Paper D24

Anticalcification treatment of glutaraldehyde fixed bovine pericardium with modified hyaluronic acid

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Abstract. Acellular bovine pericardium was treated with modified hyaluronic acid (HA-ADH) after glutaraldehyde fixation. The acellularization effect was demonstrated by H&E staining and SEM. The thermal stabilities of BP samples were characterized by measuring shrinkage temperature with DSC, and no significant deference was found between glutaraldehyde fixation bovine pericardium (GFBP) and glutaraldehyde fixation bovine pericardium treated with HA-ADH (GHBP). *In vivo* calcification tests of GHBP and GFBP were performed by implanted subdermally in male Wistar rats. The efficacy of GHBP for prevention of calcification was evaluated by SEM and ICP, and the results demonstrated that much less calcium deposition on GHBP than GFBP (0.2539±0.0771 comparing to 2.4371±0.54738 μgCa/ mg dry tissue after 8 weeks implantation). In addition, GHBP groups not only present a lower calcification degree, but also show lower Ca/P molar ratios, which correspond to amorphous calcium phosphates. The obtained results indicate that GHBP is a potential candidate for cardiac valve fabrication, since it can protecting them against calcification, and therefore, increase valve durability.

Keywords: Anticalcification; Modified hyaluronic acid; glutaraldehyde fixed bovine pericardium

1 Introduction

Glutaraldehyde fixation (GF) is the most common method of stabilizing biological tissue for the present generation of xenograft-based bioprosthetic heart valves. Although cross-linked with glutaraldehyde makes the biological tissue adequate for the manufacture of bioprostheses, late calcification after transplantation remains the most frequent cause of clinical failure and continues to be a great challenge until today.

Despite the clinical importance of the problem, the pathogenesis of calcification is incompletely understood. According to present studies, there are several currently accepted reasons resulting in calcification. One theory suggests that antigenicity due to cells, cellular components and proteins of xenogenic tissue may be the major factors contributing to the calcification of bioprosthetic heart valves [1]. Another mechanism of mineralization of GF

bioprostheses consists of attraction and precipitation of calcium upon lipid-based cell debris, in particular the acid phospholipids of cell membranes [2]. A further suggestion is that the formyl groups, which remain when the glutaraldehyde forms a mono-Schiff base, are involved in calcification [3, 4]. In addition, voids and cavities in the tissue, which created by the removal of cellular components and proteoglycans during processing, cellular degradation, or chemical treatment, afford a potential space for calcium phosphate depositing [5].

Hyaluronic acid (HA) is a natural linear polysaccharide composed of alternating disaccharide units of D-glucuronic acid and Nacetyl-D-glucosamine with β(1-4) interglycosidic linkage. HA is highly biocompatible and identical in all living organisms with a wide range of molecular weight from 1,000 to 10,000,000 Da. HA has unique viscoelastic and high water absorption properties, which makes it important for the lubrication and fill function among collagen fibers.

The aim of this study was to develop a better processing technique of GF bovine pericardium (BP) treated by chemically modified HA with effective anti-calcification, and to evaluate the anti-calcification potential. A reduction in calcification was anticipated through decreasing antigenicity by removing of cells, cellular components and acid phospholipids, inhibiting the calcificationinducing by capping of free aldehyde groups with modified HA, and filling up the voids and cavities in the tissue with HA.

2 Materials and methods

2.1 Materials

Bovine pericardium (BP) was obtained at a local slaughter house and stored in PBS (pH7.4, 50 mmol/L) at 4℃ immediately. After adherent fat was removed, the BP was cut into pieces (5×10 cm^2) for further processing.

Male Wistar rats (6-week-old, 180-220 g) were supplied by Animal Center of Jinan Medical Instruments Quality Surveillance and Inspection Centre (JiNan, China).

HA with a low molecular weight of 10,000 was donated by Shandong Freda Pharmaceutical Co., Ltd. (JiNan, China). The chemicals of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC), adipicdihydrazides (ADH), phenylmethyl-sulfonyl fluoride (PMSF), Triton X-100, Tris(hydroxymethyl)aminomethane (Tris), DNase and RNase were purchased from sigma. Glutaraldehyde (electron microscopy grade) was a 25% solution obtained from sigma. All other regents used in the paper were of analytical grade.

