Stabilized Collagen Scaffolds for Heart Valve Tissue Engineering

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Scaffolds for heart valve tissue engineering must function immediately after implantation but also need to tolerate cell infiltration and gradual remodeling. We hypothesized that moderately cross-linked collagen scaffolds would fulfill these requirements. To test our hypothesis, scaffolds prepared from decellularized porcine pericardium were treated with penta-galloyl glucose (PGG), a collagen-binding polyphenol, and tested for biodegradation, biaxial mechanical properties, and *in vivo* biocompatibility. For controls, we used un-cross-linked scaffolds and glutaraldehyde-treated scaffolds. Results confirmed complete pericardium decellularization and the ability of scaffolds to encourage fibroblast chemotaxis and to aid in creation of anatomically correct valve-shaped constructs. Glutaraldehyde cross-linking fully stabilized collagen but did not allow for tissue remodeling and calcified when implanted subdermally in rats. PGG-treated collagen was initially resistant to collagenase and then degraded gradually, indicating partial stabilization. Moreover, PGG-treated pericardium exhibited excellent biaxial mechanical properties, did not calcify *in vivo*, and supported infiltration by host fibroblasts and subsequent matrix remodeling. In conclusion, PGG-treated acellular pericardium is a promising scaffold for heart valve tissue engineering.

Introduction

IT IS ESTIMATED that the prevalence of patients requiring heart valve replacement will triple from approximately 290,000 in 2003 to more than 850,000 by 2050.¹ Thus, strategies to address these issues are bound to have a global effect. The most common treatment of valvular pathology is surgical replacement with devices including mechanical valves, valves made from chemically cross-linked biological tissues, and human allografts. Heart valve substitutes provide significant improvement in cardiac function and life expectancy but have functional limitations such as the need for life-long anticoagulation, risks of developing endocarditis, and propensity to degenerate and calcify. Most bioprosthetic valves fail within 15 to 20 years after implantation, and a second open-heart surgery to retrieve defective valves is undesirable. These risks are a particular problem in pediatric patients, in whom bioprosthetic heart valves degenerate and calcify at a faster rate.²

Strategies for heart valve tissue engineering fall into two main categories.³ The first is preparation of decellularized heart valves using enzyme and detergent methods followed by repopulation with appropriate cell types *in vitro* before implantation or relying on host cells to repopulate and re-

model the scaffolds *in vivo*. This approach has limitations, because complete valve decellularization has proven difficult to attain, and cell repopulation is difficult because of lack of adequate porosity. The second approach is assembly of synthetic biodegradable matrices populated by cells and bioreactor conditioning to express adequate properties before implantation. Although this approach seems appealing, the polymeric matrices lack sufficient mechanical strength and have not withstood the test of time under arterial pressure.³

Bovine pericardium has been widely researched as a biomaterial, including examination of its mechanical and fiber orientation properties by mapping techniques,⁴ isolation of pericardial fibroblasts and analysis of their biosynthetic abilities,^{5,6} and detailed analysis of its collagen and proteoglycan components.^{5,6} Glutaraldehyde-fixed bovine pericardium has a long history of being used in manufacturing of bioprosthetic heart valves and a good record of implantation in humans, is well characterized mechanically and biologically,^{4,7-9} and has been used in development of collapsible percutaneous heart valves¹⁰ and for mitral valve repair.¹¹ Only recently has decellularized pericardium attracted attention as a scaffold for tissue engineering.^{9,12}

Our working premise was that the ideal scaffolds for heart valve applications must function immediately after

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implantation but also need to tolerate cell infiltration and gradual remodeling. We hypothesized that moderately cross-linked collagen scaffolds prepared from acellular pericardium would fulfill these requirements. To test this hypothesis, collagen scaffolds were prepared from porcine pericardium; treated with 1,2,3,4,6-Penta-O-galloyl-beta-D-glucose (penta-galloyl glucose; PGG); and then tested for biodegradation, mechanical properties, and *in vivo* biocompatibility and remodeling. Scaffolds were also used in construction of anatomically correct heart valves. Their properties were compared with those of glutaraldehyde-fixed scaffolds. The current study showed that PGG stabilizes collagen and supports slow and progressive host cell infiltration and matrix remodeling, indicating that PGG is a promising collagen stabilization process for heart valve tissue engineering.

Materials and Methods

Materials

High-purity PGG was a generous gift from N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium (www.omnichem.be). Pure DNA, ribonuclease, glutaraldehyde (50% stock), and collagenase Type VII from *Clostridium histolyticum* were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Deoxyribonuclease I was from Worthington Biochemical Corporation (Lakewood, NJ) and bicinchoninic acid (BCA) protein assay kits from Pierce Biotech (Rockford, IL). Electrophoresis apparatus, chemicals, and molecular weight standards were from Bio-Rad (Hercules, CA), and elastase was from Elastin Products Company (Owensville, MO). All other chemicals were of highest purity available and were typically obtained from Sigma-Aldrich.

