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Evaluation of alternative glutaraldehyde stabilization strategies for collagenous biomaterials

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A range of alternative crosslinking conditions based on glutaraldehyde were examined for their effectiveness for stabilizing collagen-based materials using test samples of a collagen–polymer composite tube. Stabilization of collagen was performed with various concentrations of glutaraldehyde at acid pH, in the absence or presence of 0.7 M NaCl to control collagen swelling. For each condition, some samples were further treated at neutral pH. These test samples were compared with samples treated with glutaraldehyde at neutral pH and with samples of Omniflow vascular prosthesis. The effectiveness of the stabilization was examined by amino acid analysis, to assess the extent of modification, isometric tension analysis, to evaluate the extent of crosslinking, compliance and accelerated fatigue testing, to evaluate mechanical properties, and by a rat subcutaneous model to evaluate tissue response and propensity to calcification. The data indicated that effective crosslinking could be achieved at low pH and that this can be increased slightly by the presence of NaCl. At low pH, the extent of calcification was low compared to samples treated at pH 7. Subsequent treatment at pH 7 of samples given an initial low pH glutaraldehyde (GA) treatment generally did not alter shrinkage temperatures although the extent of lysine modification and calcification did increase. In general, a more inflammatory response was observed in samples tanned at low pH, although this was not as severe as responses to untreated tissue implants. The Omniflow vascular prosthesis showed excellent chemical and mechanical properties and did not show any calcification.

1. Introduction

One of the problems which has been reported with the use of glutaraldehyde for stabilizing collagen-based biomaterials is that calcification can occur. This problem has been of particular interest for cardiovascular devices, such as replacement heart valves, as it is detrimental to long-term function [1]. Although the mechanism for this glutaraldehyde associated calcification is not fully known, it has been proposed that it is due, in part, to the glutaraldehyde polymers which form at neutral pH and which are incorporated into the product. These polymers can subsequently degrade slowly, releasing glutaraldehyde into the surrounding tissues [2].

The reaction of glutaraldehyde with collagen is rapid at neutral pH, but fairly slow at acid pH. Nevertheless, since acidic solutions of glutaraldehyde have a low polymer content it has been suggested that the rate of stabilization of collagen by glutaraldehyde in acid conditions may be sufficient to allow stabilization without polymer incorporation [3].

In the present study, we have examined glutaraldehyde stabilization under a range of acidic conditions. The materials chosen for study were tubes of a collagen–polyester mesh composite which are used to manufacture the Omniflow vascular prosthesis (Bio Nova International, North Melbourne) [4]. The methods for stabilization used different pH conditions, different glutaraldehyde concentrations, with or without the inclusion of 0.7 M NaCl to control collagen swelling and with or without subsequent reaction at neutral pH. These approaches were compared with samples stabilized at neutral pH and with samples of Omniflow vascular prosthesis stabilized at neutral pH using a proprietary glutaraldehyde-based method.

2. Materials and methods

2.1. Production of test samples

Collagen–polymer composite test samples were made by implantation of silicone mandrels, covered with knitted polyester mesh, beneath the cutaneous trunci muscle of sheep as previously described [4]. After 12–13 weeks the collagen-covered tubes were re-

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moved, trimmed of fat and excess connective tissue and then stabilized with glutaraldehyde (GA).

Samples of the Omniflow vascular prosthesis, in 50% (v/v) EtOH, were a gift from the manufacturer, Bio Nova International (North Melbourne).

2.2. Crosslinking of test samples

Collagen-based test samples were subjected to various GA crosslinking procedures, in which pH, GA concentration and NaCl content were varied, at 25 °C for 20 h as described in Table I. For each condition, some samples were further treated with 2% GA at pH 7 for 20 h. It has previously been shown [5] that reactions reach completion within 20 h. After processing, the vascular prostheses were rinsed several times in phosphate buffered saline (PBS), pH 7.4 and stored in 50% (v/v) EtOH for up to 3 weeks prior to further analysis. For comparison, samples of Omniflow vascular prosthesis stabilized, at neutral pH using a propriety glutaraldehyde-based procedure, were used.

