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Reinforced Pericardium as a Hybrid Material for Cardiovascular Applications

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Pericardium-based cardiovascular devices are currently bound by a 10-year maximum lifetime due to detrimental calcification and degradation. The goal of this work is to develop a novel synthetic material to create a lasting replacement for malfunctioning or diseased tissue in the cardiovascular system. This study couples poly(propylene fumarate) (PPF) and a natural biomaterial together in an unprecedented hybrid composite and evaluates the composite versus the standard glutaraldehyde-treated tissue. The polymer reinforcement is hypothesized to provide initial physical protection from proteolytic enzymes and degradation, but leave the original collagen and elastin matrix unaltered. The calcification rate and durability of the hybrid material are evaluated *in vitro* and in an *in vivo* subdermal animal model. Results demonstrate that PPF is an effective support and leads to significantly less calcium deposition, important metrics when evaluating cardiovascular material. By avoiding chemical crosslinking of the tissue and associated side effects, PPF-reinforced pericardium as a biohybrid material offers a promising potential direction for further development in cardiovascular material alternatives. Eliminating the basis for the majority of cardiovascular prosthetic failures could revolutionize expectations for extent of cardiovascular repair.

Introduction

For more than 50 years, a method to replace malfunctioning or diseased tissue in the cardiovascular system has been an important area of development. Each year, over 275,000 replacement valves and 600,000 vascular grafts are implanted to correct damaged native structures.[1,2] Complex pediatric and general cardiac surgeries also utilize tissue or synthetic patch material in reconstruction and repair. When considering materials for prosthetic applications, biological tissues offer some clear advantages over synthetic substitutes. Aside from the inherent biocompatibility, biological tissues possess intelligent elastic and mechanical properties that are unable to be mimicked by manmade material.[3,4]

One biological tissue commonly used is the pericardium. Pericardial tissue has historically been selected for cardiovascular devices due to its availability, inherent strength, and elastic properties. In addition to the desirable mechanical behavior described, natural tissue often demonstrates superior fluid dynamic properties, and when compared to synthetic materials, requires less anticoagulation therapy.[4]

To capitalize on and maintain the natural properties of pericardium, previous research has focused on improving the durability and biocompatibility of the material upon implantation. However, and despite significant advances, clinical experience continues to highlight the challenges of the prolonged use of pericardium implants in the cardiovascular system.[2]

All natural tissue, including pericardium, can elicit an inflammatory and immune response from the host. To combat these inevitable events, pericardial tissue is commonly preserved in glutaraldehyde (GA), which chemically crosslinks the tissue's collagen molecules. This crosslinking process is effective at stabilizing the tissue against chemical and enzymatic degradation, as well as lessening the display of antigenic determinants.[5–7] However, the crosslinked product has been associated with local cytotoxicity and, more importantly, severe calcification of the material that can over time lead to subsequent matrix deterioration and compromised mechanical properties.[8–10]

The detailed pathways controlling calcification of cardiovascular tissue, both natural and prosthetic, are not explicitly understood, but as the most common pathology recorded in heart valve failures,[11,12] it is certainly a process under high investigation. It is observed that chemically crosslinking pericardium damages and distorts the natural structure, destroys interstitial cells, and diminishes potential for viable cell inhabitation.[13] The specialized matrix consisting of collagen, elastin, and glycosaminoglycans (GAGS) that compose the pericardium is responsible for allowing the tissue to accommodate the

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constant changes in shape and stress transfer,[13,14] and is therefore essential to maintain. Damaging this natural structure and removing native cells results in a tissue that can no longer maintain or repair itself. It has been suggested in literature that calcification and eventual ossification are the results of insufficient or irregular repair of the tissue network. In culture, myofibroblasts have been shown to undergo phenotypic differentiation into the osteoblast-like cells seen in calcified cardiac tissue, promoting calcification and bone type remodeling.[11] Others suggest that the origin of bone cells in ossified valves in unknown, but their presence is confirmed in observation of excised heart valves and tissue.[15]

Original damage leading to the irregular repair of the implanted tissue can be a result of mechanical stress, immune cell infiltration, or other pathologies, which complicates the investigation of the process. In one theory, responding immune cells are reported to secrete collagenase, among other proteolytic enzymes, which immediately begins to degrade the collagen network.[9] It is further hypothesized by some that this initial proteolysis of crosslinked collagen debris creates foci for calcium deposition to initialize.[1,10,16] Studies have shown a cooperative relationship between calcification of this type of tissue and the inflammatory response, enzymatic degradation, and microstructural failure (both independent failures and those associated with calcium deposits.)

Regardless of the mechanism directly controlling the calcium deposits, GA treatment has been shown through both *in vivo* and *in vitro* accelerated testing to destroy the surface endothelium of the tissue, autolytically disrupt interstitial cells, alter natural collagen bundles, and destroy native GAGS.[17] Despite the discrepancies in origin of the deterioration, a strong correlation between GA-treated tissue and increased calcification suggests that the chemical fixation of the tissue further inhibits the appropriate remodeling.

The above theory of calcification suggests that if an alternative to GA can be developed that is effective at blocking enzymes and other immune activators, but avoids crosslinking of the tissue, then subsequent calcification of the material will be reduced. Our principal hypothesis is that applying a paintable polymer to the surface of pericardium tissue will provide the physical support and biological block to natural degradation, while leaving the original tissue unaltered. This will result in a hybrid material that will retain mechanical integrity but will not accumulate calcium deposits. For this purpose, the synthetic polymer poly(propylene fumarate) (PPF) was selected for its demonstrated biocompatibility, biodegradability, and strength in other biomedical applications.[18,19]

PPF is an unsaturated linear polyester that is crosslinkable through UV radiation with itself or with other compatible crosslinkers through the double bonds in fumarate. PPF is biodegradable by hydrolysis of ester bonds and forms the naturally occurring byproducts fumaric acid and propylene glycol upon degradation.[20] The degradation time is largely dependent on polymer characteristics such as molecular weight, crosslinker, and crosslinked density.[20] The uncrosslinked polymer is viscous at room temperature, allowing it to be easily painted onto a surface. In addition, PPF viscosity can be reduced, without significantly altering the components of the system, with the addition of diethyl fumarate (DEF), the monomer from which PPF is synthesized. The addition of DEF to PPF can also cause variations in the crosslinked biomaterial's mechanical strength and can be utilized as a parameter to tune the characteristics of the hybrid biomaterial.[21]