Scaffold preparation

Fresh adult swine pericardial sacs obtained from Animal Technologies, Inc. (Tyler, TX) were cleaned, rinsed in sterile saline, cut into strips, and decellularized as follows. In the first step, tissues were stored in double-distilled water overnight at 4°C to induce hypotonic shock and cell lysis. After rinsing, tissues were treated with 0.25% sodium-deoxycholate, 0.15% Triton X-100, 0.1% ethylenediaminetetraacetic acid (EDTA), 0.02% sodium azide (NaN₃), in 50 mM Tris-hydrochloric acid (HCl) buffer (pH 7.8) with mild agitation for 6 days at 22°C and changes of the solution after 3 days. After rinsing with double-distilled water and 70% ethanol to remove detergents, tissues were treated with a deoxyribonuclease/ribonuclease mixture (360 mU/mL for each enzyme) at 37°C for 24 h to fully digest away nucleic acids. This was followed by rinsing twice with double-distilled water and incubation in ultrapure elastase (10 U/mL) in 50 mM Tris buffer, 1 mM calcium chloride, 0.02% NaN₃ (pH 8), at 37°C for 6 days with mild agitation. Elastase was replaced with fresh solution after 3 days. Tissues were rinsed in double-distilled water at 22°C until BCA protein assay revealed undetectable levels of soluble proteins. Scaffolds were finally rinsed with 70% ethanol and then stored in sterile saline supplemented with 0.02% NaN₃ at 4°C.

Scaffold characterization

For histological evaluation, paraffin-embedded samples were stained with hematoxylin and eosin (H&E) for general

morphology and confirmation of cell removal and with Verhoeff van Gieson to confirm removal of elastin ($n=6$ slides per group per stain). Digital pictures were taken of H&E-stained samples ($n=2$ slides per group) at 400 $\times$ magnification, and open spaces (pores) between intact collagen fibers were measured using AxioVision Release 4.6.3 digital imaging software (Carl Zeiss MicroImaging, Inc. Thornwood, NY). To further validate decellularization, total genomic DNA was extracted and purified from collagen scaffolds and from fresh pericardium as controls ($n=3$ per group) using a Fibrous Tissue DNeasy Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. DNA samples were subjected to agarose electrophoresis alongside pure DNA standards (10–100 μ g/mL) followed by densitometry using Gel-Pro Analysis Software (MediaCybernetics, Silver Spring, MD). DNA levels were calculated from the standard curve and normalized to initial tissue wet weight.

To test for the presence of soluble proteins, scaffolds and fresh pericardial samples ($n=2$ per group) were pulverized in liquid nitrogen (N₂), proteins extracted in an extraction buffer (50 mM Tris-HCl, 150 mM sodium chloride (NaCl), 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), pH 7.4, with protease inhibitor cocktail), and protein content determined using BCA assay. Samples normalized to initial dry weight were analyzed for detergent-soluble proteins using SDS polyacrylamide gel electrophoresis followed by silver staining (SilverSnap, Pierce Biotech, Rockford, IL). For detection of matrix metalloproteinase 2 (MMP-2), same-protein extracts were analyzed using an MMP-2 enzyme-linked immunosorbent assay (ELISA) kit (Amersham Biosciences, Piscataway, NJ) and results normalized to mg of soluble protein.

Chemotaxis assays were conducted using a Boyden chamber (NeuroProbe, Gaithersburg, MD) and a polycarbonate filter with 8- μ m-diameter pores, as per the manufacturer's instructions. Soluble collagen peptides (matrikines) were prepared by treating decellularized porcine pericardium with collagenase (10 U/mL) for 24 h. The supernatant was filtered through Microcon YM-3 centrifugal devices, and peptides smaller than 3 kDa were collected in the flow-through for chemotaxis assays. Rat aortic fibroblasts (10 $\times$ 10⁵/well, prepared in house using an explant technique) suspended in Dulbecco's modified Eagle medium (DMEM)/0.1% bovine serum albumin were used in these tests; undiluted fetal bovine serum (100%) was used as a positive control and DMEM/0.1% bovine serum albumin as the negative control. Cells migrated for 4 h at 37°C and 5% carbon dioxide (CO₂). After incubation, the non-migrated cells were removed using a wiper blade, and the filter was fixed and stained using a DiffQuick kit (Dade Behring Inc, Newark, DE), dried, and screened for migrated cells using an inverted microscope. Results were reported as negative (0–2 cells/10 $\times$ field), slightly positive (2–10 cells), or positive (>10 cells).