2.2 Synthesis of HA-ADH

HA-ADH was synthesized as described by Rachit Ohri and colleagues [6] with slight modifications. Briefly, 1g of HA was dissolved in 200 mL of wate. Twenty gram solid ADH was added to this solution and then mixed for 30 min. The pH of the reaction mixture was adjusted to 4.8 by the addition of 1 mol/L HCl. Then 1.8g EDC was added. The pH of the reaction mixture was maintained at 4.8 for 2h by the addition of 1 mol/L HCl. Subsequently, the reaction was stopped by raising the pH to 7.0 with 1 mol/L NaOH. The reaction mixture was

transferred to the prewashed dialysis tubing and dialyzed for 3 day, changing the medium once 12h. HA-ADH was recovered by ethanol precipitation from the solution. For further purification, HA-ADH was dissolved in water and then precipitated again with ethanol.

2.3 Cell extraction

The procedure used to remove the cellular components from bovine pericardia was based on a method developed by Courtman *et al.*[5]. The BP were initially immersed in a hypotonic tris buffer (pH 8.0) that contained a protease inhibitor (PMSF, 0.35mg/L) for 24 h at 4°C with constant stirring. Subsequently, they were immersed in a 1% solution of Triton X-100 in tris-buffered salt solution (10mmol/L , pH8.0) with protease inhibition for 24 h at 4°C with constant stirring. Samples then were thoroughly rinsed in Hanks' physiological solution (pH7.5) and digested with DNase (50U/mL) and RNase (1 U/mL) at 37°C for 1 h. They then underwent a further 24 h of extraction with Triton-X 100 in tris buffer. Finally, all samples were washed for 48 h in Hanks' solution.

The samples of cellular and acellular tissues were selected, stained using H&E staining and examined under an optical microscope.

2.4 Modification of GF acellular bovine pericardium

The sample was prepared as shown in Fig. 1. The acellular BPs were placed in 0.625% glutaraldehyde in 0.05M PBS (pH7.4), and transferred after 3 days to 0.3% glutaraldehyde in the same buffer and stored at 4°C for 2 weeks prior to use. The GFBPs were washed in distilled water to remove residual glutaraldehyde and used for the HA-ADH treatment. The GFBPs then were incubated in 1% (w/v) HA-ADH in 0.05M PBS (pH 7.4) at room temperature for 48h with constant stirring. The sample of GFBP with HA-ADH treatment was obtained (GHBP).

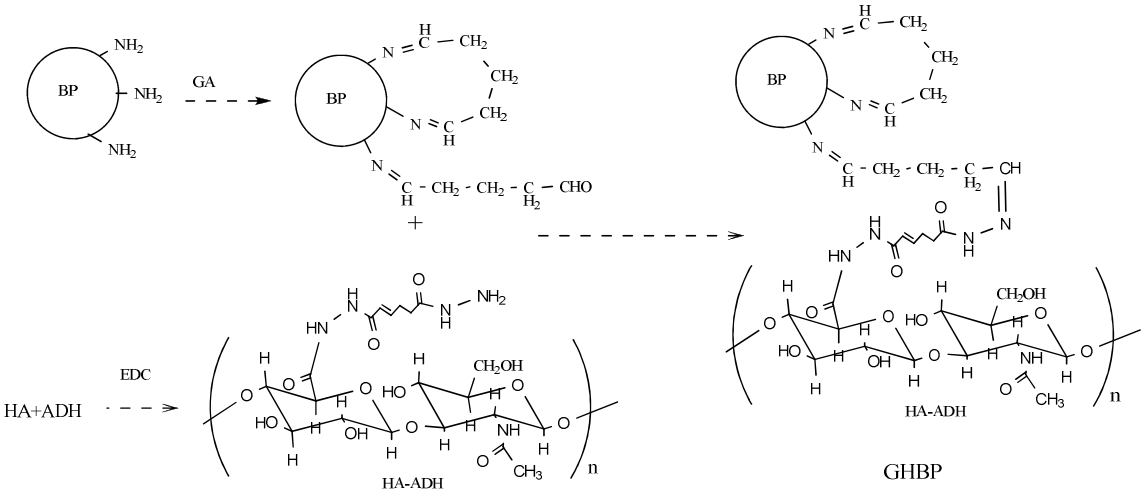

Fig.1 Schematic representation for the preparation of GHBP

2.5 Thermal stability

Shrinkage temperatures were measured for all samples using a differential scanning calori meter (DSC, 200PC, Netzsch, Germany). Samples were scanned in aluminium pans in nitrogen atmospheres, at 30–100 °C range.

