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The influence of cross-linking methods on the mechanical and biocompatible properties of vascular scaffold

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Abstract: Electrospun scaffolds fabricated from poly(epsilon-caprolactone) (PCL)/collagen composite are promising candidates for vascular graft applications. In order to improve the biocompatibility and mechanical strength of PCL/collagen scaffolds, two different cross-linking methods were applied and compared: (1) 2.5% glutaraldehyde (GA) vapor cross-linking for 6 h and (2) genipin (GN) cross-linking for 3 days in 70% ethanol. Scaffolds cross-linked with GN demonstrated higher cross-linking degree, collagen content, tensile strength and a similar stiffness compared with GA cross-linked scaffolds. Scaffolds cross-linked with GA and GN both supported endothelial and smooth muscle cell attachment and growth. However, cells seeded on GN cross-linked scaffolds displayed a faster growth rate and deeper penetration into the scaffolds than those cross-linked with GA. These data suggest that the use of GN to cross-link PCL/collagen scaffolds may enhance the cellularization of vascular scaffolds resulting in a more rapid development of integrated functional vessels in vivo. ***Published by www.inter-use.com. Available online 12-8-2013 Vol. 1 Issue 1 Page 1-7.***

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1. Introduction

The increasing rate of cardiovascular disease (CVD) is a major social problem faced by most countries [1]. The development of tissue-engineered (TE) vascular grafts (TEVG) has been proposed as a potential solution to address the limitation of current therapies, such as the lack of sufficient number of autologous grafts, high thrombosis rate for synthetic grafts [2, 3]. To fabricate TEVG, autologous cells, such as endothelial and smooth muscle cells, were cultured on supporting scaffolds ex vivo, and allowed to remodel after implantation. An ideal TEVG needs to be easily manufactured, biodegradable and resemble native blood vessel with matched mechanical and functional properties, withstanding physiological flow over time while supporting cell prolifiration and penetration [4, 5].

Electrospinning is a simple and inexpensive technique in tissue engineering to fabricate scaffolds. For TEVG applications, the scaffolds are generally electrospun from composites, in which biodegradable synthetic polymers, such as poly(epsilon-caprolactone) (PCL) [4], are mixed with natural polymers derived from extracellular matrix (ECM) proteins, such as collagen, fibrin and elastin [6-9]. The synthetic polymers provide the scaffolds with suitable mechanical properties, while the addition of natural polymers improves the attachment and proliferation of cells. The natural polymers are usually dissolvable in aqueous environment, and will be gradually released, resulting in decreased mechanical strength and biocompatibility. Chemical cross-linking of scaffolds is commonly used to address this problem. Among cross-linking agents, glutaraldehyde (GA) is by far the most clinically used due to its ability to cross-link quickly with primary amino groups of collagenous tissues. However, with the degradation of the scaffold, GA is released and has been shown to display local cytotoxicity [10, 11].

Genipin (GN) is the active compound in the extract of the gardenia fruit that has been widely used in traditional Chinese medicine as a treatment for diabetes. GN has been shown to react with amino acids in the production of food dyes [12, 13]. Several publications have reported the use of GN to cross-link gelatin [14, 15], chitosan [16] and collagenous tissues [17]. The results indicated that GN was much less

cytotoxic than GA and the stability of tissues cross-linked with GN was superior to its GA-fixed counterpart.

Our lab developed a PCL/collagen scaffold, cross-linked with GA, for use as a vascular bypass [4, 18, 19]. Previous studies showed that the PCL/collagen scaffolds cross-linked with GA displayed suitable mechanical properties and good cell biocompatibility over a 1 month time period [5]. However, the toxicity of the GA cross-linked scaffolds and its potential calcification after long-term implantation in vivo is unknown. The purpose of this study was to compare the biocompatibility and mechanical strength of PCL/collagen scaffolds cross-linked with either GA or GN in order to optimize this tissue engineering approach for generating small diameter vessels for long-term in vivo studies.

