Polyethylene glycol-grafted bovine pericardium: a novel hybrid tissue resistant to calcification

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Calcification is a frequent cause of the clinical failure of bioprosthetic heart valves fabricated from glutaraldehyde pretreated bovine pericardium (GATBP). An investigation was made of the grafting of different molecular weight polyethylene glycol (PEG 600, 1500, 4000 and 6000) via glutaraldehyde (GA) linkages to bovine pericardium (BP) and of their stability and calcification. The process of the calcification profile was studied by *in vitro* experiments via incubating pericardial samples in a metastable solution of calcium phosphate. Calcification of bovine pericardium grafted with PEG 6000 was significantly decreased compared to low molecular weight PEG grafts or Sodium dodecyl sulphate- (SDS) and GA-treated tissues. The mechanical properties of these modified tissues after enzyme (Trypsin) digestion and calcification were investigated. The biocompatibility aspects of grafted tissues were also established by monitoring the platelet adhesion, octane contact angle and water of hydration. PEG 6000-grafted tissues retained the maximum strength in trypsin buffer and calcium phosphate solutions. Scanning electron micrographs revealed that the PEG-grafted bovine pericardium had substantially inhibited the platelet–surface attachment and their spreading. It is conceivable that high molecular weight polyethylene glycol-grafted pericardium (a hybrid tissue) may be a suitable calcium-resistant material for developing prosthetic valves due to their stability and biocompatibility.

1. Introduction

Tissue degeneration associated with aggressive calcification strongly limits the durability of bioprosthetic valves manufactured using glutaraldehyde fixed bovine pericardium especially in younger patients [1]. Tissue-associated calcification occurs as an interaction of implant material factors, host biological factors and stress localization. The cross-linking of the tissue with glutaraldehyde (GA) is one of the methods to improve biostability and structural integrity of the collagen material [2]. Previous investigations demonstrated that the mineralization of the connective tissue cells of the bioprosthetic valve could be hypothesized to result from glutaraldehyde-induced cellular devitalization and the resulting disruption of cellular calcium regulation [3, 4]. However, GA remains the reagent of choice for bioprosthetic heart valve fabrication because of its superior stability and tissue thromboresistance [5]. It has also been proposed that brief exposure of GA may result in less calcification [3]. Therefore, one possible approach to the prevention of bioprosthetic valve calcification might be minimizing the use of GA.

Polyethylene glycol (PEG) has been reported to abrogate the immunogenicity of proteins while preserving their biological properties [6, 7]. The grafting of polyethylene glycol at the blood/material interface, can increase the surface hydrophilicity and subsequently can reduce protein adsorption, platelet adhesion and can act as a passive non-thrombogenic interface [8].

It has been reported that the deposition of calcium on high molecular weight PEGs is substantially less compared to their low molecular weight polymers [9]. The objective of this study was to investigate the mineralization and biostability of PEG-modified bovine pericardium (BP) in an *in vitro* model system. The versatility of high molecular weight PEG-grafted BP, as a biostable and calcium-resistant biomaterial, is proposed through these initial observations.

2. Materials and methods

2.1. Materials

Bovine pericardium was used as the tissue material for these studies, and was procured as reported elsewhere [3, 4]. Bovine hearts with intact pericardia from 12–18 month old calves, were collected fresh from the local slaughter house. Fat and excess tissues were stripped from the BP which were then processed within 4 h of slaughter [3, 4].

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PEG 600, 1500, 4000, 6000 were obtained from BDH Chemicals Ltd. Poole, UK. Glutaraldehyde and Triton X-100 were obtained from Spectrochem Bombay, India. Sodium dodecyl sulphate (SDS), trypsin and phenyl methane fluoride (PMSF) were procured from Sigma, St. Louis, MO, USA. Calcium kits were obtained from Miles India Ltd, Baroda. All other chemicals were of analytical reagent grade.

2.2. Chemical treatment of BP

Selected pericardial sacs were used for chemical treatments, where the tissues were decellularized and cross-linked, as indicated elsewhere [10, 11]. Decellularizing consists of selective removal of cellular components from the pericardial stroma, such as cells and nuclear membranes and the DNA proteins [10]. The applied procedure involves the use of two different detergents, Triton X-100 and SDS.

The triton X-100, a non-ionic surfactant, was used initially to treat the fresh pericardium at a concentration of 0.1% in saline, with the addition of 1 mM PMSF as proteinase inhibitor, to prevent degradation of the extracellular matrix. The pericardial samples were immersed in the surfactant solution and soaked overnight at room temperature. This treatment was aimed at removing the cellular membrane and proteins by disrupting their lipids [10]. After the first detergent step, the tissues were washed with distilled water to remove the traces of detergent and to prevent a possible blocking action to the second detergent used.