2.6 Subdermal implantation in rats

In vivo calcification was studied using a rat subcutaneous implantation model. All rats were anesthetized with sodium thiopentone (30 mg/kg) by the intraperitoneal injection. Eighteen samples of bovine pericardium from each group measuring 1.0 x 1.0 cm were implanted at dissected subcutaneous sites in the dorsal region of the rats. The implanted samples were retrieved at 4, 6 and 8 weeks postoperatively (n=6 rats) and were used for measurement of calcium content and morphology examination.

2.7 Measurement of calcium and phosphorus content

The retrieved tissue patches were rinsed with distilled water, dried at 105°C to constant weight, then HNO_3 and $HClO_3$ (10:1) were added to dried tissue patches and incubated on a electric platen to hydrolyse absolutely. The amount of calcium and phosphorus were determined by inductively coupled plasma (ICP, Thermo Fisher Scientific iCAP 6000) in Central Laboratory, School of Public Health, Peking University. The amount of calcium and phosphorus were expressed as μg calcium per mg dried tissue weight. Data are reported as mean ± standard error. One-way analysis of variance (ANOVA) was used to compare changes in calcium content, in different groups. All statistical analyses were accomplished using analysis software of Origin 8.0 (OriginLab, US).

2.8 Scanning electron microscopy

BP Samples of before and after implantation, approximately 0.2 cm in diameter, equilibrated in PBS were washed clearly with deionized water to remove excess salt. After freeze dried, photomicrographs were obtained with a JEOL JSM-7600F electron scanning microscope operating at 3.0 kV after sputter coating with gold.

3 Results and discussion

3.1 Ultrastructures of the cellular and acellular samples

The result of H&E staining of cellular and acellular BP are shown in Fig.1(a, b). The BP displayed numerous intact cells embedded within the connective tissue matrix before cell extraction, while the acellular BP revealed an intact connective tissue matrix with no evidence of cells or cell organelles. SEM images (Fig.1(c, d)) also revealed significant differences in the ultrastructures morphologies of the cellular and acellular BP. Fig.1 (d) showed a typical fibrous connective tissue making up of an organized network of collagen fibers with transverse stripe structure. In contrast, there were numerous cells or cellular components in cellular BP, and the transverse stripe structure of collagen fibers was not as clear as the acellular BP.

3.2 Thermal stability

Several reports have utilized DSC as an alternative method to determine the shrinkage

temperature or denaturation temperature of tanned biological tissues or leather [7, 8]. DSC has obvious advantages compared with the hydrothermal method. With DSC, shrinkage of biological tissue is accompanied by the absorption of heat, giving rise to an endothermic peak over its shrinkage temperature range. The temperature of the transition can be defined as the peak maximum.

