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Anti-calcification of Bovine Pericardium for Bioprosthetic Heart Valves after Surface Modification with Hyaluronic Acid Derivatives

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Abstract Surface modification of glutaraldehyde fixed bovine pericardium (GFBP) was successfully carried out with hyaluronic acid (HA) derivatives. At first, HA was chemically modified with adipic dihydrazide (ADH) to introduce hydrazide functional group into the carboxyl group of HA backbone. Then, GFBP was surface modified by grafting HA-ADH to the free aldehyde groups on the tissue and the subsequent HA-ADH hydrogel coating. HA-ADH hydrogels could be prepared through selective crosslinking at low pH between hydrazide groups of HA-ADH and crosslinkers containing succinimmidyl moieties with minimized protein denaturation. When HA-ADH hydrogels were prepared at low pH of 4.8 in the presence of erythropoietin (EPO) as a model protein, EPO release was continued up to 85% of total amount of loaded EPO for 4 days. To the contrary, only 30% of EPO was released from HA-ADH hydrogels prepared at pH=7.4, which might be due to the denaturation of EPO during the crosslinking reaction. Because the carboxyl groups on the glucuronic acid residues are recognition sites for HA degradation by hyaluronidase, the HA-ADH hydrogels degraded more slowly than HA hydrogels prepared by the crosslinking reaction of divinyl sulfone with hydroxyl groups of HA. Following a two-week subcutaneous implantation in osteopontin-null mice, clinically significant levels of calcification were observed for the positive controls without any surface modification. However, the calcification of surface modified GFBP with HA-ADH and HA-ADH hydrogels was drastically reduced by more than 85% of the positive controls. The anti-calcification effect of HA surface modification was also confirmed by microscopic analysis of explanted tissue after staining with Alizarin Red S for calcium, which followed the trend as observed with calcium quantification.

Keywords: anti-calcification, bioprosthetic heart valve, bovine pericardium, hyaluronic acid, crosslinking, hydrogels, protein delivery

INTRODUCTION

Pathologic calcification is the leading cause of the clinical failure of glutaraldehyde fixed bovine pericardium (GFBP) used for bioprosthetic heart valves [1]. Bioprosthetic heart valve calcification is a multi-factorial process with several causative mechanisms, including glutaraldehyde fixation of the bioprosthetic valve biomaterial, Ca^{2+} influx into fixed, non-viable cells, and so on [2,3]. Anti-calcification strategies, in the realm of bioprosthetic valves, have focused on the inhibition of the processes leading to calcification, such as non-glutaraldehyde preservation, inhibition of Ca^{2+} diffusion, and inhibition of crystal nucleation [1,4]. The anti-calcification and anti-degeneration roles of endogenous hyaluronic acid (HA) and other glycosaminoglycans (GAG) in the extracellular matrix (ECM) of bioprosthetic valve materials were recently reported by Vyavahare and colleagues [5,6]. It has also been reported that HA binds Ca^{2+} ion [7,8] and is modulated in its biological activities as a result [9,10].

HA is a natural linear polysaccharide composed of alternating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine with β(1→4) interglycosidic linkage [11]. Sodium hyaluronate, sodium salt of HA, is biodegradable, biocompatible, and viscoelastic with a wide range of molecular weight from 1,000 to 10,000,000 Da. HA is the only non-sulfated GAG which is abundant in synovial fluid and ECM [11]. HA acts to control tissue hydration and is present in hydrated networks with collagen fibers in the ECM [12]. It also constitutes the backbone of cartilage proteoglycan [13]. HA plays pivotal role in wound healing, and in promoting cell motility and differentiation during development [14]. HA has unique viscoelastic properties, which makes it important for the lubrication function of the synovial joint fluid and vitreous humor in the eye [15,16]. A number of strategies for the modification of HA through carboxyl and hydroxyl groups have been developed [17-19]. Chemical modifica-

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tion of HA provides tools for its application as a biomaterial in medicine. Because of its various functions and physicochemical properties, HA and modified HA have been extensively investigated and used for treatment of arthritis [15], ophthalmic surgery [16], drug delivery [19-21], and tissue engineering [22].