Properties of cross-linked collagen scaffolds

Scaffolds were prepared as above and separately treated with one of three methods: PGG, glutaraldehyde, or no-treatment controls. For PGG fixation, scaffolds were incubated with 0.3% PGG in 50 mM of phosphate buffer (pH 5.5) containing 4% isopropanol for 24 h at 22°C, rinsed, and stored in sterile saline. For glutaraldehyde fixation, scaffolds

were incubated in 0.6% glutaraldehyde in 50 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (pH 7.4) overnight at 22°C, rinsed, and stored in sterile saline.

To evaluate cross-linking efficacy, scaffold samples were lyophilized to obtain dry weight and then incubated in collagenase (6.25 U/mL) dissolved in 100 mM Tris buffer, 1 mM CaCl_2 , and 0.02% NaN_3 (pH 7.8) for up to 7 days at 37°C with mild agitation. At each time point, six samples from each group were rinsed and lyophilized to obtain dry weights after collagenase and percentage of digested tissue were calculated.

Three specimens from each group of control, glutaraldehyde-treated, and PGG-treated scaffolds were also subjected to thermal denaturation temperature (T_d) analysis using differential scanning calorimetry (DSC-131, Setaram Instrumentation, Caluire, France). Specimens were tested at a heating rate of 10°C/min from 20°C to 110°C in a N_2 gas environment. T_d , a well-known indicator of collagen cross-linking, was defined as the temperature at the endothermic peak.¹³

Biaxial mechanical properties of control untreated, glutaraldehyde-treated, and PGG-treated scaffolds were characterized according to a biaxial testing system using experimental techniques developed by Sacks *et al.*^{14–16} Briefly, 15-mm $\times$ 15-mm square specimens were cut from the samples with edges along fiber-preferred direction and cross-fiber-preferred direction. Each edge of the square was mounted with four stainless steel hooks attached to two suture loops. After 10-cycle equibiaxial preconditioning, specimens were subjected to biaxial tests using seven loading protocols to provide a wide range of loading states (T_{PD} : T_{XD} = 10:60, 30:60, 45:60, 60:60, 60:45, 60:30, 60:10 N/m). Sixty-N/m membrane tension was used to represent the deformation under peak diastolic load of aortic valve and to compare with other studies.¹⁵ In biaxial stress-strain testing, fiber-preferred direction stretch ratio (λ_{PD}) and cross-fiber-preferred direction stretch ratio (λ_{XD}) at 60 N/m were used as measures of tissue extensibility. Hysteresis, a parameter that reflects energy dissipation, was measured by normalizing the enclosed area of loading and unloading curves (tension vs areal strain) to the area underneath the loading curve. Areal strain is defined as $(\lambda_{PD} \lambda_{XD} - 1) \cdot 100\%$. The degree of axial cross-coupling is defined as the percentage change in peak stretch ratio along each axis as the biaxial load was changed from equibiaxial (T_{PD} : T_{XD} = 60:60) to non-equibiaxial conditions (e.g. T_{PD} : T_{XD} = 60:10) and calculated as $(\Delta\lambda/\lambda_{eq}^{peak}) \times 100\%$. Statistical analysis of mechanical data was performed using one-way analysis of variance (ANOVA) (SigmaStat 3.0, SPSS Inc., Chicago, IL). The Holm-Sidak Test for pairwise comparisons and comparisons versus a control group was used for the *post hoc* comparison. Results were considered significantly different at $p < 0.05$.

In vivo evaluation of scaffolds

Male juvenile Sprague-Dawley rats (weighing ~50 g, from Harlan Laboratories; Indianapolis, IN) were sedated using acepromazine (0.5 mg/kg, Ayerst Laboratories, Rouse Point, NJ) and maintained on 2% isoflurane during surgery. A small transverse incision was made on the backs of the rats, and two subdermal pouches (one superior and one inferior to the incision) were created. Samples were prepared for implantation

by overnight soaking in sterile saline. Controls (no cross-linking) and glutaraldehyde- and PGG-treated scaffold samples were implanted into the subdermal pouches ($n = 8$ implants per group per time point) and incisions closed using surgical staples. After surgery, the rats were allowed to recover and permitted free access to water and food. The Animal Research Committee at Clemson University approved the animal protocol, and National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication #86-23 Rev. 1996) were observed throughout the experiment.

The rats were humanely euthanized using CO_2 asphyxiation at 1, 3, and 6 weeks after surgery and samples retrieved for analysis. A small section of each explant with its associated capsule was maintained for histological evaluation. The remainder of explants were cleaned free of capsule, rinsed in saline, and divided for DNA, calcium, and MMP analysis.

