The role of protein solubilization in antigen removal from xenogeneic tissue for heart valve tissue engineering

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ABSTRACT

Decellularization techniques have been developed in an attempt to reduce the antigenicity of xenogeneic biomaterials, a critical barrier in their use as tissue engineering scaffolds. However, numerous studies have demonstrated inadequate removal and subsequent persistence of antigens in the biomaterial following decellularization, resulting in an immune response upon implantation. Thus, methods to enhance antigen removal (AR) are critical for the use of xenogeneic biomaterials in tissue engineering and regenerative medicine. In the present study, AR methods incorporating protein solubilization principles were investigated for their ability to reduce antigenicity of bovine pericardium (BP) for heart valve tissue engineering. Bovine pericardium following AR (BP-AR) was assessed for residual antigenicity, tensile properties, and extracellular matrix composition. Increasing protein solubility during AR significantly decreased the residual antigenicity of BP-AR—by an additional 80% compared to hypotonic solution or 60% compared to 0.1% (w/v) SDS decellularization methods. Moreover, solubilizing agents have a dominant effect on reducing the level of residual antigenicity of BP-AR beyond that achieved by AR additives alone. Tested AR methods did not compromise the tensile properties of BP-AR compared to native BP. Furthermore, residual cell nuclei did not correlate to residual antigenicity, demonstrating that residual nuclei counts may not be an appropriate indicator of successful AR. In conclusion, AR strategies promoting protein solubilization significantly reduced residual antigens compared to decellularization methods without compromising biomaterial functional properties. This study demonstrates the importance of solubilizing protein antigens for their removal in the generation of xenogeneic scaffolds.

1. Introduction

Over 100,000 heart valve replacements are performed annually in the United States [1]. However, residual antigenicity in glutaraldehyde-fixed bioprostheses results in chronic immune-mediated degeneration and subsequent calcification, thus limiting prosthesis lifetime [2,3]. Additionally, fixation prevents biomaterial growth with the patient [4]. Generation of a tissue-engineered heart valve from an appropriate scaffold seeded with autologous cells can potentially overcome drawbacks of glutaraldehyde-fixed bioprostheses.

Normal valve function hinges on maintained mobility, pliability, and integrity of the valve leaflets or cusps [5]. As these features are largely dependent on the extracellular matrix (ECM), selection of an appropriate tissue-engineered heart valve scaffold material is crucial. Xenogeneic biomaterials (e.g., bovine pericardium (BP)) are advantageous because they possess the appropriate complex ECM architecture (composition and structure) crucial for physiological heart valve function [6,7]. However, xenografts represent a major barrier to the use of xenogeneic scaffolds in tissue engineering. Achieving adequately low levels of biomaterial antigenicity while maintaining appropriate mechanical properties is a critical consideration that has yet to be fully addressed.

Antigen removal (AR) from xenogeneic biomaterials is required for their use as tissue engineering scaffolds. Initial attempts at scaffold production focused on cell removal (decellularization). The efficacy of osmotic lysis [8–10], chemical [8–10], enzymatic [8,10], or combinatorial [8–10] decellularization techniques for the generation of xenogeneic scaffolds has been investigated previously. These decellularization approaches make two critical assumptions: (1) xenografts are only found within the cell and (2) removal of cells as determined under light microscopy is equivalent to biomaterial non-immunogenicity. Previously reported decellularization or AR methods relied predominantly on either osmotic lysis or presence of a detergent to permeabilize the cell
membrane and thereby expose the intracellular contents of a tissue to the decellularization solution. Such approaches have universally neglected to consider the need for antigenic components of the biomaterial to be maintained in a soluble form in order to facilitate their removal into aqueous extraction solutions. Indeed, several publications have demonstrated the ability of decellularized xenogeneic biomaterials to elicit an immune response [11–15]. These findings have challenged the fundamental assumptions of decellularization and consequently prompted a shift in focus towards AR in the development of xenogeneic scaffolds for tissue engineering applications [10,16].

The use of reducing agents [17–20] and salts [21,22] to maintain protein solubility in proteomic applications is well established. However, the importance of maintaining protein solubility to achieve AR from intact tissues for tissue engineering applications has not been investigated. This is a surprising oversight since protein solubility is fundamental in permitting protein diffusion into aqueous solution and from tissue during AR. We hypothesized that failure to maintain antigenic proteins in a soluble form during the AR process will result in their precipitation from solution, preventing effective removal from the tissue. Furthermore, we hypothesized that AR methods which enhance protein solubility will reduce BP antigenicity while maintaining biomaterial functional properties. In this study, the importance of promoting protein solubilization for effective AR was evaluated by assessing residual antigenicity of BP in the presence and absence of compounds (reducing agent and salt) predicted to maintain antigenic proteins in a soluble form. The effect of each AR protocol on mechanical properties and ECM composition of BP following AR (BP-AR) was assessed by uniaxial tensile testing, biochemical quantification of ECM components, and histological analysis.