To address our overall hypothesis, the first aim was to identify measurable mechanical properties of natural pericardium and develop a composition of PPF to mirror those properties. The designed composition of the polymer was then married to the pericardium tissue, and the hybrid material was evaluated on two fronts. The first evaluation of the hybrid material was to test if PPF reinforcement was an effective support for natural pericardium enzymatic degradation. The second evaluation of the hybrid material was to investigate if PPF-reinforced pericardium would cause minimal calcification when compared to the GA-treated standard. The performance in these two areas will support the hypothesis that a topically applied polymer can biologically and mechanically support pericardium for use in cardiovascular applications.

Materials and Methods

Polymer synthesis and composition

PPF was synthesized by a two-step process as previously described.[22] Briefly, propylene glycol and DEF were combined in a 3:1 molar ratio. Zinc chloride, acting as a catalyst, and hydroquinone, as a radical inhibitor, were added in a 0.01:0.002 molar ratio. The reaction was carried out under nitrogen flow, producing ethanol as a byproduct and bis (hydroxypropyl) fumarate as the intermediate. Next, under vacuum, transesterification of the intermediate produces PPF with propylene glycol as a byproduct. Gel permeation chromatography was used to calculate the number average molecular weight (Mn) and polydispersity index (PDI) of the purified PPF. For use in this study, PPF (Mn 1150 and PDI 1.6) was mixed with the monomer DEF, and then mixed with the photoinitiator bis(2,4,6-trimethylbenzoyl) phenylphosphine oxide (BAPO), to create a UV-sensitive reaction.

To select the ratio of polymer to monomer that would be a suitable match for pericardium material, a set of weight to weight ratios of PPF to DEF was tested in thin-film formation, as shown in Table 1. A soluble fraction of the crosslinked composite was measured comparing the dry mass of the film to the dry mass after soluble components were dissolved in acetone for 12 h, using the following equation:

$$Soluble\ Fraction\ \% = \frac{m_{initial} - m_{final}}{m_{initial}} \times 100$$

Table 1. Compositions of Various Thin Films Tested

PPF (g)	*DEF (g)*
4	1
3	1
2	1
1	1
1	2
1	3

Various mass to mass ratios of PPF to DEF were made and then crosslinked into thin films from the same total volume. This change in composition of the uncrosslinked polymer solution can affect the physical properties of the crosslinked result.

DEF, diethyl fumarate; PPF, poly(propylene fumarate).

Mechanical testing of polymer compositions

PPF composite films, as previously described, were tested on an Instron for mechanical analysis using ASTM D638 to obtain 0.1% yield strength and elastic modulus. Thin films were formed between two 2×2 cm square glass plates and resulted in 0.25-mm-thick 0.15 g films (Fig. 1). This geometry is similar to the weight per surface area approximated for coating pericardium as well as a suitable shape for testing according to ASTM D882. The extension rate was set at 10 mm/min, and extension and corresponding load was recorded at a frequency of 10 Hz. A preload of 5 N was considered. The elastic modulus for each sample was calculated using MATLAB to determine the slope of the linear region of the stress–strain curve reported by the Instron. The linear region of the curve was systematically calculated using linear line fit command based on the first 10 data points. The program then continued to add data points in steps of 10 until the $R^2 < 0.97$. The slope of this region was determined to be the modulus of the sample. Strength at 0.1% yield was calculated as the intersection of the stress–strain curve with a line drawn parallel to the linear slope, whose x-axis intercept is shifted 0.01 mm/mm strain.

Sample preparation

Throughout this study, PPF reinforced pericardium was compared to GA-treated pericardium and untreated pericardium as controls. All pericardium was obtained 2 days after harvest from Innovative Research, Inc. and stored at 4°C in physiological saline. Experiments were conducted within 2 days of receiving the pericardium and because the properties of pericardium are thought to vary among individual donors, all samples for each test were taken from the same donor.

GA-treated samples were prepared by immersing small strips of pericardium (2×6 cm) in a 0.625% GA solution for 6 h at room temperature. Samples were then washed twice and stored in saline at 4°C. PPF reinforced samples were also cut into 2×6 cm strips and were lightly stretched onto wooden frames to maintain spatial conformation. The tissue was then dehydrated in ethanol dilutions, to facilitate the interaction and integration of hydrophobic PPF with pericardium and remove antigenic determinants. Dehydrated pericardium was then coated with a thin and uniform layer of PPF to completely cover the tissue strip. The coated tissue strip was then exposed to UVa light for 90 min (3.5 mW/cm^2) to crosslink the polymer. After crosslinking, tissue and polymer composite were rehydrated in a physiological buffer at room temperature for 2 h and then stored in 4°C. Pericardium samples from each group were mechanically tested, using the method described previously, as controls.

FIG. 1. Thin film of poly(propylene fumarate) (PPF) postcrosslinking. Thin films of PPF in various weight ratios with diethyl fumarate (DEF) were formed from 120 μL of polymer between 2×2 cm glass slides. Polymer crosslinking was initiated with 5 mg/g of bis(2,4,6-trimethylbenzoyl) phenylphosphine oxide (BAPO), under UVa light (3.5 mW/cm^2) for 90 min. Resulting thin films were 0.25 mm thick and weighed 0.15 g. The image shows a thin film composed of 2 g PPF to 1 g DEF (2:1).

In vitro *evaluation*

Degradation test. An *in vitro* degradation model was designed to investigate the added strength and resiliency of the PPF reinforcement as compared to untreated pericardium and GA-treated pericardium as controls. Pericardium samples were exposed to 0.4 U/mL of collagenase and shaken at 60 rpm in 37°C. The test was run over a period of 12 days, with mechanical analysis testing performed every 4 days ($n = 8$ per day), as described previously. Samples were preserved at various time points for histological analysis.