For histology, samples were placed in formalin, and paraffin sections (5 μm) were stained with H&E for general morphology. For identification of infiltrating cell types, samples tagged for immunohistochemistry were placed in formalin^{17,18} and paraffin-embedded. Sections (5 μm) were exposed to 0.1% proteinase K solution (25 U/500 mL, Qiagen DNeasy Tissue Kit) in tris buffered saline (TBS), pH = 7.5, at 22°C for 30 s. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in 0.3% normal sera (Vectastain Elite ABC kit for rabbit immunoglobulin (Ig)G, Burlingame, CA). Sections were immunostained using mouse anti-rat monoclonal antibodies to macrophages (1:200 dilution, Chemicon, Temecula, CA), vimentin (1:500 dilution, Sigma, St. Louis, MO), and prolyl-4-hydroxylase (1:200 dilution, Chemicon) at 22°C for 1 h. To minimize cross-reactivity, rat-absorbed biotinylated anti-mouse IgG was used in place of the biotinylated secondary antibody provided with the staining kit. Negative staining controls were performed with the omission of the primary antibody. A diaminobenzidine tetrahydrochloride peroxidase substrate kit was used to visualize the specific staining (Vector Laboratories, Burlingame, CA), and sections were lightly counter-stained with hematoxylin. As positive controls, we stained paraffin sections from rat spleen (macrophage control) and rat skin (fibroblast control) in parallel with the explant sections.

To visualize phenol groups within PGG-treated samples, we used an iron-based histology stain.¹⁹ Briefly, tissues were stained en-bloc with iron chloride, embedded in Tissue Tek optimal cutting temperature compound (Sakura Finetek, USA Inc., Torrance, CA), and 6- μm -thick sections were counter-stained with light green. PGG appears brown with this staining.

For DNA analysis explants ($n = 3$ /group per time point) were weighed and then subjected to DNA extraction and purification using the Qiagen Kit, and DNA content was evaluated using agarose gel electrophoresis followed by densitometry, as described above.

Explant samples ($n = 4$ per group per time point) were rinsed in saline, lyophilized to obtain dry weight, individually hydrolyzed in 6 N HCl, dried under nitrogen, and reconstituted in 1.0 mL of 0.01 N HCl. Calcium content was then measured using atomic absorption spectrophotometry as described before.^{20,21}

For MMP detection, proteins were extracted in 50 mM Tris, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, and

150 mM NaCl, with protease inhibitor mixture, pH 7.4, buffer, and protein content was determined using the BCA assay as described before.¹⁹ Samples were subjected to gelatin zymography using 6 μ g of protein per lane alongside molecular weight standards.²² Intensity of MMP bands (white bands on dark background) were evaluated using densitometry using Gel-Pro Analysis Software (MediaCybernetics, Silver Spring, MD) and expressed as relative density units normalized to protein content.

GAG content in explanted tissues was determined using a dimethylmethylene blue assay as described before.¹³ Briefly, a sample of extracted protein (described above for MMP assay) was digested with papain, and released GAGs were incubated with dimethylmethylene blue reagent and optical density (OD) read at 525 nm. GAG content was calculated from a standard curve of pure chondroitin sulfate (0–25 μ g/mL) and values expressed as μ g/mg protein.

Construction of anatomically correct valves

Whole porcine hearts collected at a local abattoir were brought to the laboratory on ice and rinsed with saline, and the aortic and pulmonary valves were dissected together with preservation of the entire anatomy of the cardiac base. Valves were then fixed for 48 h in 0.6% glutaraldehyde in 50 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (pH 7.4) at 22°C. At the onset of fixation, the aortic heart valve cusps were lightly stuffed with glutaraldehyde-imbibed cotton balls to maintain the valve in the closed position. After removal of the cotton balls and rinsing, liquid silicone (Copy Flex Liquid Silicone, Cincinnati, OH) was poured into the aortic valve through the ascending aorta and the silicone allowed to harden for 24 h at room temperature. Final molds could easily be removed through a lengthwise incision through the aorta. Collagen scaffolds prepared from porcine pericardium (see above) were placed onto the molds and air dried for 48 h in a sterile bio-hood. To test the functionality of the heart valve-shaped fibrous scaffolds, they were mounted onto a silicone mock aorta support from a home-made pulse duplicator tester system and cycled at approximately 1 Hz. Still frames were captured from digital movies using video editing software (VideoStudio 9, ULead Systems Inc., Torrance, CA).

Statistics

Results are represented as means $\pm$ standard errors of the mean (SEM). Statistical analysis was performed using one-way ANOVA, and results were considered significantly different at $p < 0.05$.

