Published in final edited form as:

Ann Thorac Surg. 2011 September ; 92(3): 858–865. doi:10.1016/j.athoracsur.2011.04.104.

Triglycidylamine Cross-linking Combined with Ethanol Inhibits Bioprosthetic Heart Valve Calcification

Jeanne M. Connolly, M.S.^{1,*}, Marina A. Bakay, Ph.D.^{1,*}, Ivan S. Alferiev, Ph.D.¹, Robert C. Gorman, M.D.², Joseph H. Gorman III, M.D.², Howard S. Kruth, M.D.³, Paul E. Ashworth, M.S.⁴, Jaishankar K. Kutty, Ph.D.⁴, Frederick J. Schoen, M.D., PhD.⁵, Richard W. Bianco, B.A.⁶, and Robert J. Levy, M.D.¹

¹Division of Cardiology, The Children's Hospital of Philadelphia, Philadelphia, PA 19104

²University of Pennsylvania School of Medicine, Philadelphia, PA 19104

³NHLBI, National Institutes of Health, Bethesda, MD 20892

⁴St. Jude Medical Inc., St. Paul, MN 55117

⁵Department of Pathology, Brigham and Women's Hospital, and Harvard Medical School Boston, MA 02115

⁶University of Minnesota, Minneapolis, MN 55455

Abstract

Background—One of the most important factors responsible for the calcific failure of bioprosthetic heart valves is glutaraldehyde cross-linking. Ethanol (EtOH) incubation after glutaraldehyde cross-linking has previously been reported to confer anti-calcification efficacy for bioprostheses. The present studies investigated the anticalcification efficacy in vivo of the novel cross-linking agent, triglycidyl amine (TGA), with or without EtOH incubation, in comparison to glutaraldehyde.

Methods—TGA cross-linking (+/– EtOH) was used to prepare porcine aortic valves for both rat subdermal implants and sheep mitral valve replacements, for comparisons with glutaraldehyde-fixed controls. Thermal denaturation temperature (Ts), an index of cross-linking, cholesterol extraction, and hydrodynamic properties were quantified. Explant endpoints included quantitative and morphologic assessment of calcification.

Results—Ts after TGA were intermediate between unfixed and glutaraldehyde-fixed. EtOH incubation resulted in almost complete extraction of cholesterol from TGA or glutaraldehyde-fixed cusps. Rat subdermal explants (90days) demonstrated that TGA-EtOH resulted in a significantly greater level of inhibition of calcification than other conditions. Thus, TGA-ethanol stent mounted porcine aortic valve bioprostheses were fabricated for comparisons with glutaraldehyde-pretreated controls. In hydrodynamic studies, TGA-EtOH bioprostheses had lower pressure gradients than glutaraldehyde-fixed. TGA-ethanol bioprostheses used as mitral valve

© 2011 The Society of Thoracic Surgeons. Published by Elsevier Inc. All rights reserved.

ADDRESS FOR CORRESPONDENCE: Jeanne M. Connolly, The Children's Hospital of Philadelphia, 702 Abramson Research Center, 3615 Civic Center Boulevard, Philadelphia, PA 19104-4318, Telephone number: 215-590-6098, Fax number: 215-590-5454, connolly@email.chop.edu.

*Joint first authors

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

replacements in juvenile sheep (150 days) demonstrated significantly lower calcium levels in both explanted porcine aortic cusp and aortic wall samples compared to glutaraldehyde-fixed controls. However, TGA-EtOH sheep explants also demonstrated isolated calcific nodules and intracuspal hematomas.

Conclusions—TGA-EtOH pretreatment of porcine aortic valves confers significant calcification resistance in both rat subdermal and sheep circulatory implants, but with associated structural instability.

Keywords

Heart valve bioprosthesis; biomaterials; calcification (heart valve); animal model