The second detergent treatment was performed with 0.5% SDS with 1 mM PMSF in saline for 72 h at room temperature in a mechanical shaker at 100 r.p.m. [11, 12]. The SDS detergent was used to dissolve the nuclear envelope and nuclear content, by unfolding their proteins, and subsequently removing them. The SDS was then completely removed by rinsing and washing several times with large amounts of distilled water. The decellularization process was immediately followed by collagen cross-linking with glutaraldehyde. Before the cross-linking process, pericardial sacs were cut into dumb-bell specimens and 1×1 cm square according to the standard ASTM 1708.

2.2.1. SDS-treated BP (SDS-BP)

Fresh pericardial tissues were subjected to double detergent extraction with Triton X-100 and SDS.

2.2.2. Glutaraldehyde-treated BP (GATBP)

Tissue fixation was carried out with 0.6% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.4) and transferred after 24 h. The tissues were then exposed to 0.2% GA for 2 wk at 4 °C, then washed with distilled water to remove residual glutaraldehyde and used for these studies.

2.2.3. PEG grafting on BP

PEG grafting on BP samples after 24 h exposure in 0.6% GA (GABP) was carried out by incubating samples in 5% solution of polyethylene glycols (PEG 600, 1500, 4000, 6000) in 0.1 M *tris*-HCl buffer (pH 5) for about 5 h. Then the PEG-grafted specimens were washed with distilled water to remove excess polyethylene glycol.

2.3. Contact angle

The PEG-modified BP tissues were mounted on microscope slides, and were supported in an inverted position in a container. The container was carefully filled with doubly distilled water until the microscope slide was completely immersed [12]. The goniometer was aligned (Kerno Instruments Co. Inc. Texas) and focused on the tissue–water interface. At this point a microsyringe, containing 99.9% pure *n*-octane, was lowered into the water. A drop was formed on the syringe tip, positioned underneath the sample surface, snapped from the tip and allowed to rise to the tissue–water interface. The apparent octane tissue contact angle was immediately measured, assuming symmetry, and at least ten angles with standard deviation [12].

2.4. *In vitro* degradation studies with trypsin

The tissue strips were immersed in trypsin solution (20 mg %) prepared in *tris*-buffer at pH 7.4 and kept at 37 °C containing 200 mg ml^{-1} antibiotics. The solution was changed every 24 h to restore the original level of enzyme activity [13]. At the end of the experimental period, the condition of the various BP samples was noted and the tensile strength was determined [13].

2.5. *In vitro* calcification experiments

The calcification experiments were performed as reported earlier [14, 15]. In brief, in this system, the calcium concentration (10.28 mg/100 ml) was similar to the mean total serum levels (10 mg/100 ml), and the ratio of Ca/PO_4 was 1.67 as in hydroxyapatite (HAP). The concentration product of calcium ($CaCl_2 \cdot 2H_2O$) and K_2HPO_4 in the incubation solution was 3.95 mM, 2.57 mM calcium and 1.54 mM phosphate. Each salt solution was prepared in 0.1 M *tris*-buffer, pH 7.4, containing 0.03% sodium azide as preservative [15]. Equal volumes of a double concentration of 2.57 mM Ca and 1.54 mM were mixed in a screw cap bottle containing the pericardial tissues.

At specified time points the tissues were removed and rinsed with water to remove excess solution and loosely attached deposits. They were oven dried (2 h, 110 °C), accurately weighed and hydrolyzed in 2 ml 6N HCl, for 24 h at 60 °C, as reported elsewhere [15]. The calcium concentration was determined from HCl hydrolysate using the colorimetric method of *o*-cresolphthalein complexone obtained as a standard kit.

2.6. Mechanical properties

The mechanical properties of the wet tissues were measured by ASTM standard method protocol

Chatillon Universal Test Stand Model UTSE- 2 [16] after trypsin digestion and *in vitro* calcification. Dumb-bell shaped specimens were prepared and they were tested under conditions, having a length between the grips of 2.5 cm and width of 0.5 cm and employing a crosshead speed of 1 in min^{-1} (~ 2.54 cm min^{-1}) and tensile strength were calculated.

2.7. Platelet adhesion studies with platelet-rich plasma (PRP)

Calf blood was collected into anticoagulant solution (1 part 3.8% sodium citrate to 9 parts whole blood) and centrifuged at 700 *g* for 15 min [17]. Supernatant PRP was then removed by aspiration and was collected in plastic tubes. The viable platelets assessed after staining the platelets with the trypan blue solution, quantitated with a haemocytometer and adjusted to give a concentration of 1.0×10^8 platelets per ml with platelet-poor plasma. After removing PRP, the haematocrit was centrifuged to 2000 *g* to obtain the platelet-poor plasma. The platelets were then exposed to various PEG-modified samples.

Platelet suspensions were exposed to the tissue surfaces at room temperature, rinsed with 0.1 M phosphate, pH 7.4, fixed with 2.5% GA and stained with Coomassie Blue G [17]. The number of platelets adhering to the tissue surface, was counted using an optical microscope (Nikon, Japan). Different vision fields were read randomly and averaged in similar fashion for all samples. A minimum of 20 fields was counted, from three separate experiments and the data were expressed as the number of platelets observed per mm^2 of the surface with the standard deviation.