2. Materials and Methods

2.1. Materials

Poly(epsilon-caprolactone) (PCL), with inherent viscosity 1.7-1.9 dl/g in CHCl3 at 30 ^{o}C, was purchased from Lactel Absorbable Polymers (Pelham, AL). Collagen type I, derived from calf skin, was supplied by Elastin Products Co. (Owensville, MO). 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and GA solution (25%) were bought from Sigma Chemical Co. (St. Louis, MO). GN was purchase from Wako Chemicals (Richmond, VA).

2.2. Preparation of scaffold

Scaffolds were fabricated from a PCL/ collagen blended solution with a weight ratio of 1:1 in HFP. Electrospinning was conducted using a typical setup as reported previously [4, 18, 19]. 5% and 15% of PCL/collagen in HFP were applied respectively to fabricate nano-fiber and micro-fiber scaffolds.

2.3. Characterization of scaffolds

The microstructure of the electrospun scaffolds was observed using a scanning electron microscopy (SEM; Model S-2260N, Hitachi Co. Ltd., Japan). Image-Pro Plus software (Media Cybernetics, Bethesda, MD) was used to measure the fiber diameters of scaffolds. The degree of cross-linking in electrospun scaffolds was determined using trinitrobenzensulfonic acid (TNBS) as reported [20, 21]. The mechanical properties of the samples were measured using an uniaxial load test machine (Model #5544, Instron Corporation, Issaquah, WA) equipped with a 10N load cell at an extension rate of 10.0 mm/min.

2.4. Cell culture

Murine MS-1 endothelial cells and 10T1/2 smooth muscle cells were obtained from American Tissue Culture Collection (ATCC). Cells were grown in Dulbecco's Modified Eagle Medium with low glucose (DMEM, purchased from Thermo Scientific) with 10% fetal bovine serum (FBS), 1% penicillin-streptomyocin (P/S), and maintained in a humidified 5% CO_2 incubator at 37 °C.

2.4.1. Cytotoxicity of cross-linkers

MS-1 and 10T1/2 cells were used to evaluate the cytotoxicity of GA and GN. 4000 cells/cm^2 of cells were cultured in 48-well plates as described above. After 4 h incubation, the cell culture medium was replaced with the new medium containing indicated amounts of GA or GN (1, 5, 10, 50, 100 ppm). After 3 days incubation with the cross-linking agents, the viability of cells was examined using an MTS assay (Promega, Madison, WI) and live/dead staining (Invitrogen, Eugene, OR) according to the manufacturers' instruction. Cells cultured without cross-linkers were used as a negative control, and the cytotoxicity was expressed as the percentage of negative control (n = 3).

2.4.2. Proliferation of cells on scaffolds

Prior to cell seeding, scaffolds were sterilized with 70% ethanol, rinsed 5X with PBS, and then incubated at 37^{o}C in 5% CO2 for 2 h. MS-1 and 10T1/2 cells were used as endothelium and smooth muscle cell sources, respectively. After 1, 3 and 5 days in culture, the number of viable proliferating cells were determined by MTS assay. Cell attachment on the scaffolds was evaluated by nuclear staining with 40,6-diamidino-2-phenylindole (DAPI) (Vector Lab., Burlingame, CA) after 5 days in culture. The morphology of cells on the scaffolds was analyzed with SEM.

2.4.3. Penetration of cells on scaffolds

10T1/2 cells were seeded on scaffolds prepared respectively from 5% and 15% PCL/collagen composites. At pre-determined time points, the cell-

seeded scaffolds were fixed with 4% paraformaldehyde, and then imbedded in paraffin for section. DAPI stained scaffold sections were observed using a Leica, DMI4000B florescent microscope (Leica Microsystems GmbH, Wetzlar, Germany). Three images, from random areas of the scaffolds were taken for each of the samples. Based on these images, the penetration depth of cells was analyzed with Image-Pro Plus software.