Calcification test. To compare *in vitro* calcification between PPF reinforced and GA-treated pericardium, samples from these groups were exposed to the physiological calcium buffer (2.6 mM calcium/1.6 mM phosphate) at 37°C, for a total period of 45 days. At each time point (5, 12, 21 days), 15 samples from each treatment group were isolated for analysis. For each treatment group, eight of the isolated samples were reserved for mechanical analysis, which was

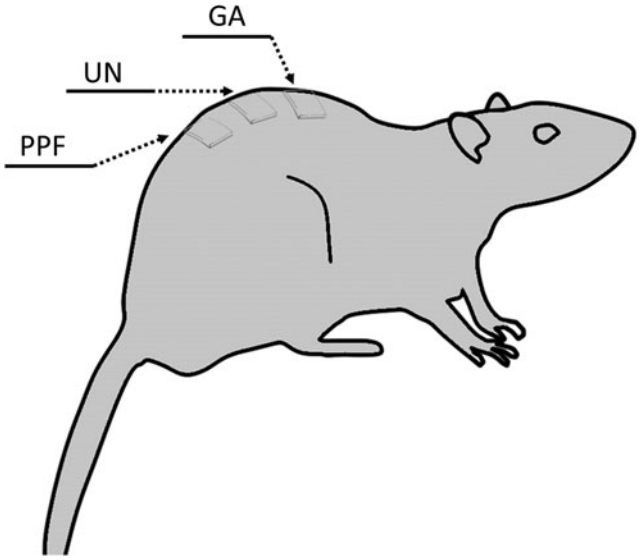

FIG. 2. Schematic of implantation for pericardium patches in a subdermal rat model. A small incision was made in the dorsal dermal layer, and a material patch (1×1cm) from each of the three groups was sutured by the corners to the underlying tissue. Each $n = 8$ animals per time point ($n = 16$ total) received one patch from each experimental group.

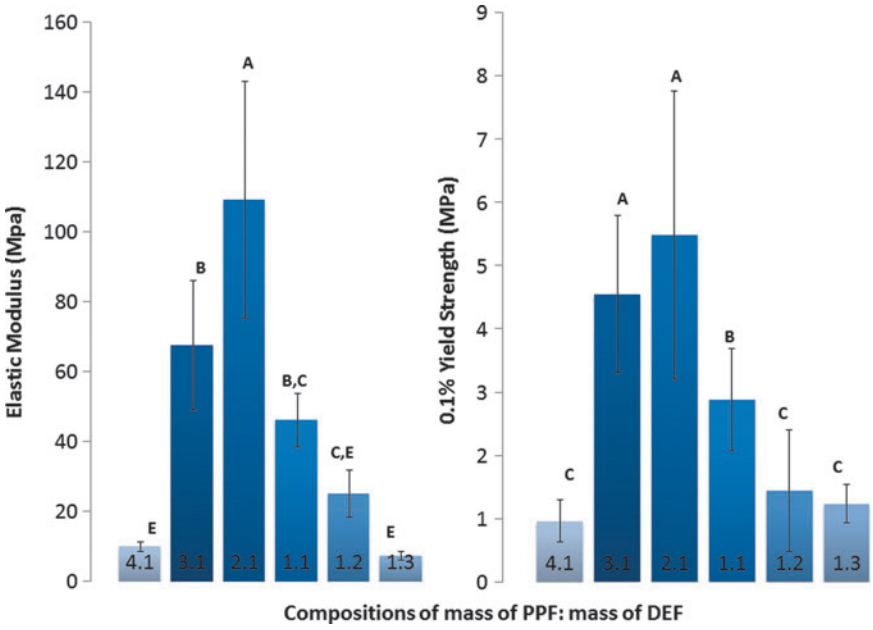

FIG. 3. Elastic modulus and 1% yield strength from thin films of various PPF:DEF composites. Thin films were tested on an INSTRON mechanical tester and extended at 10 mm/min by a 50 N load cell. $n \geq 7$ for all groups, ANOVA statistical test shows significant difference ($p < 0.05$), and Tukey's *post hoc* results are shown on graph. Groups that do not share a letter are significantly different.

performed as previously described. Two of the samples were preserved for histological study. The final five samples were used to quantify calcium deposition. These samples were first triple washed in deionized water to remove loosely attached calcium and then oven dried at 80°C for 6 h to measure dry mass. Samples were then hydrolyzed in 2 M HCl for 48 h to dissolve calcium deposits on the tissue. Solutions were then measured for calcium concentrations using a colorimetric assay. The calcium concentration at each time point was statistically compared using a Student's *t*-test.

In vivo *evaluation*

The calcification rate and material integrity of the hybrid material were evaluated in a subdermal rat model. The Institutional Animal Care and Use Committee of the University of Maryland approved the study, and all animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals. The experiment was conducted in $n = 16$ male Sprague Dawley rats, as determined by a power analysis. As seen in literature for material analysis,[23] a small incision is made in the dorsal dermal layer, and a material patch (1×1 cm) from each of the three groups is sutured by the corners to the underlying tissue (Fig. 2). At time points of 3 and 6 weeks, $n = 8$ animals were euthanized and the pericardium patches and surrounding tissue were explanted. Each explanted tissue sample was carefully sliced in half. One half of each sample was preserved in paraformaldehyde (4%) and embedded in paraffin for histological analysis to determine calcium deposition, cellular population, resulting tissue morphology, and inflammatory response.