2.8. Scanning electron microscopy

The surface and/or internal morphology of bare tissues and PEG-grafted tissues were examined using a scanning electron microscope (Hitachi S 2400). Samples were mounted on metal stumbs using double-sided adhesive tape, vacuum coated with gold film, and then observed [14].

2.9. Statistical analysis

Statistical analysis of important observations was also performed and probability values (p) for significance were calculated using student's t-test. The mean $\pm$ standard deviation t and p values are provided in Table I.

3. Results

The degradation of PEG-modified bovine pericardial tissues in the presence of trypsin digestion is studied *in vitro* in *tris*-HCl pH 7.4. Fig. 1 shows the variation in tensile strength of SDS-treated, GAT-BP and PEG-modified BP (PEG 600, 1500, 4000, 6000) tissues, with time in trypsin buffer. The original tensile strength of GAT-BP and PEG 600 BP has substantially reduced with time, while higher molecular weight PEG-grafted and SDS-treated cases do not show much reduction in their tensile strength. Among the PEG-grafted tissues, the PEG 6000 has retained its maximum strength after 70 d trypsin digestion (Fig. 1).

Fig. 2 shows scanning electron micrographs of bare and PEG-grafted bovine pericardium. The surface SDS-treated and GA-treated BP appear to be porous in nature while the PEG-modified surfaces have become smooth and compact in nature. The surface pores of the tissue have also been filled with

TABLE I Contact angle, platelet adhesion, and amount of calcium deposited on various PEG-grafted bovine pericaridium. The t-values are provided in parentheses. p values 0.05 were considered as statistically significant when all values were compared using a Student's t-test.

Surfaces[a]	Octane contact angle[b] (deg)	Mean platelets per $mm^2 \pm$ S.D.[c]	Amount of calcium deposited for 50 d (μg/mg tissue)[d]
SDS-BP	141.0 ± 3.2	82.3 ± 9.8	19.9 ± 6.7
GAT-BP	136.1 ± 1.8^{e} (3.7)	62.6 ± 7.9^{e} (6.3)	17.1 ± 0.5^{e} (1.3)
GA + PEG 600 grafted BP	143.0 ± 2.4^{f} (1.4)	47.0 ± 8.0^{e} (11.6)	15.2 ± 4.8^{g} (0.80)
GA + PEG 1500 grafted BP	145.1 ± 3.1^{h} (2.6)	52.6 ± 8.2^{e} (10.4)	13.01 ± 0.4^{f} (1.45)
GA + PEG 4000 grafted BP	144.4 ± 2.1^{h} (2.3)	32.9 ± 7.3^{e} (19.8)	15.2 ± 5.5^{g} (0.76)
GA + PEG 6000 grafted BP	151.0 ± 4.5^{e} (5.1)	27.7 ± 6.9^{e} (19.3)	11.1 ± 1.7^{h} (1.8)

[a] GA-BP were incubated with 5% PEGs for 3 h (for more details, see text).
[b] Octane contact angle expressed as mean $\pm$ S.D. (from at least ten observations).
[c] Values denoted as the average of the number of platelets attached to the surface per mm^2 with $\pm$ S.D. (at least 20 observations from triplicate experiments).
[d] Values expressed as mean $\pm$ S.D. from at least four experiments.
[e] $P \leqslant 0.005$, [f] $P \geqslant 0.05$, [g] $P < 0.25$, [h] $P \leqslant 0.05$, where the values of all PEG-modified substrates are compared with the SDS-treated cases and GA-treated BP. The t-values are provided in parentheses. p values 0.05 were considered as statistically significant when all values were compared using a Student's t-test.

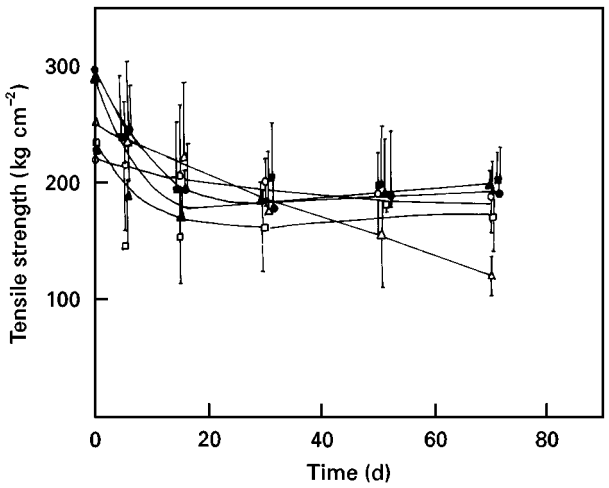

Figure 1 Effect of trypsin digestion on the tensile strength of PEG-grafted bovine pericaridium as a function of time. Bar indicates 95% confidence limits. (○) SDS-treated BP, (■) GABP-PEG 6000, (△) GATBP, (□) GABP PEG 600, (●) GABP PEG 1500, (▲) GABP PEG 4000.