2. Materials and methods

2.1. Tissue harvest

All chemicals were from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. Bovine pericardium was harvested as described by Griffiths et al. [23,24] (n = 4). Fresh BP was excised from adult cattle within 8 h of death and placed into PBS (pH 7.4) containing 0.1% (w/v) Antibiotic Antimycotic Solution (AAS). Following removal of pericardial fat and loose connective tissue, BP was stored in Dulbecco’s Modified Eagles Medium (DMEM) containing 15% (v/v) dimethyl sulfoxide (DMSO) at −80 °C.

2.2. Antiserum production

All animal procedures were conducted in accordance with the guidelines established by University of California, Davis IACUC and the Guide for the Care and Use of Laboratory Animals [25]. Anti-BP serum was generated as previously described [23,24]. Briefly, following subcutaneous injection of BP homogenate and Freund’s adjuvant at a 1:1 ratio into New Zealand white rabbits (n = 4) on days 0, 14, and 28, blood was collected at day 84. Serum was isolated following centrifugation at 3000 rpm for 10 min and stored at −80 °C.

2.3. Protein extraction

Protein extraction from minced BP was adapted from the method previously described by Griffiths et al. [23]. Minced BP (0.2 g) was incubated in standard extraction solution (10 mM Tris–HCl (pH 8.0) containing 1 mM diithiothreitol (DTT), 2 mM MgCl₂, 10 mM KCl, and 0.5 mM Pefabloc SC (Roche, Indianapolis, IN)) containing 0.1% (w/v) sodium dodecyl sulfate (SDS, Bio-Rad, Hercules, CA) at 1000 rpm, 4 °C for 1 h. Following centrifugation at 17,000 × g, 4 °C for 25 min, recovered supernatant was defined as the BP extract. The BP extract was concentrated using Amicon Ultra-0.5 centrifugal filter units with Ultracel-3 membranes (3 kDa cut-off) (Millipore, Billerica, MA) at 14,000 × g, 4 °C for 30 min and stored at −80 °C.

2.4. One-dimensional electrophoresis and Western blot (1-DE and WB)

One-dimensional electrophoresis and Western blot (1-DE and WB) was performed as previously described by Griffiths et al. [24]. Equal volumes of BP extract were separated on NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) with the XCell SureLock Mini-Cell electrophoresis unit (Invitrogen) and blotted to nitrocellulose membranes (0.2 μm pores) according to the manufacturer’s protocol using the XCell II Blot Module. Blots were blocked with Pierce Protein-Free T20 Blocking Buffer (Thermo Fisher Scientific, Rockford, IL) containing 0.05% (v/v) Tween-20 (Bio-Rad) (PFBBT) for 1 h and probed with a 1:100 dilution of rabbit serum generated against native BP in PFBBT at 4 °C, overnight. Following three 10 min washes with PFBBT, blots were incubated in a 1:5000 dilution of horseradish peroxidase-conjugated mouse monoclonal anti-rabbit IgG light chain specific antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) in 10 mM Tris-buffered saline (pH 8.8). Blots underwent five 10 min washes in 10 mM Tris-buffered saline (pH 8.8) with 0.05% (v/v) Tween-20. Protein bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and imaged using the FluorChem Xplor CCD bioimaging system and AlphaView image acquisition and analysis software (Alpha Innotech Corp., Santa Clara, CA) (Fig. 1).

2.5. Antigen removal (AR)

All steps of the AR protocol were performed in 2 mL working volume at 4 °C and 125 rpm using a method adapted from Goncalves et al. [10]. Intact pieces of BP (0.2 g, approximately 1.0 cm × 1.5 cm) were incubated in basic AR buffer (BARB; 0.5 mM Pefabloc, 1% (v/v) AAS in 10 mM Tris–HCl (pH 8.0)) for 2 d; anatomically adjacent pieces of BP subjected to 1 min incubations served as negative AR controls. Following nucleic acid digestion (2.5 Kunitz units/mL deoxyribonuclease I, 7.5 Kunitz units/mL ribonuclease A, 0.15 mM NaCl, 2 mM MgCl₂(H₂O)₆, 1% (v/v) AAS in 10 mM Tris–HCl (pH 7.6)) for 24 h and washout (0.5 mM Pefabloc, 1% (v/v) AAS in 20 mM Tris–buffered saline (pH 7.5)) for 48 h, BP post-AR (BP-AR) was stored in DMEM with 15% (v/v) DMSO at −80 °C.

