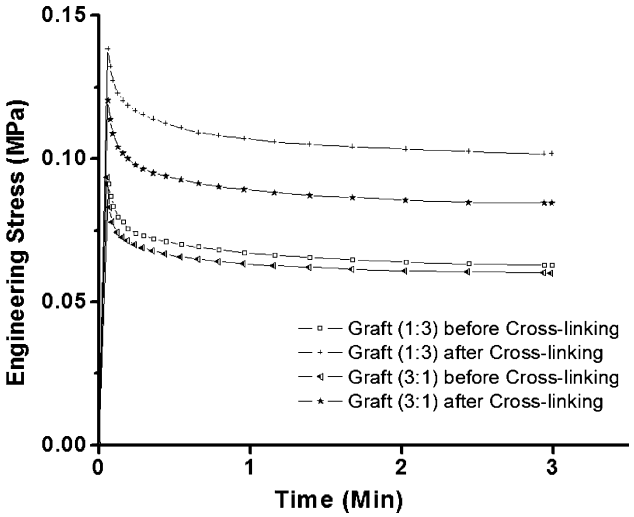

Fig. 7 Representative stress-relaxation curves

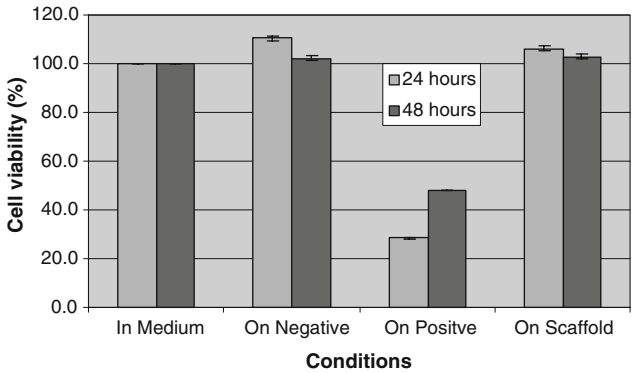

Fig. 8 MTT assay on the growth of HUVECs on the scaffolds

5 Conclusion

Bi-layer tubular hybrid scaffolds composed of PGC/PCL/proteins were prepared by electrospinning. A combination of two promising polymers, PGC and PCL enables us to tailor the mechanical properties and degradation rate of the scaffold. To prevent early disintegration of protein component blended with, EDC-crosslinking was employed (crosslinking degree = 40%). SEM images confirmed that the scaffolds survived the crosslinking treatment without losing their porous and fibrous microstructure. The graft (PGC:PCL = 1:3) did not demonstrate significant change due to crosslinking in maximum stress or strain in either longitudinal or circumferential directions. However, EDC-crosslinking increased the Young's modulus of the graft (1:3) along the longitudinal direction from 5.84 to 8.67 MPa, comparable to that of native arteries. On the contrary, the graft (PGC:PCL = 3:1) demonstrated a significant decrease only in maximum strain in both directions. The scaffolds demonstrate an anisotropic property of mechanical properties between longitudinal and circumferential directions. The HUVECs in direct contact with the surface of the scaffold for incubation periods of 24 and 48 h has reveled excellent cell-viability

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