The inflammatory response, in particular, was investigated using immunohistochemistry to stain for the presence of the macrophage cell surface antigen F4/80. Slides were incubated with PEROXIDAZED1 (Biocare), an endogenous peroxidase blocker, and BackgroundSNIPER1 (Biocare), a broad spectrum blocking reagent. The samples were then incubated with anti-F4/80 (rabbit polyclonal to F4/80; Abcam ab74384) as

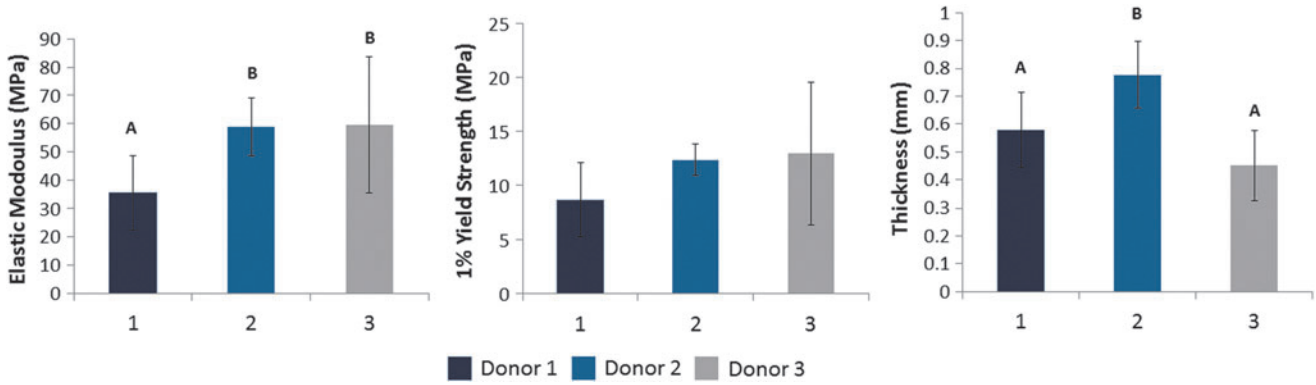

FIG. 4. Untreated pericardium physical properties. The graph displays the characteristic properties collected from pericardium from three different donors. All data were collected 2 days after harvest. Samples were cut into 1×5 cm rectangles and stored in phosphate-buffered saline until testing. ANOVA statistical tests showed significant difference in the elastic modulus and in the thicknesses ($p < 0.05$), and results from a *post hoc* Tukey's test are shown on the graphs. Groups that do not share a letter are significantly different.

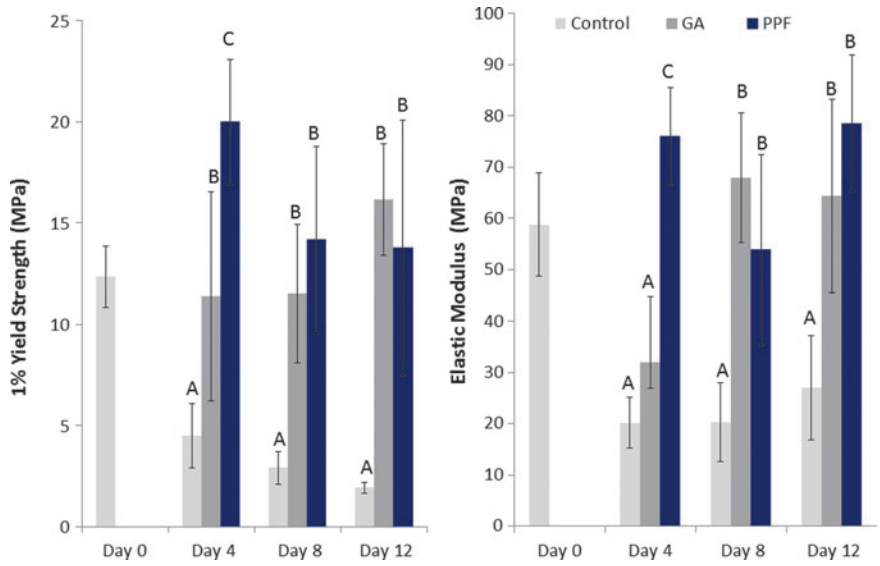

FIG. 5. Mechanical properties of pericardium samples over time in *in vitro* degradation model. 1×5 cm rectangle strips of pericardium were left untreated (control), fixed in 0.625% glutaraldehyde (GA), or reinforced with PPF (PPF). All samples were then degraded in 0.4 U/mL of collagenase, over a period of 12 days. An ANOVA statistical test shows significant difference between the treatment methods compared on the same time point ($p < 0.05$). Results from *post hoc* Tukey's test are shown on the graph. Groups that share the same letter are not statistically different. The control on day 0 has no PPF reinforcement or collagenase exposure. Control groups on the following days are exposed to collagenase but not enforced with PPF or treated with GA.

the primary antibody, followed by a biotinylated anti-rabbit IgG as the secondary antibody (Vector Laboratories). Using a horseradish peroxidase-streptavidin system, followed by a 3,3′-diaminobenzidine tetrahydrochloride chromogen, the presence of the F4/80 molecule was detected. All samples were then counterstained with hematoxylin. The macrophage population was determined through examining three standardized images from each of $n = 5$ samples per treatment group. The macrophage fraction was defined as the number of cells determined to have F4/80 staining divided by the total cells counted per image in a blinded study. Data are presented as an average of each of these fractions, and error was

