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Mechanical, Thermal and Morphological Properties of Glutaraldehyde Crosslinked Bovine Pericardium Followed by Glutamic Acid Treatment

Gilberto Goissis[a]*, *Domingo Marcolino Braile*[a]*,

Nélly Cristina Carnevalli[b], *Vladimir Aparecido Ramirez*[a]*

[a]*Braile Biomédica Ind. Com. e Repres. Ltda.*
São José do Rio Preto - SP, Brazil
[b]*Instituto de Química de São Carlos, Universidade de São Paulo – USP,*
São Carlos, SP, Brazil

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Major problems with valve bioprostheses are associated with progressive structural deterioration and calcification, directly associated with the use of glutaraldehyde (GA). This work describes the effects of GA processing and borate/glutamic acid buffer treatment on the mechanical, thermal and morphological properties of 0.5% GA crosslinked bovine pericardium (BP). The results showed that while the treatment of 0.5% GA crosslinked BP with borate/glutamic acid significantly improves the mechanical properties, it had no visible effect on surface morphology. Better surface preservation was only achieved for BP pre-treated with a lower GA concentration followed by the conventional treatment (0.5% GA). Improvements in mechanical properties probably arises from structural changes probably involving the depolymerization of polymeric GA crosslinks and an increase electrostatic interaction due to covalent binding of glutamic acid to free carbonyl groups (Schiff base). The results indicate that the treatment GA crosslinked BP with borate/glutamic acid buffer may be an attractive procedure for the manufacture of heart valve bioprostheses.

Keywords: *bovine pericardium, glutaraldehyde, crosslinking, glutamic acid, properties*

1. Introduction

Major problems with valve bioprostheses failure are associated with progressive structural deterioration and calcification[1,2]. To minimize these problems, besides the classical procedure using glutaraldehyde (GA) many alternative processes were introduced for the manufacture bioprosthetic heart valves (BHV) and includes the crosslinking with azide[3], epoxydes[4], carbodiimides[5] and diisocyanates[6], the treatment with GA in non-aqueous solvents[7], diphosphonates[8] and α-amino oleic acid treatment[9]. One emerging technique applied to the fabrication of BHV valves is tissue engineering but still in its early development stage[10,11]. Although the major problems associated with the failure of GA crosslinked BHV are attributed to the chemical characteristics of GA solution used for processing, it still is reagent of choice for the crosslinking of natural tissue intended for BHV manufacture[12].

At room temperature GA solutions are complex containing monomeric GA, mono and dehydrated forms, monomeric and polymeric cyclic hemiacetals and α and β-insaturated compounds with concentrations dependent on the temperature and pH[13]. The problems observed with BHV post implantation as a result of the complexicity of GA solutions are: 1) Impermeabilization of BP surfaces resulting from a polymeric network (Figure 1) which hinders the further crosslinking of the interstitium of the fiber leading to the formation chemically heterogeneous material[14]. This is in agreement with the fact that the number of unreacted ε-amino groups decreases with increasing concentrations of GA[15]. Associated to polymeric GA crosslinks there is also the systemic and localized cytotoxic effects observed post-implantation due to the slow release of free GA from the processed tissue[16]; 2) Incomplete glutaraldehyde binding to tissue proteins that beside the citotoxicity associated with the free aldehyde function, is also involved in the calcification process of BHV[17]. From the total monomeric GA covalently bound to the tissue approximately 60% is thorough only one of the aldehyde function (Figure 1c)[18]. Therefore one of the approaches to reduce BHV calcification is focused on the neutralization of free aldehyde groups and the removal of glutaraldehyde residuals[12].

Procedures to minimize the problems attributed to GA processed BHV observed post implantation includes the treatment with amino acids[19], particularly with Glu[20-22], ethanol[23], ethanol: Glu[24], citric acid[25] and diamines[15]. From these treatments probably the first examples of a BHV developed under the concept of tissue engineering were those processed with GA followed by the treatment with Glu solutions in acid media that were characterized by low calcification levels associated with endothelial growth[20-22]. Calcification levels are compared to those described for BHV processed with GA followed by the treatment with α-amino-oleic acid[26,27].

