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A novel crosslinking method for improved tear resistance and biocompatibility of tissue based biomaterials

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ABSTRACT

Over 300,000 heart valve replacements are performed annually to replace stenotic and regurgitant heart valves. Bioprosthetic heart valves (BHVs), derived from glutaraldehyde crosslinked (GLUT) porcine aortic valve leaflets or bovine pericardium are often used. However, valve failure can occur within 12–15 years due to calcification and/or progressive degeneration. In this study, we have developed a novel fabrication method that utilizes carbodiimide, neomycin trisulfate, and pentagalloyl glucose crosslinking chemistry (TRI) to better stabilize the extracellular matrix of porcine aortic valve leaflets. We demonstrate that TRI treated leaflets show similar biomechanics to GLUT crosslinked leaflets. TRI treated leaflets had better resistance to enzymatic degradation *in vitro* and demonstrated better tearing toughness after challenged with enzymatic degradation. When implanted subcutaneously in rats for up to 90 days, GLUT control leaflets calcified heavily while TRI treated leaflets resisted calcification, retained more ECM components, and showed better biocompatibility.

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1. Introduction

Bioprosthetic heart valves (BHVs) are used to replace stenotic and regurgitant heart valves [1–3]. BHVs do not require long-term anticoagulant therapy and also have more optimal hemodynamics, thus making it the preferred option for valve replacements. BHVs are fabricated out of two types of xenogeneic tissues: (1) Porcine aortic valve (PAV) leaflets or (2) bovine pericardium sheet. Commercially available BHVs fail due to structural degradation and/or calcification.

PAV leaflets resemble human heart valve leaflet three layered structure. The fibrosa faces the aortic side of the leaflet and contains circumferentially aligned type I collagen that provides strength to withstand high cyclic pressure environment [3–5]. Facing the opposite side towards the ventricle, the ventricularis contains radially aligned elastin along with collagen. Elastic fibers are coupled with collagen bundles and brings the collagen fiber structure back to a resting configuration between loading cycles, thus the ventricularis provides elasticity and recoil to the leaflet [6–9]. Between the fibrosa and ventricularis lies the spongiosa that primarily contains glycosaminoglycans (GAGs). This layer is assumed to mechanically function as a shearing medium between the two other layers as the GAGs absorb water and form a hydrogel-like layer [7,10,11].

PAV's are crosslinked with glutaraldehyde (GLUT) to fabricate BHV implants to make them resistant against enzymatic degradation and less-immunogenic [1,12,13]. GLUT crosslinked BHVs tear and calcify after implantation, causing valve failure within 12–15 years of use [1–3,12,14]. Furthermore, calcification and structural degradation seem to accelerate in younger patients most likely due to a more competent immune system/increased metabolism and increased physical activity [1,15]. Therefore, BHVs are less frequently used for patients under 65 years of age. It should also be noted that degeneration and calcification are not mutually exclusive and one can lead to the other. GLUT crosslinking does not stabilize components such as elastin and GAGs of the heart valve. Therefore, these major ECM components are lost from the tissue due to enzymatic degradation [10,16–18]. We have recently shown that GAGs in BHVs are strongly connected with fiber–fiber and fiber–matrix interactions at low force levels and that they may be

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important in providing a damping mechanism to reduce leaflet flutter when the leaflet is not under high tensile stress [7]. Thus, the degradation of the essential ECM components leads to the compromised mechanical function of the leaflet and this could be a major contributor to degenerative tears of BHVs.

Here we have developed a novel tissue fixative method that utilizes carbodiimide, neomycin trisulfate, and pentagalloyl glucose (PGG) crosslinking chemistry to stabilize all ECM components. We name this new method TRI as three crosslinkers were used. We demonstrate that PAV leaflets treated with TRI improve tear toughness, resist *in vivo* calcification, structural degradation, and are more biocompatible than GLUT treated leaflets.

2. Methods

2.1. Heart valve biomaterial crosslinking

Porcine aortic heart valves (PAVs) were harvested fresh from a local slaughter house and transported on 0.9% saline and ice to the laboratory. Whole PAVs were washed in 0.9% saline for 15 min. The leaflets were then cut from the aortic root and washed in 0.9% saline for 3–5 min. These leaflets were then treated with two different chemical treatment techniques: (1) GLUT – control and (2) TRI – newly proposed crosslinking technique.

2.1.1. GLUT

Fresh leaflets were treated with 0.6% glutaraldehyde in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline (pH 7.4) at room temperature with gentle shaking for 24 h, solution decanted, and replaced with 0.2% glutaraldehyde in 50 mM HEPES buffered saline (pH 7.4) and the crosslinking was continued for at least six days [6].

2.1.2. TRI

Fresh leaflets were treated with 0.5 mM neomycin trisulfate solution in 2-(N-morpholino) ethanesulfonic acid (MES) buffer for 1 h. The solution was decanted and leaflets were then incubated in a 30 mM *1-ethyl-3-(3-dimethylaminopropyl) carbodiimide* (EDC) and 6 mM *N-hydroxysuccinimide* (NHS)/0.05% PGG solution in 50 mM MES buffered saline (pH 5.5) for twenty-four hours. The solution was decanted and then the leaflets were crosslinked further in 30 mM EDC and 6 mM NHS solution in 50 mM MES buffered saline (pH 5.5) for 24 h. Following fixation, valves were placed in 20% isopropanol in 50 mM HEPES buffer (pH 7.4) for at least six days.