3. Results

3.1. Cytotoxicity of cross-linkers

We used increasing doses of both GA and GN to determine their dose-dependent cytotoxicity on 10T1/2 smooth muscle cells and MS-1 endothelial cells. Both cell types were cultured in tissue culture plates (TCP) in media containing increasing amounts of either cross-linker. After three days incubation, the status of the cells was analyzed using live/dead staining and the MTS assay. The results indicated that GA had a higher cytotoxicity than GN (Fig. 1). Exposure to 5 ppm GA showed an apparent toxic effect to both 10T1/2 and MS-1 cells, and few live cells (green color) were found on plates ≥ 5ppm GA concentration. In comparison, GN concentration up to 10 ppm showed little to no cytotoxicity. GN was found to be cytotoxic to both cell types at a concentration greater than 50 ppm (data not shown). The lesser cytotoxicity seen with GN suggests that GN cross-linked scaffolds may have better biocompatibility for the use in tissue engineered constructs.

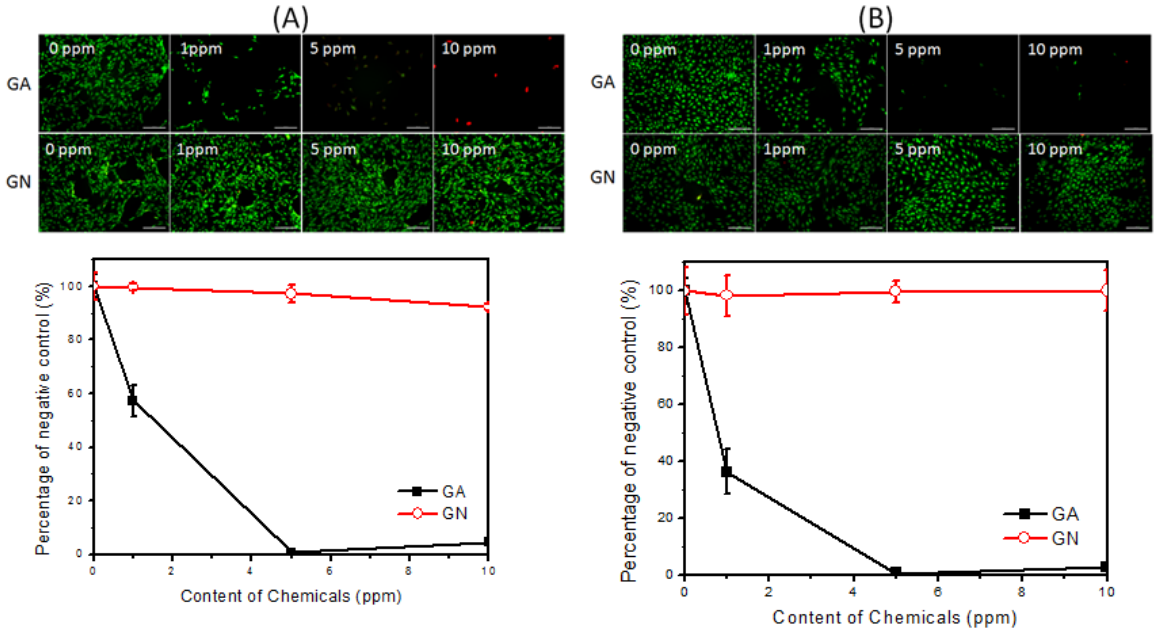

Fig. 1. Dose-dependent toxicity of GA and GN on cultured 10T1/2 and MS-1 cells. 10T1/2 (A) and MS-1 (B) cells were stained with a live/dead kit. Live (green) and dead (red) cells were observed under a fluorescent microscope (scale bar = 200 um) with quantification of cytotoxicity, as determined by MTS assays, graphed below. Bars indicate standard deviation (n = 3).

3.2. Preparation of scaffold

PCL/collagen composite scaffolds were electrospun as reported previously [4, 18]. The fiber diameter is proportional to the viscosity of the PCL/collagen solution, a higher viscosity resulting to a bigger fiber diameter. When 5% PCL/collagen solution was used, scaffolds with nano-scale fibers of about 250 nm were fabricated, while increasing the concentration to 15%, resulted in fiber diameters around 4.5 um as shown in Fig. 2. In these experiments, the collecting mandrel was set at a low rotating rate (1000 rpm), in order to generate a random orientation of the fibers.