Introduction

Bioprosthetic heart valve replacements, fabricated from either glutaraldehyde fixed porcine aortic valves or bovine pericardium, are widely used for the surgical therapy of end-stage heart valve disease [1–3]. Bioprostheses can fail because of leaflet stiffening or defects caused by intrinsic calcific deposits that develop over time in both children and adults [4]. Bioprosthetic leaflet calcification occurs by complex mechanisms that involve interactions of host and implant factors. The most important host factor has proven to be young age; children and young adults develop severe bioprosthetic calcification sooner in their postoperative course than do adults [2]. One of the most important implant factors potentiating calcification is glutaraldehyde pretreatment. Glutaraldehyde is thought to be pro-calcific because of several mechanisms. Glutaraldehyde stabilizes a fraction of the phospholipid membranes of the residual cells in the bioprosthetic tissue as well as the phosphoesters included in cellular nucleic acids; thus, sources of inorganic phosphorus, following alkaline phosphatase hydrolysis, are readily available to react with calcium from the extracellular environment [2]. Moreover, intrinsic alkaline phosphatase activity is not diminished by glutaraldehyde fixation [5]. Therefore, hydrolysis of phospho-esters, such as the residual nucleic acids present in crosslinked cells, by either intrinsic or extrinsic alkaline phosphatase, can form inorganic phosphorus, leading to calcium-phosphate mineral [2, 6]. Glutaraldehyde cross-links are also unstable, and this can lead to both material failure and local inflammation due to the leaching of glutaraldehyde from treated tissue [7–8]. Thus, a number of previous investigations have explored the use of alternatives to glutaraldehyde, such as polyepoxides [9–10], water soluble carbodiimide [11], or photooxidative cross-linking [12–13]. However, these prior strategies have not been demonstrated to be effective for preparing durable bioprostheses with calcification resistance [14–15].

We previously reported the synthesis and mechanistic characterization of an epoxy-crosslinker, triglycidyl amine (TGA) [16], that was demonstrated to result both in stable cross-linking of porcine aortic valve and bovine pericardial tissue, and significant, but not complete calcification inhibition in rat subdermal implants [6, 16–17]. These prior studies indicate that TGA anticalcification mechanisms may be due to unique alternations in collagen structure and diminished accumulation of extracellular matrix proteins associated with calcification such as tenascin-C and MMP9.

In the present studies, we explored combining TGA cross-linking with EtOH incubations following TGA exposure. Our rationale for investigating ethanol was based on our prior studies combining glutaraldehyde cross-linking with post-fixation incubations in EtOH that resulted in significant inhibition of bioprosthetic cusp calcification in both rat subdermal implants and sheep mitral valve replacements [15]. The EtOH anti-calcification mechanism is based on a number of factors including the effective extraction of virtually all of the

cuspal lipid by EtOH, thus removing lipid rich calcification initiation sites [15], and permanent alternations in collagen structure in bioprosthetic leaflet tissue, documented by FTIR[18], that confer calcification resistance in rat subdermal implant studies [15].

Therefore, the present studies investigated the hypothesis that combining TGA crosslinking with EtOH for preparing porcine aortic bioprosthetic heart valves could result in a synergistic enhancement of calcification inhibition due to the independent anticalcification mechanisms involved. The present studies investigated the following: 1) Porcine aortic valves were crosslinked with either TGA or TGA either preceded or followed by EtOH incubation. Samples were assessed for material properties and for their extent of cross-linking and cholesterol content versus glutaraldehyde fixed controls, with or without EtOH. 2) In vivo anti-calcification efficacy was first studied in rat subdermal implants. 3) TGA-EtOH porcine aortic valve bioprostheses were compared to glutaraldehyde bioprostheses in a series of mitral valve replacements in juvenile sheep, and explants were assessed for inhibition of calcification.

Material and Methods

All chemicals were obtained from Sigma-Aldrich Chemicals, St. Louis, MO unless otherwise specified. Triglycidylamine (TGA) was synthesized as previously described [16]. In brief, ammonia was reacted with an excess of epichlorohydrin in aqueous isopropyl alcohol, in the presence of ammonium triflate as a catalyst, to give Tris-(3-chloro-2-hydroxypropyl)amine. This was dehydrochlorinated to TGA by addition of aqueous NaOH.