2.3. Amino acid analysis

Samples (~ 5 mm × 5 mm) were hydrolysed with 5.8 M HCl at 108 °C for 24 h under vacuum on a Waters Pico Tag Work Station, and analysed using a Waters Amino Acid Analyser.

2.4. Isometric tension and shrinkage temperature

Shrinkage temperatures of samples were determined in PBS by isometric tension analysis according to the method of Mitchell and Rigby [6]. Samples (5 mm × 25 mm) were cut parallel and perpendicular to the tube axis and heated at 1 °C min^{-1} up to 93 °C.

2.5. Compliance

Compliance of the GA stabilized test samples in PBS was determined by ultrasound. Sample tubes, 200 mm long, sealed at one end and connected at the other end to a tap allowing connection to reservoirs giving pressures of 2.67×10^3 Pa and 2.0×10^4 Pa were supported on a horizontal perspex plate. Determinations were made for at least five points along each tube, allowing 30 mm clearance from fixtures, on at least three sample tubes. For compliance measurement, the pressure in the graft was cycled between the two pressures. The ultrasound probe was placed directly on the sample along with gel (Aquasonic 100) to ensure good sound propagation to eliminate noise problems. Signals from the outer surfaces, the polyester mesh and the inner surfaces were observed. Changes in internal diameter were used for compliance determination.

2.6. Accelerated fatigue test

Crosslinked test samples were examined for durability using an accelerated fatigue test, according to the method of Roberts *et al.* [7], in which samples were subjected to a pulsatile flow of 50% (v/v) EtOH with a pressure change of 0 to 2.33×10^4 Pa at 135 beats/min for 96 h. Sample performance was monitored by measuring the increase in porosity (ml/min 50% (v/v) EtOH at 25 kPa) through the wall at 24 h intervals [7]. At the end of the pumping (96 h) the samples were assessed to see whether a burst strength exceeding 125 kPa could still be achieved.

TABLE I Amino acid analysis, indicating extent of reaction, and shrinkage temperature, indicating extent of crosslinking for various glutaraldehyde stabilization conditions

Sample	Lys/Phe ratio[a]	Shrinkage temperature, T_s (°C)
Untreated	1.95	65.0
pH3, 1% GA	0.74	65.6
pH3, 1% GA, then pH 7, 2% GA[b]	0.34	73.0
pH3, 1% GA, 0.7 M NaCl	0.64	76.5
pH3, 1% GA, 0.7 M NaCl, then pH 7, 2% GA	0.33	76.2
pH4, 0.1% GA	0.96	75.0
pH4, 0.1% GA, then pH 7, 2% GA	0.37	76.6
pH4, 0.1% GA, 0.7 M NaCl	0.92	73.4
pH4, 0.1% GA, 0.7 M NaCl, then pH 7, 2% GA	0.33	73.6
pH4, 1% GA	0.50	74.5
pH4, 1% GA, then pH 7, 2% GA	0.35	73.4
pH4, 1% GA, 0.7 M NaCl	0.43	72.8
pH4, 1% GA, 0.7 M NaCl, then pH 7, 2% GA	0.29	73.8
pH7, 2% GA	0.38	82.0
Omniflow	0.31	81.8

Results are given as an average of two or more analysis

[a] Ratio of lysine (Lys: residue involved in crosslinks) to phenylalanine (Phe: control reference amino acid). A lower ratio indicates a greater degree of reaction.

[b] Samples were further reacted with 2% GA, pH 7, at 25 °C.

2.7. *In vivo* evaluation

Host tissue response and the degree of calcification were assessed in a rat subcutaneous model according to the method of Levy *et al.* [8], using 10 mm square samples. Prior to implantation, samples were washed in sterile saline to remove the EtOH used during storage. Rats were sacrificed after 28 and 56 days of implantation. Tissue blocks containing the test samples were excised and fixed in neutral-buffered formalin. The blocks were trimmed and radiographs were taken on Kodak Industrex R-single sided film at 25 kVp to assess mineralization. Tissues were also processed for routine histology and sections stained with haematoxylin and eosin and von Kossa stains.

3. Results and discussion

3.1. Amino acid analysis and isometric tension

The effectiveness of stabilization using various conditions (Table I) was examined by amino acid analysis, to measure the extent of lysine modification, and by

isometric tension to determine shrinkage temperature (T_s) as a marker for crosslinking.