Collagen, a major component of the extracellular matrix, provides many mechanical properties and structural support for cells and tissues. Collagen is dissolvable in aqueous solutions and therefore, without cross-linking, will dissolve out of synthetic scaffolds. In order to fix the collagen and increase the mechanical properties of the scaffolds, PCL/collagen composite scaffolds were chemically cross-linked with either GA vapor or GN. GA and GN cross-linking resulted in similar microstructure and fiber diameter (Fig. 2). The degree of cross-linking in the scaffolds was about 25% when the scaffolds were put in 2.5% GA vapor. In GN solution, the degree of cross-linking was about 85% when carried out 3 days at room temperature.

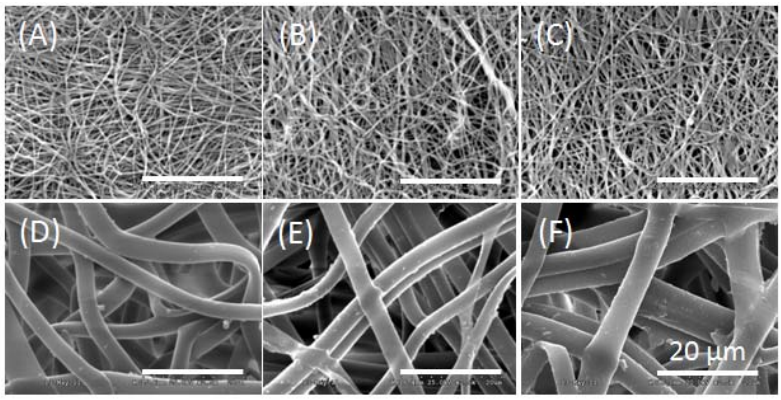

Fig. 2. SEM images of PCL/collagen scaffolds. Scaffolds were fabricated with either 5% (A-C) or 15% (D-F) PCL/collagen. Non-cross-linked (A, D), cross-linked with GA vapor for 6h at room temperature (B, E) or cross-linked with 1% GN in 70% ethanol for 3 days (C, F) scaffolds are shown. Scale bar on images is 20 μm.

3.3. Mechanical properties of scaffolds

TE vascular scaffolds should possess proper mechanical strength to withstand the physiological pressure from blood flow over a long period time. In our study, the cross-linked scaffolds were soaked in culture medium and incubated at 37 ^{o}C. At pre-determined time points, their mechanical properties were evaluated with an extension method (Fig. 3). For GA cross-linked scaffolds, stiffness changed little and the Young's modulus remained about 11MPa after three months culture. However, the tensile stress and tensile strain at break decreased for both, from 8.5 MPa and 120% for 1 day to 6.5 MPa and 70% for three months. In comparison, the scaffolds cross-linked with GN had a similar stiffness to GA cross-linked scaffolds, but higher values of tensile stress and tensile stain at break within the first month. After 3 months incubation, the mechanical strength of GN cross-linked scaffolds also decreased greatly, with the modulus changing from 11 MPa to 7.5 MPa, and tensile stress and tensile strain at break decreasing from 10.5 to 5.0 MPa and from 130% to 85% respectively. These results indicated that GN cross-linked scaffolds had stronger mechanical strength compared with scaffolds cross-linked with GA early after incubation, but the degradation of both scaffolds was similar after a longer incubation time. When compared to porcine coronary artery [4], the mechanical strength of PCL/collagen scaffolds cross-linked either GN or GA is higher than that of natural blood vessel, even after the scaffolds were incubated 3 months.

3.4. Proliferation of cells on scaffolds

To confirm that cross-linking with GN doesn't adversely affect cell growth, 10T1/2 and MS-1 cells were seeded on scaffolds cross-linked respectively with GA and GN, and cell growth rate was compared to that of cells grown on polystyrene tissue culture plates (TCP) and on latex beads (Fig. 4). As expected, both cell types grew well on TCP and did not grow on latex beads. Both cell types grew well on scaffolds cross-linked with either GA or GN, with slightly better growth on scaffolds cross-linked with GN. Cell growth was demonstrated by nuclear staining with DAPI and SEM. DAPI staining clearly showed the attachment of

10T1/2 and MS-1 cells on both scaffolds. SEM images indicated cell morphology on scaffolds after 5 days culture.

