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Chitosan modified poly(glycidyl methacrylate–butyl acrylate) copolymer grafted bovine pericardial tissue—anticalcification properties

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Abstract

Calcification is the main cause of failure of glutaraldehyde crosslinked pericardial tissue used as heart valve substitutes. In our earlier work, we have shown that the grafting of glutaraldehyde crosslinked pericardial tissue with poly[glycidylmethacrylate(GMA)–butyl acrylate(BA)] copolymer with monomer ratio of 15:7.2 resulted in 50% reduction in calcification. In the present investigation, an attempt was made to eliminate calcification of the polymer grafted glutaraldehyde crosslinked pericardial tissue by coupling with low molecular weight chitosan. Low molecular weight chitosan was prepared to improve its solubility in water containing lower concentration of acetic acid and ensure easy diffusion into the tissue fiber matrix. The effect of treatment with the prepared chitosan on the anticalcification properties of poly(GMA–BA) copolymer grafted pericardial tissue was studied. It was found that chitosan post treatment of copolymer grafted glutaraldehyde crosslinked pericardial tissue was able to reduce the susceptibility of the glutaraldehyde crosslinked pericardial tissue to negligible levels after 30 days of implantation in rat subcutaneous models.

Keywords: Bioprosthetic heart valve; Anticalcification; Chitosan coupling; Grafting; Low molecular weight chitosan

1. Introduction

Bovine pericardium fixed in buffered glutaraldehyde is presently being used as leaflet in the construction of heart valve substitutes. Calcification limits the durability of such heart valve substitutes. The mechanism of calcification is obscure and the causes of calcification are multi-factorial. Host factors, mainly younger age implant factors, such as glutaraldehyde cross-linking and mechanical stress have been identified as important reasons for pathological calcification. Anti-mineralization treatments are being widely investigated to increase the durability of bioprosthetic heart valves. The approaches made for calcification inhibition include non-aldehyde preservation (Bernacca, Dimitri, Fisher, Mackay & Wheatley, 1992; Petite, Rault, Huc, Menasche & Hergabe, 1990; Xi, Ma, Tian, Long & Xi, 1992), macromolecular binding to potential nucleation sites (Nashef Awe, 1982; 1988a,b), charge modification (Golomb & Ezra, 1991, 1992), suppressing the calcification initiation by metallic salts (Catherine, Scheon, Flowers & Tyler-Staelin, 1991; Levy, Scheon, Flowers & Tyler-Staelin, 1991), treatment of pericardial tissue with inhibitors of calcification and therapy with anticalcification agents (Carpentier, Nashef Awe, Ahmed & Goussef, 1984; Golomb, Langer, Scheon, Smith, Choi & Levy, 1986; Golomb, Dixon, Smith, Scheon & Levy, 1987; Jorge-Herrero, Gutierrez & Castillo-Olivares, 1991; Tsao, Scheon, Ravishankar, Sallis & Levy, 1988).

The use of macromolecular space-fillers for the prevention of biocalcification in the bioprosthetic heart valve (BHV) substitute materials is based on the hypothesis that the glutaraldehye crosslinking creates void spaces in the fiber matrix leading to exposure of potential binding sites for calcification (Bernacca et al., 1992). Such space-fillers should be incorporated substantively throughout the cross-section so that they are not pushed out during the flexing of heart valve. Another important prerequisite is that the space-fillers should not lead to any mechanical stress in the tissue, which would result in calcification. Nashef Awe (1982, 1988a,b) has patented a method of coupling grafting of polymer onto pericardial tissue to inhibit calcification. There is also a report that chitosan coupling prevents calcification of sodium chloride–trypsin–glutaraldehyde treated pericardium (Chanda, 1994).

In our earlier work, we have shown that it was possible to reduce the susceptibility of glutaraldehyde crosslinked pericardial tissue (GCPC) for calcification by the incorporation of a copolymer of poly(GMA–BA) employing graft

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copolymerization technique (Shanthi & Panduranga Rao, 1997). The calcification was found to be reduced by more than 50% by graft copolymerization of the glutaraldehyde crosslinked pericardial tissue with GMA and BA in the molar ratio of 15:7.2. Calcium content in the grafted GCPC after 30 days implantation period in rat subcutaneous models was found to be 52.2 μg/mg of dry tissue compared to 100.1 μg/mg found in the control GCPC. This value is more than the minimum amount of calcium, 34 μg/mg of dry tissue, found in the failed human mitral valve and only slightly less than 67 μg/mg found in failed aortic valve (Scheon, Kujovich, Webb & Levy, 1987). These levels of calcium in the failed human valves were reached 7 years after BHV implantation but after only 30 days implantation period in the rat subcutaneous model. Hence, further improvement in the anticalcification properties is necessary before the grafted GCPC could find use as heart valve substitutes. Hence in the present work, an attempt has been made to achieve this objective by coupling of polymer grafted GCPC with chitosan.