To examine the extent of Lys modification, its yield was compared with that of phenylalanine, an amino acid unaffected by GA treatment. The maximum Lys/Phe ratio, of 1.95, was obtained for unmodified samples, whereas samples reacted with 2% GA at neutral pH, where GA fixation is known to be effective, had a Lys/Phe ratio of 0.38. It has been suggested that the 18% of Lys residues which remain unreacted lie in the overlap zone of the collagen structure and are inaccessible due to steric constraints [9]. At low pH, the Lys/Phe ratio was higher, indicating that less reaction had taken place than at pH 7; for low pH samples, a greater level of reaction with 1% GA took place at pH 4 than at pH 3, while reducing the concentration of GA at pH 4 lead to reduction in the extent of reaction, with only about 50% of the total Lys residues having reacted, about 62% of the available Lys. At each pH, inclusion of 0.7 M NaCl, which reduces the extent of collagen swelling, led to an increase in the amount of Lys which reacted. However, this was minimal at pH 4, 0.1% GA, where the extent of reaction without NaCl was least effective. In all cases, with and without inclusion of NaCl, when samples were further reacted at pH 7 with 2% GA, additional Lys modification took place and all showed similar final extents of reaction, comparable to samples which had been reacted directly under these conditions. A sample of Omniflow vascular prosthesis showed a similar, high level of Lys modification.

Amino acid analysis can only tell the extent of Lys modification, but not the extent to which this leads to crosslink formation. To examine whether crosslinking is taking place, the T_s of samples were determined using the isometric tension method (Table I).

The lowest value for T_s, 65 °C, was obtained for untreated material. A similar low value was obtained for samples treated with 1% GA at pH 3, suggesting that the majority of the Lys modification which had been observed, about 75% of the available Lys, was not involved in crosslinking. At pH 3, GA would be expected to be mainly a monomeric dialdehyde and collagen molecules are markedly swollen, which would favour weaker intramolecular crosslinks and very few intermolecular stable crosslinks. Increasing the pH to 4 led to an increase in T_s of about 10 °C, indicating that cross-linking was taking place. At pH 4, reduction of the GA concentration to 0.1% did not lead to a lower T_s, suggesting that while the extent of Lys reaction was lower under these conditions, the reactions which did occur led to effective crosslink formation. At pH 4, addition of 0.7 M NaCl did not lead to an increase in shrinkage temperature for either GA concentration, suggesting that collagen swelling was not limiting the extent of crosslinking at this pH; rather, a slight fall was observed suggesting that NaCl addition may have limited accessibility for the GA. However, at pH 3, inclusion of 0.7 M NaCl did lead to an increase in T_s, giving a value comparable to those obtained at pH 4, indicating the importance of swelling at this lower pH. The presence of NaCl would suppress the degree of swelling which could then allow the monomeric GA dialdehyde to effectively form intermolecular crosslinks. When samples treated at pH 3 in the absence of NaCl were further treated at pH 7 with 2% GA, the T_s was also increased as was the extent of Lys modification. However, the other samples showed no significant changes in T_s on further GA treatment, despite an increase in the extent of Lys modification. For samples which were treated directly with 2% GA at pH 7, and for samples of Omniflow vascular prosthesis, a higher T_s was observed. Since T_s is dependent on the chemical nature of the crosslinking and the accessibility of the structure to water, as well as the number of crosslinks present, it is likely that the higher T_s values obtained with reaction at pH 7 reflect the higher level of polymeric material which would be incorporated into the materials during the initial crosslinking reaction rather than solely a greater extent of crosslink formation. At acid conditions the extent of polymer inclusion in crosslinks would be less, and polymers introduced by the second reaction would be readily removed by washing.

Samples for isometric tension were taken both parallel and perpendicular to the tube axis. In all cases, no differences in T_s were found between these directions. However, the extent of the force generated by samples parallel to the axis was greater (generally more than 2.5-fold) than that for equivalent samples cut perpendicular to the axis. These data are consistent with previous observations which show that the collagen in the test samples is highly oriented along the direction of the axis of the tube [9, 10].