The purpose of the present study was to evaluate the anti-calcification potential of surface modified GFBP with HA derivatives. A reduction in biomaterial calcification was anticipated *via* inhibition of the calcification-inducing processes, namely *via* capping of free aldehyde groups, Ca^{2+} sequestration by HA, and HA-mediated inhibition of inflammatory response [23]. At first, GFBP was surface modified with adipic dihydrazide (ADH) grafted HA and then coated with HA-ADH hydrogels. The characteristics of HA-ADH hydrogels were compared with HA hydrogels crosslinked with divinyl sulfone in terms of degradation by hyaluronidase (HAse) and protein drug delivery. The osteopontin-null *in vivo* calcification model mice were used to evaluate the surface modified GFBP with HA derivatives. Previous work by Steitz *et al.* in our group showed that significantly higher calcification levels of glutaraldehyde-fixed aortic valve (GFAV) tissue were obtained following subcutaneous implantation in osteopontin-null mice, as compared to wild-type counterparts [24]. The degree of calcification after implantation of surface modified GFBP with HA-ADH and HA-ADH hydrogels was determined colorimetrically *via* the *o*-cresolphthalein complexone method.

MATERIALS AND METHODS

Materials

GFBP was kindly provided from Edwards Life Sciences Inc. (Irvine, CA, USA) and sodium salt of high molecular weight HA from Genzyme (Boston, MA, USA). HA with a low molecular weight of 25,000 was purchased from Denkikagaku Kogyo Co. (Tokyo, Japan). The chemicals of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC), ADH, and ethanol were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The crosslinker for HA hydrogel preparation, polyethyleneglycol-succinimidyl butanoate (PEG-SBA), was kindly supplied from NEKTAR (San Carlos, CA, USA), bis(sulfosuccinimidyl) suberate (BS^3) from Pierce (Rockford, IL, USA), and divinyl sulfone from Aldrich. Dialysis kit with MW cut off of 3,500 was purchased from Pierce. For punching circular sections of the bovine pericardium tissue, sterile 8-mm biopsy punches were obtained from Miltex Instrument Co. Inc. (Lake Success, NY, USA). Alizarin Red S and the kit for the colorimetric determination of calcium quantification by the *o*-cresolphthalein complexone method, and bovine testicular HAse were obtained from Sigma (St. Louis, MO, USA).

HA-ADH Synthesis

HA-ADH was synthesized as described elsewhere [1,21]. The preparation protocol was slightly modified for direct HA-ADH recovery by ethanol precipitation from HA-ADH solution [1]. The degree of substitution by ADH was determined by ^{1}H-NMR analysis [25].

HA Hydrogel Synthesis with DVS

One mmol of HA was dissolved in 10 mL of 0.2 N NaOH solution (pH=13). After stirring for 30 min, DVS was added into the HA solution [17,23]. The final molar ratio of HA to DVS was 8:5. A strong gel was formed within 30 min. The hydrogel was allowed to react completely for 1 h before the hydrogel was neutralized with 0.2 N HCl and washed with phosphate buffer solution (PBS, pH=7.4, 10 mM).

HA-ADH Hydrogel Synthesis

HA-ADH (100 mg, MW=25,000) with ADH modification of *ca.* 70% was dissolved for 2 h in 660 μL of distilled water. Then, 100 μL of acetate buffer (pH=4.8, 100 mM) and 200 μL of EPO (5 mg/mL in PBS) were added to the solution. After mild mixing for 30 min, 192 μg of the solution was inserted to head-cut syringe. As a specific crosslinker to hydrazide at low pH, 8.0 mg of BS^3 (MW=572.4) was dissolved in 40 μL of water and 8 μL of its solution was added to each syringe containing HA-ADH solution. The final precursor solution was mixed with a needle and incubated at 37°C to complete the crosslinking reaction for HA-ADH hydrogel preparation. The amount of BS^3 added was 10 mol% of ADH groups in HA repeating units. In a similar way, HA-ADH hydrogels were also prepared at different pH of 7.4 in PBS.