2.2. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to measure the thermal denaturation temperature (T_d) of collagen. Samples (3–8 mg) from each of the TRI and GLUT groups were carefully cut from generally the same region of the leaflet, blotted dry, and placed flat in hermetically sealed pans. Samples were tested using a DSC 2920 (TA Instruments, Newcastle, DE). A pilot run verified the T_d range for GLUT compared to previous results, and the remainder of samples were equilibrated at 20 °C, and heated at 10 °C/min. The denaturation temperature was recorded as the maximum value of the endotherm peak.

2.3. In vitro enzymatic challenge studies

2.3.1. Resistance to collagenase and elastase

PAV leaflets were cut in half symmetrically, rinsed in nanofiltered water, blotted dry, frozen, lyophilized, and weighed. Half leaflets were incubated in 1.2 mL of 5 U/mL porcine pancreatic elastase (Elastin Products Co. Inc., Owensville, MO) in 100 mM Tris, 1 mM $CaCl_2$, 0.02% NaN_3 (pH 7.8) for 24 h, or 75 U/mL collagenase (Type I, Sigma, St. Louis, MO) in 50 mM Tris, 10 mM $CaCl_2$, 0.02% NaN_3 (pH 8.0) for 48 h at 37 °C while shaking at 650 RPM while other half was incubated with buffer only. Enzyme liquid was saved for further studies, digested cusps rinsed in nanofiltered water, blotted dry, frozen, lyophilized, and weighed. Percent weight loss was calculated. For storage studies, samples were stored for 21, 60, or 180 days in 0.2% glutaraldehyde solution (GLUT samples) or 20% isopropanol in HEPES buffer (TRI samples), taken out of storage, washed in saline 3× for 15 min, and challenged with the aforementioned protocol with collagenase or elastase and percent weight loss calculated.

2.3.2. Resistance to GAGases

PAV leaflets were carefully excised from the aortic wall, and washed thoroughly in 100 mM ammonium acetate buffer (AAB) at pH 7.0 3× for 5 min. Leaflets were cut symmetrically, one half incubated in 1.2 mL GAG degrading enzyme solution (5 U/mL hyaluronidase (Sigma, St. Louis, MO), 0.1 U/mL chondroitinase (Sigma, St. Louis, MO) in 100 mM AAB, pH 7.0) while the other half incubated in AAB. Samples were shaken vigorously for 24 h at 37 °C. Following 24 h incubation, leaflets were washed in distilled water 3× for 5 min, frozen, and lyophilized. Total hexosamine content was measured using the hexosamine assay as previously reported [6]. Elson and Morgan's modified hexosamine assay was used to measure GAG-related hexosamines [18]. Lyophilized GAG digested samples were weighed, digested in 2 mL 6 N HCl (24 h, 96 °C), and dried under nitrogen gas. Samples were resuspended in 2 mL 1 M sodium chloride and reacted with 2 mL of 3% acetyl acetone in 1.25 M sodium carbonate. 4 mL ethanol and 2 mL of Ehrlich's reagent (0.18 M p-dimethylaminobenzaldehyde in 50% ethanol containing 3 N HCl) were added, and solutions left for 45 min to allow the color to develop. A pinkish-red color is indicative of tissue hexosamine quantities, and the absorbance was read at 540 nm. A set of D(+)-glucosamine standards were used. For storage studies, samples were stored for 21, 60, or 180 days in 0.2% glutaraldehyde solution (GLUT samples) or 20% isopropanol in HEPES buffer (TRI samples) taken out of storage, washed in saline 3× for 15 min, and challenged with the aforementioned protocol with collagenase or elastase and percent weight loss calculated.

2.4. Mechanical testing

Tissue specimens (n = 7 per group) were tested under biaxial tension using a custom-built biaxial device [19,20]. Roughly 1 cm by 1 cm sections were taken from the central belly region of the leaflets and mounted to the device with the circumferential and radial directions along the device axes. The specimens were then loaded to a maximum membrane tension of 60 N/m over a period of 15 s following a preconditioning step [21,22]. The strain was determined via four fiducial markers were glued to the central region of the specimen [22]. All mechanical testing were performed in PBS at room temperature.

2.5. Suture pull-out test

PAV leaflets were evaluated for structural integrity and its ability to resist tearing. Fresh, GLUT, and TRI, leaflets were cut with a die in rectangular 40 mm × 4.0 mm sections and were then treated with GAGase, porcine pancreatic elastases, or no enzyme treatment (n = 10, 9 groups total). For suture pull-out test, half of the samples were oriented circumferentially (with collagen alignment) and half samples were aligned radially (perpendicular collagen alignment). One side of tissue samples was attached to a bottom clamp in a tensile tester (MTS, Minneapolis, MN). A single suture

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