Chitosan is the deacetylated derivative of chitin, which is one of the most abundant natural polysaccharides containing nitrogen. The primary unit of chitin is 2-acetamido-2-deoxy-D-glucose while that of chitosan is 2-amino-2-deoxy-D-glucose linked by β,1-4 glucosidic linkage (Muzzarelli, 1985). Chitosan is insoluble in water, alkali and many organic solvents but is soluble in many dilute aqueous solutions of organic acids, of which the most commonly used are formic and acetic acid. Chitosan is an ideal biopolymer with a wide variety of biomedical and industrial applications. Chitosan's major attractions include its biocompatibility and its acceptable biodegradation properties by virtue of its biopolymer origin. The hydrolysis of chitosan by lysozyme and chitinase enables the use of chitosan as a base material in biomedical and biotechnological fields (Hirano, Tsudida & Nagao, 1989).

In the present study, attempts were made to prepare low molecular weight chitosan for coupling onto polymer grafted glutaraldehyde crosslinked pericardial tissue to prevent calcification. Low molecular weight chitosan was prepared in order to increase the solubility of chitosan in water containing a minimum concentration of acetic acid and also to ensure better diffusion and uniform distribution throughout the fiber matrix. The molecular weight of the chitosan was reduced to about 2000 Da by degradation with hydrogen peroxide. The epoxy ring in the grafted GMA offers itself as an ideal site for a coupling reaction with chitosan.

The animal implant study with the treated pericardial tissue has been carried out in rat subcutaneous model and the results are reported.

2. Materials and methods

2.1. Materials

Pericardium from ox of South Indian origin aged 3 years was obtained from the local slaughterhouse. Chitosan (80% deacetylation, soluble in 5% acetic acid) was obtained as a gift from the Central Institute of Fisheries Technology (Cochin, India). Glycidylmethacrylate (Fluka, Switzerland), butylacrylate (SD FineChem Ltd.India) were purified by distillation. Ceric ammonium nitrate (CAN), analytical grade was obtained from Qualigens, India. All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Preparation of low molecular weight chitosan

Chitosan (I) (3 g) was dispersed in 40 ml of distilled water and the pH was adjusted to 11.5 with 2.5 M sodium hydroxide. The above mixture was heated in a water bath to 70°C and 2 ml of 30% hydrogen peroxide was added dropwise over a period of 2 h to this mixture. Subsequently, 1 ml of 30% hydrogen peroxide was added dropwise over a further hour. The reaction was allowed to continue for 4 h. The above treated chitosan in solid form (Chitosan II) was filtered and dried in a desiccator. The procedure was repeated with Chitosan II to prepare a further degraded chitosan termed Chitosan III. The molecular weight of Chitosans I–III were determined using an Ostwald viscometer in 5% acetic acid at 25°C. The molecular weight was calculated using the Mark–Houwink equation (Billmeyer, 1984), $\eta = KM_w^{\alpha}$ where η is the intrinsic viscosity, M_w the molecular weight and K and α are constants with values of 8.9×10^{-4} and 0.71, respectively.

2.2.2. Preparation of glutaraldehyde crosslinked pericardial tissue (GCPC) and poly(GMA–BA) grafted GCPC

Bovine pericardium was removed from the heart and placed immediately in iced sterile saline. After dissection of superficial fat from the external surfaces, pieces of $3 \times 3\ cm^2$ were cut. The cut pieces of pericardial tissues were placed in 0.2% glutaraldehyde in 0.05 M *N*-2-hydroxyethylpiperazine *N*-2 ethanesulfonic acid (HEPES) buffer of pH 7.4 for 7 days and then stored at 4°C in the same buffer.

2.2.3. Grafting procedure

Polymeric side chains were grafted to the GCPC using CAN as initiator as reported earlier. The procedure is summarized briefly as follows. GCPC was placed in 100 ml of distilled water containing 0.2% sodium dodecyl sulfate. The initiator and monomeric mixtures were added dropwise over half an hour to the reaction flask containing GCPC. The mixture was stirred continuously in a nitrogen atmosphere. The reaction was allowed to proceed for 3 h at 28°C. After the grafting was over, the homopolymers were removed by repeated extractions with acetone. Polymerization was repeated with varying concentrations of monomers as listed in Table 1 keeping the initiator concentration constant at 7.5×10^{-3} mol l^{-1}.