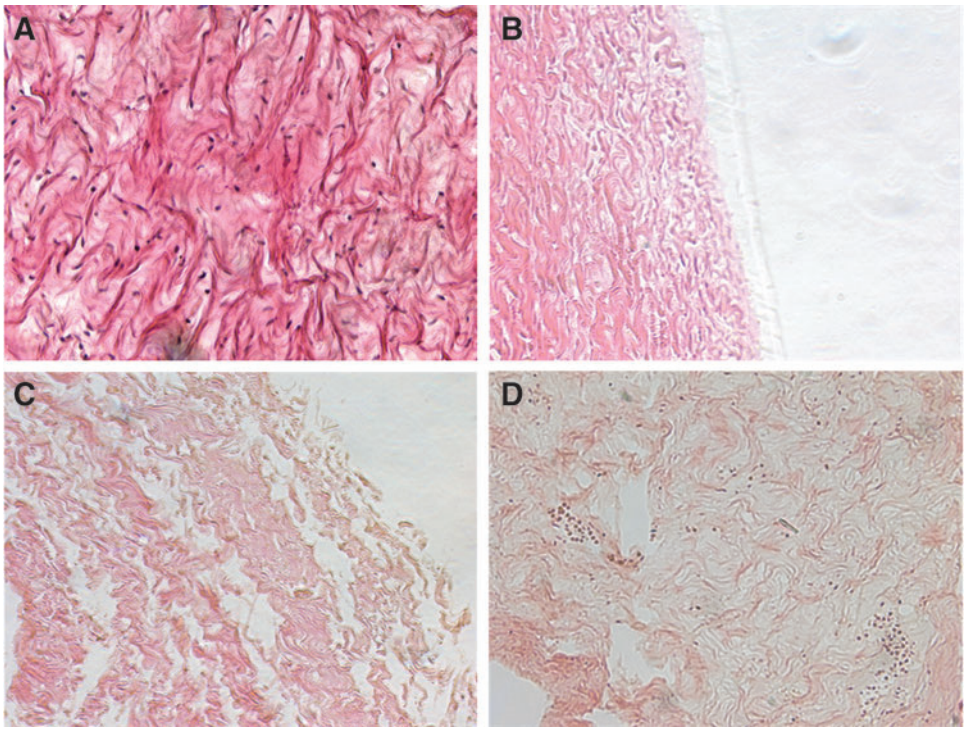

FIG. 6. Histological analysis of pericardium samples from *in vitro* degradation. Samples shown are stained with hematoxylin and eosin to display structure of collagen network. Control pericardium appears to have lost structural integrity after collagenase degradation **(D)**, as did the EtOH dehydrated sample **(C)**, while the sample with PPF reinforcement **(B)** appears unaltered from the natural pericardium **(A)**.

calculated as the standard deviation between all images in each group.

The other half of each sample was dried in a 60°C oven for 18 h and then hydrolyzed in 2 M HCl for 48 h. A colorimetric assay was used to determine the calcium concentration of each sample.

Statistical analysis

All quantitative assessments were statistically compared using a one-way ANOVA test ($p < 0.05$), followed by a *post hoc* Tukey's test, unless otherwise noted.

Results

We have developed a biohybrid material that consists of a polymer reinforcement later painted on the natural biomaterial pericardium. The mechanical characteristics of the polymer directly affect those of the hybrid material and were therefore the determining factor in selecting a polymer composite. Following mechanical analysis of the thin films composed of different formations of PPF:DEF ratios, the 2:1 dilution was selected for future work. The yield strength of this composition (5.48 ± 2.27 MPa) was found to be the greatest, and therefore the closest to the tested values for untreated and GA fixed pericardium (11.32 ± 4.24 and 13.01 ± 4.35 MPa, respectively). A comparison of yield strength and modulus of the thin films is shown in Figure 3. The modulus of elasticity and yield stress of pericardium are known to vary between donors based on age and other physiological features, but the values exhibited by the 2:1 films in elasticity measurements are sufficiently high to support the pericardium within the range tested. Figure 4 displays mechanical analysis data from untreated pericardium from three separate donors, as a reference. The soluble fraction of thin sheets of 2:1 PPF to DEF was calculated as $13.3\% \pm 2.7\%$ ($n = 9$).

As mentioned, the 2 PPF:1 DEF composition was selected for further evaluation in the hybrid material model. After physiological degradation *in vitro*, mechanical yield strength and elastic modulus of PPF reinforced pericardium are not significantly different than the gold standard GA-treated pericardium ($\alpha = 0.05$) (Fig. 5). By day 8, the strength and elasticity of both of these materials still remains above 11.50 ± 3.42 MPa 0.1% yield strength and 54.00 ± 12.67 MPa elastic modulus. These values are comparable to the untreated control on day 0, measured at 12.34 ± 1.49 MPa and 58.80 ± 10.17 MPa, respectively. However, by day 8, the untreated sample had fallen as low as 2.91 ± 0.80 MPa and 20.29 ± 7.76 MPa. These results of reduced or maintained material strength are supported by observations from histological photographs of the pericardium samples, which contrast a highly disordered fresh pericardium fiber network with a more ordered and dense PPF reinforced pericardium sample (Fig. 6).

In addition to mechanical property retention, calcium deposition was also evaluated *in vitro*. The time-dependent graph in Figure 7 displays calcium deposition onto GA-treated and PPF reinforced tissue from an *in vitro* calcification model. The level of calcification of GA-treated tissue is significantly higher than the level of calcification of PPF reinforced tissue at each time point (except day 0) ($p < 0.05$). Mechanical data did not show any distinct differences between groups.

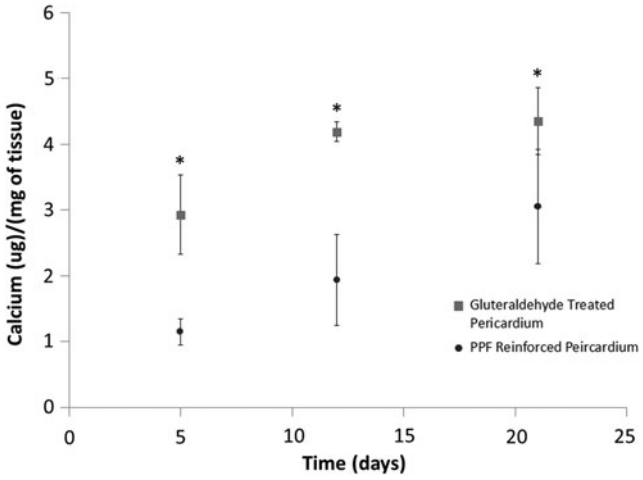

FIG. 7. *In vitro* calcification of pericardium samples: quantified results. Pericardium samples were left untreated, fixed in 0.625% GA, or reinforced with PPF. All samples were then stored in a calcium phosphate buffer, and calcium deposition was measured on 0, 5, 12, and 21 days. A Student's *t*-test shows significant difference between the calcium contents for all time points, denoted *, ($p < 0.05$).

