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Stepwise solubilization-based antigen removal for xenogeneic scaffold generation in tissue engineering

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ABSTRACT

The ability of residual antigens on decellularized tissue to elicit the immune response upon implantation motivates development of a more rigorous antigen removal (AR) process for xenogeneic scaffold generation. Antigen removal strategies promoting solubilization of hydrophilic proteins (predominantly cytoplasmic) enhance the reduction of hydrophilic antigenicity in bovine pericardium (BP); however, the diversity of protein antigens within a tissue necessitates development of AR strategies capable of addressing a spectrum of protein antigen solubilities. In the present study, methods for promoting solubilization of lipophilic proteins (predominantly membrane) were investigated for their ability to reduce lipophilic antigenicity of BP when applied as a second AR step following our previously described hydrophilic AR method. Bovine pericardium following AR (BP-AR) was assessed for residual hydrophilic and lipophilic antigenicity, removal of known lipophilic xenoantigens, tensile properties, and extracellular matrix structure and composition. Facilitating hydrophile solubilization (using dithiothreitol and potassium chloride) followed by lipophile solubilization (using amidosulfobetaine-14 (ASB-14)), in a two-step sequential, differential AR strategy, significantly reduces residual hydrophilic and lipophilic antigenicity of BP-AR beyond that achieved with either one-step hydrophilic AR or decellularization using 1% (w/v) sodium dodecyl sulfate. Moreover, use of 1% (w/v) ASB-14 for lipophilic AR eliminates the two most critical known barriers to xenotransplantation (galactose-α(1,3)-galactose and major histocompatibility complex I)) from BP-AR without compromising the structure–function properties of the biomaterial. This study demonstrates the importance of a sequential, differential protein solubilization approach to reduce biomaterial antigenicity in the production of a xenogeneic scaffold for heart valve tissue engineering.

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1. Introduction

The ability of xenoantigens to elicit an immune response represents the critical barrier in scaffold generation from xenogeneic tissues for tissue engineering and regenerative medicine applications [1]. The decellularization paradigm attributes xenograft antigenicity to the cellular component of a tissue and utilizes histological acellularity as the primary indicator of satisfactory reduction in biomaterial xenoantigenicity. Implantation of decellularized porcine valve tissue into sheep, rats and dogs showed a minimal immunogenic response for up to 1 year [2,3], encouraging confidence in decellularization methods. However, rapid failure of SynerGraft decellularized porcine heart valves in juvenile patients, attributed to inadequate xenoantigen removal by decellularization [4], highlights flaws in the fundamental principles of the decellularization approach. Identification of extracellular matrix (ECM)-associated xenoantigens within bovine pericardium (BP) [5] invalidates the assumption that antigens are purely cellular in origin. Moreover, histological acellularity did not equate to removal of known xenoantigens from BP [6] or murine aortic valves [7]. Finally, in our previously published work, no correlation between residual nuclei counts and residual hydrophilic antigenicity could be identified following removal of hydrophilic antigens from BP [8]. Taken together, these studies demonstrate that the fundamental assumptions of the decellularization paradigm are flawed; decellularization is inadequate as both a method and a metric for reduction of biomaterial antigenicity. Thus, recent efforts in xenogeneic scaffold generation have shifted towards achieving antigen removal (AR) and assessing residual biomaterial antigenicity.

A critical error in previous decellularization approaches was focusing merely on cell disruption [9,10], disregarding the need for antigenic molecules to be solubilized for their effective removal from xenogeneic tissue. We have demonstrated previously that solubilization of hydrophiles (predominantly cytoplasmic components) significantly enhances reduction in residual hydrophilic antigenicity of BP following AR (BP-AR) [8]. Our solubilization-based AR approach reduced residual hydrophilic antigens by an

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additional 80% compared to hypotonic solution and 60% compared to 0.1% (w/v) sodium dodecyl sulfate (SDS) decellularization methods while maintaining biomaterial structure–function properties [8]. However, the diversity in amino acid composition of tissue proteins suggests that no single solution can simultaneously solubilize all antigenic proteins in a given tissue for removal [11–13]. In promoting the solubilization of only hydrophiles, lipophilic antigens (predominantly membrane-associated components) likely persist within the tissue. Thus, a sequential, differential protein solubilization approach designed to facilitate removal of hydrophilic antigens, followed by removal of lipophilic antigens in a stepwise manner, may reduce the overall residual antigenicity of BP-AR.

Differential protein solubility has frequently been exploited for the serial extraction of protein fractions from cell lysate [14–18] or homogenized tissue [5,17,19–21] for downstream proteomic analysis. Since proteins can only be extracted into solutions in which they are soluble [15,17], application of a series of solutions promoting protein solubilization along a spectrum of solubilities is critical for sequential, differential extraction protocols. Traditionally, proteins are solubilized, and subsequently extracted, based on increasing difficulty of solubilization in aqueous solutions (i.e. hydrophile extraction followed by lipophile extraction) [14–19]. In spite of the effectiveness with which sequential, differential solubilization approaches facilitate protein extraction from homogenized tissues, the importance of adapting the same principle to AR from intact tissues during xenogeneic scaffold generation has not been investigated. This is a surprising oversight, given the complex composition of protein antigens within a tissue requiring removal prior to implementation in tissue engineering applications [5]. Therefore, we hypothesized that application of a series of solutions, each promoting the solubilization and subsequent removal of a different subset of proteins, will significantly enhance overall AR from BP. Furthermore, we hypothesized that such a sequential, differential AR strategy will maintain native biomaterial structure–function properties. In this study, several lipophile solubilization agents were applied as a second step of AR following initial hydrophile solubilization and assessed for their ability to reduce residual lipophilic antigenicity of the resultant BP-AR. The ability of the resulting two-step sequential, differential strategy to reduce the residual lipophilic antigenicity of BP-AR while maintaining native structure–function properties was compared to a one-step AR strategy (hydrophile solubilization) [8] and the literature gold standard for decellularization (1% (w/v) SDS) [9,10,22].

2. Materials and methods

2.1. Tissue harvest

All chemicals were from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. Fresh BP was harvested from adult cattle as previously described [8] ($n = 3$).

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* [23]. Anti-native BP serum was generated from New Zealand white rabbits ($n = 4$) as previously described [5,8,19]. 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 until used on Western blots (Section 2.4).

2.3. Protein extraction

Protein extraction from minced BP-AR was adapted from a method described previously [8,19]. All centrifugation steps were performed at 17,000*g*, 4 °C for 25 min. Briefly, minced BP-AR was incubated in standard extraction solution (10 mM Tris–HCl (pH 8.0) containing 1 mM dithiothreitol, 2 mM magnesium chloride hexahydrate, 10 mM potassium chloride and 0.5 mM Pefabloc SC (Roche, Indianapolis, IN)) containing 0.1% (w/v) SDS (Bio-Rad, Hercules, CA) at 1000 rpm, 4 °C for 1 h. Following centrifugation, recovered supernatant was defined as residual hydrophilic protein extract. The insoluble pellet was washed twice in standard extraction solution containing 0.1% (w/v) SDS at 1400 rpm, 4 °C for 30 min and then incubated in standard extraction solution containing 1% (w/v) SDS at 1400 rpm, 4 °C for 1 h. Following centrifugation, recovered supernatant was defined as residual lipophilic protein extract. All extracts were stored at −80 °C.

2.4. One-dimensional electrophoresis and Western blot

One-dimensional electrophoresis and Western blot was performed as previously described [8], using equal volumes of residual hydrophilic or lipophilic protein extract per group.

2.5. Antigen removal

Antigen removal was adapted from a method previously described [8]. All steps were performed in a 2 ml working volume at 4 °C and 125 rpm unless otherwise stated. Briefly, intact pieces of BP (0.2 g, approximately 1.0 cm × 1.5 cm) were subjected to hydrophile solubilization for 2 days as the first step of AR. This was followed by lipophile solubilization at room temperature for 2 days as the second step of AR. For each AR sample, an anatomically adjacent piece of BP subjected to AR for 1 min served as a negative AR control for biological tissue variability and effects of AR additives. Following nucleic acid digestion for 24 h and washout for 48 h, BP-AR was stored in Dulbecco's modified Eagle's medium with 15% (v/v) dimethyl sulfoxide at −80 °C. All AR experiments were conducted with $n = 6$ per group.