The isometric tension curves showed a clear difference between samples which had been stabilized at pH 3 and those which had been treated at higher pH values. For the samples treated at pH 4 and pH 7, as well as untreated material, a sharp shrinkage transition was observed. At pH 3, however, only a gradual shrinkage transition was observed, over a range of about 10–15 °C, before a steady, linear increase of force with temperature increase was observed. This type of curve was still observed when NaCl was present and after further GA treatment at pH 7. In all cases, T_s was determined by extrapolation of the initial linear part of the trace to the temperature where zero force was generated. Thus at pH 3, where the extrapolated T_s is similar to untreated material, the initial stages of shrinkage are taking place at a lower temperature than observed with untreated tissue, suggesting that the Lys modification, without substantial crosslinking may be destabilizing the tissue structure by partially fixing the swollen conformation.

3.2. Compliance

The ultrasound method used for compliance evaluation allows an internal diameter to be determined for the tubes; this had not been possible with cantilever-based methods [7]. Thus, although all samples, including the Omniflow vascular prosthesis, had been prepared using the same 6 mm OD silicone mandrel, initial diameter measurements at 2.67×10^3 Pa pressure showed a range of diameters (Fig. 1). Treatment with 1% GA at pH 4 showed smaller diameters than

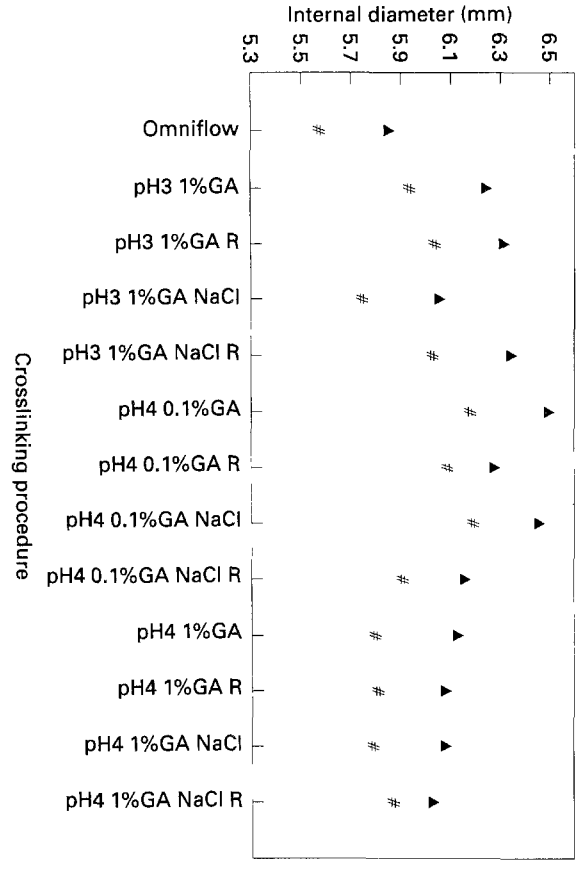

Figure 1 Compliance of test collagen samples after various GA stabilization strategies. Initial tube diameter (#) and final tube diameter (▲) at a low and high pressure. R = further treated at pH 7.

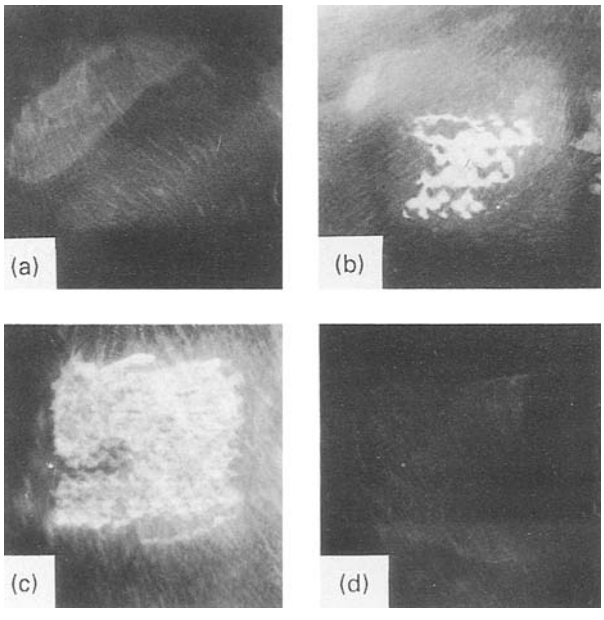

Figure 2 Radiographs of explanted samples from rats after 28 days implantation. Calcification appears as white on a black background. (a) Untreated control showing no calcification. (b) Test sample treated at pH 4, 1% GA and further treated at pH 7, 2% GA. (c) Test sample treated at pH 7, 2% GA (d) The Omniflow vascular prosthesis.