Table 1
Effect of monomer concentration on percent grafting (reaction time: 3 h)

S. No.	Initiator conc. ($\times 10^{-3}$ mol l^{-1})	GMA conc. ($\times 10^{-2}$ mol l^{-1})	BA conc. ($\times 10^{-2}$ mol l^{-1})	Grafting (%)
1	7.5	15.0	7.2	16.6
2	7.5	11.3	10.5	29.1
3	7.5	7.5	14.0	47.6

2.2.4. Chitosan modification of poly(GMA–BA) grafted GCPC and ungrafted GCPC

Chitosan III, due to its complete solubilization in water containing a low concentration of acetic acid (0.5%), has been selected for coupling with polymer grafted GCPC. One percent solution of chitosan III was prepared with 0.5% acetic acid. The polymer grafted GCPC was placed in chitosan solution in a bottle and the contents were shaken for 1 h. Then the pH of the mixture was raised to 8.5 with 5% sodium carbonate solution and the contents were shaken thoroughly for another 3 h. The tissue was washed well in distilled water after this treatment.

The chitosan coupling was carried out for ungrafted GCPC in the same fashion as described above.

2.3. Characterization of chitosan modified pericardial tissues

2.3.1. Estimation of hexosamine content

The GCPC, chitosan coupled GCPC, polymer grafted GCPC and chitosan coupled polymer grafted GCPC were dried to constant weight. A known amount of tissue from the above four samples was hydrolyzed in 6 N hydrochloric acid for 18 h at 110°C. The hydrolysate in each case was diluted to a known volume and the hexosamine content was determined by Elson–Morgan method (Elson & Morgan, 1933).

2.3.2. Measurement of shrinkage temperature for the chitosan coupled polymer grafted GCPC

The stability of chitosan coupled polymer grafted GCPC was measured in terms of hydrothermal shrinkage temperature using the Theis shrinkage meter (manufactured by Project Promoters, India). The shrinkage meter consists of two clamps for holding the sample, stirrer, temperature indicating dial and a heating device. The clamps are mounted vertically 2.5 cm apart with the stationary one at the bottom. To the upper or movable clamp is attached an indicating device which will maintain the sample under slight tension, and will indicate any preliminary swelling in the sample. The indicating device will accurately detect the point at which the shrinkage begins and indicate by a glowing display ‘test over’. The tissue of length 3 cm and width 1 cm was fixed between the two clamps of the instrument. The tissue was immersed in water taken in the beaker and the instrument was started. The rate of heating was 3–5°C min^{-1}. The temperature at which the test specimen shrinks is indicated as described above.

2.3.3. Differential scanning calorimetry (DSC) of chitosan coupled polymer grafted GCPC

The stability of the chitosan coupled polymer grafted GCPC was also measured by recording DSC thermograms for the tissues and compared with the polymer grafted GCPC. Pieces of pericardial tissues were rinsed in distilled water. Tissue specimens were blot dried with filter paper for 1 min and immediately sealed in aluminum pans. The thermogram was recorded using V4 1C DuPont 2000 at the rate of 10°C min^{-1}.

2.3.4. Tensile strength measurement for chitosan coupled polymer grafted GCPC

Tensile strength was measured using an Instron Universal testing machine, which consists of a movable cross head for applying deformations, a load cell for detecting forces and a chart recorder. The dumb-bell shaped samples were cut from the tissues and firmly held between the jaws of the tensile strength tester. The width of the sample before break is 5.15 mm at the center of the dumb bell. The tensile strength tester was started with a programmed uniform speed of separation of the jaws of 10 mm min^{-1}. The load at which the specimen breaks is noted and the tensile strength is calculated using the equation

$$\text{Tensile strength (MPa)} = \frac{\text{Breaking load (N)}}{\text{Thickness (mm)} \times \text{width (mm)}}$$

2.3.5. Implantation and retrieval of pericardial tissues in rats

Two sub-dermal pouches separated by at least 2 cm were dissected in the abdominal walls of ether anesthetized female weaning rats of albino-Wister strain weighing about 70–80 g. Each group consisted of six rats. A 1×1 cm^2 piece of grafted GCPC was sterilized in 70% ethanol for 2 h, rinsed extensively in sterile distilled water and implanted in each pocket. After implantation the wound was sutured. The rats were sacrificed 30 days after the implantation by an overdose of ether and the implants were retrieved. The implantation studies were also carried out for 90 days evaluation in rat subcutaneous model.

2.3.6. Morphological studies of GCPC and polymer grafted GCPC

The samples of PC retrieved from animals were fixed immediately in 10% neutral buffered formalin, dehydrated in increasing concentrations of alcohol, cleared in xylene and embedded in paraffin wax according to standard

Table 2
Molecular weights of chitosan samples

Sample	Molecular weight (Da)
Chitosan I	92 880
Chitosan II	2964
Chitosan III	1766

procedures (Gordon, 1982). Sections, 5 μm thick, were cut using an automated microtome, stained specifically for calcium with Von Kossa stain and micrographed using Leica Reichert Polyvar 2 optical microscope attached to a camera.