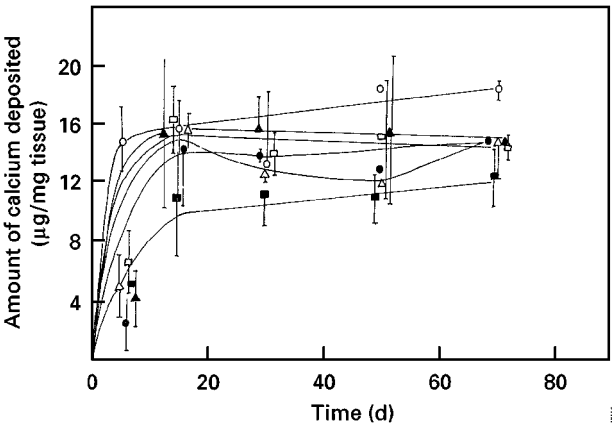

Figure 3 Amount of calcium deposited on PEG-grafted bovine pericardium as a function of time, on exposure to calcium phosphate solution. Bar indicates 95% confidence limits. (○) SDS-treated BP, (△) GATBP, (□) GABP-PEG 600, (▲) GABP-PEG 4000, (●) GABP-PEG 1500, (■) GABP-PEG 6000.

polyethylene glycols on grafting, as is evident from Fig. 2.

The amount of calcium deposited on various PEG-grafted BP, incubated in calcium phosphate solution, with time is depicted in Fig. 3. In general, the calcium concentration in SDS- and GA-treated cases is significantly higher than in PEG 6000-modified tissues at all incubation times.

Scanning electron micrographs of calcified (PEG-grafted bovine pericardium) tissue, surface morphology are shown in Fig. 4. The calcium phosphate crystals are evident on the surface as plaque-like deposits. However, the PEG grafting has variably reduced calcium nodulation on BP. It is also evident that the grafting of PEG 6000 has substantially inhibited the calcium deposition on the pericardial tissue (Fig. 4).

Fig. 5 shows the scanning electron micrographs of the platelets on the bare, and PEG-grafted bovine