those at pH 3 or with lower GA concentration, where amino acid analysis and isometric tension had suggested that the stabilization was not as effective. The Omniflow vascular prosthesis, which has very effective GA stabilization, showed the smallest diameter. Despite this range in resultant diameters, all samples including the Omniflow prosthesis showed very similar compliance values.

3.3. Accelerated fatigue test (AFT)

An *in vitro* measure of material durability is its resistance to fatigue. The durability of the sample tubes was determined by intermittent measurements of the vessel porosity after continuous, elevated stress. Since the tube samples were of natural origin, some intrinsic variation in porosity between samples would be expected, in addition to variations due to the trimming effecting wall thickness. Therefore, to allow significant comparisons of the effect of GA treatment on absolute porosity, very large numbers of samples would be needed. Therefore, in the present study, the changes in porosity for an individual sample were measured. The observed changes in porosity were small ($< 15\%$ per 24 h) and comparable with previously reported values for the Omniflow prosthesis [7]. In the final burst strength evaluations, all test samples withstood pressures > 125 kPa.

3.4. *In vivo* analysis

The extent to which the samples were susceptible to calcification was examined in a rat subcutaneous model [8]. All test samples showed varying degrees of calcification as assessed by radiography (Fig. 2) and von Kossa staining (Fig. 3). The radiography showed negligible to low calcification for all low pH stabilization approaches, with an increase on further reaction with GA at pH 7. However, calcification was more prominent in samples reacted directly at pH 7. Both the unmodified and Omniflow samples showed no evidence of calcification, after both 28 and 56 days. Similar results were observed by von Kossa staining. In the test samples, calcification was negligible in samples stabilized at pH 3 with 0.7 M NaCl with only occasional speckled deposits around the polyester. Calcification was more prominent in samples stabilized at pH 4, although this was variable and in most instances was not severe. Calcification was most evident with samples treated at pH 7. Samples from low-pH GA treatment which were further treated at pH 7, showed a minor increase of calcification, generally speckled in definition, but showed less calcification than the samples treated directly at pH 7. No sample performed better than the Omniflow vascular prosthesis, where no calcification was apparent in any of the replicate samples (Figs 2 and 3).

The tissue response to the implanted materials was also assessed (Fig. 4). As expected, untreated samples showed a moderate to intense infiltration of mononuclear cells, particularly at 28 days. A mild to severe inflammatory cell invasion was associated with all other samples (Fig. 4). Moderate infiltration of cells around the polyester fibre bundles occurred uniformly in most samples. At 28 days, more severe cellular infiltration was observed in low-pH stabilized samples compared with those treated with GA at neutral pH.