Bioprosthetic materials preparation

Fresh porcine aortic valve leaflets, or aortic roots with attached leaflets, were shipped overnight on ice from St Jude Medical (Minneapolis, MN). Upon arrival, all were rinsed extensively in sterile normal saline solution, and fixed with either glutaraldehyde (Glut) or with TGA. For material studies and subdermal implants, Glut fixed tissues were treated with 0.6% Glut (EM grade, Polysciences, Inc, Warrington, PA) in 50mM HEPES/0.9% NaCl buffer, pH 7.4, at room temperature for seven days with one change, then stored in 0.2% buffered Glut at room temperature until use. Tissues fixed with TGA were treated with 100mM TGA in borate mannitol buffer (25 mM sodium tetraborate decahydrate, pH 7.4) at room temperature with daily changes of the TGA solution for 7 days, then for 24 hours with 100mM thiosulfate in borate mannitol buffer to neutralize residual epoxy groups. For differential scanning calorimetry, cholesterol analyses or rat subdermal implants, a subset of each material was subsequently treated for 24 hours with 95% absolute EtOH in borate mannitol buffer, and after extensive rinsing, stored in 20% absolute EtOH in borate mannitol buffer (pH 7.4). An additional group was prepared by first treating with 95% EtOH for 24 hours, then following the 7 day TGA and 1 day thiosulfate neutralization procedure as above. Prior to rat subdermal implantation, all materials were rinsed in an excess of sterile normal saline.

Bioprosthetic heart valves for sheep orthotopic mitral valve replacement were prepared using either Glut or TGA-pretreated porcine aortic leaflets with an attached aortic root segment, following both the cross-linking and EtOH pretreatment protocols described above. Santoprene stuffers with the anatomic curvature of aortic leaflets were placed in contact with the aortic side of each porcine aortic cusp during fixation to maintain the physiologic conformation of each cusp. Subsequently, sterilized TGA valves were treated with 95% EtOH for 24 hours and stored as described above; Glut fixed valves were terminally sterilized and stored in buffered 0.2% Glut as previously described [14]. The cusp-stuffer assemblies were then shipped to St. Jude Medical for fabrication as stent-mounted trileaflet valves, using the St. Jude Epic valve mounting stent, 23mm diameter, per

GMP procedures. These stent mounted bioprostheses were used in both the hydrodynamic studies and the sheep mitral valve replacement studies.

Differential Scanning Calorimetry (DSC) analysis

Porcine aortic valve cusps were crosslinked with Glut, Glut-EtOH, TGA, or TGA-EtOH as described above and shrink temperature (Ts) determined using a DSC7 (Perkin-Elmer, Inc., Wilson, CT) with temperature ramping from 60C to 100C. Analyses were performed in quadruplicate.

Cholesterol determination

Materials prepared as above were extracted as previously described [15]. In brief, 10 samples of each group were extracted with chloroform/methanol 2:1 (vol/vol) [19]. Total cholesterol was determined enzymatically with fluorometric detection, [20] and expressed as nanomoles per milligram of dry tissue weight

Hydrodynamic testing

Stent-mounted porcine aortic bioprosthetic valves were subjected to testing under pulsatile flow using a pneumatic actuated left-sided heart model, which includes a mechanical mitral valve and the bioprosthetic aortic valve under evaluation. Controlling and tuning of the system was performed using a custom National Instrument LabView™ acquisition system that measures flow, and pressure readings at the inflow and outflow of the aortic valve over 10 cardiac cycles [21]. Parameters measured at 70 pulses per minute, 100mmHg aortic mean pressure and 5 liters per minute flow included backpressure, stroke volume, regurgitation, pressure gradient, and effective orifice area (EOA). Under steady forward flow testing, pressure drop across valves at discrete flow rates was measured. Quadruplicate valves of each type were tested.

Rat Subdermal Implants

90 day rat subdermal implants were performed under isoflurane anesthesia in compliance with NIH guidelines pertaining to the care and use of laboratory animals [16] using porcine aortic valve cusps prepared as described above, in full accordance with an Institutional Animal Care & Use Committee (IACUC) approved protocol at The Children's Hospital of Philadelphia. Flunixin was administered sc (2.2mg/kg) as an analgesic immediately postoperatively. Upon euthanasia, explants were rinsed in sterile saline, stored at 4C for later processing for Ca analysis. 6 rats per group were included in all experiments, each with 2 implants.

Sheep Mitral Valve Replacement

It should be noted that because of the high costs of sheep open heart surgery with long term survival, only the most effective anticalcification pretreatment per the rat subdermal results could be studied in comparison with the current standard of care, a glutaraldehyde-fixed porcine aortic valve bioprosthesis. Sheep were used in this study in compliance with NIH guidelines pertaining to the care and use of laboratory animals per IACUC approval (University of Minnesota, University of Pennsylvania) and fed age appropriate diets containing the recommended daily calcium intake. Animals used were either Dorset or Colombian crossbred female or castrated male sheep (4 to 5 months of age, 30–55 kg) (Neaton Farms). Briefly, sheep were subjected to general anesthesia (isoflurane) and placed on cardiopulmonary bypass, and porcine bioprosthetic valves (St Jude Medical, Inc, as above; size, 23 mm) were implanted into the mitral position via a standard left lateral thoracotomy through the fourth intercostal spaces previously described[15]. Sheep were heparinized during the surgical procedure with protamine neutralization at the conclusion.