2.5.1. Effect of hydrophile solubilization (one-step AR)

Residual lipophilic antigenicity of BP-AR was assessed after hydrophile solubilization with either basic AR buffer (BARB; 10 mM Tris–HCl (pH 8.0) containing 0.5 mM Pefabloc and 1% (v/v) antibiotic antimycotic solution) or optimized solubilizing AR buffer (opt SARB; BARB containing 100 mM dithiothreitol, 2 mM magnesium chloride hexahydrate and 100 mM potassium chloride) containing: no additional additive, 134 mM 3-(benzyldimethylammonio) propanesulfonate (NDSB-256) or 0.1% (w/v) SDS.

2.5.2. Effect of sequential hydrophile and lipophile solubilization (two-step AR)

Both residual hydrophilic and lipophilic antigenicity of BP-AR were assessed after two-step AR (Fig. 1). Pieces of BP underwent hydrophile solubilization with opt SARB, followed by lipophile solubilization in opt SARB containing: no additional additive; 134 mM NDSB-256 and 1% (w/v) n-dodecyl-β,D-maltoside (Griffiths solution) [5,19]; 8 M urea (Bio-Rad), 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2% (w/v) N-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB 3-10) and 1% (w/v) 3-[N,N-Dimethyl(3-myristoylaminopropyl)ammonio]propanesulfonate (ASB-14) (Cordwell solution) [17]; or 10% (v/v) isopropanol (Thermo Fisher Scientific) and 5% (v/v) glycerol (Leimgruber solution) [24]. These samples were compared to a literature control of BARB containing 0.1% (w/v) SDS for

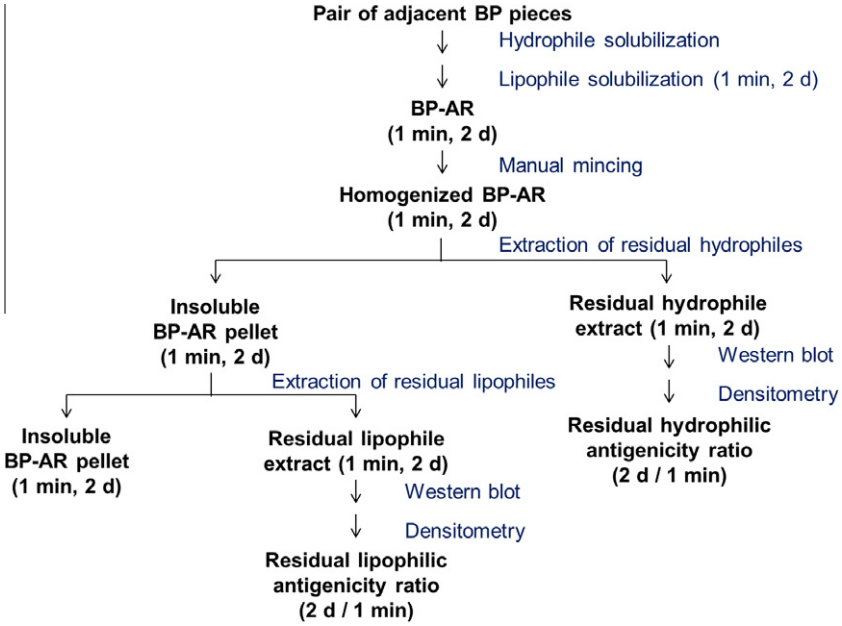

Fig. 1. Schematic of AR and assessment of residual antigenicity. Residual hydrophiles and lipophiles extracted from BP-AR were subjected to Western blot and probed with rabbit serum generated against native bovine. Residual hydrophilic and lipophilic antigenicity ratios were defined as the intensity of banding following 2 days of AR divided by 1 min of AR.

hydrophile solubilization, followed by 1% (w/v) SDS for lipophile solubilization [19].

2.5.3. Effect of Cordwell solution components

Residual lipophilic antigenicity of BP-AR was assessed after hydrophile solubilization with opt SARB, followed by lipophile solubilization in opt SARB containing: no additional additive; 8 M urea and 2 M thiourea; 2% (w/v) CHAPS; 2% (w/v) SB 3-10; 1% (w/v) ASB-14; or the entire Cordwell solution during the second step of AR.

2.6. Assessment of residual antigenicity following AR

Assessment of residual BP-AR antigenicity was performed using a method previously validated [5] and described [8] on residual hydrophilic or lipophilic protein extracts (Fig. 1). Briefly, residual hydrophilic or lipophilic protein extracts ($n = 6$ per group) were subjected to electrophoresis and Western blot, probed with anti-native BP serum and assessed for IgG positivity, with band intensity quantified by densitometry. Residual antigenicity of BP-AR was defined as the ratio of the banding intensity from 2 days of AR to that of the 1 min AR control. Residual antigenicity ratios for each AR treatment were then normalized to the negative AR control within each experiment (BARB for one-step AR or opt SARB alone for two-step AR).

2.7. Uniaxial tensile testing

Tensile properties of BP were determined as previously described [8]. Strips of BP (15×3 mm) were cut from separate 0.2 g pieces of native BP and BP-AR ($n = 6$ per group) and made into dogbone shapes. Samples from adjacent anatomical locations were used for each replicate of AR treatment and control tissue. BP mounted under zero strain was subjected to a constant strain rate of 0.1 mm s^{-1}. The initial gauge length was set at 2 mm. The initial gauge width and thickness were determined from BP images using ImageJ 1.42q software (Wayne Rasband, National Institutes of Health, USA). For each sample, a stress–strain curve was generated from the load–elongation curve, and the Young's modulus and ultimate tensile stress (UTS) were determined.

2.8. Quantitative biochemistry

The ECM composition of native BP and BP-AR was determined as previously described [8] from two 5 mm disks (taken from the initial 0.2 g piece, $n = 6$ per group). One disk was subjected to papain digestion for quantification of collagen content per dry weight (DW) using a modified colorimetric hydroxyproline assay [25] and sulfated glycosaminoglycan (GAG) content per DW using the Blyscan sulfated GAG assay (Biocolor Ltd., Carrickfergus, UK). The second disk was subjected to hot oxalic acid extraction for quantification of elastin content per DW using the Fastin elastin assay (Biocolor Ltd.).

2.9. Histology

Histological assessment of native BP and BP-AR was performed as previously described [8] from two 1 mm wide strips (taken from the initial 0.2 g piece, $n = 6$ per group). Formalin-fixed, paraffin-embedded sections underwent Verhoeff van Gieson staining (VVG) for assessment of gross collagen and elastin organization and hematoxylin and eosin (H&E) staining. Assessment of ECM fiber morphology was performed for all six replicates per AR treatment throughout the full thickness of the tissue (from the parietal surface to the mediastinal surface of the pericardium). Quantification of residual nuclei was performed on six randomized high-powered fields (HPFs) per slide. Correlation between nuclei counts and residual lipophilic antigenicity was determined by plotting average nuclei counts against residual lipophilic antigenicity for each AR method.

2.10. Immunohistochemistry

Paraffin-embedded sections of native BP and BP-AR samples ($n = 6$ per group) were deparaffinized in xylene (Fisher Scientific, Pittsburgh, PA) and rehydrated through an ethanol gradient.

Endogenous peroxidase was quenched through incubation in peroxidase block (Dako, Carpinteria, CA, USA) for 5 min, followed by antigen retrieval using proteinase K (Dako) for 10 min. Slides were blocked in 5% normal goat serum (NGS) (Jackson ImmunoResearch Laboratories, Inc.) for 30 min and then incubated in primary antibody for 60 min. Following incubation in EnVision+ anti-mouse HRP-labelled polymer (Dako) for 30 min, bound primary antibodies were detected using EnVision+ DAB+ chromogen (Dako) for 10 min. Finally, slides were counterstained with Mayer's hematoxylin for 5 min, dehydrated through an ethanol gradient, cleared in xylene, mounted in Permount (Fisher Scientific) and coverslipped. The two primary antibodies used were anti-galactose-α(1,3)-galactose (α-gal) clone M86 (Enzo Life Sciences, Plymouth Meeting, PA) at a 1:5 dilution in 5% NGS and anti-major histocompatibility complex I (MHC I) heavy chain (Abcam, Cambridge, MA) at a 1:250 dilution in 5% NGS. Sections from all six replicates per AR treatment were assessed for the presence of α-gal and MHC I antigens throughout the full thickness of the tissue (from the parietal surface to the mediastinal surface of the pericardium).