In vitro Release Test of EPO

In vitro release tests of EPO from two different HA-ADH hydrogels (triplicate test samples) prepared at pH=4.8 and 7.4 were carried out in 2 mL of PBS (pH=7.4, 10 mM) at 37°C using a shaking incubator. After sampling 200 μL of supernatant at the pre-determined time intervals, 200 μL of fresh PBS was added to the vial. The released amount of EPO was quantified by RP-HPLC analysis.

Degradation of HA Hydrogels with Hyaluronidase

Ten milligrams of HA hydrogel was added to 10 mL of PBS containing 100 U HAse. At pre-determined time intervals, 0.5 mL of supernatant was sampled with the subsequent addition of 0.5 mL of fresh PBS. The degradation of the hydrogels was determined by measuring the amount of glucuronic acid released from HA hydrogels after degradation with HAse through the carbazole assay [26].

Surface Modification of Bovine Pericardium

Fresh tissue of circular section (8 mm diameter) of

GFBP was dipped into one percent HA-ADH solution (MW of HA=2 million). After a day, the tissue was recovered, washed with PBS, sonicated for 30 min, and then washed again with PBS. Finally, the tissue was kept in PBS before use. Meanwhile, HA-ADH hydrogel coating was carried out as follows. HA-ADH (20 mg) was dissolved for 2 h in 1.8 mL of PBS (pH=7.4). Then, GFBP was dipped into HA-ADH solution for HA-ADH grafting. The next day, 0.2 mL of PEG-SBA solution in PBS (20 mg/mL) was added as a crosslinker for HA-ADH hydrogel preparation. After incubation for 30 min, GFBP was taken out from the precursor solution and incubated for additional one hour at room temperature. HA-ADH crosslinking reaction with PEG-SBA was completed in an hour. HA-ADH hydrogel coated GFBP was washed with PBS several times for the complete removal of the remaining PEG-SBA. Finally, the tissue was kept in PBS before implantation.

Subcutaneous Implantation in Osteopontin-null Mice and Explantation

Swiss-Black osteopontin-null mice were generated as described before by Liaw *et al.* [27]. The knock-out mice litters were generated by inbreeding osteopontin knock-out parents. Genotyping was performed using polymerase chain reaction (PCR). Female mice between 5 and 6 weeks of age were used for subcutaneous implantation of surface modified GFBP with HA derivatives. They were housed and used in specific-pathogen-free (SPF) facilities according to the protocol approved by the Institutional Animal Care Use Committee (IACUC). The mice were anesthetized by injecting them with a mixture of xylazine (0.01 mg/g mouse) and ketamine (0.15 mg/g mouse) in saline solution. Circular sections (8 mm diameter) of the GFBP tissue with and without surface modification were implanted subcutaneously in the backs of the mice (two tissue sections per animal). Three groups of the osteopontin-null mice were used for the implantation. The first group was implanted with the HA-ADH grafted GFBP, another group with HA-ADH hydrogel coated GFBP, and the other group with untreated GFBP. After 2 weeks, euthanasia was performed on the mice by injecting an overdose of nembutal prior to explantation of the bovine-pericardium. Following explantation, the circular tissue sections were cut in half for calcium quantification and histological staining, respectively. NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) have been followed.

Analysis for Calcium Quantification

The calcium content of the explanted GFBP tissue was determined colorimetrically *via* the *o*-cresolphthalein complexone method. The reaction of calcium with *o*-cresolphthalein in alkaline medium produces a red complex with an absorbance maximum at 575 nm and the intensity of the color measured at 575 nm is directly proportional to the calcium concentration in the sample. The explanted tissue was lyophilized overnight, weighed and subsequently decalcified by incubating in 0.6 N HCl at 37°C for 24 h. The calcium content of the 0.6 N HCl supernatant was then determined using the Sigma kit. The total calcium content of each tissue section was normalized to its dry weight.