Initially, efficiency of AR using no additive, 134 mM 3-(benzyl(dimethyl)ammonio) propane sulfonate (NDSB-256), or 0.1% (v/v) SDS in either BARB or BARB containing 100 mM DTT, 2 mM MgCl₂, and 10 mM KCl (solubilizing antigen removing buffer 1, SARB-1) was compared (Table 1 – A1, A2). In phase 1 of DTT optimization, AR efficiency of 134 mM NDSB-256 in SARB-1 containing different concentrations of DTT (0, 1, 25, 100, 200 mM) was assessed (Table 1 – B1, B2, B3, B4, B5, B6) (Fig. 1). In phase 2 of DTT optimization, AR efficiency using no additive, 134 mM NDSB-256, or 0.1% (v/v) SDS in either SARB-1 or BARB containing 100 mM DTT, 2 mM MgCl₂, and 10 mM KCl (solubilizing antigen removing buffer 2, SARB-2) was compared (Table 1 – C1, C2). In phase 1 of KCl optimization, AR efficiency of 134 mM NDSB-256 in SARB-2 containing different concentrations of KCl (0, 10, 50, 100, 200, 500 mM) was assessed (Table 1 – D1, D2, D3, B4, D5, D6). In phase 2 of KCl optimization, AR efficiency using no additive, 134 mM NDSB-256, or 0.1% (v/v) SDS in either SARB-2 or BARB containing 100 mM DTT, 2 mM MgCl₂, and 100 mM KCl (optimal solubilizing antigen removing buffer, opt SARB) was compared (Table 1 – E1, E2). All AR experiments were conducted with n = 6 per group (Fig. 2).

For tensile testing, quantitative biochemistry, and histology of BP-AR, AR (using no additive, 134 mM NDSB-256, or 0.1% (v/v) SDS in either BARB or opt SARB) was performed on 0.2 g pieces of BP which were then trimmed into smaller pieces for downstream analysis (n = 6 per group for all analyses).

2.6. Assessment of residual antigenicity following AR

Proteins extracted from BP-AR (as described in section 2.3 above) were defined as residual antigens. To assess the level of residual antigenicity of BP-AR, extracted proteins from minced BP were separated on NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) with the XCell SureLock Mini-Cell electrophoresis unit (Invitrogen) and blotted to nitrocellulose membranes (0.2 μm pores) according to the manufacturer’s protocol using the XCell II Blot Module. Blots were blocked with Pierce Protein-Free T20 Blocking Buffer (Thermo Fisher Scientific, Rockford, IL) containing 0.05% (v/v) Tween-20 (Bio-Rad) (PFBBT) for 1 h and probed with a 1:100 dilution of rabbit serum generated against native BP in PFBBT at 4 °C, overnight. Following three 10 min washes with PFBBT, blots were incubated in a 1:5000 dilution of horseradish peroxidase-conjugated mouse monoclonal anti-rabbit IgG light chain specific antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) in 10 mM Tris-buffered saline (pH 8.8). Blots underwent five 10 min washes in 10 mM Tris-buffered saline (pH 8.8) with 0.05% (v/v) Tween-20. Protein bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and imaged using the FluorChem Xplor CCD bioimaging system and AlphaView image acquisition and analysis software (Alpha Innotech Corp., Santa Clara, CA) (Fig. 1).
Table 1
BARB supplements used in AR. Various concentrations of DTT, MgCl₂, and KCl were used to supplement BARB in a series of AR experiments (treatments not connected by the same letter were assessed in different experiments) to demonstrate the importance of protein solubility for AR. For each experiment, the buffer demonstrating a significant reduction in residual antigenicity compared to baseline was named (SARB-1, SARB-2, opt SARB) and used as the negative control in the next experiment to determine whether further improvements could be made.

<table>
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<th>Buffer</th>
<th>[DTT]</th>
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<td>A2 (SARB-1)</td>
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proteins were subjected to 1-DE and WB, probed with anti-native BP rabbit serum and assessed for IgG positivity. Densitometry was used to quantify the banding pattern intensity. Residual antigenicity of BP-AR was defined as the ratio of banding intensity for extracts following 2 d of AR to the 1 min AR control. Overall residual antigenicity for each AR treatment.

2.7. Uniaxial tensile testing

Tensile properties of native BP and BP-AR (n = 6 per group) were determined using a uniaxial materials testing system (Instron Model 5565, Canton, MA) with a 50 N load cell. Strips of BP (15 × 4 mm) were cut from separate 0.2 g pieces of native BP and BP-AR along the circumferential and longitudinal anatomical axes with samples from adjacent anatomical locations used for each replicate of the AR methods and control tissue. A 2 mm diameter biopsy punch was used to subtract semicircles from the midpoint of each 15 mm side, creating a dog bone-shaped piece of BP. BP was mounted under zero strain and subjected to a constant strain rate of 5% per sec². Initial gauge length, width, and thickness were determined.