2.11. Statistical analysis

Normalized residual antigenicity ratios were compared between experimental AR groups and the negative solubilization control (BARB for one-step AR and opt SARB alone for two-step AR). Values determined from tensile testing, biochemical assays and histology were compared to those for control tissues (native BP). Non-repeated measures analysis of variance and Tukey–Kramer HSD post hoc analysis were performed on sample means. Correlation was determined using bivariate fit analysis. All data are presented as mean ± standard deviation from the mean. Statistical significance was defined at $p < 0.05$.

3. Results

3.1. Antigen removal

3.1.1. Effect of hydrophile solubilization (one-step AR)

Promotion of hydrophile solubilization with opt SARB did not significantly change the residual lipophilic antigenicity of BP-AR compared to BARB for any of the additives assessed: no additional additive (0.86 ± 0.14 vs. 1.00 ± 0.51), 134 mM NDSB-256 (0.51 ± 0.12 vs. 0.58 ± 0.24) or 0.1% (w/v) SDS (0.35 ± 0.12 vs. 0.42 ± 0.03) (Fig. 2).

3.1.2. Effect of sequential hydrophile and lipophile solubilization (two-step AR)

Promotion of lipophile solubilization during a second step of AR with Griffiths, Cordwell or Leimgruber solution did not significantly change the residual hydrophilic antigenicity of BP-AR compared to opt SARB alone (0.79 ± 0.28, 0.13 ± 0.11, 0.72 ± 0.27, 1.00 ± 0.15, respectively) (Fig. 3A). However, use of opt SARB during the first of two AR steps reduced residual hydrophilic antigenicity significantly – by 75% – compared to that remaining following two-step AR with 0.1 and 1% (w/v) SDS in BARB (4.03 ± 2.27, $p < 0.0005$).

Promotion of lipophile solubilization during a second step of AR significantly decreased residual lipophilic antigenicity of BP-AR with respect to that treated with opt SARB alone (1.00 ± 0.22) (Fig. 3B). Compared to the use of opt SARB alone, the per cent reduction of residual lipophile antigenicity achieved was 44% with the Griffiths solution (0.66 ± 0.14, $p < 0.05$), 91% with the Cordwell solution (0.09 ± 0.04, $p < 0.0001$) and 33% with the Leimgruber solution (0.67 ± 0.11, $p < 0.05$). Use of 1% (w/v) SDS in BARB resulted in a 37% decrease in residual lipophilic antigenicity (0.63 ± 0.15, $p < 0.01$) compared to opt SARB alone. Furthermore, use of the Cordwell solution in opt SARB during the second step of AR significantly reduced residual lipophilic antigenicity by 54% compared to with 1% (w/v) SDS in BARB ($p < 0.0001$).

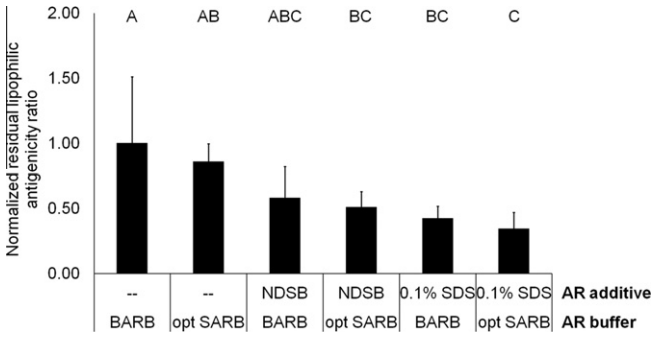

Fig. 2. Residual lipophilic antigenicity of bovine pericardium following one-step AR. Residual lipophilic antigenicity is not significantly decreased with hydrophile solubilization (opt SARB vs. BARB) containing no additional additive, 134 mM 3-(benzyldimethylammonio) propanesulfonate (NDSB-256) or 0.1% (w/v) SDS. Results are plotted as mean ± standard deviation. Groups not connected by the same letter are significantly different, $p < 0.05$ ($n = 6$ per group).

3.1.3. Effect of Cordwell solution components

Promotion of lipophile solubilization during a second step of AR in opt SARB reduced the residual lipophilic antigenicity of BP-AR compared to that treated with no additional additive (1.00 ± 0.19, $p < 0.0001$) significantly – by 85% with 8 M urea and 2 M thiourea (0.15 ± 0.11), 60% with 1% (w/v) ASB-14 (0.40 ± 0.12) and 94% with the entire Cordwell solution (0.06 ± 0.02) (Fig. 4A). However, treatment with 1% (w/v) ASB-14 in opt SARB did not reduce the residual lipophilic antigenicity of BP-AR to the level achieved with the entire Cordwell solution ($p < 0.05$). The residual lipophilic antigenicity of BP-AR treated with opt SARB containing 8 M urea and 2 M thiourea was not significantly different from that achieved using either 1% (w/v) ASB-14 or the entire Cordwell solution.

3.2. Gross tissue morphology

3.2.1. Effect of hydrophile solubilization (one-step AR)

No change in BP-AR thickness was observed following AR with opt SARB (containing no additional additive, 134 mM NDSB-256, or 0.1% (w/v) SDS) compared to BP-AR generated using BARB, 1 min AR controls or native BP (data not shown).

3.2.2. Effect of sequential hydrophile and lipophile solubilization (two-step AR)

Treatment with either 1% (w/v) SDS in BARB or the Cordwell solution during the second step of AR resulted in gross morphological thickening of BP-AR compared to that generated using opt SARB alone (Fig. 3C). Moreover, BP-AR subjected to the Cordwell solution curled upward, rather than remaining flat. No significant change in BP-AR thickness was observed following treatment with the Griffiths or Leimgruber solution.

3.2.3. Effect of Cordwell solution components

Treatment with either 8 M urea and 2 M thiourea in opt SARB or the entire Cordwell solution during the second step of AR resulted in gross morphological thickening and curling of BP-AR compared to that generated using opt SARB alone (Fig. 4B). No significant change in BP-AR thickness was observed following treatment with opt SARB containing 2% (w/v) CHAPS, 2% (w/v) SB 3-10 or 1% (w/v) ASB-14.