2.3.7. Von Kossa staining procedure for sectioned pericardial tissue

The retrieved pericardial sections are covered with 5% silver nitrate solution and then exposed to bright sunlight for 5–10 min. Then the slides are rinsed well with distilled water. The slides are then immersed in 5% sodium sulfite for 20 s, dehydrated, cleared and mounted.

2.3.8. Calcium analysis of retrieved pericardial tissues

The retrieved samples were rinsed with copious amount of triple distilled water and dried to constant weight and the amount of calcium was determined by atomic absorption spectroscopy Perkin–Elmer Model 3110. Twenty milligrams of dried tissue was digested with 5 ml of acid mixture (nitric acid, sulfuric acid and perchloric acid in the ratio 5:3.5:11.5) which were then diluted in acidified lanthanide solutions. The amount of calcium was determined by atomic absorption spectroscopy calibrated using standard calcium (Sigma Chemicals) in acidified lanthanide solution. The calcium content was expressed as microgram per milligram of dry weight of tissue.

3. Results and discussion

Since several factors are believed to be involved in the calcification of biological prostheses, many new chemical treatments of the tissue are being tried by various research groups to eliminate calcification. In an earlier work (Shanthi & Panduranga Rao, 1997), the graft copolymerization of poly(GMA–BA) onto GCPC was carried out to block the probable nucleation sites on GCPC for biocalcification. In this study, an attempt was made for the total elimination of calcification using GCPC grafted with synthetic polymers and subsequent coupling with chitosan.

3.1. Preparation and characterization of low molecular weight chitosan

To achieve solubility in water containing lower concentration of acetic acid and better penetration of chitosan into the fiber matrix, low molecular weight chitosan was prepared. The molecular weights of Chitosans I–III are listed in Table 2. Chitosan I–III were soluble in 5, 1 and 0.5 aqueous solutions of acetic acid, respectively. In the coupling experiments onto grafted GCPC, Chitosan III of molecular weight 1766 Da was chosen due to its better solubility.

3.2. FT-IR spectroscopy of poly(GMA–BA) grafted GCPC

The presence of epoxy group peak around 910 cm^{-1} in poly(GMA–BA), copolymer grafted GCPC was confirmed by attenuated total reflection infrared spectroscopy (Fig. 1).

3.3. Mechanism of binding chitosan to GCPC and polymer grafted GCPC

Chitosan was coupled to GCPC using two types of

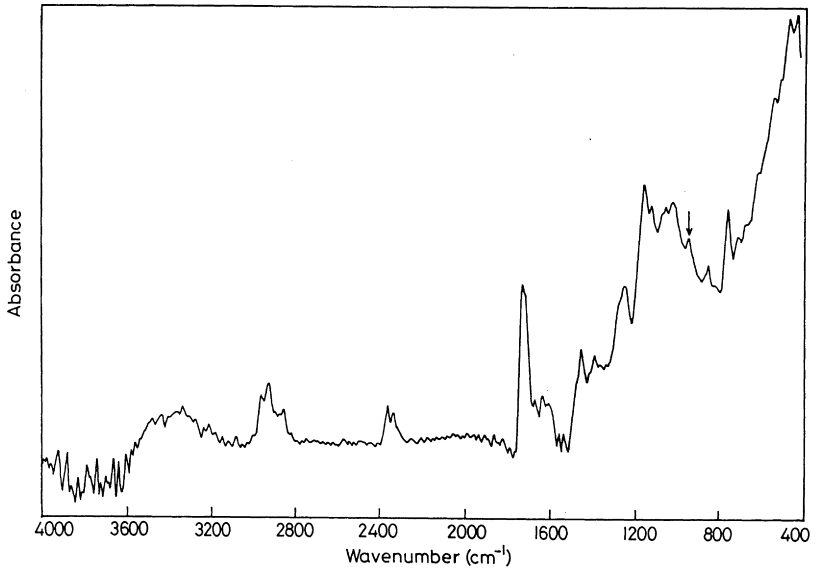

Fig. 1. Attenuated total reflection infra red spectrum of poly(glycidylmethacrylate–butylacrylate) grafted glutaraldehyde crosslinked pericardial tissue.