Calcification was next further evaluated in the *in vivo* model. Calcification of PPF reinforced pericardium was significantly less than GA-treated samples in the quantitative assessment ($p < 0.05$) at each time point. The graph in Figure 8 shows calcium deposition per mass of dried tissue weight compared between the three experimental groups. These results were confirmed by the Von Kossa histological stain (Fig. 9), in which calcium phosphate depositions are observed in GA-treated samples on week 3 and 6, but little to no staining is observed on PPF reinforced samples or on untreated samples. The staining in these last two groups appears unchanged from the unimplanted controls (day 0), a result that is confirmed by the maximum variance of ± 1 μg of calcium/mg of tissue from the day 0 level over the 6-week period.

In histological samples stained with Masson's trichrome (Fig. 10), we observe that PPF reinforced tissue remains intact up to 6 weeks, with regions of cellular infiltration. GA-treated tissue appears to have limited cell movement into the implanted tissue, but instead has a line of cells near the surface of the implant. Furthermore, GA-treated pericardium is observed to exist in a dense network. We further observed that untreated tissue was difficult to identify as a distinct material within the surrounding tissue.

Using immunohistological staining techniques, it was confirmed that cells expressing the F4/80 surface molecule, a known marker of macrophages, are present at or near the implanted tissue in all treatment groups (Fig. 11). Regions of tissue with dense cell packing also present high amounts of F4/80 stain. In particular, it was observed that the dense cell line seen near the edge of the GA-treated tissue has a high presence of F4/80 stain. This is also observed in the other tissue samples, but in small pockets and dispersed throughout the implanted region and neighboring soft tissue. The macrophage fraction of the total cell population in each sample is displayed in Figure 12. This fraction is greatest in GA-treated samples, $36.3\% \pm 18.7\%$ of the population, and

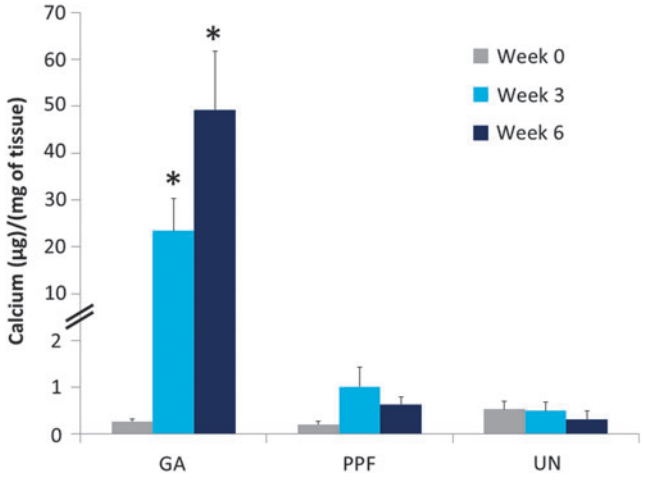

FIG. 8. Calcification of pericardium samples from *in vivo* subcutaneous model: quantified results. The calcium content was quantified on samples explanted after 3 and 6 weeks and compared to nonimplanted controls. Calcium content from each sample group was compared for each time point using an ANOVA test ($p < 0.01$). A *post hoc* Tukey's test showed significant difference in the GA-treated group, as denoted *, ($p < 0.05$).

is followed by the untreated samples at 29.0% ± 12.4%, and finally, by the PPF reinforced samples at 18.2% ± 8.0%.

Discussion

Utilizing pericardium as a naturally strong and elastic biomaterial offers clear benefits for building cardiovascular devices. To utilize pericardium's strengths for a cardiovascular implant, steps need to be taken to preserve the tissue in a foreign body environment. GA treatment is effective at preventing degradation of the collagen network and preventing immune cell infiltration by crosslinking the collagen fibers. However, this process renders the tissue nonviable, which forces the body to resort to unnatural healing mechanisms to repair inevitable fractures and defects in the tissue. In this process, the natural mechanical properties and hemodynamic capabilities are lost or altered.

To combat the issues caused by GA fixation, strategies that target either therapy with anticalcification agents or biomaterial modifications are among approaches commonly investigated. Biomaterial modifications focus on improving or modifying the GA fixation through altering the reactivity or concentration of the chemical, or using an altogether different chemical process. This last route has included options such as epoxy compounds, carbodiimides, and acyl azides, all of which eliminate the use of GA, but still hinge on a chemical crosslink of the tissue. Some of those methods have shown successful results in the laboratory, but have had little translation into clinical practice.[2]

In this study, it was hypothesized in this study that a thin coating of PPF would sufficiently prevent enzymatic degradation of the pericardium tissue, and yet by completely avoiding alteration of the tissue through the use of chemical crosslinker, result in less calcium deposits.

As demonstrated initially in *in vitro* degradation, PPF reinforced pericardium did not lose mechanical strength (yield strength or elastic modulus) after collagenase exposure. In fact, the retained elastic modulus and yield strength were not significantly different from the gold standard control GA-treated. This retained strength as demonstrated in mechanical testing and retained physical structure observed in histological images support the superior durability of PPF reinforced pericardium when compared to the untreated control in a physiological model. This indicates a thin layer of PPF is successful at protecting pericardium