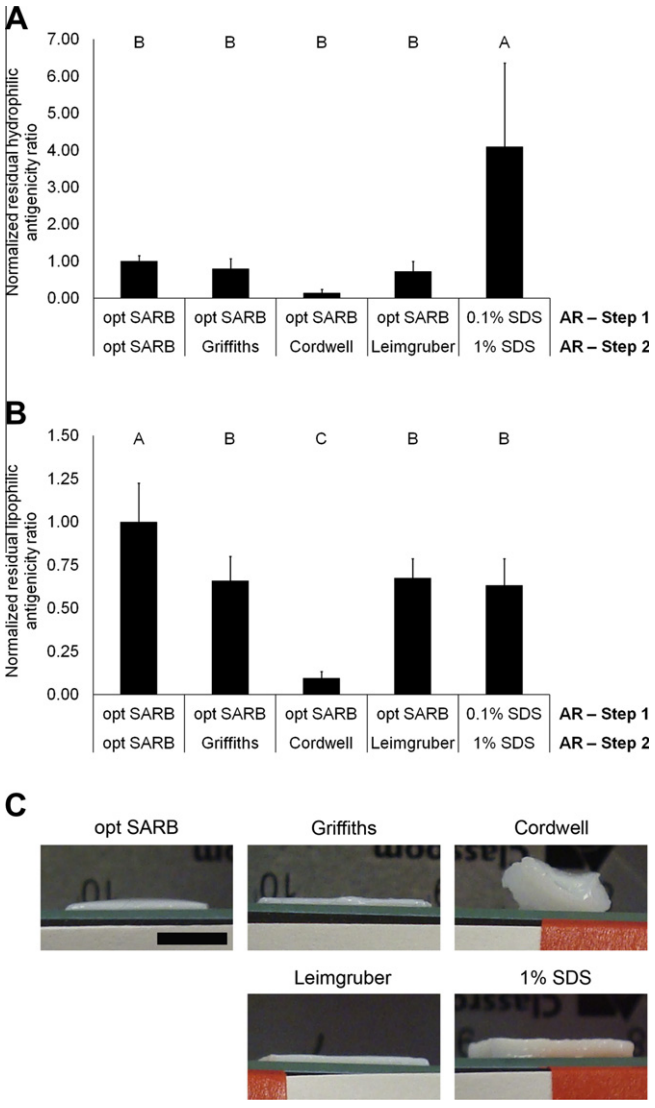

Fig. 3. Residual hydrophilic and lipophilic antigenicity and gross morphology of bovine pericardium following two-step AR. Hydrophilic antigenicity is not decreased further following addition of a lipophile solubilization step (A). Lipophilic antigenicity is significantly decreased following addition of lipophile solubilization (B). Results are plotted as mean ± standard deviation. Groups not connected by the same letter are significantly different, $p < 0.05$ ($n = 6$ per group). Use of the Cordwell solution in opt SARB or 1% (w/v) SDS in BARB dramatically alters gross tissue morphology (C). The scale bar represents 1 cm.

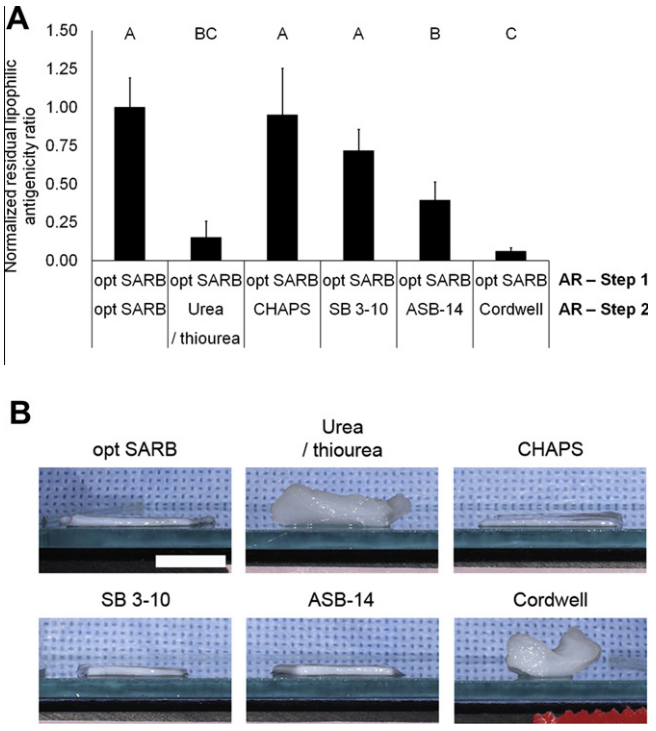

Fig. 4. Residual lipophilic antigenicity and gross morphology of bovine pericardium following two-step AR with either the entire Cordwell solution or its individual components. Use of opt SARB containing 8 M urea and 2 M thiourea, 1% (w/v) ASB-14 or the entire Cordwell solution significantly reduces residual lipophilic antigenicity compared to opt SARB alone (A). Results are plotted as mean ± standard deviation. Groups not connected by the same letter are significantly different, $p < 0.05$ ($n = 6$ per group). Treatment with 8 M urea and 2 M thiourea in opt SARB or the entire Cordwell solution drastically changes gross tissue morphology (B). The scale bar represents 1 cm.

3.3. Uniaxial tensile testing

No significant differences in Young's modulus and UTS were observed between native BP (14.25 ± 6.87 and 8.25 ± 2.64 MPa, respectively) and BP-AR generated with opt SARB alone (16.43 ± 7.01 and 9.86 ± 2.14 MPa, respectively), 1% (w/v) ASB-14 in opt SARB (11.36 ± 3.94 and 7.52 ± 2.22 MPa, respectively) or 1% (w/v) SDS in BARB (8.87 ± 4.23 and 5.48 ± 2.26 MPa, respectively) (Fig. 5). Use of 8 M urea and 2 M thiourea in opt SARB significantly decreased the Young's modulus and UTS (1.35 ± 0.55 and 1.70 ± 0.68 MPa, respectively) of BP-AR compared to native BP.

3.4. Quantitative biochemistry

The water content of BP-AR generated using opt SARB containing no additional additive (74.36 ± 3.67%) or 1% (w/v) ASB-14 (78.74 ± 1.39%) during the second step of AR was not significantly different from that of native BP (74.73 ± 2.42%) (Fig. 6A). However, use of 8 M urea and 2 M thiourea in opt SARB (83.43 ± 0.59) or 1% (w/v) SDS in BARB (82.61 ± 2.58%) significantly increased the water content of BP-AR compared to that of native BP ($p < 0.0001$).

The collagen content of BP-AR was not significantly different than that of native BP (34.98 ± 14.75% per DW) following any of the tested lipophile solubilization treatments: opt SARB alone (48.10 ± 21.07% per dry weight (DW)), 1% (w/v) ASB-14 in opt SARB (55.40 ± 28.60% per DW), 8 M urea and 2 M thiourea in opt SARB (31.56 ± 3.99% per DW) or 1% (w/v) SDS in BARB (28.75 ± 9.73% per DW) (Fig. 6B).

The elastin content of BP-AR generated using opt SARB containing no additional additive (2.58 ± 1.04% per DW) or 1% (w/v) ASB-14 (2.16 ± 1.02% per DW) during the second step of AR was not significantly different from that of native BP (3.09 ± 0.56% per DW) (Fig. 6C). However, use of 8 M urea and 2 M thiourea in opt SARB (0.70 ± 0.27% per DW) significantly decreased the elastin content of BP-AR compared to that of native BP ($p < 0.0005$). The elastin content of BP-AR generated using 1% (w/v) SDS in BARB was also significantly reduced, to a level below the limit of detection of the assay ($p < 0.0001$).

The GAG content of BP-AR was significantly different from that of native BP (0.75 ± 0.05% per DW) following treatment with opt SARB containing: no additional additive (0.52 ± 0.09% per DW), 1% (w/v) ASB-14 (0.25 ± 0.10% per DW) or 8 M urea and 2 M thiourea (0.44 ± 0.08% per DW) during the second step of AR ($p < 0.0005$) (Fig. 6D). The presence of residual SDS in BP-AR subjected to 1% (w/v) SDS in BARB during the second step of AR interfered with the Blyscan assay (data not shown).

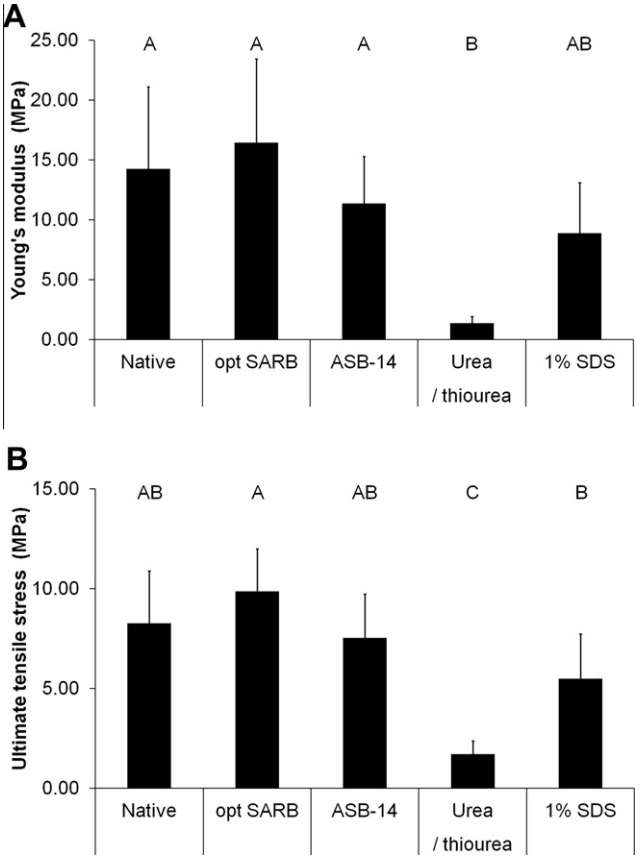

Fig. 5. Tensile properties of BP. Young's modulus (A) and UTS (B) of BP following two-step AR using no additive or 1% (w/v) ASB-14 in opt SARB, or 1% (w/v) SDS in basic AR buffer are not significantly different from those of native BP. A second step of AR using 8 M urea and 2 M thiourea in opt SARB results in a significant decrease in Young's modulus and UTS. Results are plotted as mean ± standard deviation. Groups not connected by the same letter are significantly different, $p < 0.05$ ($n = 6$ per group).