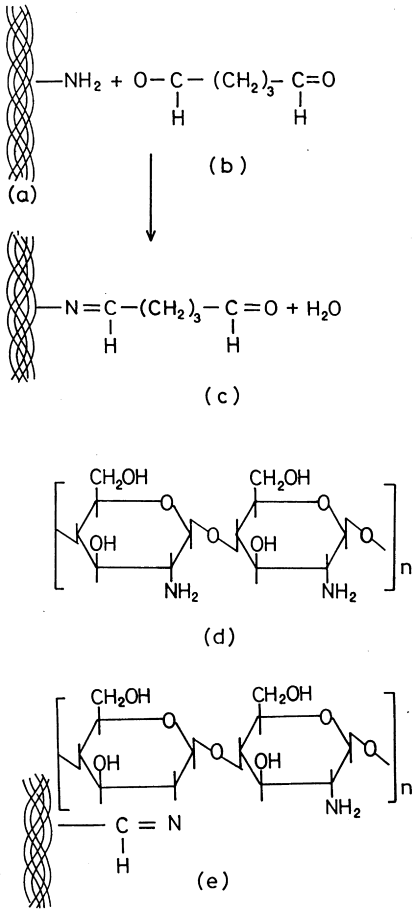

Fig. 2. Scheme showing coupling of residual aldehyde present in glutaraldehyde crosslinked pericardial tissue to chitosan through its amino group: (a) collagen (pericardial tissue); (b) glutaraldehyde; (c) glutaraldehyde crosslinked pericardial tissue with residual aldehyde; (d) chitosan; (e) chitosan coupled with residual aldehyde.

mechanism: (1) a residual aldehyde groups present in GCPC were directly utilized for coupling to the amino groups present in chitosan; and (2) the coupling reaction was carried out through the epoxy groups present in GCPC after grafting with the poly(GMA–BA) copolymer.

Many investigators reported that some residual aldehyde would always be available on GCPC. This residual aldehyde in GCPC is thought to be one of the reasons for calcification (Gendler, Gendler & Nimni, 1984; Gong, Scifter, Factor & Frater, 1991; Grimn et al., 1991; Levy, Scheon, Levy, Nelson, Howard & Oshry, 1983). Moreover, residual aldehyde in tissue valves may damage blood cells, which then may adhere to the surface of valves and act as a promoter of calcification (Dunn & Marmon, 1985). In the present study, an effort was made to use these residual aldehyde groups for binding chitosan through its amino groups. The mechanism of binding of chitosan to pericardial tissue is given as a scheme in Fig. 2.

Even though it is possible to utilize the residual aldehyde groups directly by coupling to chitosan, it was thought worthwhile first to graft the GCPC with biocompatible synthetic polymers and then couple chitosan. This will serve a dual purpose, the polymers fill the gaps in glutaraldehyde crosslinked collagen structure and subsequently coupling with chitosan removes any residual aldehyde groups present on the substrate in addition to filling the gaps further. In total, it is expected that this should work better than directly using the residual groups for coupling. As expected, first grafting GCPC with polymers and subsequent coupling with chitosan gave the best results with anticalcification of tissues (Table 4). The coupling of chitosan is more efficient by using grafted GCPC since the residual aldehyde groups present on the GCPC backbone as well as the epoxy groups present on poly(GMA) side

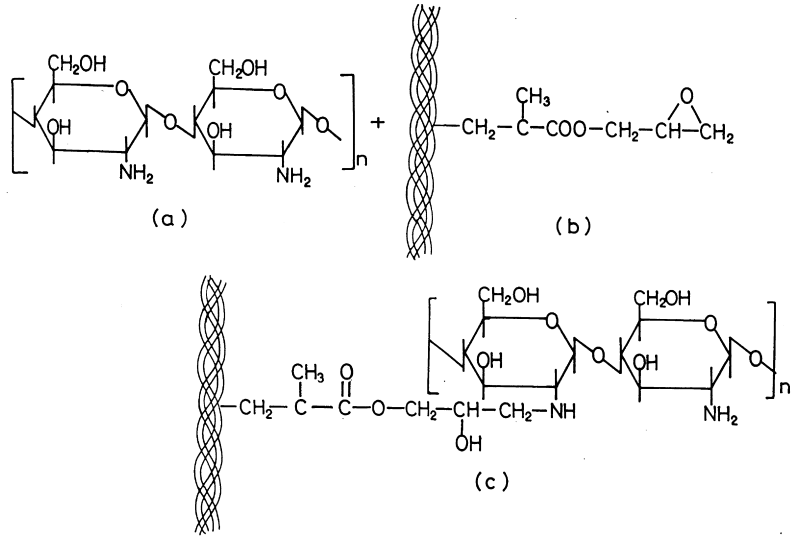

Fig. 3. Schematic diagram of chitosan coupling to epoxy group of glycidylmethacrylate grafted to glutaraldehyde crosslinked pericardial tissue: (a) chitosan; (b) poly(glycidylmethacrylate–butylacrylate) grafted glutaraldehyde crosslinked pericardial tissue; (c) chitosan coupled to epoxy group of glycidylmethacrylate.

Table 3
Estimation of hexosamine content in polymer grafted GCPC and chitosan coupled polymer grafted GCPC (GCPC, glut. crosslinked pericardial tissue; C-GCPC, chitosan coupled GCPC; P-GCPC, polymer grafted GCPC; CP-GCPC, chitosan coupled polymer grafted GCPC)

Sample	Hexosamine (%)
GCPC	0.670
C-GCPC	0.801
P-GCPC	0.574
CP-GCPC	1.615

chains participate in the coupling reaction to chitosan through its amino groups (Fig. 3).