In spite of the favorable post implantation effects described for BHV processed with GA followed by the treatment with Glu, some properties of these materials have not yet been described since they were performed on commercially available commercial BHV. This work reports the study on the mechanical and thermal stability properties, the stability to collagenase hydrolysis and surface morphology by scanning electron microscopy of BP crosslinked with GA before and after the treatment with Glu solution.

2. Experimental

2.1. Solvents and reagents

Except for GA all reagents and solvents were ACS grade and collagenase type V, 435U.mg^{-1} was purchased from Sigma. GA, 25% from Union Carbide was purified before use by treatment with activated charcoal[28] and the criteria for acceptance was given by the

*e-mail: ggoissis@terra.com.br, domingo@braile.com.br, vladimir@braile.com.br

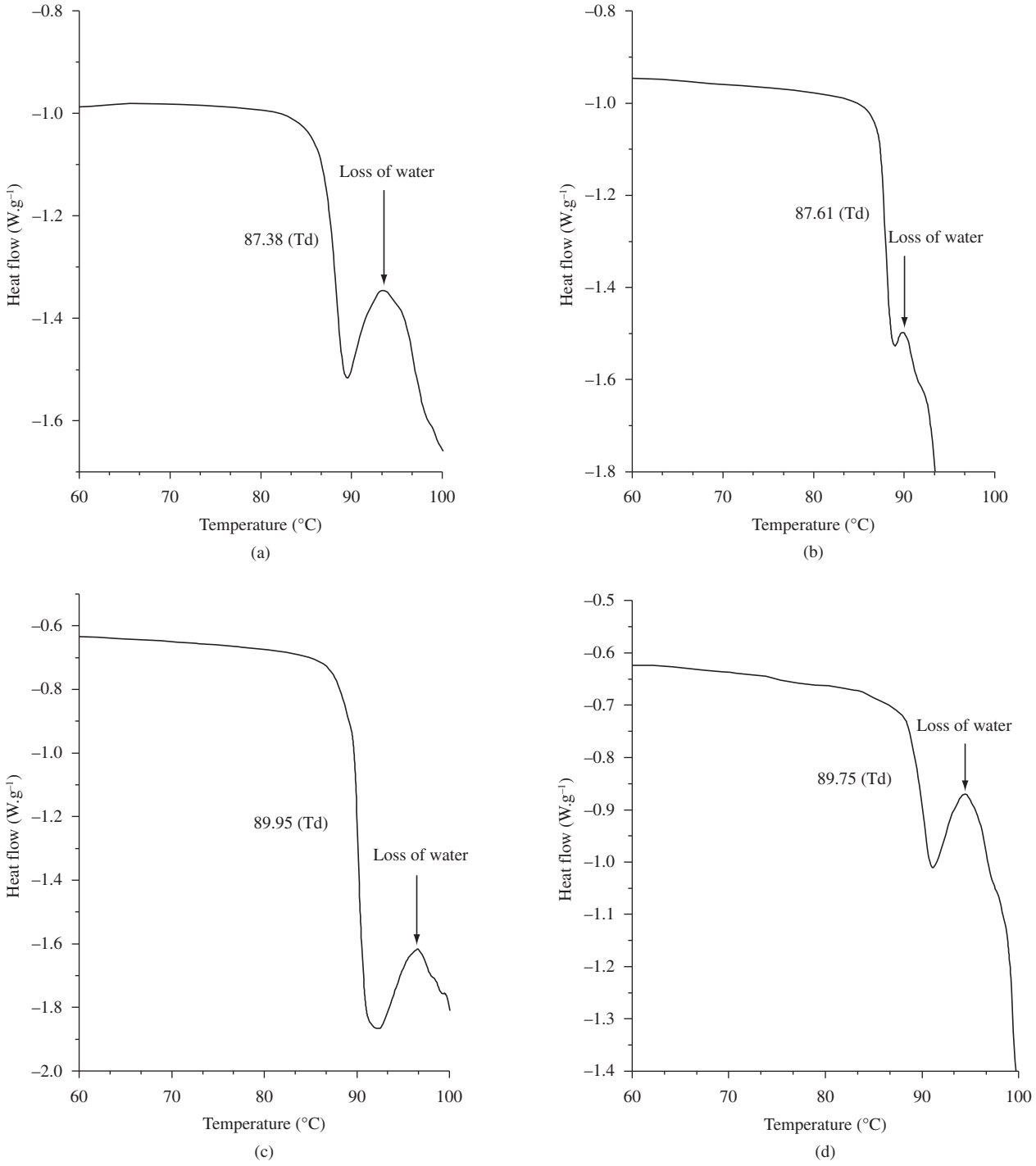


Figure 1. Thermogram of bovine pericardium materials after crosslinking with glutaraldehyde (GA) under different conditions: a) 0.5% GA for 10 days; b) 0.5% GA for 10 days followed by glutamic acid/borate buffer; c) Pre-treatment with GA 0.05% + 0.5% GA for 10; d) Pre-treatment with GA 0.05% + 0.5% GA for 10 days followed by glutamic acid/borate buffer.

ratio from the absorbances 280/235 nm (acceptance: > 1.50). Bovine pericardium (BP) was kindly supplied by Braile Biomédica Ind. Com. e Repres. Ltda S.A.

2.2. Crosslinking of bovine pericardium with GA

Conventional processing: 12 fresh BP with a thickness between 0.25 and 0.30 mm were fixed in circular plastic supports (16 cm in diameter) followed by crosslinking with 0.5% GA solution in 0.13 mol.L^{-1} phosphate buffer solution, pH 7.40, (PB) for a period of 10 days according to the routine procedure used by Braile Biomédica Ind. Com. e Repres. Ltda S.A for the manufacture of commercial BHV[28].

Pre-treatment with 0.05% GA solution followed by conventional processing: 6 fresh BP as described for the conventional processing were treated for 48 hours with a 0.05% GA in PB followed by six washes with the same buffer. The resulting material