Alizarin Red S Staining for Calcium

The 2-week explants were fixed with methyl carnoys solution (methanol:acetic acid=3:1) and embedded in paraffin. Alizarin Red S staining was used to visualize calcification in the 5-μm thick sections [1].

RESULTS AND DISCUSSION

Surface Modification of GFBP with HA Derivatives

HA is a biodegradable, biocompatible, non-immunogenic, and non-inflammatory natural polysaccharide which can be used for surface modification of various artificial tissues [11]. Fig. 1 shows the schematic representation of surface modification of GFBP by grafting high molecular weight HA-ADH on the surface of the tissue and the subsequent crosslinking with PEG-SBA for HA-ADH hydrogel coating. At first, HA was chemically modified by introducing ADH into the carboxyl group of HA backbone. With increasing degree of ADH modification, the degradation of HA has been reported to be proportionally reduced, since the carboxyl groups on the glucuronic acid residues are recognition sites for HA degradation by HAse [11,28]. The degree of HA-ADH modification was determined by ^{1}H-NMR analysis. The peak assignment for HA-ADH was carried out according to Pouyani *et al.* [25]. The hydrazide content in HA-ADH could be controlled by adjusting the molar equivalent ratio of HA to EDC and the highest degree of HA-ADH modification was about 80%. After HA-ADH synthesis, its grafting to the aldehyde groups on GFBP was successfully carried out by hydrazone bond formation *via* the Schiff base reaction. This bond is relatively stable compared to the easily reversible Schiff base interaction of an amine with an aldehyde [29], thus obviating the need for an additional reduction step for stabilization. Subsequently, HA-ADH grafted GFBP was successfully coated with HA-ADH hydrogel. The crosslinking reaction between HA-ADH and PEG-SBA was completed in an hour. During HA-ADH hydrogel coating for the surface modification of GFBP, anti-calcification proteins, such as osteopontin, may be encapsulated for their controlled release from the HA-ADH hydrogels.

Characteristics of HA-ADH Hydrogels

Two different HA hydrogels were prepared and compared; HA hydrogels crosslinked with DVS and HA-ADH hydrogels crosslinked with bis-succinimmidyl derivatives. At first, HA hydrogels were successfully prepared by the crosslinking reaction of DVS with hydroxyl groups of HA. It was observed that, as soon as the crosslinking reagent