One of the monomers, GMA, is a difunctional monomer having epoxy group intact after graft copolymerization. By utilizing the epoxy functionality of the grafted copolymer in GCPC, chitosan was coupled through its amino groups. This reaction was performed at pH 8.5. Generally chitosan solution in acetic acid precipitates at alkaline pH; however in the present study the chitosan having very low molecular weight (M_w of the degraded chitosan 1766 Da) was not precipitated even at pH 8.5. Hence, the coupling reaction could be conducted in solution media.

3.4. Characterization of chitosan coupled GCPC and polymer grafted GCPC

3.4.1. Hexosamine content

The hexosamine content of GCPC, chitosan coupled GCPC, polymer grafted GCPC and chitosan coupled polymer grafted GCPC was determined and the results are given in Table 3. Hexosamine content of chitosan coupled GCPC was higher than that of GCPC confirming the incorporation of chitosan in the tissue. In the case of chitosan coupled polymer grafted GCPC, the value was much higher than that of GCPC coupled with chitosan. These results confirm that the epoxy groups present in grafted GCPC are involved in the coupling reaction with the amino groups of chitosan.

3.4.2. Stability of the chitosan modified pericardial tissues

The stability of the polymer grafted GCPC before and after chitosan coupling was determined by the measurement of shrinkage temperature in the hydrothermal method and denaturation temperature in the DSC method. The shrinkage temperature data are presented in Table 4. Shrinkage is inherently a molecular phenomenon. The interactions between adjacent polypeptide chains namely electrovalent and coordinate linkages are considered to be responsible for most of the shrinkage properties of collagen. Any influence that modifies the interchain attractions will modify the shrinkage properties of collagen (Nayudamma, 1978). Shrinkage temperature has been generally accepted as a practical measure of degree of crosslinking in collagen. It is that temperature at which intramolecular forces in proteins become greater than the diminishing intermolecular forces. When collagen fibers are heated in an aqueous medium, shrinkage to about one-third of the original length takes place at a rather sharply defined temperature. With the shortening in length, the fiber increases in thickness and acquires a glue-like feel and rubber like elasticity. The shrinkage temperature of GCPC was found to be 85 ± 1°C as compared to 65°C for the untreated pericardial tissue. Chitosan coupled GCPC showed the same degree of stability (i.e. 84.3 ± 1.5°C), whereas polymer grafted GCPC showed 83.8 ± 0.6°C. On the other hand, when the same tissue was coupled with chitosan, the shrinkage temperature rose from 83.8 to 86.3 ± 0.6°C. This may be attributed to some type of stabilization brought about by coupling chitosan to the epoxy group in poly(GMA) grafted to the pericardial tissue.

The DSC thermograms of GCPC, poly(GMA–BA) grafted GCPC and chitosan coupled poly(GMA–BA) grafted GCPC are given in Fig. 4a–c, respectively. On grafting of polymer to GCPC, the endothermic peak has been shifted to higher temperature (Fig. 4b). The DSC recorded for chitosan coupled polymer grafted GCPC shows three endothermic peaks (Fig. 4c). The appearance of first endothermic peak around 95°C may be due to dehydration, since the water retention of chitosan coupled polymer grafted GCPC may be more due to the hydrophilic nature of coupled chitosan. The second and third endothermic peak around 125 and 135°C could be due to denaturation of polymer grafted collagen and chitosan, respectively.

3.4.3. Measurement of tensile strength properties of chitosan modified GCPCs

The tensile strength properties of GCPCs are presented in Table 4. GCPC having tensile strength of 7.5 ± 1.7 MPa has

Table 4
Effect of chitosan treatment on GCPC and polymer grafted GCPC on shrinkage temperature, tensile strength and calcification (mean ± s.d., $n = 6$. GCPC, glut. crosslinked pericardial tissue; C-GCPC, chitosan coupled GCPC; P-GCPC, polymer grafted GCPC; CP-GCPC, chitosan coupled polymer grafted GCPC. Means with different superscripts are significantly different at $p < 0.01$)

Sample	Shrinkage temperature (°C)	Tensile strength (MPa)	Calcification (μg/mg)
GCPC	85.0 ± 1.0	7.5 ± 1.7	100.1 ± 1.2^a
C-GCPC	84.3 ± 1.5	9.7 ± 2.2	21.2 ± 0.3^b
P-GCPC	83.8 ± 0.6	10.7 ± 3.8	52.2 ± 0.2^c
CP-GCPC	86.3 ± 0.6	12.6 ± 4.8	3.6 ± 0.4^d