2.8. Quantitative biochemistry

To determine whether AR methods change ECM composition, native BP and BP-AR samples (approximately a quarter of the initial 0.2 g piece by area, n = 6 per group) were weighed, frozen at −20 °C overnight, lyophilized for 72 h, and weighed again. Lyophilized BP samples were digested in phosphate buffer containing 125 μg/ml papa, 5 mM EDTA, and 5 mM N-acetyl-cysteine at 65 °C for 18 h. Collagen content per dry weight (DW) was quantified using a modified colorometric hydroxyproline assay [28]. Sulfated glycosaminoglycan (GAG) content per DW was quantified from papain digests using the Blyscan sulfated GAG assay (Biocolor Ltd., Carrickfergus, UK). Lyophilized BP samples were incubated in 20 volumes of 0.25 n Molar acetic acid at 100 °C for 60 min. After cooling to room temperature, samples were centrifuged at 3000 rpm for 30 min and supernatant containing soluble collagen was collected. Oxalic acid extraction was repeated 3 times. Supernatant from all steps was pooled and collagen content per DW was quantified using the Fastin elastin assay (Biocolor Ltd.).

2.9. Histology

Approximately one quarter of the initial 0.2 g piece (by area) of each native BP and BP-AR sample (n = 6 per group) was histologically processed for Verhoeven van Gieson staining (VGV) and hematoxylin and eosin (H&E) staining. For both stains, slides were viewed under light microscopy (Nikon Eclipse E600). Images were captured of six random high-powered fields (HPFs) using a Retiga 1300 Q-imaging camera and processed with Simple PCI software v5.2.1 (Compix Inc., Cranberry Township, PA). Gross ECM structure in BP was assessed with VGV (collagen and elastin) staining. Slides stained with H&E were used to assess general tissue morphology and quantify residual nuclei on native BP and BP-AR (n = 6 per group, 6 HPFs per slide). Correlation between nuclei counts and overall residual antigenicity was determined by plotting average nuclei counts against overall residual antigenicity for each AR treatment.

2.10. Statistical analysis

Densitometry values were compared between experimental AR groups and negative AR control (1 min). Values determined from tensile testing, biochemical assays, and histology were compared to those for control tissues (native BP). Non-repeated measures ANOVA and Tukey–Kramer HSD post hoc analysis were performed on sample means. Correlation was determined using bivariate fit analysis. All data are presented at mean ± standard deviation from the mean. Significant significance was determined at p < 0.05.

3. Results

3.1. Antigen removal

3.1.1. Basic versus solubilizing antigen removal buffer 1 (BARB vs. SARB-1)

The residual antigenicity of BP-AR using 134 mM NDSB-256 in BARB (NDSB/BARB, 1.08 ± 0.23) was not significantly different than that of BP-AR using BARB alone (1.36 ± 0.22). However, residual antigenicity following use of 0.1% (w/v) SDS in BARB (SDS/BARB, 0.64 ± 0.20) was significantly reduced compared to both BARB (p < 0.0001) and NDSB/BARB (p < 0.05). The presence of 1 mM DTT, 2 mM MgCl₂, and 10 mM KCl in SARB-1 significantly reduced residual antigenicity of BP-AR following treatment with no additive (SARB-1, 0.85 ± 0.23, p < 0.0005) and 134 mM NDSB-256 (NDSB/SARB-1, 0.66 ± 0.17, p < 0.05), but not 0.1% (w/v) SDS (SDS/SARB-1, 0.55 ± 0.18), compared to in BARB (Fig. 3).

3.1.2. DTT optimization

In phase 1, increasing DTT concentration in solutions containing 134 mM NDSB-256, 2 mM MgCl₂, and 10 mM KCl resulted in BP-AR with decreasing residual antigenicity (p < 0.0001), with a plateau at 100 mM DTT (Fig. 4A). In phase 2, AR in the presence of 100 mM DTT (SARB-2) significantly reduced residual antigenicity of BP-AR compared to 1 mM DTT (SARB-1) for no additive (1.17 ± 0.24 to 0.75 ± 0.23, p < 0.0005), 134 mM NDSB-256 (0.96 ± 0.13 to 0.51 ± 0.11,
with decreasing residual antigenicity ($p < 0.0001$), the effect saturating at 100 mM KCl (Fig. 5A). In phase 2, AR in the presence of 100 mM KCl (opt SARB) significantly reduced residual antigenicity of BP-AR compared to 10 mM KCl (SARB-2) for no additive (1.07 ± 0.26 to 0.69 ± 0.15, $p < 0.05$) and 134 mM NDSB-256 (0.94 ± 0.19 to 0.50 ± 0.10, $p < 0.01$), but not 0.1% (w/v) SDS (0.77 ± 0.25 to 0.55 ± 0.17). Moreover, residual antigenicity of BP-AR was not significantly different following treatment with opt SARB, 134 mM NDSB-256 in opt SARB (NDSB/opt SARB), or 0.1% (w/v) SDS in opt SARB (SDS/opt SARB) (Fig. 5B).