Results

Properties of collagen scaffolds

Our technique for decellularizing porcine pericardium involved hypotonic shock, detergent extractions, nuclease digestions, and elastase treatment. Results showed that an acellular structure of histologically intact pure wavy collagen fibers with long inter-fibril open spaces (pores) 8 to 30 μ m in diameter was obtained (Fig. 1a–d). Most of the fibers were insoluble because strong detergent extractions yielded few extractable proteins as evidenced by electrophoresis and silver-staining (Fig. 1e). Decellularization was confirmed ac-

cording to DNA extraction and evaluation using agarose gel electrophoresis (Fig. 1f) followed by densitometry (Fig. 1g), which showed a more than 94% reduction in DNA content. ELISA measurements showed significantly lower levels of remnant MMP-2 activity in scaffolds than in fresh pericardium (Fig. 1h). Fibroblasts exhibited a strong positive chemotaxis toward collagen peptides obtained from decellularized pericardium (Fig. 1i), suggesting that scaffold degradation products could encourage repopulation by acting as matrikines.

Cross-linking evaluation

Collagen scaffolds were treated with glutaraldehyde, a known protein cross-linker,²³ and PGG, a compound known to interact with proline-rich proteins such as collagen.²⁴ Samples were then tested *in vitro* for resistance to collagenase, mechanical properties, and thermal denaturation characteristics. Collagenase was used as an accelerated model for degradation and a widely accepted test that reveals extent of collagen cross-linking.^{25,26} Results showed that collagenase almost completely digested control, untreated scaffolds in 1 to 2 days (Fig. 2a), suggesting that decellularized porcine pericardium is highly biodegradable. As expected, glutaraldehyde-treated collagen was highly resistant to collagenase at all time points, indicating that glutaraldehyde fixation of collagen is strong and practically irreversible. PGG-treated collagen scaffolds exhibited excellent resistance to collagenase at 1 and 2 days, with a slight but significant ($p < 0.05$) increase in loss of mass at 7 days (almost 15% degradation by collagenase). These data suggest that PGG treatment is a good short-term collagen fixative.

Differential scanning calorimetry analysis showed T_d values of $76.9 \pm 1.0^\circ\text{C}$ for control scaffolds, $86.9 \pm 0.2^\circ\text{C}$ for glutaraldehydetreated scaffolds, and $78.2 \pm 0.6^\circ\text{C}$ for PGG-treated scaffolds. All values were statistically different between groups (ANOVA, $p < 0.05$), with the exception of PGG versus control ($p = 0.45$).

Samples from each scaffold fixation group were subjected to a series of biaxial tests as described before for heart valve cusps (Fig. 3a).^{27,28} Maximum stretch ratios at 60 N/m equibiaxial tension (physiologic peak diastolic load of aortic valve) were evaluated in the fiber-preferred direction and the cross-fiber-preferred directions (Fig. 3b). No significant changes were observed between the PGG-treated group and native scaffolds. The PGG-treated group showed a slightly higher hysteresis (Fig. 3c), suggestive of direct PGG-collagen interactions. Mechanical coupling of the stretch axes was not affected in the PGG-treated group. Overall, the data indicate that PGG induced only minor changes in the biaxial mechanical properties of the collagen scaffolds.

In vivo biocompatibility studies

To study the biocompatibility and cell repopulation potential of our scaffolds, we implanted samples subdermally in juvenile rats and analyzed them at 1, 3, and 6 weeks post-implantation. H&E staining (Fig. 4a–d) revealed a time-dependent increase in cell infiltration in control (untreated) scaffolds associated with visible collagen fiber degeneration, confirming that the collagen scaffold is degradable *in vivo*. Glutaraldehyde-fixed collagen scaffolds showed good collagen fiber preservation and significantly less cell infiltration

FIG. 1. Characteristics of acellular porcine collagen scaffolds. (a) Hematoxylin and eosin staining of acellular scaffold showing wavy collagen fibers and complete lack of cell staining, compared with native tissue (b) in which cells are outlined by black arrows. (c) Verhoeff van Gieson staining showing fine elastin fibers (black arrow) throughout the native pericardial tissue, which were completely removed (d) by elastase treatment. (e) Presence of detergent-soluble proteins in fresh pericardium and acellular collagen scaffolds was evaluated using electrophoresis and silver staining. (f) Agarose and ethidium bromide gel electrophoresis of DNA extracted from fresh pericardium and the acellular collagen scaffolds; (g) densitometry of DNA bands in (f). (h) Enzyme-linked immunosorbent assay for matrix metalloproteinase 2 in fresh pericardium and the acellular collagen scaffolds, (i) chemotaxis of fibroblasts toward collagen peptides obtained from acellular pericardium; inset in (i) shows a cell undergoing mitosis after chemotaxis, (j) positive chemotaxis control, (k) negative control. Color images available online at www.liebertonline.com/ten.