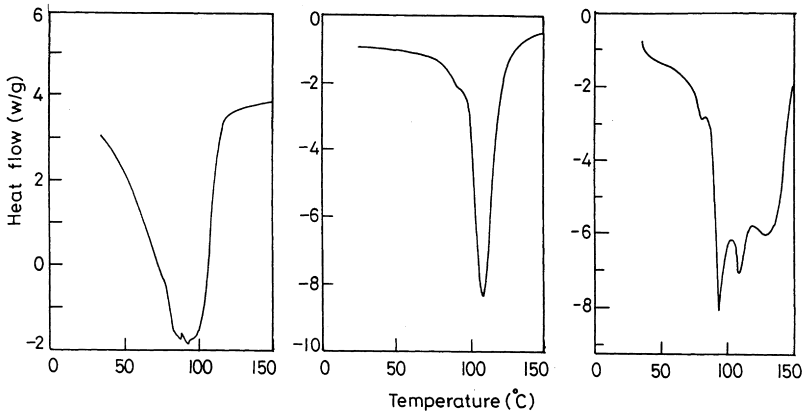

Fig. 4. Differential scanning thermogram of: (a) glutaraldehyde crosslinked pericardial tissue; (b) poly(glycidylmethacrylate–butylacrylate) grafted glutaraldehyde crosslinked pericardial tissue; (c) chitosan coupled polymer grafted glutaraldehyde crosslinked pericardial tissue.

increased its tensile strength to 9.7 ± 2.2 MPa by direct coupling with chitosan through residual aldehyde groups. In the case of polymer grafted GCPC having tensile strength of 10.7 ± 3.8 MPa, its tensile strength value is increased to 12.6 ± 4.8 MPa on coupling with chitosan. The increase in tensile strength values by coupling with chitosan onto GCPCs can be attributed to the lubricating effect of chitosan on the fiber matrix.

3.4.4. Calcification studies of retrieved pericardial tissues

3.4.4.1. Morphological studies. Since morphological studies indicate the in-depth modification of the tissue by various treatments, all the GCPC samples were analyzed morphologically. The results of the morphological studies are presented in Fig. 5a–d. The calcium present in the retrieved tissues appeared as black spots when stained