3.5. Histology

Qualitatively, no differences in collagen and elastin content and organization were observed between VVG-stained sections of native BP and BP-AR following a second step of AR in opt SARB containing no additional additive or 1% (w/v) ASB-14 (Fig. 7). However, BP-AR generated with 8 M urea and 2 M urea in opt SARB or 1% (w/v) SDS in BARB exhibited a marked loss of collagen fiber organization and elastin content. Minor differences in staining intensity were attributed to processing artifacts and not a change in collagen and elastin organization.

In H&E-stained BP sections, ECM morphology was grossly maintained following a second step of AR using no additive or 1% (w/v) ASB-14 in opt SARB compared to native BP (Fig. 7). However, treatment with 8 M urea and 2 M thiourea in opt SARB or 1% (w/v) SDS in BARB resulted in marked disruption of native ECM morphology.

All two-step AR treatments significantly reduced the number of nuclei per HPF compared to native BP (107.42 ± 21.95) ($p < 0.0001$) (Fig. 8). Use of opt SARB containing no additional additive (14.81 ± 23.44) or 1% (w/v) ASB-14 (3.81 ± 6.47), or 1% (w/v) SDS in BARB (0.75 ± 1.11), significantly reduced residual nuclei per HPF in BP-AR compared to 8 M urea and 2 M thiourea in opt SARB (67.56 ± 27.27) ($p < 0.0001$). Moreover, two-step AR using 1% (w/v) ASB-14 in opt SARB reduced residual nuclei per HPF to a similar degree to 1% (w/v) SDS in BARB. No statistically significant correlation was observed between residual nuclei counts per HPF and the normalized residual lipophilic antigenicity ratio of BP-AR following two-step AR ($p = 0.2740$, $R^2 = 0.5270$).

3.6. Immunohistochemistry

Immunohistochemical staining revealed the presence of α-gal and MHC I antigens in native BP and BP-AR using opt SARB alone (Fig. 7). Low levels of α-gal and MHC I antigens localized to vascular structures were observed in BP-AR generated with 8 M urea and 2 M thiourea in opt SARB. Although no α-gal antigens were detected in BP-AR using 1% (w/v) SDS in BARB, a low level of MHC I antigens was observed. No detectable α-gal and MHC I antigens were found in BP-AR treated with 1% (w/v) ASB-14 in opt SARB or the negative primary antibody controls.

4. Discussion

There were four objectives to this study: (i) to determine if solubilization of one protein subset affects the residual antigenicity of a second protein subset (i.e. is residual lipophilic antigenicity reduced with hydrophile solubilization, or is residual hydrophilic antigenicity reduced with lipophile solubilization?); (ii) to determine whether a two-step sequential, differential protein solubilization AR strategy (hydrophile solubilization, followed by lipophile solubilization) reduces xenogeneic tissue antigenicity beyond that achieved by a one-step AR method (hydrophile solubilization alone) or the positive literature control (decellularization with 1% (w/v) SDS); (iii) to identify which of the tested lipophile solubilizing factors most effectively reduce residual lipophilic antigenicity of BP-AR in a two-step AR strategy; and (iv) to assess whether two-step sequential, differential solubilization-based AR methods adversely affect biomaterial structure–function properties, defined as uniaxial tensile properties and ECM structure and composition. We demonstrate that: (i) promotion of hydrophile or lipophile solubilization does not significantly alter residual lipophilic or hydrophilic antigenicity, respectively; (ii) promotion of hydrophile solubilization, followed by lipophile solubilization, in a two-step sequential, differential AR procedure enhances the removal of antigens from intact BP beyond that achieved using a one-step AR method; and (iii) 1% (w/v) ASB-14 enhances the removal of lipophilic antigens from BP, eliminating the two most critical known barriers to xenotransplantation (α-gal and MHC I), without compromising biomaterial structure–function properties.

Previously, we reported that hydrophile solubilization using a reducing agent and salt (opt SARB) enhances removal of hydrophilic antigens from BP [8]. In the current study, we demonstrate that hydrophile solubilization has no effect on the removal of lipophilic antigens from BP. Additionally, we show that application of lipophile solubilization as a second AR step has no effect in further reducing residual hydrophilic antigenicity of BP following one-step AR. Furthermore, the 75% reduction in residual hydrophilic antigenicity observed with opt SARB compared to 1% (w/v) SDS is comparable to our previously published results for residual hydrophilic antigenicity observed with opt SARB compared to 0.1% (w/v) SDS [8]. This suggests that the increase in concentration of SDS from 0.1% (w/v) to 1% (w/v) does not remove markedly more hydrophilic antigens. In sum, these findings are in agreement with the observation that protein extraction from homogenized tissue can only occur into a solution in which the particular protein subset of interest is soluble [17]. Therefore, removal of antigenic proteins from intact tissue is heavily dependent on the ability of the AR buffer to effectively solubilize the protein antigen subset(s) of interest.

Persistence of lipophilic antigens following one-step AR underscores the need for lipophile solubilization in a sequential AR strategy. Incorporation of lipophile solubilizing factors into a second AR step facilitates a significant reduction in the residual lipophilic antigenicity of BP-AR compared to hydrophile solubilization alone (opt SARB alone). Additionally, lipophile solubilization reduces