FIG. 2. (a) Resistance to collagenase of treated collagen scaffolds: a time-course study. Percentage mass loss after collagenase treatment is shown for untreated scaffolds (control) and for glutaraldehyde and penta-galloyl glucose (PGG)-treated scaffolds. Control values were all significantly higher than other groups. Analysis of variance statistical significance between groups. (b) Chemical structure and three-dimensional model of the PGG molecule. The central derivatized glucose core (blue) and the external gallic acid residues (pink circles) are depicted at left. Atoms in the three-dimensional model are C = black, O = red, H = light gray. Color images available online at www.liebertonline.com/ten.

(Fig. 4c), validating that glutaraldehyde-treated collagen is not an ideal substrate for cell-mediated remodeling. PGG-treated scaffolds (Fig. 4d) exhibited clear signs of collagen fiber degradation, visibly less than control untreated collagen, and more than glutaraldehyde-fixed collagen. Similarly, larger numbers of infiltrating cells were found in PGG-treated collagen than in glutaraldehyde-fixed collagen but less than in untreated controls. Cell infiltration in PGG-treated scaffolds increased with time, showing that PGG treatment is not cytotoxic. Histology data were confirmed using quantitative DNA analysis (Fig. 4e). Despite considerable biological variability between samples, data showed significant DNA present at all time points in most tissues, with less DNA present in glutaraldehyde-fixed tissues at 3 weeks ($p < 0.05$). Comparative analysis of 6-week and 3-week data revealed that DNA content apparently reached a plateau after 3 weeks, indicating that cell infiltration is complete within this time frame in subdermal implants.

Phenol staining showed tight binding of PGG to collagen (Fig. 4g), which appeared to be maintained even after 3 weeks of subdermal implantation (Fig. 4h), suggestive of stable PGG–collagen interactions.

A large majority of cells infiltrating collagen scaffolds were vimentin-positive cells resembling fibroblasts (Fig. 5a). Cell density apparently increased with time of implantation for control and PGG scaffolds, whereas cell infiltration in glutaraldehyde-fixed scaffolds was significantly lower at all time points. Infiltrating cells were also positive for proline-hydroxylase, an enzyme involved in collagen synthesis

FIG. 3. (a) Representative equibiaxial tension protocol for acellular collagen scaffolds. (b) Peak stretch ratios along the fiber-preferred direction and cross-fiber-preferred direction for control untreated scaffolds and for glutaraldehyde and penta-galloyl glucose-treated scaffolds. (c) Hysteresis values calculated for the four scaffold groups. (d) Comparison of axial coupling of fiber-preferred direction for the four scaffold groups. *Analysis of variance statistical significance between groups.

FIG. 4. Histological analysis of subdermally implanted scaffolds. Representative hematoxylin and eosin micrographs from 3-week samples showing (a) pre-implant collagen scaffolds, (b) explanted control scaffolds, (c) explanted glutaraldehyde-treated scaffolds, (d) explanted penta-galloyl glucose (PGG)-treated scaffolds. Black arrows point to cells present within implants. (f) Phenol staining showing collagen-bound PGG (brown) before implantation and (g) 3 weeks after subdermal implantation. Inset in (f) represents phenol staining of a negative control (scaffold not treated with PGG). Color images available online at www.liebertonline.com/ten.

(Fig. 5b). Macrophage infiltration was scarce in all implants at all time points (Fig. 5c). Calcium analysis in explanted scaffolds (Table 1) revealed significantly high levels in glutaraldehyde-fixed collagen, whereas control, untreated collagen scaffold samples and PGG-treated scaffolds did not accumulate any significant amounts of calcium, indicating that, despite effective collagen stabilization, PGG treatment may not induce collagen calcification. MMP activity was measured in explants from all time points using gelatin zymography (Fig. 6a). Two major proteases were identified in tissue extracts, namely MMP (migrating at ~ 90 – 95 kDa) and MMP-2 (65–80 kDa). Band intensity was measured for each sample (normalized to protein content) and represented as relative density units (Fig. 6a). Similar trends were observed for MMP-9 and MMP-2 (high enzyme levels at 1 and 3 weeks leveling off at 6 weeks). Activity of MMP-2 was approximately 10 times as high as MMP-9 in all samples. The total MMP enzyme activity distribution resembled that of the DNA content. In addition, GAG levels within all implant groups

showed a mean overall value of 175 ± 30 $\mu\text{g}/\text{mg}$ extracted protein but without showing statistically significant difference between treatment groups and time points ($p > 0.05$).