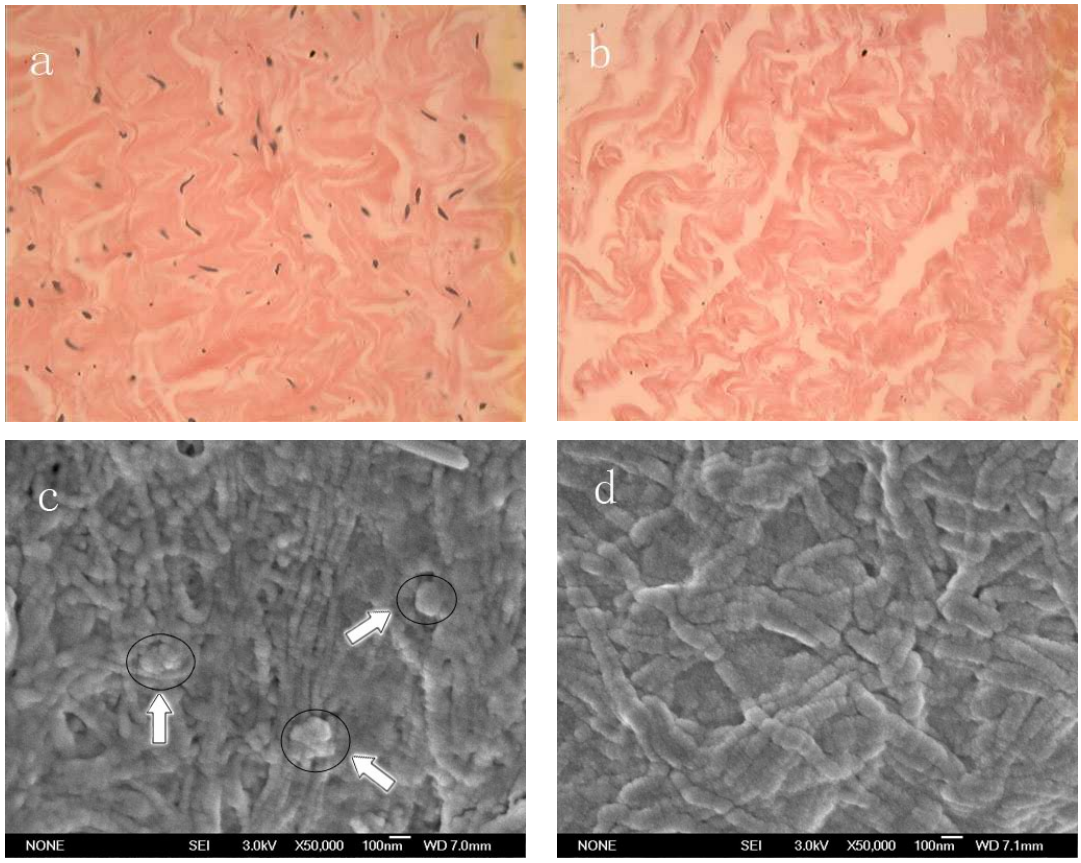

Fig. 1 HE staining (a, b; ×200 magnification), and SEM micrographs (c, d; ×50,000 magnification) of cellular (a, c) and acellular (b, d) BP.

The results of shrinkage temperature measured by DSC were showed in Fig.2. No significant deference in shrinkage temperature of GFBP treated with HA-ADH (84.7°C) was observed comparing with GFBP (84.9°C). Therefore, the effect of HA-ADH treatment on shrinkage temperature was slight.

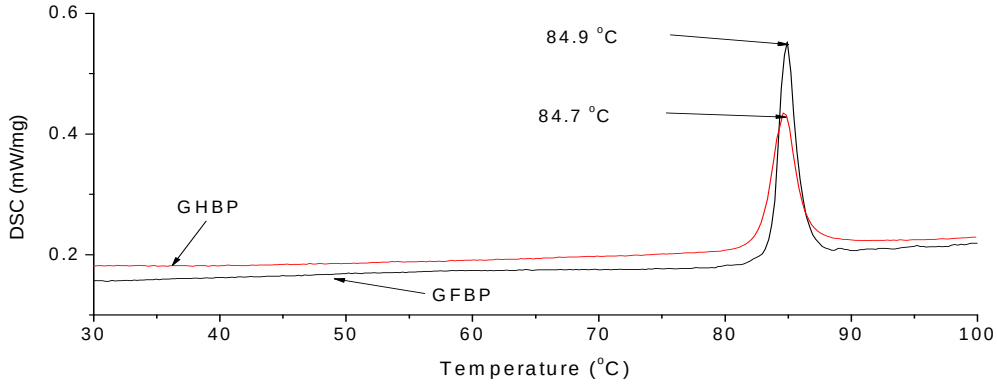

Fig. 2. DSC curves of GFBP and GHBP

3.3 In vivo calcification

In vivo calcification was measured using a rat subcutaneous implantation model. The GFBP and GHBP were implanted in male rats for 4, 6, 8 weeks. Calcium contents of retrieved matrices measured by ICP were showed in Fig.3. The significant differences ($p<0.01$) were found between the control GFBP and the GHBP group after 4, 6, 8 weeks implantation. In fact, the calcium contents of GHBP group were around 10 times lower than that of the GFBP group after 8 weeks implantation (0.2539±0.0771 comparing to 2.4371±0.54738 μgCa/ mg). In addition, after 8 weeks implantation, GFBP group increase calcium content 2-fold than 4 weeks (1.386±0.0542 to 2.4371±0.5473 μgCa/ mg), while no significant differences ($p>0.05$) were found among GHBP group with the increase of implantation time. It may be hypothesized that the treatment of HA-ADH can fill the voids and cavities among collagen fibers, blocks the potential binding sites, and thus makes the process impermeable to host-plasma calcium [9], therefore lead to the decrease of calcium content.