was crosslinked with GA by the conventional processing and stored in the refrigerator until use.

Treatment with glutamic acid: 6 samples random samples removed from BP were crosslinked as described above and after washes with PB buffer and individually treated with a solution made 2.5 10^{-2} mol.L^{-1}/Glu and 5.0 x 10^{-3} mol.L^{-1} sodium borate. After adjusting the pH to alkaline conditions with 0.1 mol.L^{-1} sodium hydroxide the reaction was allowed to stand for 24 hours at room temperature. After this period the samples were washed 6x with PB and kept in the refrigerator.

2.3. Material characterization

Stability to collagenase hydrolysis[29]: six 8 mm discs were removed from BP crosslinked by the conventional processing, with pre-treatment with 0.05% GA before and after Glu treatment, totalizing 24 x 8 mm discs. After the removal of the excess buffer with filter paper they were frozen in liquid nitrogen, introduced in screw cap tube and lyophilized until constant weight. The average weight of the discs was 30 mg. To each tube was added a calculated volume of a solution of collagenase (Sigma - type V, 435 U.mg^{-1}) in 5.0 x 10^{-2} mol.L^{-1} 5 Tris-HCl/10 x 10^{-2} $CaCl_2.2H_2O$ mol.L^{-1}, pH 7.4 in such a way that in all tubes the enzyme concentration was 17.4 U.mg^{-1} of BP. The reaction was performed for 144 hours at 37 °C and stopped by heating the tubes in boiling water for 5 minutes. After centrifugation the supernatant was discarded, the remaining residue frozen in liquid nitrogen, lyophilized and weighted. The extent of hydrolysis was calculated by the relationship: (initial mass- mass of residue after lyophylization) / initial mass x 100.

Denaturation temperature (Td): Td was determined on computer-interfaced differential scanning calorimeter (DSC) from TA Instruments, model DSC-2010, USA after calibration with indium standard. BP samples of about 10 mg were previously equilibrated in PB buffer and introduced in sealed aluminum pans. The rate of heating was 5 °C/min from 25 to 150 °C under nitrogen atmosphere.