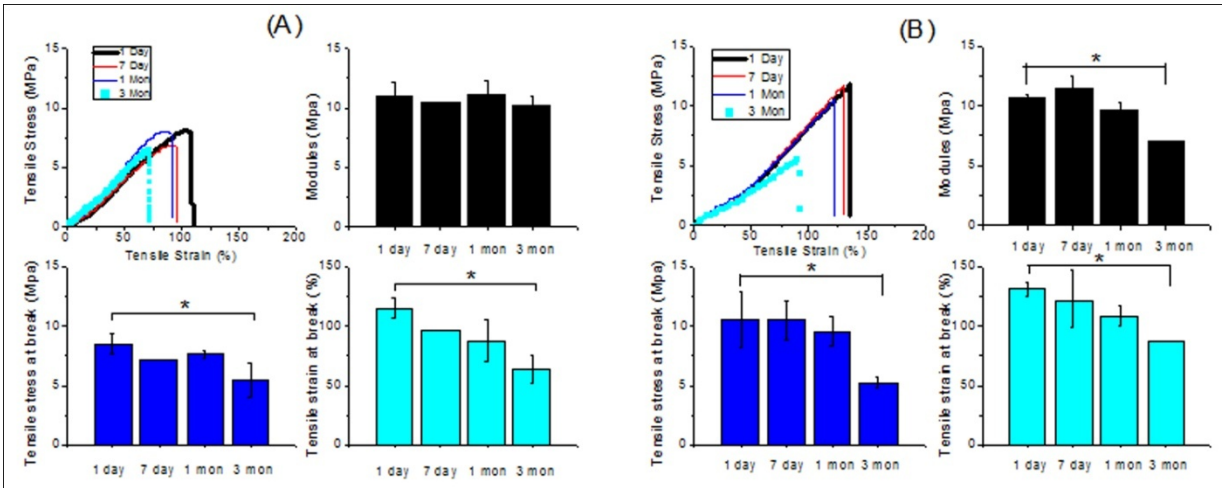

Fig. 3. Mechanical properties of scaffolds cross-linked with GA (A) and GN (B). Bars indicate standard deviation (n = 3). * indicates significant difference ($P < 0.05$).

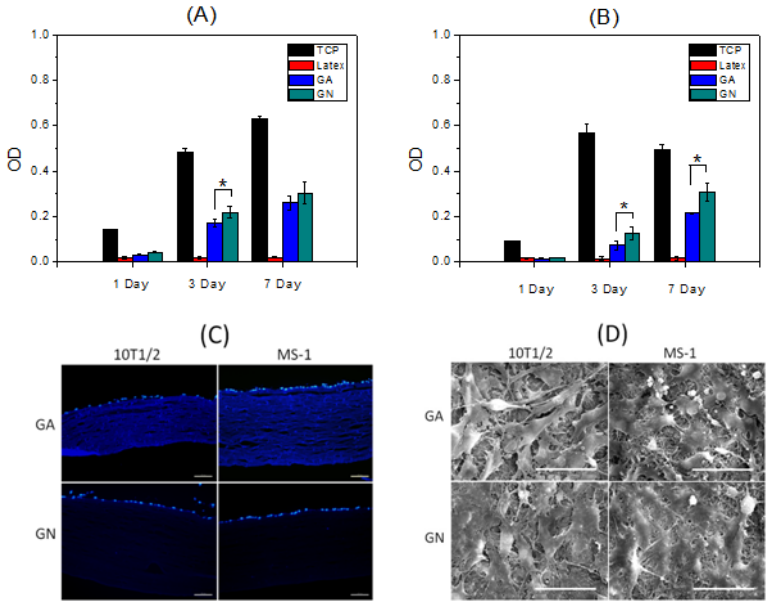

Fig. 4. The proliferation and morphology of cells on scaffolds. The viability of 10T1/2 (A) and MS-1 (B) cells were evaluated with MTS assay and bars indicate standard deviation (n = 3). Cells were stained with DAPI and observed with a fluorescent microscopy (C) (scale bar = 200 um), and cell morphology on the scaffolds was observed with SEM (D) (scale bar = 50 um) after 5 days culture. * indicates significant difference ($P < 0.05$).