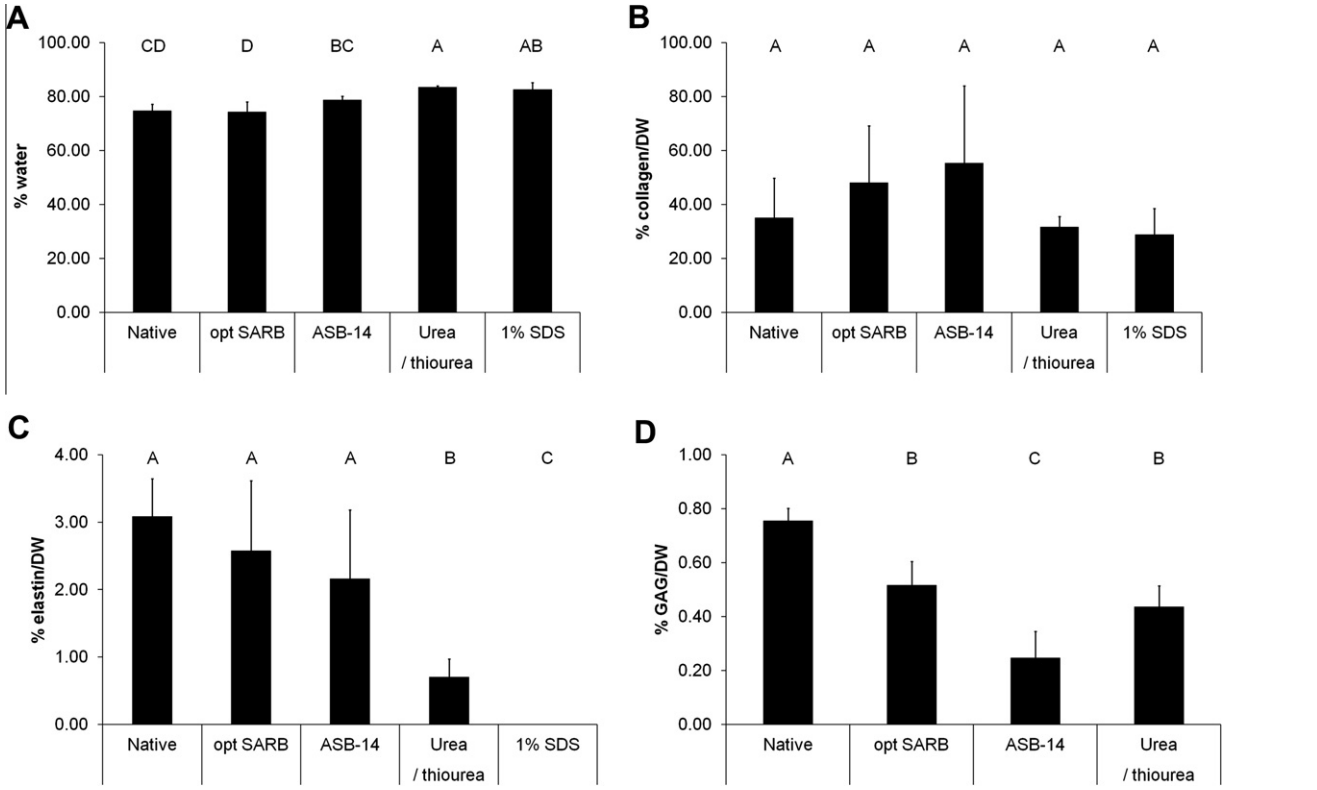

Fig. 6. Quantitative biochemical analysis of BP composition. Water content is maintained following two-step AR in opt SARB containing no additional additive or 1% (w/v) ASB-14 compared to native BP (A). A second step of AR using either 8 M urea and 2 M thiourea in opt SARB or 1% (w/v) SDS in BARB significantly increases water content. The collagen content per DW is not significantly different following two-step AR compared to native BP (B). The elastin content per DW is maintained following two-step AR using opt SARB containing no additional additive or 1% (w/v) ASB-14 compared to native BP (C). Use of 8 M urea and 2 M thiourea in opt SARB significantly decreases the elastin content per DW. The elastin content per DW for samples treated with 1% (w/v) SDS in BARB is below the limit of detection of the assay. GAG content per DW is significantly decreased following two-step AR compared to native BP (D). Results are plotted as mean ± standard deviation. Groups not connected by the same letter are significantly different, $p < 0.05$ ($n = 6$ per group).

residual lipophilic antigenicity of BP-AR to a degree comparable to (Griffiths [5,19] or Leimgruber [24] solutions) or beyond (Cordwell solution [17]) that achieved by the current most commonly used decellularization agent (1% (w/v) SDS) [9,10,22]. Furthermore, use of opt SARB for hydrophile solubilization significantly reduces residual hydrophilic antigenicity compared to 1% (w/v) SDS. Consequently, a two-step sequential, differential AR strategy using opt SARB, followed by lipophile solubilization is more effective than 1% (w/v) SDS at reducing both residual hydrophilic and lipophilic antigenicity of BP-AR. Further studies will be necessary to determine if the sequence in which solubilization is promoted has any effect on the residual antigenicity of BP-AR. These findings highlight the importance of a two-step sequential, differential AR approach, consisting of hydrophile solubilization followed by lipophile solubilization, for effective reduction of residual BP antigenicity compared to a one-step AR methodology or positive decellularization control.

After validating the need for lipophile solubilization, we sought the best candidate for use in two-step AR. Cordwell solution, the only tested two-step AR treatment to significantly reduce residual lipophilic antigenicity compared to the literature control (1% (w/v) SDS), reduces the lipophilic antigenicity of BP-AR impressively – by 91% – compared to opt SARB alone, but drastically alters the gross morphological appearance of BP-AR. Assessment of the individual components of Cordwell solution revealed that chaotropes (8 M urea and 2 M thiourea) reduce residual lipophilic antigenicity – by 85% – compared to opt SARB alone. However, chaotropes were also found to the responsible for the gross morphological disruption of BP-AR observed with the entire Cordwell solution. In contrast, 1% (w/v) ASB-14 reduces residual lipophilic BP-AR antigenicity – by 60% – compared to opt SARB alone, while avoiding the detrimental changes in gross morphology of BP-AR. Additionally, lipophile solubilization using 1% (w/v) ASB-14 eliminates detection of the two most critical known barriers to xenotransplantation (α-gal and MHC I) from resultant BP-AR. The α-gal epitope is a carbohydrate moiety present on glycolipids and glycoproteins within the cell membrane [26] and the principal determinant of hyperacute rejection in discordant xenotransplants [27]. The cell surface molecule MHC I is the most ubiquitously known stimulator of both innate and adaptive xenogeneic immune responses [27,28]. Thus, removal of these known cell membrane-associated xenoantigens is likely to be crucial in reducing recipient immune response to xenogeneic biomaterials. Conversely, persistence of α-gal and MHC I antigens in the absence of lipophile solubilization (opt SARB alone) suggests that BP-AR undergoing only one-step AR would likely be subject to a substantial immune response. Similarly, detectable α-gal and/or MHC I antigens in BP-AR generated with 8 M urea and 2 M thiourea or 1% (w/v) SDS suggest that the resultant scaffolds would be unlikely to avoid the immune response. At first glance, these immunohistochemical findings appear to run counter to the Western blot findings when, in fact, it is likely that they are complementary. The rabbit serum used to assess residual lipophilic antigenicity of BP-AR via Western blot comprises polyclonal antibodies to a broad range of antigens, representing the global lipophilic antigenicity of BP-AR. Immunohistochemical analysis of an individual known antigen using a monoclonal antibody represents a semi-quantitative assessment of residual antigenicity for a specific epitope. Since removal of indi-