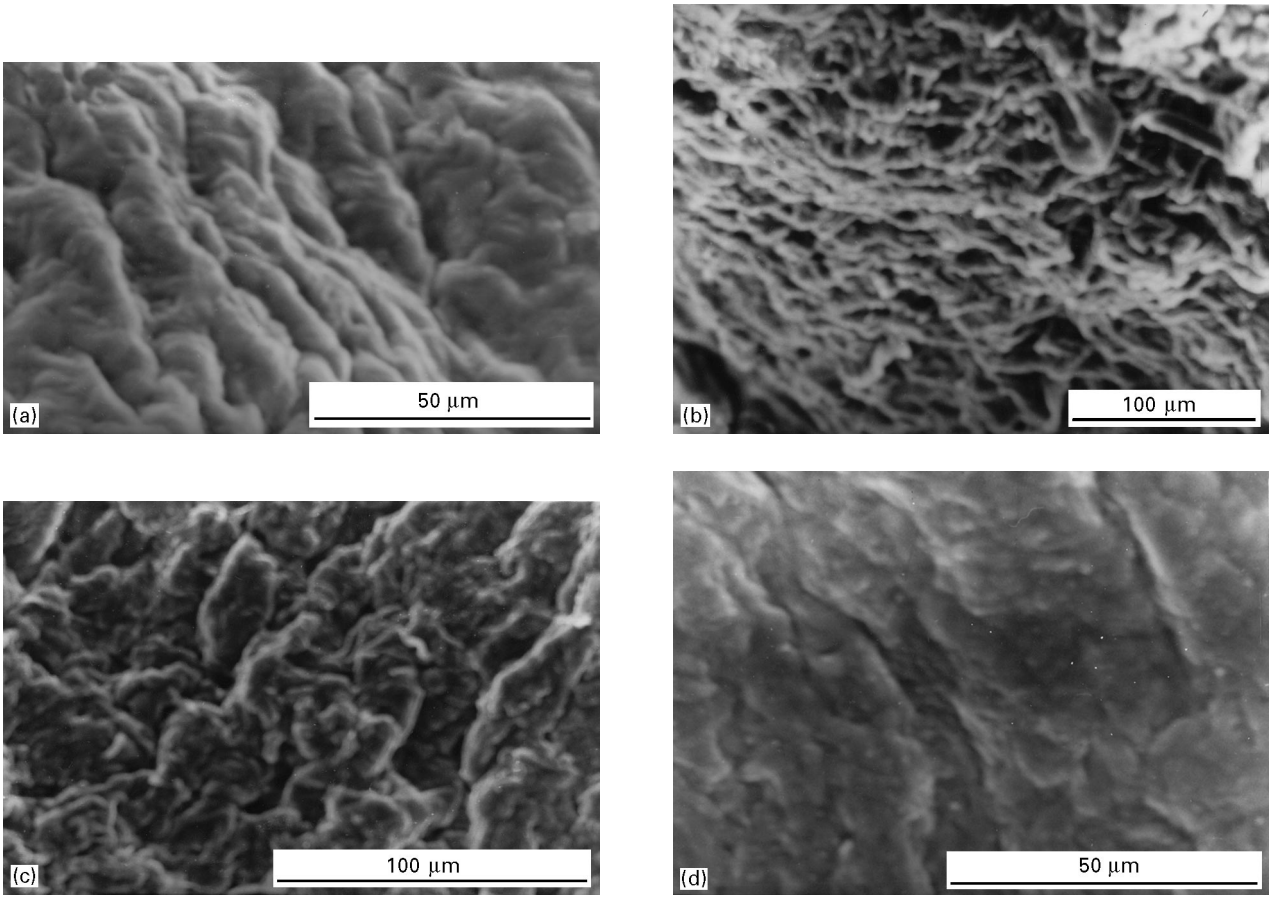

Figure 2 Scanning electron micrographs of bovine pericardium surface morphology of (a) SDS-treated, (b) GA, and (c,d) PEG grafted (PEG 4000 and 6000, respectively) tissues.

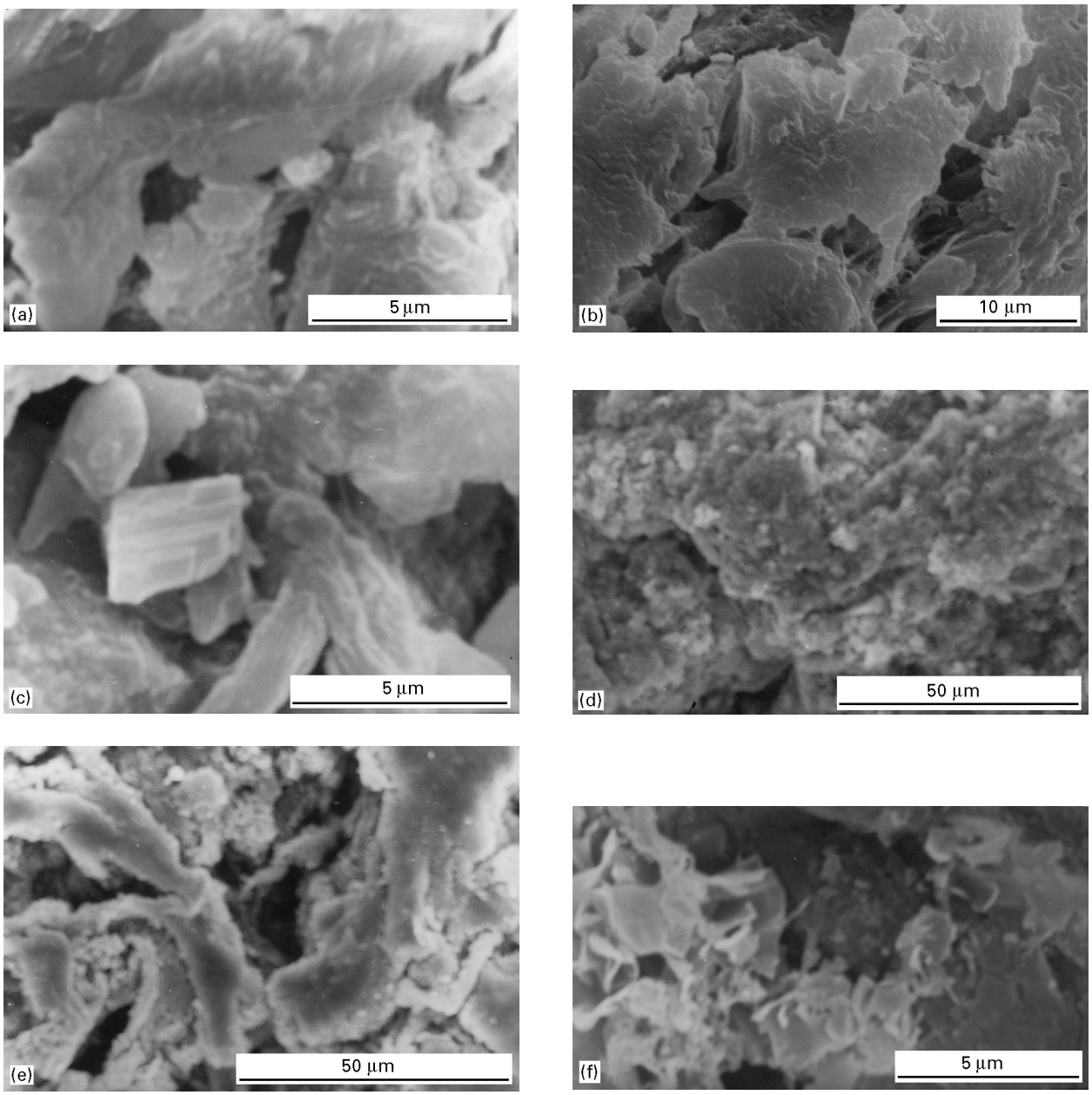

Figure 4 Scanning electron micrographs of bovine pericardium after 30 d incubation in calcium phosphate solution. Surface morphology of (a) SDS-treated, (b) GA-treated and (c–f) PEG-grafted (PEG 600, 1500, 4000, 6000, respectively) tissues.

pericardium. It appears that platelet count decreases on the PEG-grafted surfaces compared to bare samples. Platelets on SDS- and GA-treated surfaces have extended their pseudopods, leading to complete spreading, while most of the platelets on the PEG-grafted surface retained their discoid shapes. Platelet spreading is maximum on GA-treated surfaces, and in some cases a few aggregates were also observed.