However, the animals were not administered anticoagulants postoperatively. Analgesics administered postoperatively include a transdermal fentanyl patch (1.8–4mcg/kg) for the first 72 hours, followed by 0.1–0.03mg/kg buprenorphine as needed. After 150 days, sheep were euthanized, a complete necropsy was done including examination of organs for emboli, and the heart was placed in neutral buffered formalin. Ten valve replacement surgeries in each of two implant groups were carried out.

Explant morphology

After gross pathologic examination of the whole heart, explanted valves were dissected free of the heart, photographed and analyzed grossly, and representative samples were processed for histological analyses. Valve dissection involved careful removal of the entire tissue complex (cusps and associated aortic wall) from the stent as an intact unit, and then taking radial sections through cusp and associated wall for each cusp for light microscopic examination. Remaining portions of cusps and aortic wall were excised at their commissural junctions, individually labeled, and sent for calcium and phosphorus content (described below). Routine processing for light microscopy included dehydration through graded alcohols and embedding of specimens in paraffin. Sections 5–6 μ m were stained with hematoxylin and eosin for overall morphology and with von Kossa stain for calcium phosphates.

Explant Calcium and Phosphorous analyses

Explanted samples, freeze-dried to a uniform weight, were hydrolyzed in 6N HCl at 100°C for 24 hours. Each hydrolysate was appropriately diluted for either calcium or phosphorous concentration determination using atomic absorption spectroscopy (Perkin-Elmer 2380) or ammonium-molybdate complexation assay respectively [22].

Statistical Analysis

Data are reported as mean $\pm$ SE. One-Way ANOVA analysis was used to compare differences between groups, followed by post hoc Dunn's or Holm-Sidak analysis. Comparisons between individual test groups were made using Student's t-test or the Mann-Whitney rank sums test as appropriate. Surgical outcomes were assessed using a z-test for differences of sample proportions. All statistical analyses were accomplished using SigmaStat (ver. 3.0, SPSS, Inc., Chicago, IL) analysis software. $p \leq 0.05$ was considered significant.

Results

Differential scanning calorimetry—Comparisons of Glut and TGA

Thermal denaturation temperatures (T_s) were obtained using differential scanning calorimetry as an index of cross-linking, comparing TGA-EtOH porcine aortic leaflets to leaflets treated with either glutaraldehyde alone, Glut-EtOH, or using only TGA cross-linking without EtOH incubation, with a comparison to untreated (native) leaflets. These studies revealed that TGA cross-linking resulted in T_s that were significantly lower ($p < 0.001$) than those obtained following glutaraldehyde or Glut-EtOH cross-linking (Figure 1). While EtOH incubations following either Glut or TGA cross-linking did not significantly affect T_s , TGA cross-linking regardless of EtOH resulted in significantly higher T_s than that of native (untreated) leaflets ($p < 0.001$).

Cholesterol extraction due to EtOH pretreatment

Total cuspal cholesterol measurements were obtained as an index of lipid content and extraction due to exposure to the glutaraldehyde, TGA, and EtOH incubations (Figure 2).

TGA-EtOH treatment resulted in the removal of nearly all of the cuspal cholesterol (0.02 ± 0.006 nmol/mg, vs. pre-EtOH value of 7.6 ± 3.7 nmol/mg), and this level of cholesterol extraction was not significantly different than that observed following glutaraldehyde fixation and EtOH post-treatment. Pretreatment with EtOH prior to TGA fixation also efficiently extracted cholesterol (Figure 2).

Rat rat subdermal implant results

Calcium and phosphorus analyses of ninety days duration subdermal explants from these rat studies revealed that a number of the conditions studied significantly inhibited the otherwise severe calcification of glutaraldehyde pretreated cusps (Figure 3). TGA-EtOH, with EtOH incubations following TGA fixation, resulted in the most significant inhibition of calcification (Figure 3). Interestingly, incubation in EtOH first followed by TGA crosslinking resulted in less effective inhibition of calcification (Figure 3). Other pretreatment protocols studied, i.e. the use of TGA alone, and Glut-EtOH, while significantly inhibiting bioprosthetic cusp calcification, were not as effective as TGA-EtOH. Phosphorus determination of these explants (Figure 3B) showed comparable trends to the calcium analyses (Figure 3A).