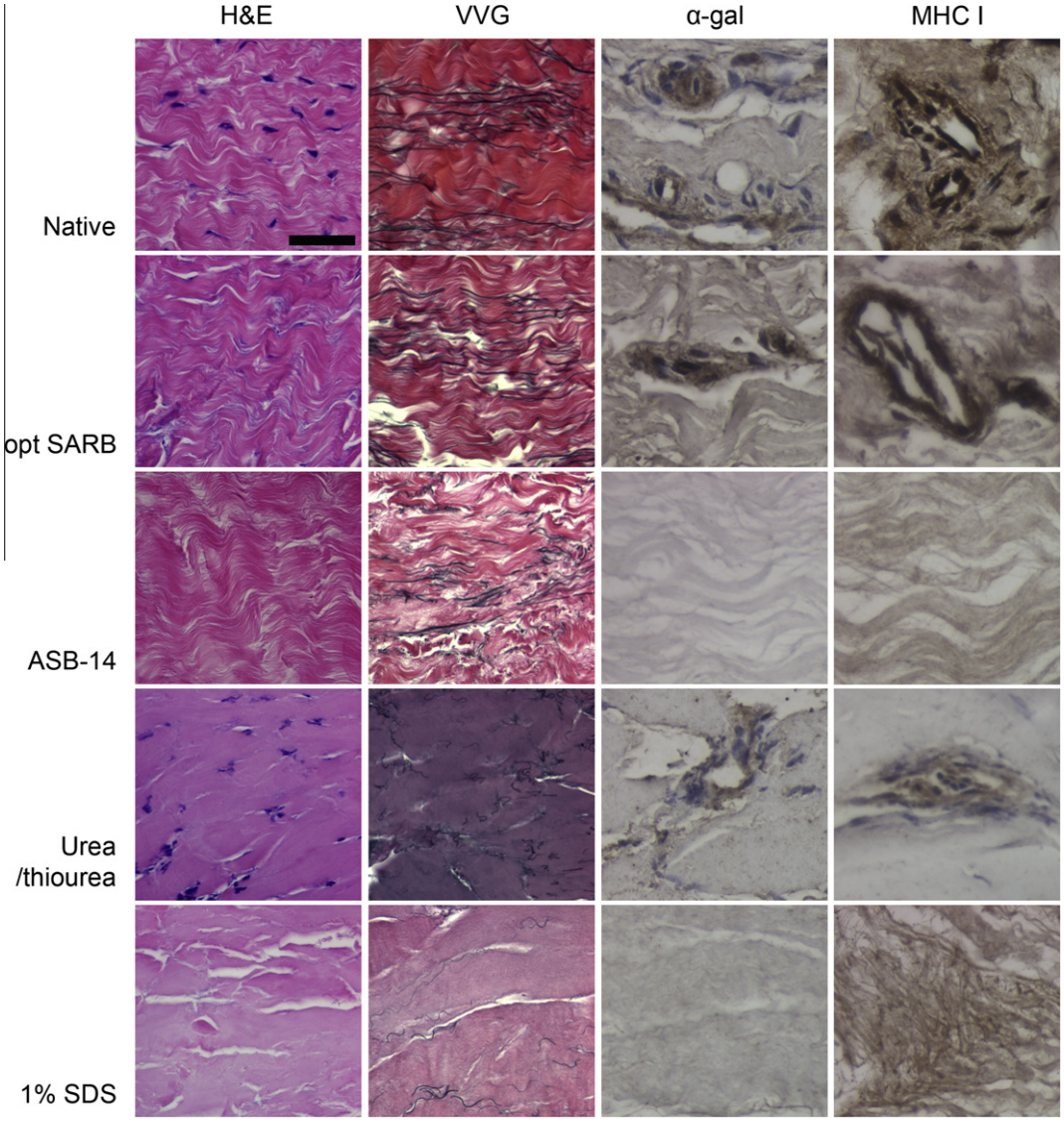

Fig. 7. Gross histological morphology and residual known xenoantigens in representative images of BP. H&E staining reveals both preservation of histological ECM morphology and reduction in residual nuclei following two-step AR using 1% (w/v) ASB-14 in opt SARB. Treatment with 8 M urea and 2 M thiourea in opt SARB or 1% (w/v) SDS in BARB does not maintain histological ECM morphology. Verhoeff van Gieson staining indicates that gross collagen and elastin structure is preserved following two-step AR using no additive or 1% (w/v) ASB-14 in opt SARB for lipophile solubilization. Treatment with 8 M urea and 2 M thiourea in opt SARB or 1% (w/v) SDS in BARB does not maintain gross collagen and elastin organization. Immunohistochemical staining reveals that no α-gal antigens persist in BP treated with 1% (w/v) ASB-14 in opt SARB or 1% (w/v) SDS in BARB. Residual α-gal antigens are observed in BP subjected to no additive or 8 M urea and 2 M thiourea in opt SARB. Immunohistochemical staining indicates that no MHC I antigens persist in BP treated with 1% (w/v) ASB-14 in opt SARB. Residual MHC I antigens are observed in BP subjected to no additive or 8 M urea and 2 M thiourea in opt SARB or 1% (w/v) SDS in BARB. The scale bar represents 50 μm.

vidual antigens is dependent on their solubility in the AR solution used, it is conceivable for a large proportion of an individual antigen to be removed while global antigenicity is minimally affected or a small proportion of an individual antigen be removed while global antigenicity is significantly reduced. Additional studies will be necessary to determine if this potential mechanism accurately accounts for the observed discrepancy between our Western blot and immunohistochemistry results. Furthermore, future in vivo studies will be crucial in determining what level of xenogeneic scaffold residual antigenicity (including insoluble components) is compatible with development of immune tolerance upon implantation, both for the presented small animal model and ultimately in human patients. Nonetheless, the ability of 1% (w/v) ASB-14 to significantly reduce residual lipophilic antigenicity and eliminate cell membrane-associated xenoantigens known to facilitate immune rejection of xenogeneic tissue makes it a strong candidate for lipophile solubilization in two-step AR, warranting further characterization of structure–function properties for the resultant scaffold.

A successful two-step AR strategy must preserve xenogeneic scaffold structure–function properties. Use of 1% (w/v) ASB-14 for lipophile solubilization resulted in BP-AR with tensile properties, ECM composition and ECM organization indistinguishable from native BP. This is likely attributed to the ability of zwitterionic detergents such as ASB-14 to accomplish lipophile solubilization while maintaining native protein conformation [29]. The presence of both hydrophilic and hydrophobic domains on detergent molecules enables their incorporation into the cell membrane, leading to bilayer destabilization and fragmentation and, ultimately, the solubilization of resultant detergent–protein complexes [30]. In contrast, chaotropes such as 8 M urea and 2 M thiourea have been reported to achieve lipophile solubilization through protein dena-

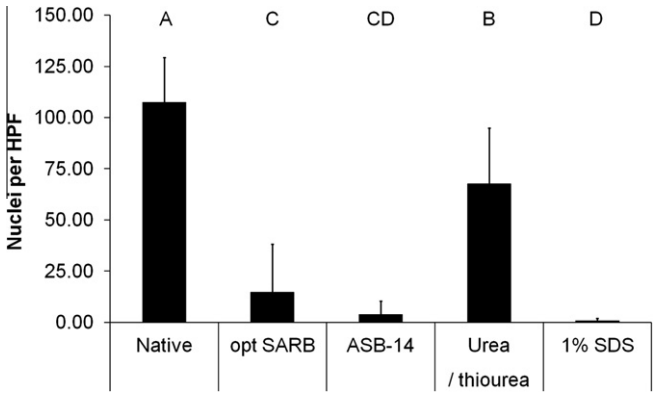

Fig. 8. Residual nuclei per HPF in BP. Following two-step AR, 1% (w/v) ASB-14 in opt SARB or 1% (w/v) SDS in BARB reduces nuclei most significantly compared to native BP. Results are plotted as mean ± standard deviation. Groups not connected by the same letter are significantly different, $p < 0.05$ ($n = 6$ per group).

turation [31–33]. Denaturation, but not loss, of collagen may account for disrupted collagen fiber organization, and subsequent gross thickening, increased water content and altered tensile properties of BP-AR following AR using 8 M urea and 2 M thiourea [32,33]. Additionally, elastin denaturation, manifested as a loss of elastin organization and content, may also contribute to the unacceptable loss of structure–function properties in chaotrope-treated BP-AR. In aortic valve leaflets, elastin fibers maintain leaflet structure–function properties by facilitating changes in collagen fiber configuration throughout the cardiac cycle [34]. By compromising elastin-mediated pre-stress on the collagen fibers in BP-AR, 8 M urea and 2 M thiourea may adversely alter collagen fiber organization and, ultimately, the structure–function properties of BP-AR. Similarly, lipophile solubilization using 1% (w/v) SDS was also found to result in significant alterations to biomaterial structure–function properties. Increasing concentrations of SDS increases collagen swelling [35,36] due to destabilization of the triple helical domain [37]. Thus, SDS-mediated disruption of collagen architecture and removal of elastin fibers may explain the noticeable gross tissue thickening and increased water content observed in the resultant BP-AR. The structural and compositional alterations associated with 1% (w/v) SDS may account for tensile properties trending lower than those of native BP, although this finding failed to reach statistical significance. Amongst the antigen removal agents tested for two-step AR, 1% (w/v) ASB-14 demonstrates the most promise in xenogeneic scaffold generation by achieving significant reduction in residual lipophilic antigenicity while maintaining structure–function properties comparable to native BP. Future studies will be necessary to determine the compatibility of scaffolds generated using a two-step AR process (opt SARB, followed by 1% (w/v) ASB-14 in opt SARB) with recellularization, in vivo physiological function and in vivo recipient immune response.

Previously, we questioned the appropriateness of using residual nuclei counts as the sole indicator of sufficient AR after demonstrating that overall residual hydrophilic antigenicity does not correlate significantly with residual nuclei counts [8]. The lack of significant correlation was not surprising as one would not expect one-step AR, solely promoting hydrophile solubilization, to efficiently solubilize the nuclear membrane. Thus, residual nuclei counts were expected to better represent residual lipophilic antigenicity of BP-AR. While lipophile solubilization reduces residual nuclei counts in BP-AR significantly, no significant correlation was found between residual lipophilic antigenicity and residual nuclei counts. As residual nuclei counts merely serve as an indicator of DNA that persists within the tissue, they do not reflect the level of either residual hydrophilic or lipophilic antigenicity within the biomaterial. Thus, assessment of biomaterial decellularization does not provide an accurate assessment of AR from xenogeneic biomaterials.