TABLE 1. CALCIUM LEVELS IN EXPLANTED COLLAGEN SCAFFOLDS

Sample	<i>Ca content ($\mu\text{g}/\text{mg}$ dry weight $\pm$ standard error of the mean, $n = 4$)</i>		
	1 week	3 weeks	6 weeks
Control	1.4 ± 0.5	1.2 ± 0.3	0.5 ± 0.1
glutaraldehyde	2.8 ± 1.4	$5.4 \pm 3.8^{a,b}$	$4.6 \pm 2.6^{a,b}$
Penta-galloyl glucose	1.4 ± 0.4	1.5 ± 0.5	1.1 ± 0.2

^aStatistical significance between time points within each fixation group ($p < 0.05$).

^bStatistical between among groups at each time point ($p < 0.05$).

FIG. 5. Immunohistochemical identification of infiltrating cells. (a) Vimentin stain showing extensive fibroblast-like infiltrating cells in all implant groups at 1 week (1w), 3 weeks (3w), and 6 weeks (6w) after implantation. (b) Proline-hydroxylase immunohistochemical staining; arrowheads point to positively stained cells. (c) Specific staining showing few macrophages (arrowheads). Inset in (c) shows macrophage staining of rat spleen sections used as a positive control. Sections were counterstained with hematoxylin (nuclei dark blue). Bars are 50 μ m in all micrographs. Color images available online at www.liebertonline.com/ten.

Overall results suggest the presence of cells actively involved in matrix remodeling.

Testing of anatomically correct valves

The long-term aim of our studies is to create anatomically correct scaffolds to be used as off-the-shelf constructs for heart valve tissue engineering. For this purpose, we created silicone molds from porcine aortic heart valves and then modeled decellularized porcine pericardium into anatomically correct scaffolds (Fig. 6b). After being dried in their molds, the scaffolds acquired the shape of the aortic valve, which could then be preserved by exposure to PGG. Functionality testing of the heart valve-shaped scaffolds showed good leaflet coaptation

upon closure and good opening characteristics (data not shown).

Discussion

Replacement or regeneration of heart valves, exquisite examples of optimum hemodynamics, durability, design, and adaptability, has challenged engineers and surgeons for the last 40 years. Among the different heart valve substitutes, the most physiologic valve replacements are the pulmonary autograft valves (whereby the patient's own pulmonary valve is transplanted into the aortic position) and the human allograft valves (sterilized, cryopreserved cadaveric valves obtained from humans). These exhibit excellent durability but are not

FIG. 6. (a) Matrix metalloproteinase (MMP) activities in explanted scaffolds. Densitometry of active MMP bands are expressed as relative density units normalized to protein content. MMP-9 (a) and MMP-2 (b) were identified from their relative migration on gelatin containing 10% polyacrylamide gels (insets show representative zymograms). (b) Anatomically correct heart valve scaffolds. Silicone molds of porcine aortic valves were made by casting. (1) Note a portion of the ascending aorta, the emergence of the two coronary arteries (RC, LC) and the three cusps (A, B, C), which closely mimic valve anatomy. Decellularized porcine pericardium scaffolds were modeled into heart valve-shaped constructs using the molds and penta-galloyl glucose treated to cross-link and maintain shape (2) followed by immersion in saline (3). The position of the three cusps is marked by A, B, C. Color images available online at www.liebertonline.com/ten.

readily available and represent only a small proportion of total valve replacements.

It is vital that implanted heart valves function immediately after implantation but also support host cell infiltration and remodeling. It is our working hypothesis that such properties can be achieved by developing partially cross-linked collagen scaffolds. Full cross-linking would reduce cell infiltration and remodeling capacity to a minimum and thus might prevent tissue regeneration. Conversely, un-cross-linked scaffolds may degrade too rapidly after implantation and thus reduce functionality of the tissue-engineered heart valves. Moderately cross-linked scaffolds would allow for slow but sustained cell infiltration with temperate remodeling of the scaffold, without alterations in valve properties. In the current study, we evaluated non-glutaraldehyde fixation methods such as PGG for development of novel scaffolds for heart valve tissue engineering.

Porcine pericardium was successfully decellularized using hypotonic shock, detergent extraction, and nuclease digestion. This is an accepted decellularization approach for fibrous connective tissues.²⁹ To obtain pure collagen scaffolds with constant and predictable composition and properties, decellularized pericardium was further treated with elastase. Removal of elastin created supplementary pores for cell infiltration and assisted with tissue decellularization (data not shown).

Native, untreated scaffolds degraded quickly *in vitro* and *in vivo*, were rapidly invaded by host cells, and showed signs of remodeling and lack of calcium deposition. To evaluate cross-linking possibilities, we treated decellularized pericardium with PGG and glutaraldehyde and compared their properties.