Table I gives the contact angle, platelet adhesion, and deposition of calcium (after 50 days of calcification) to various PEG-modified pericardiam. Octane contact angles to these tissues indicate an increase with PEG graftings in the order GAT-BP < SDS-treated < PEG 600 < PEG 4000 < PEG 1500 < PEG 6000. In other words, the GA-treated BP shows the lowest octane contact angle. A reverse pattern is observed with the number of platelets seen on the surface; the platelet attachment reduces with the PEG grafting of BP.

4. Discussion

Calcification is the principal cause of the clinical failure of tissue valves, and it was duplicated in both circulatory and subdermal models [3, 16]. Recently, an *in vitro* model was developed by Golomb *et al.* [15] to study the effect of biomaterial-associated calcification, and this *in vitro* model was quantitatively compared with *in vivo* models [16]. This *in vitro* model consisted of tissue incubation in calcium phosphate metastable solution, resulting in calcium phosphate precipitation and deposition. The validity of the proposed model to study the effect of biomaterial-related properties on calcification as a prescreening method in

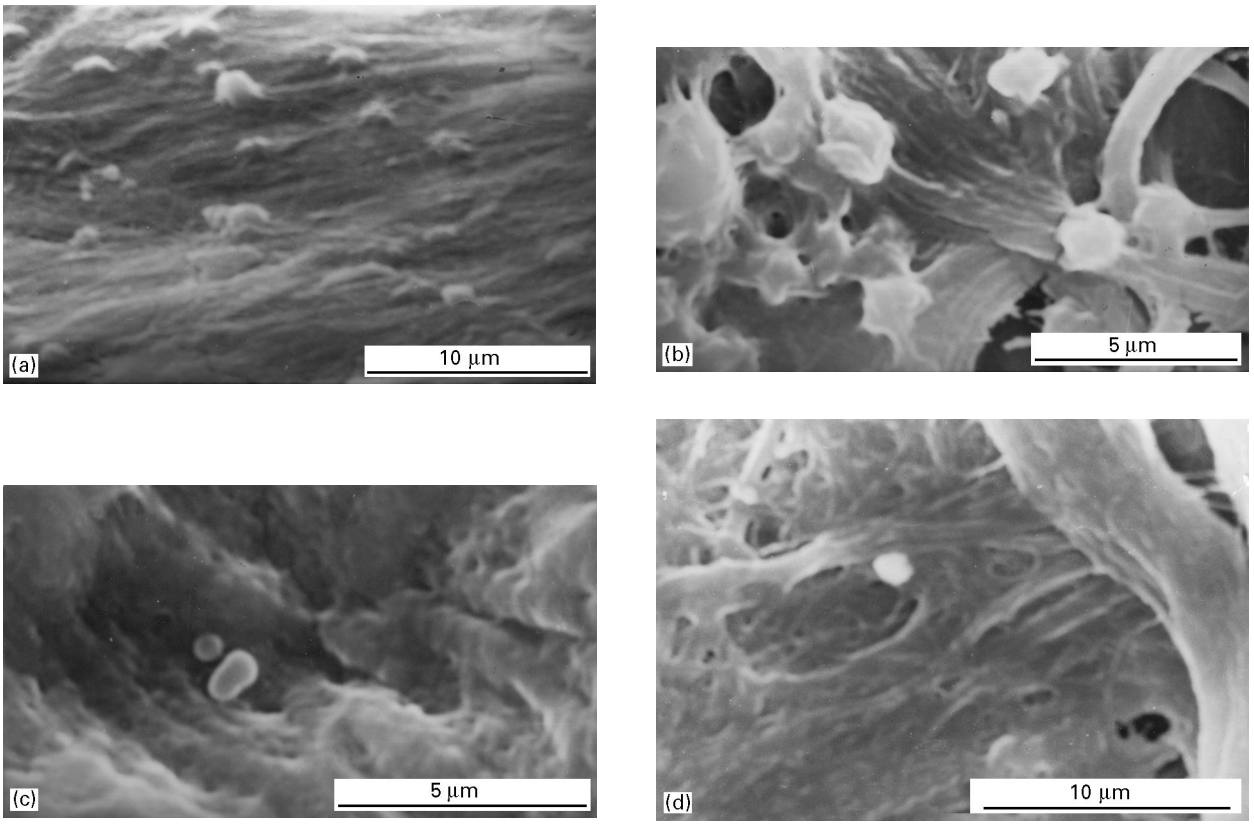

Figure 5 Scanning electron micrographs of platelets adhered to bovine pericardial surfaces: (a) SDS-treated, (b) GA-treated, and (c, d) PEG-grafted (PEG 4000 and 6000, respectively).

evaluating the propencity of different biomaterials to calcify, as well as anticalcification measures, was demonstrated [19].