5. Conclusions

By targeting the solubilization of multiple protein subsets using a sequential, differential approach (first removing hydrophiles, then lipophiles), biomaterial antigenicity can be more efficiently reduced compared to a single solution that only solubilizes one protein antigen subset. Sequential application of opt SARB, followed by 1% (w/v) ASB-14 in opt SARB, to BP reduces residual hydrophilic antigenicity by an additional 75% compared to that achieved by 1% (w/v) SDS in BARB and residual lipophilic antigenicity by an additional 60% compared to that achieved by opt SARB alone. Excitingly, this two-step AR method eliminates the presence of the two most critical known barriers to xenotransplantation (α-gal and MHC I) without significantly compromising structure–function properties of the resultant scaffold. In sum, these findings illustrate that facilitating the sequential, differential solubilization of hydrophiles and lipophiles in a two-step AR strategy, utilizing opt SARB followed by 1% (w/v) ASB-14 in opt SARB, (i) significantly reduces the residual hydrophilic and lipophilic antigenicity of BP-AR, and (ii) maintains biomaterial structure–function properties. Beyond the generation of BP-derived scaffolds for heart valve tissue engineering, application of this stepwise AR strategy to other tissues or organs of the body may represent a more efficient alternative to decellularization for the generation of immune system-tolerant, tissue engineering scaffolds from xenogeneic tissues.

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References

[1] Platt J, DiSesa V, Gail D, Massicot-Fisher J. Recommendations of the National Heart, Lung, and Blood Institute Heart and Lung Xenotransplantation Working Group. Circulation 2002;106:1043–7.
[2] Goldstein S, Clarke DR, Walsh SP, Black KS, O'Brien MF. Transpecies heart valve transplant: advanced studies of a bioengineered xeno-autograft. Ann Thorac Surg 2000;70:1962–9.
[3] Iwai S, Torikai K, Coppin CM, Sawa Y. Minimally immunogenic decellularized porcine valve provides in situ recellularization as a stentless bioprosthetic valve. J Artificial Organs 2007;10:29–35.
[4] Simon P, Kasimir MT, Seebacher G, Weigel G, Ullrich R, Salzer-Muhar U, et al. Early failure of the tissue engineered porcine heart valve SYNERGRAFT(TM) in pediatric patients. Eur J Cardiothorac Surg 2003;23:1002–6.
[5] Griffiths LG, Choe LH, Reardon KF, Dow SW, Christopher Orton E. Immunoproteomic identification of bovine pericardium xenoantigens. Biomaterials 2008;29:3514–20.
[6] Goncalves A, Griffiths LG, Anthony R, Orton EC. Decellularization of bovine pericardium for tissue-engineering by targeted removal of xenoantigens. J Heart Valve Dis 2005;14:212–7.
[7] Meyer SR, Chiu B, Churchill TA, Zhu L, Lakey JRT, Ross DB. Comparison of aortic valve allograft decellularization techniques in the rat. J Biomed Mater Res A 2006;79A:254–62.
[8] Wong ML, Leach JK, Athanasiou KA, Griffiths LG. The role of protein solubilization in antigen removal from xenogeneic tissue for heart valve tissue engineering. Biomaterials 2011;32:8129–38.
[9] Zhou J, Fritze O, Schleicher M, Wendel H-P, Schenke-Layland K, Harasztosi C, et al. Impact of heart valve decellularization on 3-D ultrastructure, immunogenicity and thrombogenicity. Biomaterials 2010;31:2549–54.
[10] Cebotari S, Tudorache I, Jaekel T, Hilfiker A, Dorfman S, Ternes W, et al. Detergent decellularization of heart valves for tissue engineering: toxicological effects of residual detergents on human endothelial cells. Artif Organs 2010;34:206–10.
[11] Cedano J, Aloy P, Pérez-Pons JA, Querol E. Relation between amino acid composition and cellular location of proteins. J Mol Biol 1997;266:594–600.

[12] Nozaki Y, Tanford C. The solubility of amino acids and related compounds in aqueous urea solutions. J Biol Chem 1963;238:4074–81.
[13] Nozaki Y, Tanford C. The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions. J Biol Chem 1971;246:2211–7.
[14] Lehner I, Niehof M, Borlak J. An optimized method for the isolation and identification of membrane proteins. Electrophoresis 2003;24: 1795–808.
[15] Molloy MP, Herbert BR, Walsh BJ, Tyler MI, Traini M, Sanchez J-C, et al. Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. Electrophoresis 1998;19:837–44.
[16] Ramsby ML, Makowski GS, Khairallah EA. Differential detergent fractionation of isolated hepatocytes: biochemical, immunochemical and two-dimensional gel electrophoresis characterization of cytoskeletal and noncytoskeletal compartments. Electrophoresis 1994;15:265–77.
[17] Cordwell SJ. Sequential extraction of proteins by chemical reagents. Methods Mol Biol 2008;424:139–46.
[18] Cordwell SJ, Nouwens AS, Verrills NM, Basseal DJ, Walsh BJ. Subproteomics based upon protein cellular location and relative solubilities in conjunction with composite two-dimensional electrophoresis gels. Electrophoresis 2000;21:1094–103.
[19] Griffiths LG, Choe L, Lee KH, Reardon KF, Orton EC. Protein extraction and 2-DE of water- and lipid-soluble proteins from bovine pericardium, a low-cellularity tissue. Electrophoresis 2008;29:4508–15.
[20] Beers MF, Bates SR, Fisher AB. Differential extraction for the rapid purification of bovine surfactant protein B. Am J Physiol 1992;262:L773–8.
[21] Wilson R, Bateman JF. A robust method for proteomic characterization of mouse cartilage using solubility-based sequential fractionation and two-dimensional gel electrophoresis. Matrix Biol 2008;27:709–12.
[22] Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. Biomaterials 2006;27:3675–83.
[23] National Research Council. Institute of Laboratory Animal Resources Commission on Life Sciences. Guide for the Care and Use of Laboratory Animals. Washington, DC: National Academy Press; 1996.
[24] Leimgruber RM. Extraction and solubilization of proteins for proteomic studies. In: Walker JM, editor. The Proteomics Protocols Handbook. Totowa: Humana Press; 2005. p. 1–18.
[25] Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 1961;93:440–7.
[26] Galili U. The [alpha]-gal epitope (Gal[alpha]1-3Gal[beta]1-4GlcNAc-R) in xenotransplantation. Biochimie 2001;83:557–63.
[27] Yang Y-G, Sykes M. Xenotransplantation: current status and a perspective on the future. Nat Rev Immunol 2007;07:519–31.
[28] Cascalho M, Platt JL. The immunological barrier to xenotransplantation. Immunity 2001;14:437–46.
[29] Nagy JM, Lipka A, Pereira F, Marlin N, Hassard S. Sample preparation for 2D electrophoresis and CE-based proteomics. Sample preparation in biological mass spectrometry. New York: Springer; 2011. p. 41–60.
[30] le Maire M, Champeil P, Moller JV. Interaction of membrane proteins and lipids with solubilizing detergents. Biochim Biophys Acta 2000;1508:86–111.
[31] Frank HS, Franks F. Structural approach to the solvent power of water for hydrocarbons; urea as a structure breaker. J Chem Phys 1968;48:4746–57.
[32] Hua L, Zhou R, Thirumalai D, Berne BJ. Urea denaturation by stronger dispersion interactions with proteins than water implies a 2-stage unfolding. Proc Natl Acad Sci U S A 2008;105:16928–33.
[33] Gordon JA, Jencks WP. The relationship of structure to the effectiveness of denaturing agents for proteins. Biochemistry 1963;2:47–57.
[34] Vesely I. The role of elastin in aortic valve mechanics. J Biomech 1997;31:115–23.
[35] Krejčí J. Interaction of mixture of anionic surfactants with collagen. Int J Cosmetic Sci 2007;29:121–9.
[36] Henriquez M, Lissi E, Abuin E, Ciferri A. Assembly of amphiphilic compounds and rigid polymers. 1. Interaction of sodium dodecyl sulfate with collagen. Macromolecules 1994;27:6834–40.
[37] Samouillan V, Dandurand-Lods J, Lamure A, Maurel E, Lacabanne C, Gerosa G, et al. Thermal analysis characterization of aortic tissues for cardiac valve bioprostheses. J Biomed Mater Res 1999;46:531–8.