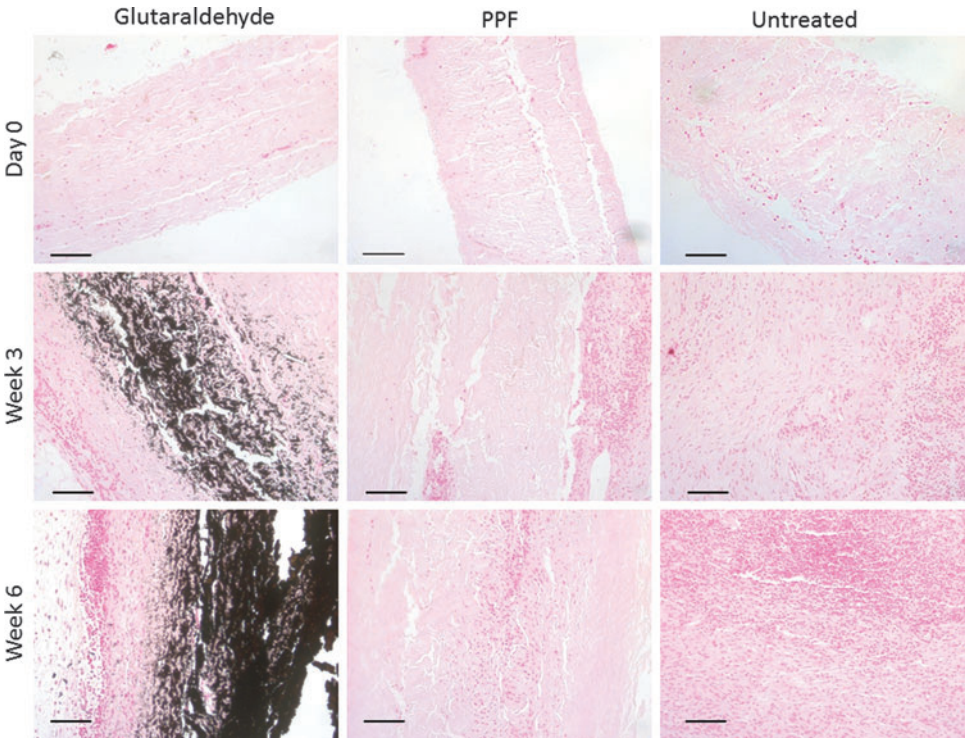

FIG. 9. Calcification of pericardium samples from *in vivo* subcutaneous model: histological analysis. Samples explanted at 3 and 6 weeks were fixed and prepared for histological analysis using Von Kossa stains. The *dark black* region indicates calcium phosphate deposits. This region is seen significantly darker in GA-treated samples after 3 and 6 weeks of implantation and not observed in PPF reinforced or untreated samples. Scale bars represent 100 μm.

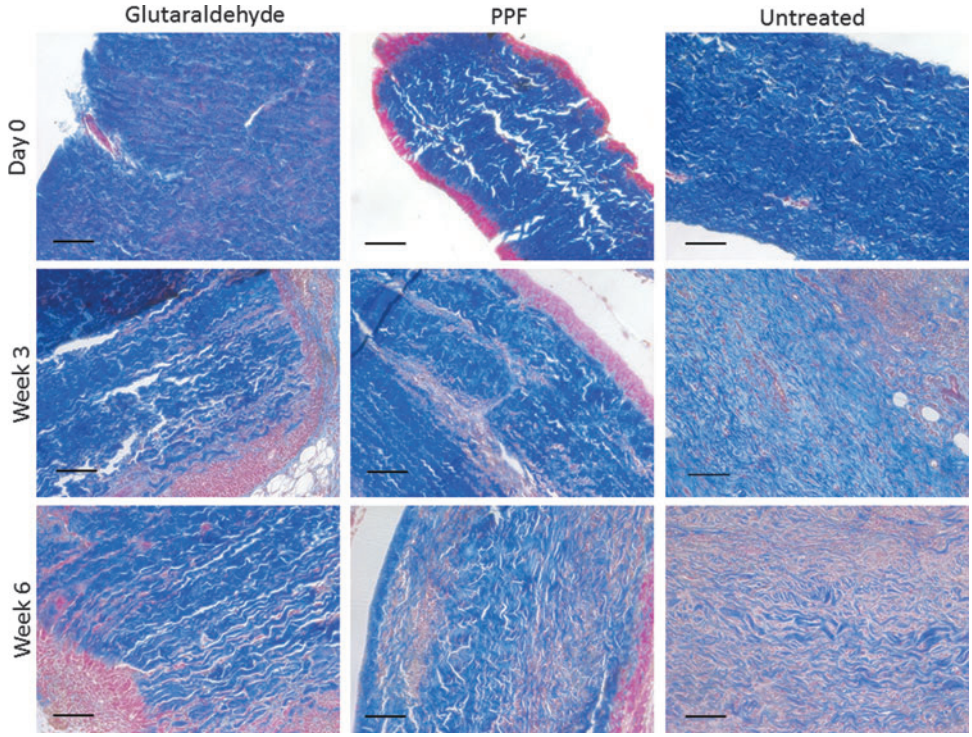

FIG. 10. Histological analysis of pericardium samples from an *in vivo* subcutaneous model. Cellular response to implanted samples was analyzed using Masson's trichrome stain. *Blue* regions indicate collagen; *pink* indicates cell bodies. Staining shows local cell populations relative to implanted tissue. Untreated pericardium displays dense cellular regions, suggesting an acute inflammatory response. PPF reinforced and GA-treated tissue does not display such a response. Scale bars represent 100 μm.

from structural deformation due to enzyme activity. This claim is supported further by results from the subdermal model. The rapid loss in strength and the observed loss in organization of natural pericardium are visually and statistically distinct from the PPF reinforced and GA-treated pericardium after enzymatic degradation.

Furthermore, the inflammatory response characteristics differ between the PPF reinforced and GA-treated cases, as observed with immunohistochemical staining for the macrophage presence. In addition to the overall lower total macrophage density in PPF reinforced samples, macrophages are homogenously spread throughout the implant. In GA-treated samples, macrophages are seen in a distinct and dense cell line surrounding the exterior.

More importantly, the calcium deposition in the reinforced pericardium was significantly less than the GA-treated