There are many limitations to this *in vitro* calcification model compared to that of *in vivo* conditions. The *in vivo* calcification model is also not representative of the impairment of bioprosthetic valve durability and calcification because mineralization occurring in this model is mostly restricted to the surface. The available amount of calcium phosphate precipitate in the *in vitro* model system is limited and depends on the initial working solution concentrations. In contrast, body homeostasis generates relatively unlimited amounts of calcium phosphate in the subdermal milieu. Moreover, calcium phosphate precipitation in the *in vitro* system is random compared to the pathological calcification. Therefore, the nature of the crystalline mineral phase, in the *in vitro* system, is completely unpredictable and may not bear any resemblance *in vivo*. Further, there is conclusive evidence to support the role of degraded cells in extrinsic calcification; the proposed model might be used to explain the formation of the subsurface deposits and gross deposits which show no evidence of cellular involvement. However, the proposed model might be helpful in explaining the formation the surface deposits and gross deposits which show no evidence of cellular involvement.

In our studies, the calcification was compared on pericardial tissues and polyethylene glycol-grafted tissues incubated in calcium phosphate solutions and their stability was examined. The mechanical tests clearly showed a substantial decrease of strength properties in all the tested samples after 70 d trypsin digestion and calcification (Figs 1 and 2). However, the loss of mechanical property was less evident in PEG 6000-grafted bovine pericardium. Thus, it appears that PEG grafting may increase the stability of the tissue prosthesis and subsequently improve their mechanical properties. Doillon *et al.* [8] have conjugated different concentrations and molecular weights of activated PEG to collagen materials, and their degradation *in vivo* was evaluated. It was also reported that PEGs conjugated on to collagen sponges stabilize the porous structure without deactivating the biological properties of collagen. Thus, it is conceivable that the PEG-grafted pericardial tissue could also stabilize the porous structure of collagen and subsequently increase its stability.

The deposition of calcium was less with PEG-grafted surfaces compared to treated surfaces (Fig. 2). The reports suggest that the porosity provides a large surface area and volume, which facilitate increased permeation of calcium or phosphate ions yielding greater calcification. Further, more calcium deposits were observed on thick and porous materials *in vitro* and *in vivo* [15, 16]. Surface morphology (Fig. 2a) of SDS- and GA-treated pericardium appeared to be porous in nature. However, the PEG-modified pericardium appears with less porosity (Fig. 2e and f). Hossainy and Hubbell [9] have studied the calcification of polyethylene glycols, using a rat subcutaneous model, and proposed that calcification of PEGs was dependent on molecular weight and PEG cross-linking. Han *et al.* [7] and Doillon *et al.* [8] also indicated that PEGs conjugated with proteins exhibit a decrease

in biodegradation and immunogenicity. These observations further support our present studies on PEG-modified pericardium towards their biostability and resistance to calcium deposition.

Courtman *et al.* [11] and Pasquino *et al.* [10] have developed a cell extraction process for BP-free cells and soluble proteins, leaving a framework of largely insoluble collagen and elastin. It appears that the detergent extraction of the tissues adopted in this study can remove the cell components such as phospholipids and the nuclear proteins which are considered to be the sites of first calcium nucleation. Further, the double detergent extraction of BP indicated a reduction in tensile strength of untreated BP, $241.9 + 27.6\,kg\,cm^{-2}$ to $221.0 + 59.4\,kg\,cm^{-2}$. This loss of tensile strength might be due to the decellularization of BP, as reported elsewhere [10, 11]. The platelet attachment studies on various PEG-modified pericardia have shown that the cell adhesion was substantially reduced with PEG grafting (Table I). Scanning electron micrographs also revealed that the surface platelet attachment was less with PEG modifications and the cellular morphology was also retained. However, the platelet attachment and spreading was higher with SDS- and GA-treated pericardia (Fig. 2a and b). Marguerie *et al.* [20] have indicated that platelets possess membrane receptors for a wide variety of agents, including collagen. Thus it appears that the binding of PEG to the pericardium modifies or masks the platelet receptor sites for collagen, and causes the reduction of platelet density on the surface.

Table I also provides information related to octane contact angle for various PEG-modified tissues. The octane angles of PEG-modified surfaces were higher compared to GA-treated tissues (Table I). The octane contact angle technique has been widely used to study the nature of the surface by various investigators, and has been correlated with blood compatibility [13]. This can provide information related to the hydrophilic and hydrophobic nature of the surfaces, where a higher angle shows an increase in hydrophilic character. Hence, PEG-modified pericardium had become variably hydrophilic, although the platelet adhesion was substantially reduced (Table I). Earlier studies have shown that polyethylene glycol, a hydrophilic polymer, can improve the blood compatibility and can reduce platelet adhesion [9]. Thus it appears that the grafting of PEG to pericardium dramatically inhibited the cell adhesion and spreading.