Fig. 4 shows the phosphorous content in different groups pericardium samples (GFBP and GHBP) retrieved after 4 weeks, 6 weeks and 8 weeks implantation. After 4 weeks implantation, GHBP group (0.9313±0.1934) showed similar phosphorous content compared to the GFBP group (0.8943±0.0215). The phosphorous content increased significantly ($p< 0.01$) in the GFBP group at both implantation times (6 weeks and 8weeks) compared to 4 weeks. However, when phosphorous content of GHBP group was analyzed, 6 weeks and 8 weeks presented a much various change. After 6 weeks implantation, the phosphorous content of GHBP (1.3850±0.3119) was mach higher ($p< 0.05$) than that of 4 weeks (0.9313±0.1934). In contrast, after 6 weeks implantation the phosphorous content decreased to 0.8320±0.2810 ($p< 0.01$).

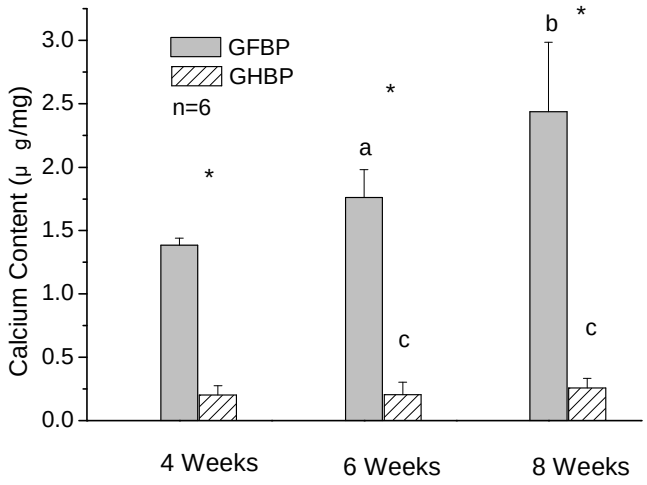

Fig.3 Calcium content (μg Ca/ mg of dry tissue) in BP samples after 4, 6, 8 weeks implantation Data represented as mean ±standard error. * Significantly deferent between GFBP and GHBP at each implantation time ($p< 0.01$). a: Significantly deferent compared to GFBP at 4 weeks ($p< 0.01$). b: Significantly deferent compared to GFBP at 4 weeks ($p< 0.05$).c: No significantly deferent compared to GHBP at 4 weeks ($p> 0.05$).

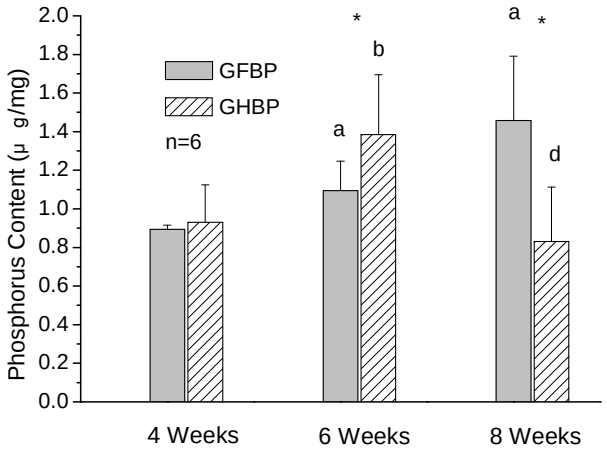

Fig.4 Phosphorus content (μg P/ mg of dry tissue) in BP samples after 4, 6, 8 weeks implantation Data represented as mean ±standard error. * Significantly deferent between GFBP and GHBP at each implantation time ($p< 0.01$). a: Significantly deferent compared to GFBP at 4 weeks ($p< 0.01$). b: Significantly deferent compared to GHBP at 4 weeks ($p< 0.05$). d: Significantly deferent compared to GHBP at 6 weeks ($p< 0.01$)