Scanning Electron Microscopy (SEM): BP Samples of approximately 1 cm in diameter equilibrated in PB buffer were washed 3x with deionized water to remove excess salt. After lyophylization photomicrographs were obtained in a Zeiss® SEM 960 electron scanning microscope operating at 20 keV after sputter coating with gold in a Balsers mod. SDC 050 equipment.

Mechanical properties[28]: These were evaluated by Braile Biomédica Ind. Com. e Repres. Ltda according to ASTM-638 in a MTS equipment, Model Qtest/1L, serial nº M-206170/102398.

3. Result and Discussion

The values for the tensile strength, elongation and toughness for BP crosslinked with 0.5% GA before and after the exposition to Glu/borate buffer (Table 1) were respectively 1.7 ± 0.4 Kgf.mm^{-2}, 14.2 ± 4.4% and 1379 ± 6.6 and, 2.2 ± 0.4 Kgf.mm^{-2},12.8 ± 2.3% and 15.5 ± 5.0 suggesting that, except for an increase of approximately 30% observed in the tensile strength, no others significant changes were observed in mechanical properties after the exposition of 0.5% GA crosslinked BP to Glu/borate buffer. Elongations for materials before and after the exposure to Glu/borate buffer were similar and of respectively 14.2 ± 4.4 and 12.8 ± 2.3. Nevertheless, significant increases in tensile strength and toughness were observed for BP previously crosslinked with 0.05% GA + conventional processing (0.5% GA for 10 days) (Table 1) followed by Glu/borate buffer, in comparison to materials crosslinked only with 0.5% GA.

In this case, the values determined tensile strength and toughness were respectively 2.5 ± 0.8 Kgf.mm^{-2} and 20.5 ± 5.0 and 1.4 ± 0.6 and 10.4 ± 4.7 corresponding in the same order to 78.5 and 96.1% increase in these properties. These results indicate that the exposure BP previously crosslinked with 0.05% GA followed by 0.5% GA and Glu/borate buffer significantly improves the mechanical properties of BP intended for the manufacture of BHV.

Table 1. Mechanical properties[a] of bovine pericardium crosslinked with glutaraldehyde under different conditions and with or without exposure to glutamic acid treatment.

	Crosslinking conditions			
	With 0.5% + GA for 10 days		With 0.05% GA + 0.5% GA for 10 days	
	BG	AG	BG	AG
Parameters				
Tensile strength (kgf.mm^{-2})	1.7 ± 05	2.2 ± 0.5	1.4 ± 0.6	2.5 ± 0.8
Elongation (%)	14.2 ± 4.4	12. 8± 2.3	16.2 ± 2.4	15.8 ± 2.0
Toughness	13.8 ± 6.6	15.5 ± 5.0	10.4 ± 4.7	20.5 ± 4.8
BG/AG Ratios				
Tensile Strength	1.3		1.7	
Elongation (%)	0.9		1.0	
Toughness	1.1		1.9	

[a]Values correspond to the average of six independent determinations. [b]BG and AG, before and after the treatment with Glu/borate buffer.

Although the changes in mechanical properties would suggest changes in structure, particularly in the case of BP previously crosslinked with 0.05% GA followed by 0.5% GA for 10 days, this was not confirmed by differential scanning calorimetry data (Table 2) since no significant change were detected in Td values for BP processed under the same conditions before or after the exposure to Glu/borate buffer. Td values for BP crosslinked with 0.5% GA before and after the exposition to Glu/borate buffer (Table 2) were respectively 87.7 ± 0.5 and 87.8 ± 0.9 °C in comparison to 90.5 ± 0.8 and 90.2 ± 0.5 °C determined for BP previously crosslinked with 0.05% GA.