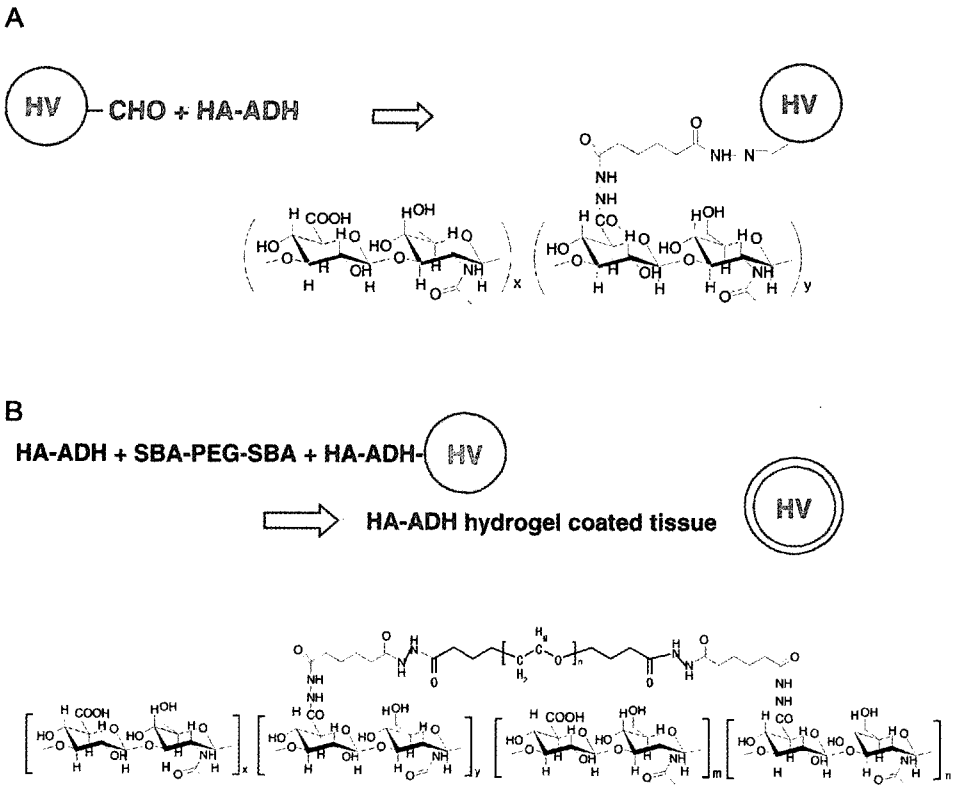

Fig. 1. Schematic representation for A, HA-ADH grafting to glutaraldehyde fixed bovine pericardium (GFBP) and B, HA-ADH hydrogel coating on GFBP.

of DVS was added to the HA solution, the solution became viscous and hydrogels started to form within 5 min. The crosslinking reaction was completed within 15 to 30 min. However, sensitive molecules such as protein drugs could not be loaded *in situ* during HA hydrogel preparation, as the polymer network hydrogels were prepared in highly alkaline solution, which could lead to protein denaturation [17,21]. In addition, DVS may cause the denaturation of protein, too. In order to encapsulate proteins in HA hydrogels, we investigated the specific reactivity of succinimmidyl to hydrazide, as the difference in pKa between amine group (pKa>9) of protein and hydrazides (pKa=3.0) of HA-ADH may contribute to their selective crosslinking at low pH without protein denaturation. Fig. 2 shows *in vitro* release of EPO (model protein) from HA-ADH hydrogels prepared at different pHs. When HA-ADH hydrogels were prepared at low pH of 4.8 in the presence of EPO, EPO release was continued up to 85% of total amount of encapsulated EPO for 4 days. To the contrary, only 30% of EPO was released from HA-ADH hydrogels prepared at pH of 7.4, which might be due to the denaturation of EPO during the crosslinking reaction. From these results, we could confirm the selective reactivity of succinimmidyl to hydrazide rather than amine group at low pH.

The degradation characteristics of two different HA hydrogels were also investigated. As shown in Fig. 3, HA-DVS hydrogels were degraded rapidly, but HA-ADH

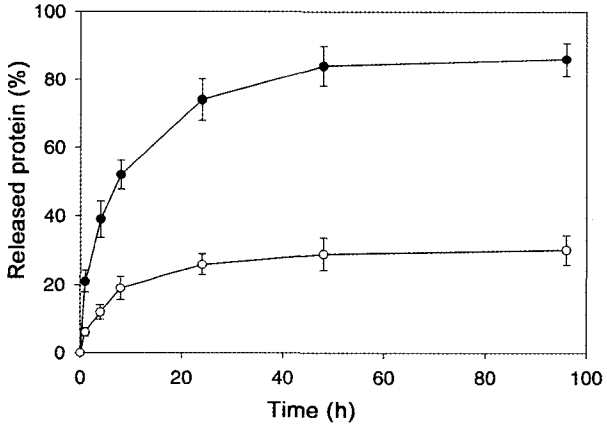

Fig. 2. *In vitro* release of a model protein from selectively crosslinked HA-ADH hydrogels at two different pHs. (●) pH=4.8, (○) pH=7.4.

hydrogels degraded relatively slowly. Although the degradation rate of HA hydrogels depended on the amount of added HAse, we could observe the relative stability of HA-ADH hydrogels compared with HA-DVS hydrogels. The results confirmed that the chemical modification on the carboxyl group of HA contributed to the slow degradation of HA hydrogels, since the carboxyl groups on the glucuronic acid residues are recognition sites for HA deg-