### 3.1.4. Overall AR

The level of residual antigenicity in BP-AR following treatment with BARB was defined as 100.00%. The relative amount of residual antigens following AR compared to BARB was determined to be 79.98% with NDSB/BARB, 47.50% with SDS/BARB, 25.89% with opt SARB, 18.74% with NDSB/opt SARB, and 20.86% with SDS/opt SARB.

### 3.2. Tensile testing

Tensile properties of circumferentially- and longitudinally-oriented BP-AR (generated by BARB, NDSB/BARB, SDS/BARB, opt SARB, NDSB/opt SARB, and SDS/opt SARB) were compared to native BP control using uniaxial tensile testing. No significant differences in Young’s modulus, ultimate tensile stress, and ultimate tensile strain were observed between circumferentially-oriented native BP (32.10 ± 1.03 MPa, 2.41 ± 1.03 MPa, and 0.38 ± 0.02, respectively) and AR treatments.
and BP-AR generated by any AR buffer (Fig. 6). Similarly, no significant differences in Young's modulus, ultimate tensile stress, and ultimate tensile strain were observed between longitudinally-oriented native BP (28.51 ± 10.15 MPa, 2.12 ± 0.60 MPa, and 0.36 ± 0.02, respectively) and BP-AR generated by any AR buffer (Fig. 6). No significant differences were observed between circumferentially- and longitudinally-oriented native BP.

3.3. Quantitative biochemistry

The water content of BP-AR generated using BARB (74.83 ± 1.38%), NDSB/BARB (75.24 ± 0.87%), SDS/BARB (71.50 ± 4.01%), opt SARB (74.41 ± 0.66%), NDSB/opt SARB (76.17 ± 1.16%), or SDS/opt SARB (76.22 ± 1.66%) was not significantly different from that of native BP (73.79 ± 1.97%) (Fig. 7A).

The collagen content per DW of BP-AR was not significantly different than that of native BP (33.94 ± 7.71% per DW) following treatment with BARB (45.15 ± 18.21% per DW), NDSB/BARB (41.69 ± 11.03% per DW), SDS/BARB (33.68 ± 6.62% per DW), opt SARB (34.05 ± 6.52% per DW), NDSB/opt SARB (42.48 ± 18.94% per DW), or SDS/opt SARB (38.46 ± 9.33% per DW) (Fig. 7B).

The elastin content per DW of BP-AR following treatment with BARB (10.08 ± 3.36% per DW), NDSB/BARB (8.04 ± 1.89% per DW), opt SARB (7.54 ± 3.25% per DW), or NDSB/opt SARB (7.40 ± 1.20% per DW) was not significantly different from that in native BP (7.59 ± 1.41% per DW). However, elastin in BP-AR was significantly reduced compared to that in native BP when SDS/BARB (3.14 ± 2.23% per DW, p < 0.05) or SDS/opt SARB (3.70 ± 1.77% per DW, p < 0.05) was used for AR (Fig. 7C).

The GAG content per DW of BP-AR was not significantly different from that of native BP (0.55 ± 0.04% per DW) when treated with BARB (0.52 ± 0.05% per DW). However, AR using opt SARB (0.39 ± 0.05% per DW), NDSB/BARB (0.47 ± 0.04% per DW), or NDSB/opt SARB (0.34 ± 0.05% per DW) significantly decreased GAG content per DW compared to that in native BP (0.87 ± 0.04% per DW, p < 0.0001, respectively) (Fig. 7D). The presence of residual SDS in BP-AR subjected to SDS/BARB and SDS/opt SARB interfered with the Blyscan assay (data not shown).

3.4. Histology

Presence of nuclei was assessed in H&E-stained native BP and BP-AR sections (generated by BARB, NDSB/BARB, SDS/BARB, opt SARB, NDSB/opt SARB, and SDS/opt SARB) (Fig. 8). All AR treatments significantly reduced the number of nuclei per HPF compared to native BP (85.72 ± 20.35) (p < 0.005) except for BARB (83.28 ± 19.66). In both BARB and opt SARB, the number of persistent nuclei following treatment in 0.1% (w/v) SDS (18.83 ± 9.78 with BARB and 29.72 ± 14.12 with opt SARB) was significantly less than that from 134 mM NDSB-256 (64.61 ± 16.07 with BARB, p < 0.0001; 58.00 ± 16.44 with opt SARB, p < 0.0001), which was significantly less than that with no additive (83.28 ± 19.66 with BARB, p < 0.0001; 69.94 ± 16.44 with opt SARB, p < 0.0001). Although opt SARB resulted in significantly fewer nuclei than BARB (p < 0.05), there was not a significant difference in nuclear count between BARB and opt SARB in the presence of 134 mM NDSB-256 or 0.1% (w/v) SDS (Fig. 9). No statistically significant correlation was observed between nuclei counts and overall residual antigenicity of BP-AR (p = 0.239, R² = 0.3234) (Fig. 10). Qualitatively, differences in collagen and elastin content per DW and organization were not observed between native BP and BP-AR in VVG-stained sections (Fig. 11).