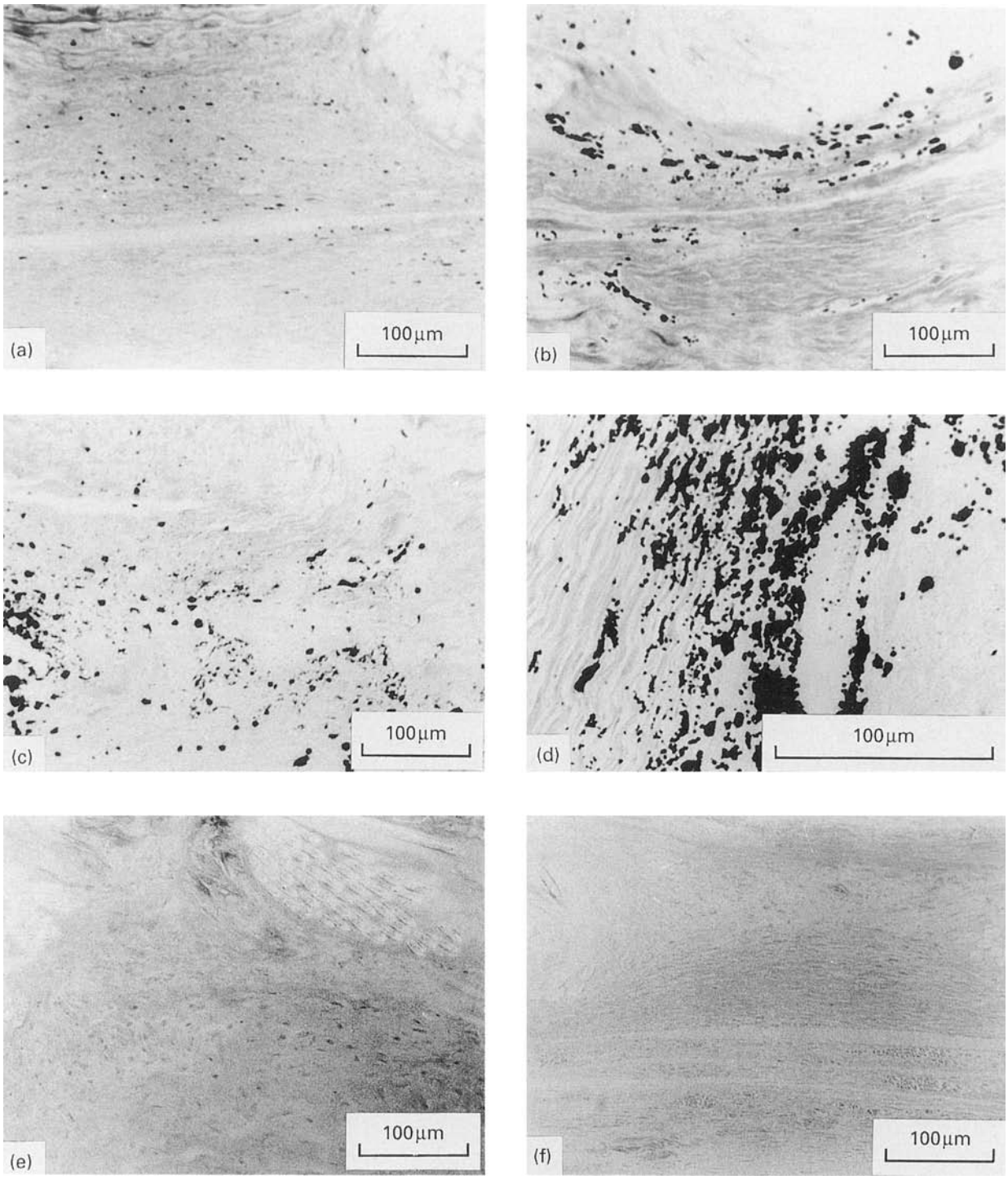

Figure 3 Evaluation of the extent of calcification by von Kossa staining of explanted samples from rats after 28 days implantation. Calcification appears as black deposits on a grey background. (a) Test sample treated at pH 3, 1% GA, 0.7 M NaCl showing only speckled staining. (b) Test sample treated at pH 3, 1% GA, 0.7 M NaCl and further treated at pH 7, 2% GA, showing mild staining. (c) Test sample treated at pH 4, 1% GA 0.7 M NaCl and further treated at pH 7, 2% GA, showing mild staining. (d) Test sample treated at pH 7, 2% GA showing extensive staining. (e) Untreated control. (f) The Omniflow vascular prosthesis.

By 56 days, most test samples were associated with only a mild tissue response. The Omniflow vascular prosthesis had a low uniform infiltration of inflammatory cells in all sections examined.

Overall, the data indicated that effective cross-linking could be achieved at low pH and that the extent of calcification was low compared to samples treated at pH 7. The Omniflow vascular prosthesis showed excellent chemical and mechanical properties and did not show any calcification. Calcification has not been reported as a problem for this device in clinical application. Thus, while an acid pH stabilization strategy would not improve this device, it could be useful for other collagen-based biomaterials where different functional requirements exist.