Hydrodynamic testing of TGA-EtOH vs glutaraldehyde pretreated mitral valve prostheses

In view of the rat subdermal results, TGA-EtOH, the most effective anticalcification pretreatment, was chosen for use in sheep mitral valve replacements. Thus, TGA-EtOH porcine aortic valve bioprostheses were fabricated for comparisons with clinical grade, stent mounted glutaraldehyde pretreated porcine aortic valve bioprostheses. In vitro hydrodynamic testing studies used steady state flow velocities ranged from 5 liters per minute (lpm) to 30 lpm. TGA-EtOH pretreated bioprostheses demonstrated lower pressure gradients at all flow rates studied (Figure 4). TGA-EtOH mean pressure gradients ranged from 1.1 to 25.5mm over the flow rates studied compared to glutaraldehyde pretreated bioprostheses that demonstrated gradients ranging from 1.5 to 32.9mm. The proportionate increase in gradients with increasing applied flow was 20% higher for Glut than that of TGA-EtOH valves (average linearized slopes of gradients vs. the square of the flow velocity are 0.029 and 0.023, respectively). Using a pulsatile-flow system (Table 1), at a rate of 70 cycles/min which corresponds roughly to 20 liters/minute, backpressure was higher, the pressure gradient was lower, and the EOA higher ($p=0.04$, $p=0.026$, and $p=0.022$, respectively) in TGA-EtOH valves compared to Glut pretreated bioprostheses.

Juvenile sheep mitral valve replacements

Mitral valve replacements in juvenile sheep resulted in an operative survival of 7 of the 10 animals receiving glutaraldehyde pretreated porcine aortic valve bioprosthetic valves, and 8 of the 10 animals that underwent mitral valve replacement with porcine aortic valve bioprostheses that were prepared with TGA-EtOH. Of the 7 glutaraldehyde pretreated implants, 5 survived to 150 days for elective sacrifice and 2 were terminated prematurely due to valve dysfunction causing left-sided heart failure. The postoperative duration of the glutaraldehyde-pretreated bioprosthetic implants in sheep euthanized prematurely due to severe symptoms were 60 and 119 days. None of the animals that underwent mitral valve replacements with TGA-EtOH bioprostheses were symptomatic or required termination prior to elective euthanasia at 150 days post-operation.

Although none of the cusps of the eight TGA-EtOH explant leaflets were grossly calcified (Figure 5A and 5E), isolated calcified cuspal nodules were present in one of the cusps of each of two TGA-EtOH treated bioprostheses (Figure 5B and 5F). The cause of the nodules was not apparent from pathologic examination. Three of the eight TGA-EtOH explants also demonstrated intracuspal hematomas, that were grossly (Figure 5B) and microscopically

evident (Figure 5G); these included the 2 bioprosthetic valves mentioned above with isolated calcific nodules on the surface of the cusps (Figure 5B). In contrast, all of the glutaraldehyde pretreated bioprostheses had grossly apparent calcifications involving all of the cusps (Figure 5C). Quantitative calcium and phosphorus analyses demonstrated significant inhibition of leaflet calcification in the TGA-EtOH group compared to the high calcium levels present in the leaflet tissues of the glutaraldehyde pretreated explants (Figure 6). Aortic wall segments were dissected free of the valve support-stent structure and were also analyzed for both calcium and phosphorus. TGA-EtOH aortic wall demonstrated significantly lower calcium and phosphorus levels than did the aortic wall samples obtained from the explanted glutaraldehyde pretreated bioprostheses, that were extensively calcified (Figure 6). Furthermore, the aortic wall calcium levels for the TGA-EtOH explants were not significantly different than those observed in the TGA-EtOH cusps ($p=0.16$).

Comment

The results of these studies show that while TGA-EtOH pretreatment of porcine aortic bioprosthetic cusps can inhibit calcification more effectively than glutaraldehyde or the other inhibitory pretreatments studied (TGA alone, glutaraldehyde-EtOH), the structural instability observed, intra-cuspal hematomas, tears, and extrinsic calcific nodules, are a major concern. The structural instability is consistent with the Ts data (Figure 1), that demonstrated that while TGA crosslinking techniques resulted in Ts significantly greater than uncrosslinked, native cusps, these TGA Ts data were not at the level achieved with glutaraldehyde.