Our present studies indicated that the PEG 6000-grafted BP subcutaneously inhibited platelet adhesion and spreading, and reduced the calcium deposition, while retaining biostability. The involvement of cellular debris and the matrix components for calcium nodulation was reported [20, 21]. Further, one proposed mechanism for calcification is that platelets or cell deposits on the implant surface act as sites for subsequent calcium phosphate deposition [9]. In this context, PEG 6000- or other high molecular weight polyethylene glycol-modified pericaridium appear to be promising matrices for inhibiting calcium accumulation.

The stability of the PEG-modified tissue might be linked to the repulsive properties of PEGs after which their covalent binding to the amino groups of the protein stabilize the tertiary structure of the protein [10]. Hence, the present observations have potential applications for developing calcium-resistant and biostable implants.

In summary, we find that a PEG-grafted surface produces highly blood compatible and anticalcifying interfaces, and its immunogenecity and stability is appreciable. In addition, it is conceivable that a tissue/PEG material, in combination with site-specific drug, may have promise for developing implants. However, these preliminary studies do not provide information relating to how much longer a heart valve, *in vivo* treated in this manner, will last before failing due to calcification. More *in vivo* studies are needed to elucidate these observations.

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References

1. R. J. LEVY, F. J. SCHOEN, W. B. FLOWERS and S. T. STAELIN, *J. Biomed. Mater. Res.* **25** (1991) 905.
2. R. J. LEVY, F. J. SCHOEN, J. T. LEVY, A. C. NELSON, S. L. HOWARD and L. J. OSHRY, *Am. J. Pathol.* **113** (1983) 143.
3. G. I. GOLOMB, F. J. SCHOEN, M. S. SMITH, J. LINDEN, M. I. DIXON and R. J. LEVY, *ibid.* **127** (1987) 122.
4. F. J. SCHOEN, J. W. TSAO and R. J. LEVY, *ibid.* **123** (1986) 134.
5. C. A. PEREIRA, J. M. LEE and S. A. HABERER, *J. Biomed. Mater. Res.* **24** (1990) 345.
6. K. BERGSTROM, E. OSTERBERG, K. HOLMBERY, A. HOFFMAN, T. P. SCHUMAN, A. KOZLOWSKI and J. M. HARRIS, *J. Biomater. Sci. Polym. Edn.* **6** (1994) 123.
7. D. K. HAN, S. Y. JEONG and Y. H. KIM, *J. Biomed. Mater. Res. Appl. Biomater.* **23** (1989) 211.
8. C. J. DOILLON, M. R. COLE, K. PIETRUCHA, G. LAROCHA and R. C. GAUDREAULT, *J. Biomater. Sci. Polym. Edn.* **6** (1994) 715.
9. S. F. A. HOSSAINY and J. A. HUBBELL, *Biomaterials* **15** (1994) 921.
10. E. PASQUINO, S. PASCALE, M. ANDREON, S. RINALDI, F. LABORDE and M. GALLONI, *J. Mater. Sci. Mater. Med.* **3** (1992) 272.
11. D. W. COURTMAN, C. A. PEREIRA, V. KASHEF, D. McCOMB, J. M. LEE and G. J. WILSON, *J. Biomed. Mater. Res.* **28** (1994) 655.
12. R. D. BAGNALL, J. A. D. ANNIS and S. J. SHERLIKER, *ibid.* **14** (1980) 1.
13. T. CHANDY and C. P. SHARMA, *Biomaterials* **12** (1991) 677.
14. T. CHANDY, B. AJITHKUMAR and C.P. SHARMA, *J. Appl. Biomater.* **5** (1994) 245.

15. G. GOLOMB and D. WAGNER, *Biomaterials* **12** (1991) 397.
16. D638-80 Annual Book of ASTM standards, American Society for Testing and Materials, Philadelphia, PA, 1982, p. 250.
17. T. CHANDY and C. P. SHARMA, *Thromb. Res.* **33** (1984) 105.
18. G. GOLOMB, *J. Mater. Sci. Mater. Med.* **3** (1992) 272.
19. D. K. HAN, K. D. PARK, S. Y. JEONG, Y. H. KIM, U. Y. KIM and B. G. MIN, *J. Biomed. Mater. Res.* **27** (1993) 1063.
20. G. A. MARGUERIE, N. ARCLAILLON, G. CHEREL, E. I. PLOW, *J. Biol. Chem.* **257** (1982) 11 872.
21. I. B. ROSANOVA, B. P. MISCHENKO, V. V. ZAITSEV, S. L. VASIN and V. I. SEVASTIANOV, *J. Biomed. Mater. Res.* **25** (1991) 277.

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