3.5. Penetration of cells into scaffolds

As reported previously, the penetration of cells into scaffolds is dependent on the pore size of the scaffold [18, 22] Generally, scaffolds with a big fiber diameter, have smaller fiber packing density and a larger pore size compared with scaffolds with a smaller fiber diameter [18]. As expected, for the nano-fiber scaffolds prepared from 5% PCL/collagen, 10T1/2 cells were limited to the surface of scaffolds cross-

linked with either GA or GN, even after 4 weeks in culture (Fig. 5). For macro-fiber scaffolds prepared from 15% PCL/collagen composite, most cells remained on the surface of the scaffolds during the first week but migrated through a 1/4 of the scaffold's wall by 2 weeks. By 4 weeks, a percentage of cells had migrated completely through the scaffold (about 800 um) in scaffolds cross-linked with GN. Cell penetration distance at 4 weeks was significantly larger in GN cross-linked scaffolds when compared to GA cross-linked scaffolds, in which cells penetrated only to a distance of 600 μm. These results suggest that cross-linking with GN may be slightly better for cell migration and penetration, compared with GA cross-linking.

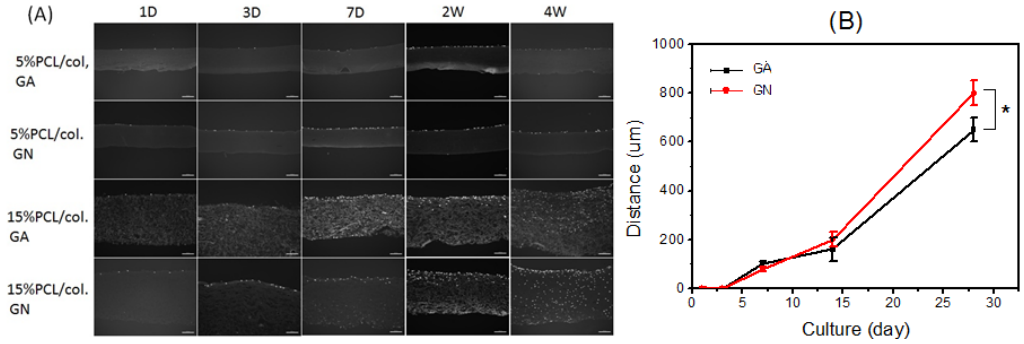

Fig.5. Penetration of 10T1/2 cells into scaffolds. (A) Cells were stained with DAPI and observed using fluorescent microscopy (scale bar = 200 um). Scaffolds were prepared with 5% or 15% PCL/collagen and cross-linked with either GA or GN as labeled. (B) Penetration depth of 10T1/2 cells on scaffolds cross-linked with GA and GN over 4 weeks, bars indicate standard deviation (n = 3). * indicates significant difference ($P < 0.05$).

4. Conclusion

PCL/collage scaffolds with nano- and micro-fibers were fabricated using electrospinning methods, and cross-linked with either GN and GA. GN cross-linked scaffolds had a higher cross-linking degree and a stronger tensile strength compared with GA cross-linked scaffolds. Due to the degradation of collagen and/or PCL, the mechanical strength of scaffolds decreased over 3 months in culture in conditions. The introduction of GN did not change the surface wettability of scaffolds, while GA induced a slight increase of wettability. The scaffolds cross-linked with GN showed better cyto-compatibility, and cells displayed a higher growth rate on nano-fiber scaffolds and a deeper penetrating distance on micro-fiber scaffolds, when compared with the scaffolds cross-linked with GA. Experimental results suggested that GN cross-linking is a promising method for cross-linking PCL/collagen scaffolds for vascular graft applications.

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