4. Discussion

The objectives of this study were (1) to determine whether strategies promoting protein solubility could reduce the antigenicity of xenogeneic tissue beyond that achieved by decellularization methods, (2) to identify under which conditions protein solubilization-mediated AR was most effectively achieved, and (3) to assess whether these AR methods adversely affect the xenogeneic biomaterial. We have demonstrated that (1) promotion of protein solubility enhances AR without compromising biomaterial properties, (2) solubilizing factors are critical determinants of AR efficiency, overwhelming the effect of AR additives (hypotonic solution, 134 mM NDSB-256, or 0.1% (w/v) SDS) in the level of...
residual antigenicity, and (3) the number of residual nuclei may not be an appropriate assessment of residual antigenicity.

Dithiothreitol and KCl have previously been utilized to facilitate protein extraction from homogenized BP into aqueous solution [23,24], however solubilizing agents have yet to be incorporated into AR protocols. In this study, AR with hypotonic solution, 134 mM NDSB-256, and 0.1% (w/v) SDS were assessed for their ability to reduce BP antigenicity in the presence and absence of compounds predicted to maintain antigenic proteins in a soluble form. Hypotonic solution [8][10] and 0.1% (w/v) SDS [29] correspond to osmotic lysis and detergent-based decellularization, respectively. Use of 134 mM NDSB-256 was motivated by previous studies which demonstrated the effectiveness of the compound in protein extraction protocols [23,24]. The addition of low levels of reducing agent and salt (1 mM DTT, 2 MgCl₂, and 10 mM KCl) to BARB (SARB-1) significantly reduced the residual antigenicity of BP-AR treated with no additive or 134 mM NDSB-256. In contrast, insignificant improvement in AR observed with 0.1% (w/v) SDS in SARB-1 can be explained by the relatively high efficiency of SDS/BARB to remove antigens, owing to the ability of the anionic detergent to readily denature and solubilize proteins. Low levels of DTT and KCl are able to achieve a reduction in residual antigenicity that is not significantly different from that of 0.1% (w/v) SDS. These findings support the hypothesis that maintaining proteins in solution facilitates their removal from intact BP.

The effect of DTT and KCl concentration on AR enhancement was investigated in a stepwise fashion as a series of two-phase studies. Phase 1 aimed to determine the effect of increasing DTT or KCl concentration on BP-AR residual antigenicity. The optimal concentration was then tested in phase 2 for its ability to influence AR by BARB, NDSB/BARB, and SDS/BARB. For each study, the concentration determined to be most effective for AR in the previous study served as the new negative control to be improved upon, such that the results reflect a progressive improvement in AR beyond that achieved in the previous step (Fig. 2). Solutions containing 100 mM DTT (SARB-2) were found to significantly reduce residual antigenicity of BP-AR compared to 1 mM DTT (SARB-1). These data indicate that not only can the efficiency of AR be modulated based on dose of reducing agent, but that a reducing agent can improve the degree of AR accomplished by the gold standard for decellularization agents (SDS). The intracellular environment is maintained in a reduced state by thioredoxin and glutathione systems [30]. Water-soluble cytoplasmic proteins are consequentially maintained in a reduced state within the cell. Following cell lysis in the decellularization process, the cellular reducing environment can no longer be maintained. Oxidation of cytoplasmic proteins increases the tendency for disulfide bond formation to occur between proteins, resulting in protein aggregation and precipitation from solution [31]. By discouraging the formation of intermolecular disulfide bridges [17,20,31], presence of a reducing agent prevents disulfide bridge-mediated aggregation of proteins and their subsequent precipitation from solution [19]. The beneficial effect of DTT on AR suggests that maintenance of a reducing environment during cell lysis is critical in facilitating water-soluble antigenic protein solubility and removal into aqueous AR solutions.

The effect of altering KCl concentration on AR enhancement was assessed for solutions containing the previously identified optimal concentration.