Why does TGA not achieve the same level of crosslinking as glutaraldehyde? While TGA has been shown to react with a broader array of amino acids than glutaraldehyde, which only reacts with lysine residues via the amino side-chain, the TGA reaction kinetics vary markedly with thiol-containing amino acids reacting most rapidly, and amino groups reacting relatively slowly [17]. In general TGA crosslinking proceeds more slowly than glutaraldehyde, and requires daily changes of depleted reactants, each day for a week [17]. Glutaraldehyde solutions react more rapidly, and do not become depleted after one week's time [23].

The present studies have a number of limitations. The coauthors acknowledge that the accelerated calcification of glutaraldehyde crosslinked bioprostheses observed in juvenile sheep over the five months or less duration period of implants, occur far more rapidly than calcific failure in clinical subjects which commonly occurs after two or more years time in children [24], or more than a decade in adults [25]. Nevertheless, the structural defects noted in the TGA-EtOH explants are of concern, since they reflect hemodynamic effects that would be comparable between sheep and human subjects. It is possible that the structural abnormalities noted in the TGA-EtOH were related to a host immune response, perhaps accentuated by insufficient crosslinking, that would make the bioprosthetic cusps more susceptible to inflammatory mechanisms that could lead to extracellular matrix degradation. However, investigation of this possibility is beyond the scope of the present studies.

Conclusions

TGA-EtOH demonstrates significantly greater inhibition of the calcification of porcine aortic valve bioprosthetic leaflets than either TGA alone, or glutaraldehyde with EtOH; this enhanced efficacy was demonstrated in both rat subdermal implants and juvenile sheep mitral valve replacements. However, the level of crosslinking achieved with TGA-EtOH is significantly less than with glutaraldehyde, and thus poses the risk of primary structural failure, despite calcification inhibition.

Acknowledgments

This study was funded by NIH grants, HL74731, HL101820, HL007915, The Kibel Foundation, and the William J. Rashkind Endowment of the Children's Hospital of Philadelphia.