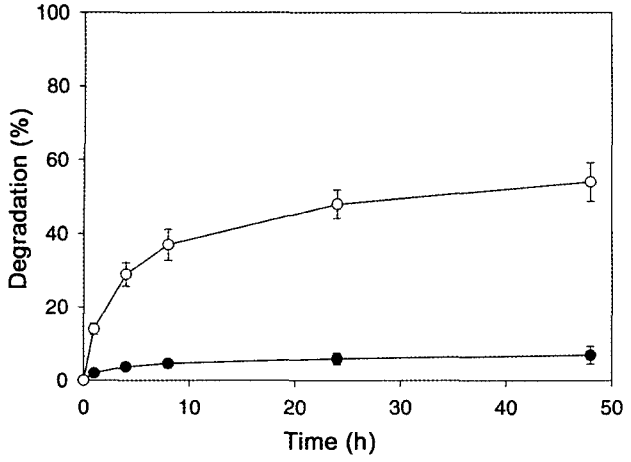

Fig. 3. Degradation of two different HA hydrogels with bovine testicular hyaluronidase (10 U/mL). (●) HA-ADH hydrogel crosslinked with PEG-SBA, (○) HA hydrogel crosslinked with divinyl sulfone.

radation by HAse [11,28]. According to Bulpitt *et al.*, HA-ADH hydrogels with 25% of ADH modification degraded with an eight fold faster rate than that with 65% of ADH modification. The result shows that the degree of HA modification critically affects the sensitivity of the hydrogels to enzymatic degradation [28].

Effect of Surface Modification of GFBP on the Calcification

The anti-calcification potential of surface modified GFBP with HA derivatives was evaluated by subcutaneous implantation in osteopontin-null mice as *in vivo* calcification test model. Steitz *et al.* in our group previously established that significantly higher calcification level of glutaraldehyde-fixed aortic valve tissue was obtained following subcutaneous implantation in osteopontin-null mice, compared to wild-type counterparts [24,30]. Using the osteopontin-null mice, the subsequent study showed that clinically significant levels of calcification were obtained for GFBP tissue after implantation for 7 days or longer. Based on these results, the osteopontin-null *in vivo* calcification model was used for the present study. A drastic reduction in calcification higher than 85% was observed for the surface modified GFBP with HA-ADH and HA-ADH hydrogels crosslinked with PEG-SBA, compared to untreated control tissue after 2-week implantation (Fig. 4). HA-ADH hydrogels showed slightly higher anti-calcification effect. In addition to mitigating effect of HA-ADH hydrogels on tissue calcification, it is worth noting here the opportunity to pursue controlled release of anti-calcification biomolecules from the hydrogels. As a model protein, EPO could be released up to 85% of the loaded amount from HA-ADH hydrogels without any denaturation (Fig. 2). Controlled release matrices made of HA present several advantages such as biocompatibility, biodegradability, and lack of immunogenicity [11]. Based on the *in vitro* release test of EPO, *in vitro* and *in vivo* release study of natural

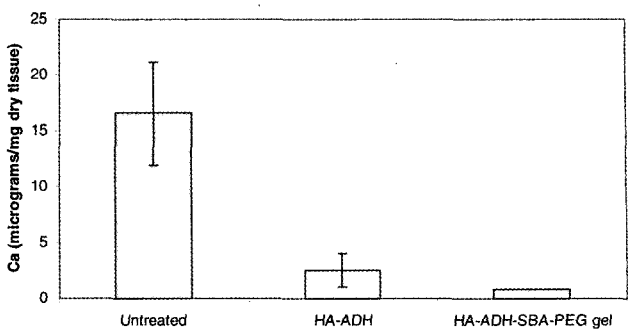

Fig. 4. Anti-calcification of glutaraldehyde-fixed bovine pericardium (GFBP) after surface modification with HA derivatives. Explants were obtained on day 14. The number of mice used was : $n=7$ for untreated control, $n=5$ for HA-ADH grafted GFBP, and $n=3$ for HA-ADH hydrogel coated GFBP.