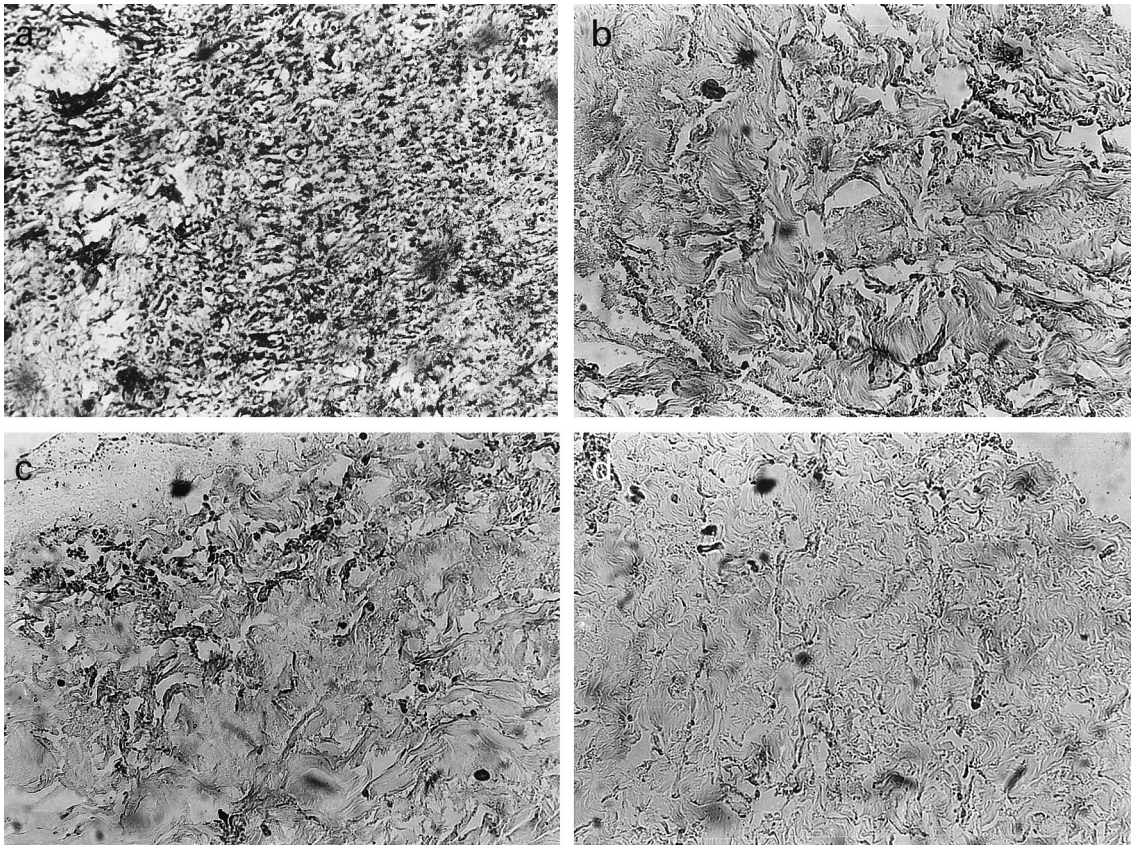

Fig. 5. Optical micrographs of sections of implanted pericardial tissue with Von Kossa stain. Calcium deposits seen as black spots: (a) glutaraldehyde crosslinked pericardial tissue; (b) 16.6% polymer grafted glutaraldehyde crosslinked pericardial tissue; (c) chitosan coupled glutaraldehyde crosslinked pericardial tissue; (d) chitosan coupled polymer grafted glutaraldehyde crosslinked pericardial tissue.

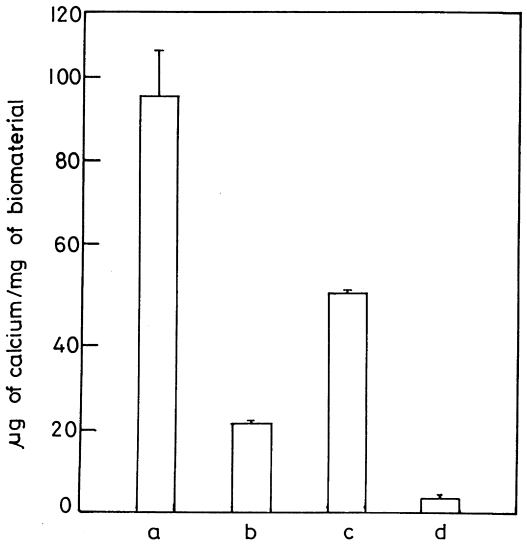

Fig. 6. Distribution of calcium uptake in pericardial samples calcified in vivo (rat subcutaneous implant model): (a) glutaraldehyde crosslinked pericardial tissue; (b) chitosan coupled glutaraldehyde crosslinked pericardial tissue; (c) 16.6% polymer grafted glutaraldehyde crosslinked pericardial tissue; (d) chitosan coupled polymer grafted glutaraldehyde crosslinked pericardial tissue.

with Von Kossa stain. Chitosan treated GCPC had markedly less calcification than that of GCPC (Fig. 5a and b). Calcification is negligible in the case of chitosan coupled polymer grafted GCPC as seen in Fig. 5d. Chitosan coupled GCPC was found to be calcified in the interstitial spaces of the pericardial tissues. This might be due to the presence of some void spaces in GCPC even after coupling with chitosan. In the case of polymer grafted GCPC, calcification is minimal in the interstitial space and is more pronounced in the edges whereas calcification is negligible in chitosan coupled polymer grafted GCPC. These results clearly indicated that by filling the voids in GCPC and by blocking all the residual groups, it is possible to reduce the calcification to the minimum. By coupling with chitosan, it is possible to give the modified pericardial tissue, the required flexible properties.

3.4.4.2. Analysis of calcium by atomic absorption spectroscopy. Calcium contents in the retrieved tissues as determined by atomic absorption spectroscopy are given in Table 4 (Fig. 6). The chitosan treatment in both modifications has been found to reduce the tendency of calcification in GCPC. The calcium content of GCPC is 100.1 μg/mg of dry tissue whereas the tissues grafted with 16.6% copolymer of GMA–BA had 52.2 μg/mg. There was 50% reduction of calcification of GCPC grafted to a very low extent of copolymer. The ungrafted GCPC and polymer grafted GCPC when coupled with chitosan gave calcium content of 21.2 and 3.6 μg/mg, respectively. These values were much lower than that of GCPC. The anticalcification studies were initially carried out for 30 days to know the trend of the results discussed above. From these initial studies, it was found that GCPC grafted with poly(GMA–BA) copolymer and subsequently coupled with chitosan gave best results. To have better biological evaluation, the calcification studies were continued in polymer grafted GCPC with coupled chitosan for 90 days. The calcium content after 90 days implantation for chitosan coupled polymer grafted GCPC was found to be 16.58 ± 0.79 μg/mg of dry tissue (at significance of $p < 0.01$) compared to 160.72 ± 22.95 μg/mg in case of GCPC. The in vivo study in rat models clearly indicated a substantial decrease in calcification of chitosan coupled polymer grafted GCPC when compared to GCPC.

4. Conclusions

Low molecular weight chitosan has been found to be a good chemical agent for the elimination in the grafted GCPC. The calcium content in the chitosan treated polymer grafted GCPC was found to be 3.6 ± 0.4 and 16.58 ± 0.79 μg/mg of dry tissue grafted with GMA and BA in the mole ratio of 15:7.2.

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