References

1. Schoen FJ. Evolving concepts of cardiac valve dynamics: the continuum of development, functional structure, pathobiology, and tissue engineering. *Circulation*. 2008; 118:1864–1880. [PubMed: 18955677]
2. Schoen FJ, Levy RJ. Calcification of tissue heart valve substitutes: progress toward understanding and prevention. *Ann Thorac Surg*. 2005; 79:1072–1080. [PubMed: 15734452]
3. Turina J, Hess OM, Turina M, Krayenbuehl HP. Cardiac bioprostheses in the 1990s. *Circulation*. 1993; 88:775–781. [PubMed: 8339436]
4. Schoen FJ. Cardiac valves and valvular pathology: update on function, disease, repair, and replacement. *Cardiovasc Pathol*. 2005; 14:189–194. [PubMed: 16009317]
5. Maranto AR, Schoen FJ. Alkaline phosphatase activity of glutaraldehyde-treated bovine pericardium used in bioprosthetic cardiac valves. *Circ Res*. 1988; 63:844–848. [PubMed: 3139323]
6. Rapoport HS, Connolly JM, Fulmer J, Dai N, Murti BH, Gorman RC, Gorman JH, Alferiev I, Levy RJ. Mechanisms of the in vivo inhibition of calcification of bioprosthetic porcine aortic valve cusps and aortic wall with triglycidylamine/mercapto bisphosphonate. *Biomaterials*. 2007; 28:690–699. [PubMed: 17027944]
7. Schoen F. Cardiac valves and valvular pathology: Update on function, disease, repair, and replacement. *Cardiovasc Pathol*. 2005; 14:189–194. [PubMed: 16009317]
8. Giachelli C. Ectopic calcification: gathering hard facts about soft tissue mineralization. *Am J Pathol*. 1999; 154:671–675. [PubMed: 10079244]
9. Sung HW, Shen SH, Tu R, Lin D, Hata C, Noishiki Y, Tomizawa Y, Quijano RC. Comparison of the cross-linking characteristics of porcine heart valves fixed with glutaraldehyde or epoxy compounds. *ASAIO J*. 1993; 39:M532–536. [PubMed: 8268592]
10. Xi T, Ma J, Tian W, Lei X, Long S, Xi B. Prevention of tissue calcification on bioprosthetic heart valve by using epoxy compounds: a study of calcification tests in vitro and in vivo. *J Biomed Mater Res*. 1992; 26:1241–1251. [PubMed: 1429769]
11. Girardot JM, Girardot MN. Amide cross-linking: an alternative to glutaraldehyde fixation. *J Heart Valve Dis*. 1996; 5:518–525. [PubMed: 8894992]
12. Bianco RW, Phillips R, Mrachek J, Witson J. Feasibility evaluation of a new pericardial bioprosthesis with dye mediated photo-oxidized bovine pericardial tissue. *J Heart Valve Dis*. 1996; 5:317–322. [PubMed: 8793684]
13. Schoen F, Levy RJ. Calcification of tissue heart valve substitutes: progress toward understanding and prevention. *Ann Thorac Surg*. 2005; 79:1072–1080. [PubMed: 15734452]
14. Vyavahare NR, Chen P, Joshi P, Lee P, Hirsch P, Levy P, Schoen M, Levy M. Current progress in anticalcification for bioprosthetic and polymeric heart valves. *Cardiovasc Pathol*. 1997; 6:219–229.
15. Vyavahare NR, Hirsch D, Lerner E, Baskin JZ, Schoen FJ, Bianco R, Kruth HS, Zand R, Levy RJ. Prevention of bioprosthetic heart valve calcification by ethanol preincubation: efficacy and mechanisms. *Circulation*. 1997; 95:479–488. [PubMed: 9008467]
16. Connolly JM, Alferiev I, Clark-Gruel JN, Eidelman N, Sacks M, Palmatory E, Kronsteiner A, Defelice S, Xu J, Ohri R, Narula N, Vyavahare N, Levy RJ. Triglycidylamine crosslinking of porcine aortic valve cusps or bovine pericardium results in improved biocompatibility, biomechanics, and calcification resistance: chemical and biological mechanisms. *Am J Pathol*. 2005; 166:1–13. [PubMed: 15631995]
17. Alferiev I, Connolly JM, Levy RJ. A novel mercapto-bisphosphonate as an efficient anticalcification agent for bioprosthetic tissues. *J Organomet Chem*. 2005; 690:2543–2547.
18. Vyavahare NR, Hirsch D, Lerner E, Baskin JZ, Zand R, Schoen FJ, Levy RJ. Prevention of calcification of glutaraldehyde crosslinked porcine aortic cusps by ethanol preincubation:

- Mechanistic studies of protein structure and water-biomaterial relationships. *J Biomed Mater res.* 1998; 40:577–585. [PubMed: 9599034]
19. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem.* 1957; 226:497–509. [PubMed: 13428781]
 20. Gamble W, Vaughan M, Kruth HS, Avigan J. Procedure for determination of free and total cholesterol in micro-or nanogram amounts suitable for studies with cultured cells. *Journal Lipid Res.* 1978; 19:1068–1070. [PubMed: 731127]
 21. Shipkowitz T, Ambrus J, Kurk J, Wickramasinghe K. Evaluation technique for bileaflet mechanical valves. *J Heart Valve Dis.* 2002; 11:275–282. [PubMed: 12000172]
 22. Vyavahare N, Jones PL, Hirsch D, Schoen FJ, Levy RJ. Prevention of glutaraldehyde-fixed bioprosthetic heart valve calcification by alcohol pretreatment: further mechanistic studies. *J Heart Valve Dis.* 2000; 9:561–566. [PubMed: 10947050]
 23. Golomb G, Schoen FJ, Smith MS, Linden J, Dixon M, Levy RJ. The role of glutaraldehyde-induced cross-links in calcification of bovine pericardium used in cardiac valve bioprostheses. *Am J Pathol.* 1987; 127:122–130. [PubMed: 3105321]
 24. Sanders SP, Levy RJ, Freed MD, Norwood WI, Castaneda AR. Use of Hancock porcine xenografts in children and adolescents. *Am J Cardiol.* 1980; 46:429–438. [PubMed: 7415989]
 25. Hammermeister KE, Sethi GK, Henderson WG, Oprian C, Kim T, Rahimtoola S. A comparison of outcomes in men 11 years after heart-valve replacement with a mechanical valve or bioprosthesis. Veterans Affairs Cooperative Study on Valvular Heart Disease. *N Engl J Med.* 1993; 328:1289–1296. [PubMed: 8469251]

Figure 1. Thermal denaturation temperatures (Ts) of porcine aortic leaflets. After crosslinking with Glut, Glut-EtOH, TGA, or TGA-EtOH, Ts is significantly higher than that of untreated (native) tissue. §p<0.001 vs. native, *p<0.001 vs Glut). [mean± S.D.]