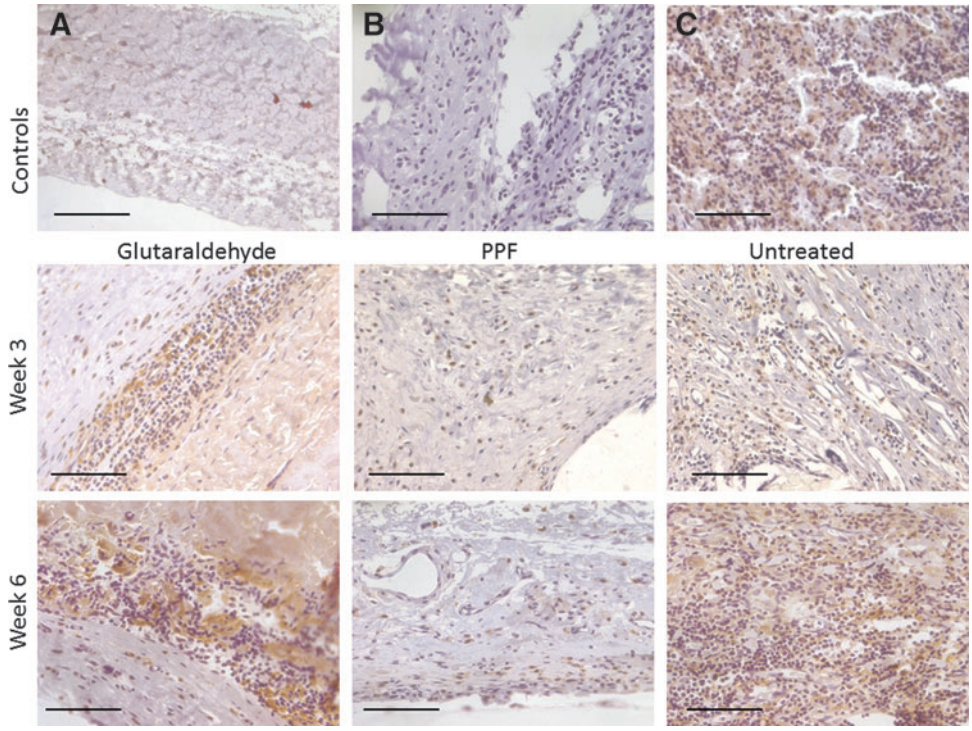

FIG. 11. Immunohistochemical analysis of pericardium samples from an *in vivo* subcutaneous model. Cell populations in the region of the implanted tissues were stained for F4/80, a macrophage surface marker. Staining shows a correlation between dense cell regions and the F4/80 stain, indicating an inflammatory response. A dense line of macrophages is seen near the edge of the GA-treated tissue, while in the PPF reinforced and untreated samples, macrophages are seen in smaller regions throughout the sample. On the other hand, there was no significant F4/80 expression in day 0 samples **(A)** and samples lacking the primary antigen from week 6 **(B)**. A rat spleen stained by the same procedure is shown in **(C)** as a positive control. Scale bars represent 100 μm.

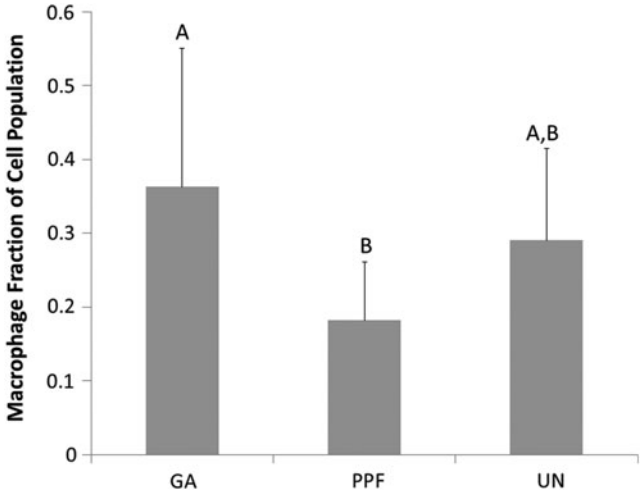

FIG. 12. Macrophage percent of cell population from an *in vivo* subcutaneous model. Macrophage population was investigated by examining three standardized images from each of $n=5$ samples per treatment group. Macrophage fraction was defined as the number of cells determined to have F4/80 staining divided by the total cells counted per image in a blinded study. Data are presented as an average of each of these fractions, and error was calculated as the standard deviation between all images in each group. Population fractions from the GA-treated samples and the PPF reinforced samples are significantly different, as determined by a one-way ANOVA test, followed by a *post hoc* Tukey's test ($p<0.05$). Groups that share the same letter are not statistically different.

samples in both the *in vitro* and *in vivo* models. The accumulated calcium on the PPF reinforced samples was in fact so low that it was not significantly higher than either the untreated samples or any of the unimplanted day 0 samples. This result suggests that the calcium deposits are not necessarily related to implanting xenographic tissue, but agrees with the theory that detrimental calcium deposition seen on GA-treated implants is related directly to the crosslinking of the tissue. By eliminating the crosslinking component, we have demonstrated an approach that could block calcification of implants before the unnatural healing mechanism starts.

As we have shown here, the process of dehydrating and coating the pericardium with the biocompatible polymer PPF interacts with the extracellular matrix in a physical and not chemical or otherwise transformative way. Histological images in Figure 10 display cellular infiltration from the edges of the samples, indicating that PPF reinforcement leaves the protein matrix suitable for cell viability. These observations can be directly compared to GA-treated samples, where little to no cells have migrated into the tissue. This difference suggests that PPF crosslinks into a network on the surface of the pericardium that is mechanically linked in the surface network of proteins and does not disturb the natural composition. This alternate outcome from the processing of pericardium may explain why the PPF reinforced pericardium appears to retain more of the original structure (Fig. 6), as compared to the dense protein packing in GA-treated tissue, as well as why it does not accumulate significant calcium deposits. Overall, the results presented support that the innovative and unprecedented combination of a synthetic polymer with a natural biomaterial may avoid the detrimental endpoints of GA-treated pericardium.

Conclusion

We have established for the first time, a hybrid material that preserves the natural properties of pericardium and adds support with a biocompatible polymer, while avoiding crosslinking of the tissue. We have shown that the pericardium composite is protected against enzymatic degradation by the paintable application of the polymer, and that the addition of this polymer causes less calcification than the GA-treated pericardium. Calcification of cardiovascular devices remains a forefront of concerns. The lower amount of calcium deposition on PPF reinforced pericardium is an important improvement when compared with the GA-treated calcium accumulation. These results suggest that PPF can be applied to reinforce pericardium instead of GA-treated tissue, which is habitually used despite its dangerous and inevitable failure. By eliminating crosslinking with GA, not only would this fate be avoided but also the probability of improved healing and maintenance of the injured or diseased state would be gained. A material with a lowered calcification rate would significantly improve the material options and revolutionize expectations for the extent of cardiovascular repair.

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Disclosure Statement

No competing financial interests exist.

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