The slightly higher values in Td values (around 2.0 °C) observed for BP previously treated with 0.05% GA are in agreement with the exposure of BP to higher GA concentrations that gives rise to more thermal stable materials[14,29] .As shown by the DSC profiles (Figure 1), independent from processing conditions, all resulting materials were homogeneous in the sense that only one thermal transition was observed in all cases ND and apparently no significant changes in the structure of BP crosslinked with GA were induced by the exposure to Glu/borate buffer.

The only difference observed in the thermograms was that, independent from the crosslinking conditions relative to GA concentration, the width of the transitions for BP materials exposed to Glu/borate buffer was smaller. While for BP exposed to Glu/borate buffer the width of the transition averaged 2.7 °C for non exposed materials this values was 4.9 °C suggesting on a comparative basis that materials exposed to Glu/borate buffer are more homogeneous.

Evidences of structural changes induced by exposure of GA crosslinked BP to Glu/borate buffer were detected by collagenase hydrolysis of the materials under study (Table 2). While no differences were observed in the extent of collagenase hydrolysis of PB previously crosslinked with 0.05% GA followed by 0.5% GA, before (8.9 ± 0.4%) or after exposure to Glu/borate buffer (8.8 ± 0.9%) for PB crosslinked only with 0.5% GA the extent of hydrolysis after exposure to Glu/borate were respectively 15.4 ± 0.5% and 9.2 ± 0.6% suggesting the occurrence of changes in GA crosslinked BP that

results in a material more stable to collagenase hydrolysis. These changes may be responsible for the increase observed in mechanical properties in BP crosslinked with GA after the exposure to Glu/borate buffer (Table 1).

The results above suggests that the changes in chemical characteristics and/or structure observed in GA crosslinked PB after the exposure to Glu/borate buffer may results from two independent effects: a) the first associated with the cleavage of polymeric GA crosslinks (Figure 2b-a') which are known to be formed after the processing of native tissue with GA solutions[13,16,30,31] which are cleaved under acidic[20-22,25] or basic conditions[32]; b) the second associated with the neutralization of remaining free aldehyde groups within the PB matrix as a result of Schiff base formation by reaction of the carbonyl function with α-amino group of Glu (Figure 2d-a' and b').

Table 2. Denaturation temperature and stability to collagenase hydrolysis[a] of bovine pericardium crosslinked with glutaraldehyde under different conditions, with or without the exposure to glutamic acid/borate buffer.

Property	Crosslinking procedure			
	With 0.5% + GA for 10 days		With 0.05% GA + 0.5% GA for 10 days	
	BG[b]	AG	BG	AG
Td (°C)	87.7 ± 0.5	87.8 ± 0.9	90.5 ± 0.8	90.2 ± 0.5
Hydrolysis (%)	15.4 ± 0.5	9.2 ± 0.6	8.9 ± 0.4	8.8 ± 0.9

[a]Values correspond to the average of six independent determinations; [b]BG and AG, before and after the treatment with Glu/borate buffer. Higher values in Td values (around 2.0 °C) observed for BP previously treated with.