Figure 2. Cholesterol analysis of porcine aortic valve leaflets. EtOH treatment significantly reduces cholesterol content of porcine aortic leaflets, whether the leaflet is first crosslinked with either Glut or TGA, or if initially treated with EtOH. (**p<0.001 vs untreated tissue; NS between EtOH groups.)

Figure 3. 90 day rat subdermal explants. (A) Calcium (Ca) and (B) Phosphorus (P) analysis of explanted porcine aortic valve leaflets, showing significant suppression of calcification by all treatments compared to Glut (§p<0.001 by ANOVA). Further significant differences exist between TGA and TGA-ETOH (**p=0.001), and between TGA-EtOH and Glut-EtOH (*p=0.016). Differences between P levels were statistically significant, and showed a difference between Glut and all other treatments by ANOVA (§p<0.001 by ANOVA).

Figure 4. Hydrodynamic testing results, using steady flow conditions. TGA-EtOH valves exhibit lower pressure drops than Glut valves at flow rates ranging from 5 to 30 liters per minute (lpm), an average difference which increases as the flow rate increases. (* $p \leq 0.05$ vs Glut; ** $p < 0.01$ vs. Glut)

Figure 5.

Morphologic findings in 150 day sheep mitral bioprosthetic valve explants. Gross photographs A-C; photomicrographs D-G, with original magnification 100x. A. Typical TGA-EtOH valve, showing no gross abnormalities, and (E) cuspal microscopic qualities comparable to an unimplanted leaflet cusp (D). B. Atypical TGA-EtOH valve explant showing small calcific nodule (black arrow) and hematoma (red arrow), in addition to a tear believed to be the result of necropsy error in upper right quadrant. F and G show von Kossa stain of the calcific nodule and H&E of hematoma respectively. C. Typical Glut150 day sheep mitral bioprosthetic valve explant, with large gross calcifications on all three leaflets, one of which is specifically indicated (black arrow). D, E, and F stained with hematoxylin and eosin, F with von Kossa stain. Micrograph scalebars = 100µM.

Figure 6. Quantification of Ca and P of 150 day sheep mitral valve explants. Both leaflet and wall segments of the TGA-EtOH trileaflet bioprostheses had significantly lower calcification than Glut-prepared materials. A: Ca analysis. B: P analysis. (**p<0.001, *p=0.006 TGA-EtOH vs. Glut leaflet and wall respectively)

Table 1

TGA-EtOH Bioprosthetic Heart Valves Compared to Glutaraldehyde-Fixed: Hydrodynamic Testing Results (70 cycles per minute, flow rate of 5 liters per minute)

Sample ID	Cardiac Output (lpm)	Backpressure (mm Hg)	Stroke Volume (ml/pulse)	Regurgitation (ml/pulse)	Pressure Gradient (mm Hg)	Effective Orifice Area (cm ²)
TGA-EtOH-1	5.1	105.9	76.4	-3.6	10.8	1.56
TGA-EtOH-2	5.0	101.7	76.1	-4.3	9.9	1.67
TGA-EtOH-3	4.9	101.4	75.0	-4.2	8.6	1.82
TGA-EtOH-4	5.1	103.0	76.6	-3.7	10.7	1.65
Mean±S.D.	5.0±0.1	103.0±2.1	76.0±0.7	-3.9±0.4	10.0±1.0	1.68±0.11
Glut-1	5.0	99.3	76.3	-4.4	12.4	1.47
Glut-2	4.9	99.8	74.7	-5.1	10.7	1.57
Glut-3	4.8	95.7	72.5	-3.8	12.7	1.47
Glut-4	4.9	101.3	75.1	-4.6	12.4	1.42
Mean±S.D.	4.8±0.1	99.0±2.4	74.7±1.6	-4.5±0.5	12.0±0.9	1.48±0.06
p Value Between groups	0.070	0.044	0.181	0.136	0.026	0.022