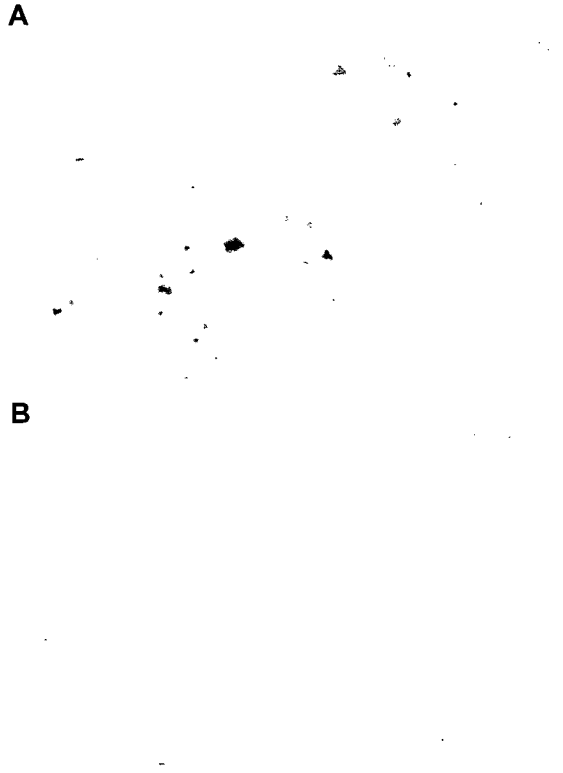

Fig. 5. Microscopic observation of glutaraldehyde-fixed bovine pericardium after Alizarin Red S staining for calcium. Explants were obtained on day 14. A, Control, without any surface modification B, After surface modification with HA-ADH hydrogels.

biomolecular inhibitors of calcification, such as osteopontin, will be carried out in near future. The reduction in calcification for the HA-modified GFBP was also confirmed by Alizarin Red S staining (Fig. 5). The Alizarin Red S staining followed the trend as observed with calcium quantification.

This study showed the inhibition of calcification by

surface modification of GFBP with HA derivatives. The remaining free aldehyde groups on the surface of the tissue after glutaraldehyde fixation are known to be one of the main cause for calcification [2,3]. At the same time, HA-induced Ca^{2+} chelation and HA-induced inhibition of an inflammatory/immunogenic response may also contribute to the anti-calcification effect. Although it is not clear to explain, therefore, the mechanism of the observed anti-calcification effect could be ascribed to some intrinsic properties of HA as well as the capping of free aldehyde groups on the tissue. The relevance of endogenous GAGs in preventing the degeneration and calcification of bioprosthetic valve material has been well established in recent work by Vyavahare *et al.* [5,6]. Inhibition of calcification following surface-grafting of GAGs, such has heparin, has also been demonstrated in a recent study [31].

CONCLUSION

A novel surface modification of GFBP for bioprosthetic heart valves was developed by grafting HA-ADH on the surface of the tissue and the subsequent crosslinking with PEG-SBA for HA-ADH hydrogel coating. Chemical modification of HA through the carboxyl group appeared to cause the delayed degradation of HA, because the carboxyl groups on the glucuronic acid residues are recognition sites for HA degradation by HAse. We could confirm the selective crosslinking at low pH between HA-ADH and crosslinkers containing succinimmidyl moieties with minimized protein denaturation. When HA-ADH hydrogels were prepared at low pH of 4.8 in the presence of EPO, EPO release was continued up to 85% of total amount of loaded EPO for 4 days. Following a two-week subcutaneous implantation in osteopontin-null mice, the calcification of surface modified GFBP with HA-ADH and HA-ADH hydrogels was drastically reduced by more than 85%, when compared to the control. The anti-calcification effect of HA surface modification was also confirmed by microscopic analysis of explanted tissue after staining with Alizarin Red S for calcium. Based on this study, controlled release of osteopontin from HA-ADH hydrogels and its effect on tissue calcification will further be investigated.

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