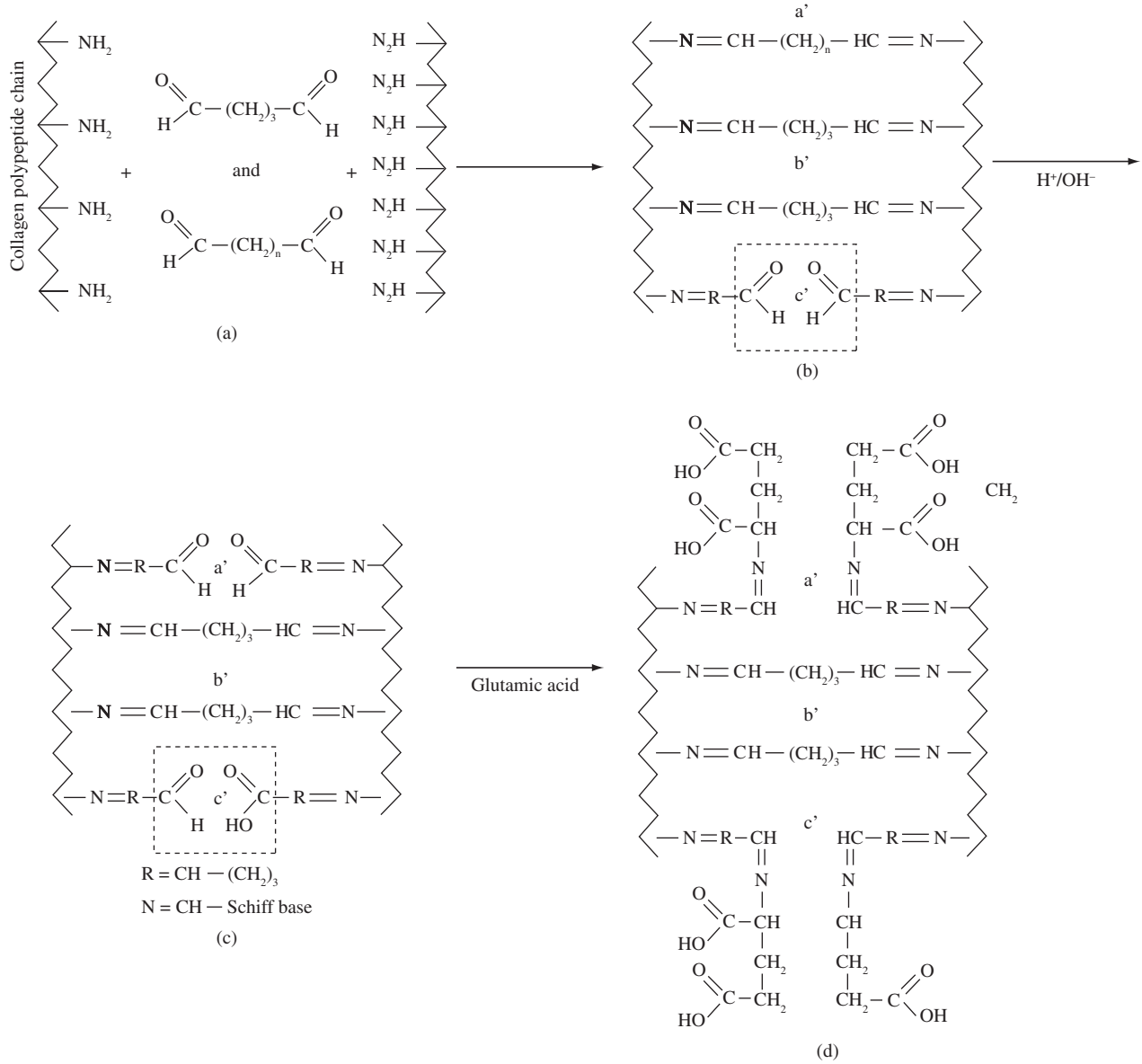


Figure 2. Schematic representation of the reaction glutaraldehyde (GA) solutions a) with natural tissues leading to b) desirable monomeric GA type of crosslinks a') polymeric GA crosslinks in red; b') bivalent monomeric glutaraldehyde type of crosslink; and c') residual free aldehyde groups from incomplete initial GA reaction or resulting from the c) depolymerization process and d) its neutralization by glutamic acid.

This chain of chemical events promotes an increase of two covalently bound carboxyl group/Glu residue resulting in an increase electrostatic interaction that could partially explain the improvements on the mechanical properties (Table 1). This increase may be significative since it has been described that from all covalently bound GA, 60% still preserves one free aldehyde group[18].