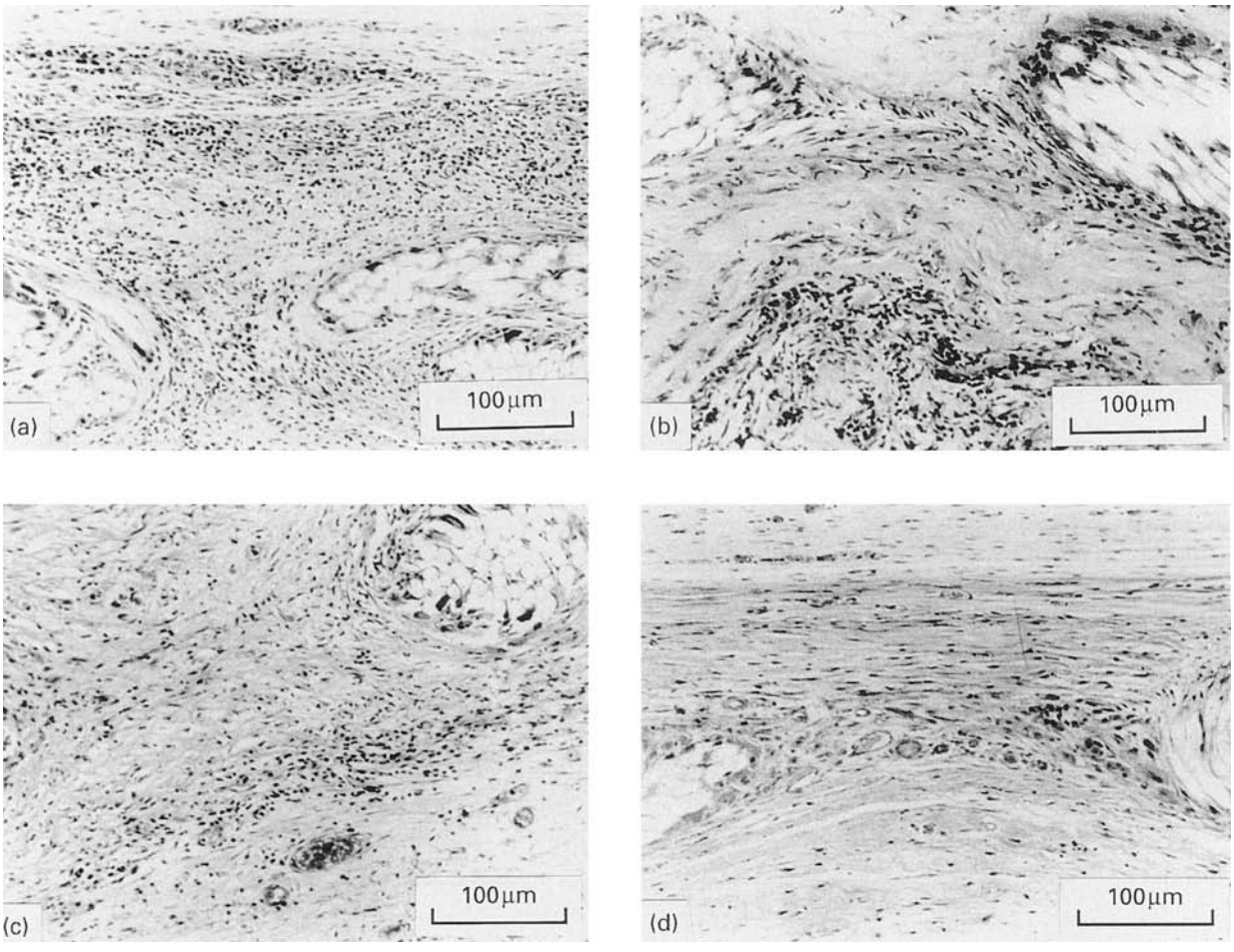

Figure 4 Host inflammatory response to explanted samples from rats after 28 days implantation, by haematoxylin and eosin staining (a) Untreated sample showing intense cell infiltration. (b) Test sample treated at pH 3, 1% GA, 0.7 M NaCl showing pockets of cell infiltration around polyester fibre bundles and adjacent tissue. (c) Test sample treated at pH 7, 2% GA showing moderate areas of cell infiltration. (d) the Omniflow vascular prosthesis showing minimal cell infiltration.

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