3.4 Characterization of calcium deposits in implanted BP

Table 1 shows the Ca/P molar ratios in BP samples after 4, 6, 8 weeks implantation. Ca/P molar ratios in GFBP groups changed slightly (from 1.2 to 1.3). According to previous studies [10, 11], these values could correspond to brushite (Ca/P=1; $CaHPO_4.2H_2O$) or to octacalcium

phosphate [Ca/P=1.33; $Ca_8(PO_4)_6H_2.5H_2O$], that maybe transient precursor phases in the formation of apatite deposits (Ca/P=1.67; $Ca_5(PO_4)_3OH$). Ca/P molar ratios in GHBP groups were lower significantly ($p<0.01$) (0.1699±0.0605, 0.1191±0.0643 and 0.2578±0.0981 after 4 weeks, 6 weeks and 8weeks implantation, respectively) compared to GFBP. These calcium deposits must correspond to amorphous calcium phosphates.

Table 1 Ca/P molar ratios in BP samples after 4, 6, 8 weeks implantation.

Groups	Ca/P (molar ratio)		
	4 Weeks	6 Weeks	8 Weeks
GFBP	1.1976±0.0265	1.2447±0.0503	1.2933±0.0664
GHBP	0.1699±0.0605*	0.1191±0.0643*	0.2578±0.0981*

* Significant differences ($p<0.01$) were found between the GHBP group and the GFBP group after 4 weeks, 6 weeks and 8 weeks implantation.

3.5 Scanning electron microscopy

SEM micrographs of bovine pericardium samples after in vivo calcification tests (6 weeks) are exhibited in Fig. 5. Both GFBP and GHBP all presented deposits of calcium phosphate in collagen fibers. However, the size of calcium deposits in GFBP was obviously larger than that in GHBP. The SEM results were accord with the data of calcium content measurement.

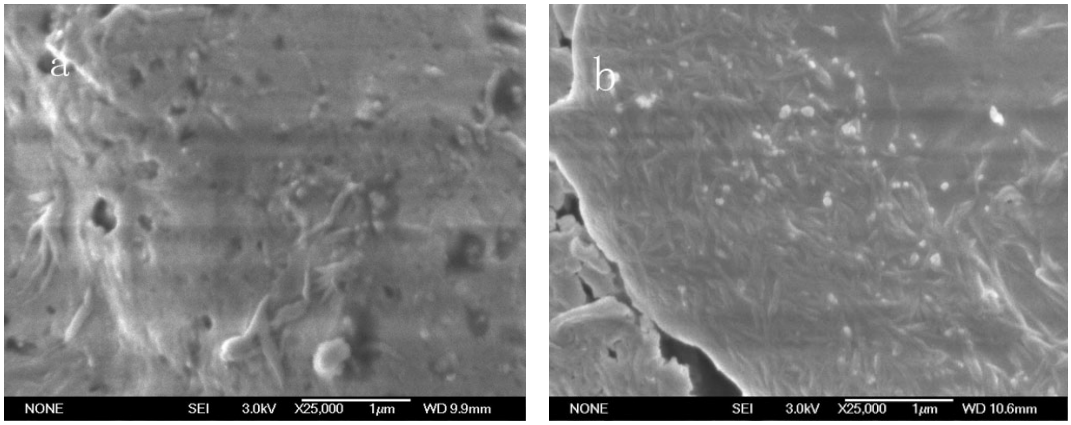

Fig.5 Scanning electron micrographs of GFBP (a) and GHBP (b) after in vivo calcification (6 weeks). ×25,000

4 Conclusions

In the study, a novel method for the preparation of glutaraldehyde fixation bovine pericardium was investigated. Cellular BP was cross-linked with glutaraldehyde and treated with HA which was modified by coupling ADH on $-COO^-$. The thermal stabilities were measured using DSC, and the results showed no significant deference between GFBP and GHBP. In vivo calcification was studied using a rat subcutaneous implantation model. From the results of calcium content measurement and SEM, calcium deposited on GHBP was much less than those of GFBP. The results of Ca/P molar ratios indicated that the characterization of

calcium deposits between GFBP and GHBP were different evidently. The calcium deposits in GFBP were crystalline, but amorphous in GHBP. Based upon the obtained results, the GFBP has the potential to be used for anticalcification and biocompatible matrix patches for cardiovascular applications.

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