Glutaraldehyde treatment effectively stabilized decellularized pericardium against collagenase, an expected

feature known in the bioprosthetic heart valve field.² Moreover, glutaraldehyde-fixed scaffolds exhibited typical mechanical properties and high T_d (compared with native collagen). Upon implantation, glutaraldehyde-fixed collagen calcified to some extent but not to levels reported for bioprosthetic heart valves analysis, in which glutaraldehyde-fixed pericardium showed calcification levels of approximately 100 $\mu\text{g}/\text{mg}$.³⁰ This may be because of absence of pericardial cells that are presumably the initial calcification nucleation sites.³¹ Cell infiltration was noted to occur to some extent in glutaraldehyde-fixed scaffolds, but cell numbers did not increase with time, and despite the fact that some infiltrating cells were fibroblast-like cells expressing MMPs, proline-hydroxylase, and GAGs, it is unlikely that these scaffolds would undergo remodeling after implantation.

PGG treatment revealed substantial initial collagen stabilization against the action of collagenase *in vitro*, with the potential for reversibility. The *in vitro* collagenase system is an accelerated model for degradation, because collagenase activities *in vivo* are not expected to be so harsh.³² Differential scanning calorimetry analysis showed a slightly higher T_d than in native scaffold and lower than glutaraldehyde (but not statistically different), partially confirming collagenase results. This phenomenon of tissue resistance to collagenase in the absence of high T_d values was reported previously for the Photofix stabilization procedure.³³ Mechanical properties of PGG-treated scaffolds were similar to untreated and glutaraldehyde-treated samples except for a higher hysteresis. Despite effective collagen stabilization, PGG-treated collagen did not calcify *in vivo*, suggesting that the nature of the cross-linker may determine the outcome of collagenous implants. Host cells infiltrated implants relatively rapidly, indicating that PGG-treated collagen is not cytotoxic. Moreover, these fibroblast-like cells secreted MMPs, expressed proline-hydroxylase, and secreted GAGs and thus may exhibit true potential for remodeling. Subdermal implantation in juvenile rats serves as a model for calcification,³⁴ as well as for degradation of matrix components and remodeling.^{35,36} To create structures that mimic the heart valve anatomy, we used silicon molds from porcine heart valves. Scaffolds dried onto such molds adopted the valve structure down to very fine details. This shape was maintained after rehydration and PGG stabilization and functioned acceptably in a pulse duplicator.

Mechanisms of PGG-induced collagen stabilization are not fully understood. PGG is a naturally derived polyphenol characterized by a D-glucose molecule derivatized at all five hydroxyl moieties by gallic acid (3,4,5-trihydroxybenzoic acid) (Fig. 2B). Polyphenols have a hydrophobic internal core and numerous external hydroxyl groups. By virtue of this structure, they react with proteins, specifically binding to hydrophobic regions³⁷ but also establishing numerous hydrogen bonds, showing particularly high affinity for proline-rich proteins³⁸ such as collagen and elastin.³⁹ In addition, they are efficient antibacterial agents and reduce inflammation and antigenicity.⁴⁰ Recently, we showed that peri-arterial delivery of PGG to rat abdominal aorta prevented aneurysm formation or progression and did not elicit any detectable changes in serum liver enzyme activities or liver histology, showing that PGG was not toxic at the local or systemic level.¹⁹ Moreover, extractables obtained from PGG-fixed tissues exhibited low

in vitro cytotoxicity toward fibroblasts and smooth muscle cells⁴¹ and thus can be used safely in tissue-engineering applications.^{42–46} In current studies, we have shown that PGG binds strongly to pericardial collagen and that this binding is stable for at least 3 weeks *in vivo*.

Conclusions

Decellularized pericardium fulfills many properties required for use in valvular tissue engineering, including adequate mechanical properties, minimal cytotoxicity, excellent cell repopulation potential, and propensity for matrix remodeling. Stabilized collagen scaffolds could be shaped into anatomically correct valve-shaped constructs that may function as heart valves. Such collagen scaffolds are superior to polymer scaffolds in terms of mechanical and biological properties but also maintain a natural tendency to degenerate rapidly *in vivo* unless stabilized. Glutaraldehyde cross-linking fully stabilizes collagen but does not allow for tissue remodeling and also induces calcification when implanted subdermally in rats. Conversely, PGG is a mild, non-toxic, reversible collagen-stabilizing agent capable of controlling tissue degradation. PGG-treated collagen does not calcify *in vivo* and supports host cell infiltration and matrix remodeling. In conclusion, PGG treatment is a promising collagen stabilization process for heart valve tissue engineering. Ongoing work in our group is focused on development and testing of cell-seeded PGG-stabilized collagen-based constructs for heart valve tissue engineering.

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Disclosure Statement

No competing financial interests exist.

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