Fig. 7. Quantitative biochemical analysis of BP composition. Water, collagen, elastin, and GAG content per DW in BP. Water (A) and collagen (B) content per DW of BP-AR was not significantly different following AR. Elastin content per DW was significantly decreased in BP-AR with 0.1% (w/v) SDS treatment (C). GAG content per DW was significantly decreased in BP-AR following treatment with opt SARB and/or 134 mM NDSB-256 (D). Results plotted as mean ± standard deviation. Groups not connected by same letter are significantly different, p < 0.05 (n = 6 per group).
DTT concentration (100 mM). The addition of 100 mM KCl (opt SARB) significantly reduced residual antigenicity of BP-AR compared to 10 mM KCl (SARB-2) with no additive or 134 mM NDSB-256. Salt concentration is known to affect protein solubility in aqueous solutions, with protein solubility initially increasing as salt is added to a solution (salting in) and decreasing as additional salt is added (salting out) [21]. By incorporating a physiologically relevant concentration of salt [32] in opt SARB (100 mM KCl), most biological proteins are maintained between the points of salting in and salting out, mitigating the aggregation of protein molecules via protein–protein interactions [21], permitting maintenance of protein

![Fig. 8. Hematoxylin and eosin staining of BP. Representative images of H&E-stained BP sections showing preserved gross tissue morphology and presence of residual nuclei with all AR treatments. Scale bar represents 20 μm.](image)

![Fig. 9. Residual nuclei per high-powered field in BP. Following AR, SDS/BARB and SDS/ opt SARB most significantly reduced nuclei compared to native BP. Results plotted as mean ± standard deviation. Groups not connected by same letter are significantly different, p < 0.05 (n = 6 per group).](image)

![Fig. 10. Residual nuclei per high-powered field vs. overall residual antigenicity. Residual nuclei counts per high powered field do not correlate to the overall residual antigenicity of BP-AR (p = 0.239, R² = 0.3234). Nuclei counts represent the average value from 6 replicates per AR treatment group (6 HPFs per replicate). Residual antigenicity was calculated from n ≥ 6 replicates per AR treatment group.](image)
molecules in solution for subsequent removal from BP. Taken together, the results for the addition of both DTT and KCl to the AR buffer support the hypothesis that maintaining solubility of water-soluble antigens is critical to achieving efficient AR from intact BP. Remarkably, differences in AR efficiency by BARB, NDSB/BARB, and SDS/BARB were mitigated by addition of 100 mM DTT, 2 mM MgCl$_2$, and 100 mM KCl, suggesting that factors promoting solubility were more influential than AR additives in reducing the level of residual antigenicity of BP-AR.

A successful AR process must maintain biomaterial mechanical properties in a range appropriate for physiological function. Closed heart valves experience planar tension as a result of blood flow reversal [33]. The organization of ECM components confers directionality to heart valves important for their function. Circumferentially-oriented collagen fibers provide strength to the heart valve and the ability to accommodate the load imparted by blood flow during the cardiac cycle [34]. Radially-aligned elastin molecules modulate the radial strain during valve opening and closing [35]. In the present study, Young’s modulus, ultimate tensile stress, and ultimate tensile strain of BP-AR were not significantly different from native BP control (for both circumferential and longitudinal axes), suggesting that AR using opt SARB does not compromise BP tensile properties. Moreover, the values measured for BP-AR generated using opt SARB are comparable to or exceed those of cadaveric human aortic and pulmonary valves along the circumferential and radial axes determined by Stradins et al. [36]. Since tensile properties of BP-AR approximate those of native human heart valve, we reason that BP-AR generated using opt SARB will be capable of withstanding the tensile forces experienced by heart valves. Tension represents one important type of loading experienced by heart valves. Future studies using cyclical loading, stress relaxation, and biaxial tensile testing will better inform how BP-AR will perform under in vivo conditions.

To ensure the structure–function relationship was preserved, ECM composition of BP-AR was assessed. Following AR with opt SARB, collagen content per DW of BP-AR was not observed to be significantly different from that in native BP. This is in agreement with the lack of significant difference in ultimate tensile stress between native BP and opt SARB-treated BP-AR, and further supports the promise of generating BP-AR using opt SARB without compromising the functional properties of native BP. Additionally, AR using opt SARB did not significantly alter the amount of elastin in BP. However, the elastin content per DW of BP-AR was significantly decreased in the presence of 0.1% (w/v) SDS during AR. As elastin is necessary for the large deformations heart valves undergo during the cardiac cycle, this result represents a potential detrimental effect of protocols utilizing 0.1% (w/v) SDS for AR. It should be noted, however, that differences in elastin content per DW did not appear to translate to differences in ultimate tensile strain of BP-AR following 0.1% (w/v) SDS treatment compared to native BP. Antigen removal with opt SARB resulted in a significant decrease in GAG content per DW compared to that in native BP. Despite this

![Fig. 11. Verhoeff van Gieson staining of BP. Representative images of VVG-stained BP sections showing gross collagen and elastin structure was preserved following AR. Scale bar represents 20 μm.](image-url)
observed reduction in GAGs, significant differences in water content were not observed between native BP and BP-AR, likely due to the fact that GAGs are not a major contributor to the DW of native BP (0.55 ± 0.04% per DW). The GAG content per DW of BP-AR could not be determined for 0.1% (w/v) SDS treated samples due to assay incompatibility with residual SDS in BP-AR extracts (data not shown). Overall, AR methods involving opt SARB do not drastically compromise the collagen, elastin, or GAG content per DW of BP-AR.

Previous studies have utilized residual nuclei counts as the primary outcome measure for success of decellularization [8,9,29,37–39]. In the current study, cell nuclei were uniformly distributed throughout sections of native BP. No significant decrease in number of nuclei was noted following AR using BARB. Although treatment with opt SARB and/or 134 mm NDSB-256 significantly reduced nuclei on BP-AR compared to native BP, treatment with 0.1% (w/v) SDS significantly reduced residual nuclei further. Following the reasoning used in decellularization studies, these results suggest that AR buffers containing 0.1% (w/v) SDS are the most effective at producing a non-immunogenic biological material, regardless of the presence or absence of a reducing agent and salt. However, this conclusion is in stark contrast to the findings from the assessment of residual antigenicity for BP-AR which utilized a more specific, antibody-based assay. The IgG-based assessment employed in this study provides a rigorous determinant of biomaterial antigenicity as it probes for residual antigens using specific antibody—antigen interactions that elicit the immune response in vivo. Moreover, since B-cell isotype switching to IgG production requires T-helper cell cosimulation [40], limiting assessment to IgG positivity indicates the residual antigens assessed in this study are capable of inducing both a humoral and cell-mediated immune response. The absence of a significant correlation between residual nuclei counts and the overall residual antigenicity suggests that the presence or absence of residual cell nuclei may not be an appropriate indicator of biomaterial antigenicity. Following decellularization of rat aortic valve leaflets by hypotonic lysis or Triton X-100, Meyer et al. reported the presence of residual major histocompatibility complex molecules inspire of apparent tissue acellularity on H&E-stained sections [41]. Kasimir et al. observed persistent galactosyl α-1,3-galactose (α-gal) xen-antigens and cellular debris in commercially-available SynerGraft decellularized porcine grafts [16] despite being branded as an acellular graft [37]. The present study confirms that assessment of cellularity under light microscopy as an indication of reduction in biomaterial antigenicity is prone to both underestimation (e.g., opt SARB and NDSB/opt SARB) and overestimation (e.g., SDS/BARB) of AR efficiency. It is becoming increasingly clear that a nuclei-based assessment of a xenogeneic scaffold may be an insufficient measure of its residual antigenicity. Overestimation of AR efficiency by assessment of residual nuclei counts is concerning as high levels of residual antigenicity in an apparently acellular scaffold may be capable of eliciting an immune response following in vivo implantation. Underestimation of AR efficiency by assessment of residual nuclei counts also raises important questions regarding the effect of residual nuclei on the immunological potential of the scaffold following in vivo implantation. Future in vivo implantation studies are therefore required to elucidate how xenogeneic scaffold residual antigenicity and residual nuclei counts translate to the immunological potential of the biomaterial.

5. Conclusions

This present study indicates that AR buffers promoting protein solubility (opt SARB) significantly reduce residual antigens in BP-AR compared to previously reported decellularization and/or AR buffers that do not maintain protein solubility. By accounting for the solubility of antigenic proteins to be removed, the total residual antigenicity of BP-AR can be reduced beyond that attained by common decellularization protocols. Factors promoting solubility (i.e., reducing agent and salt) have a dominant effect on AR efficiency beyond that achieved by the presence of a particular additive alone. Antigen removal using opt SARB permitted maintenance of BP-AR tensile properties similar to those of native BP. Moreover, the collagen and elastin content per DW are unchanged in BP-AR generated using opt SARB. However, AR with 0.1% (w/v) SDS significantly decreased elastin content per DW. Furthermore, the primary endpoint of decellularization methods (absence of cells under light microscopy) may not be an appropriate measure for the removal of antigenic proteins from xenogeneic materials. Taken together, these results demonstrate that by promoting protein solubilization, AR methods utilizing opt SARB (1) yield BP-AR with significantly reduced residual antigenicity compared to current methods of decellularization and (2) do not compromise the functional properties of the biomaterial. Adaptation of the presented AR strategy to other tissues of the body has potential for the generation of xenogeneic scaffolds with an immunogenic potential significantly lower than that achieved by current decellularization methods.

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