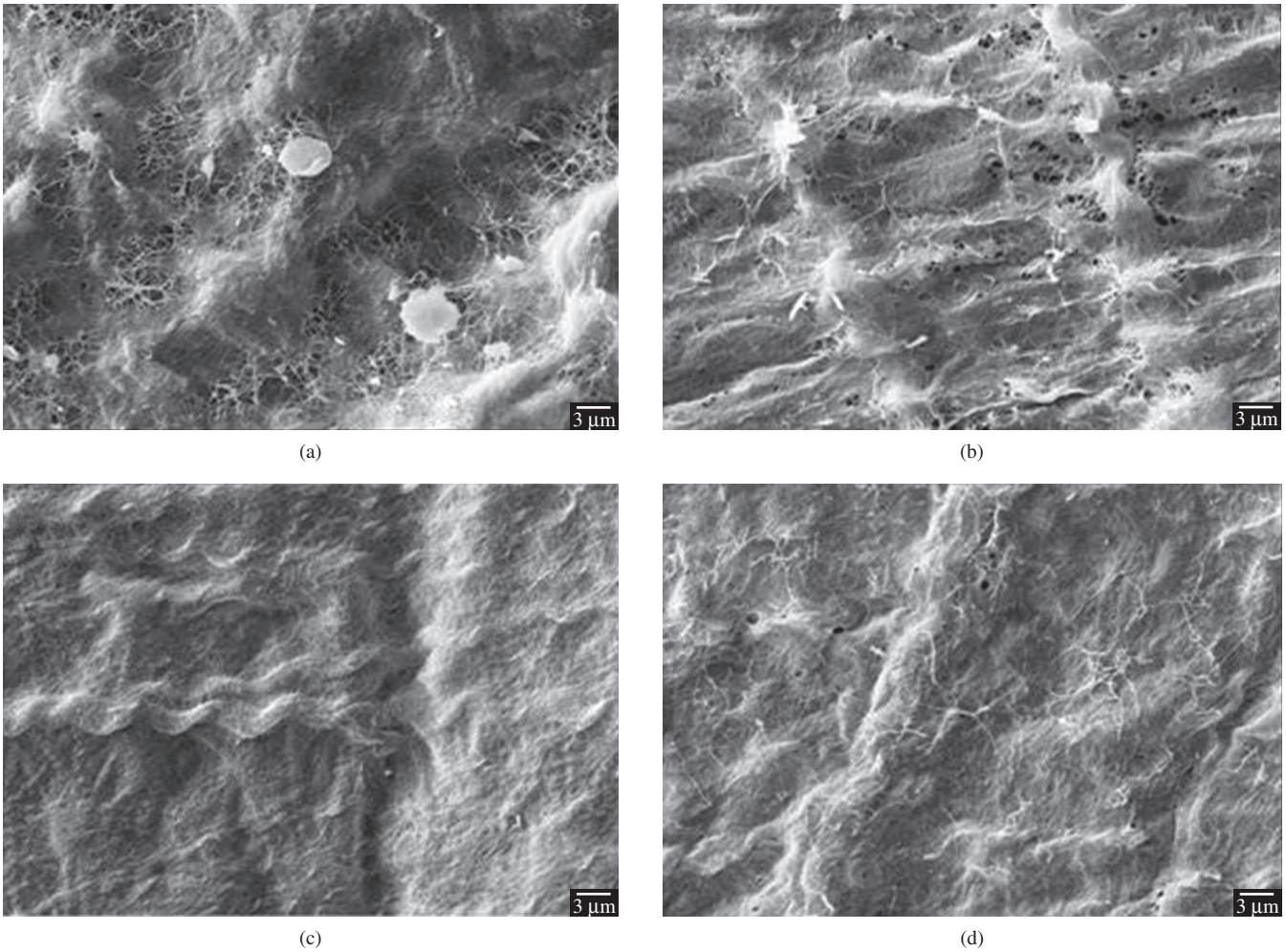
Coincidentally and in support to the chain of chemical events (Figure 2) proposed to explain the changes in properties described for GA crosslinked BP after exposure to Glu/Borate buffer is that most processes introduced to reduce BHV calcification were only effective[12] when GA crosslinked BP were exposed to: a) to acidic pH, a condition which is known to remove GA polymeric crosslinks[25]; b) treatment under acidic or alkaline[26] pH associated with reagents that neutralize the exceeding carbonyl function such as, Glu[20-22], ethanol/Glu[24], diamines[15] and 2-amino-oleic acid[26] existent free aldehyde groups. In support to this is the fact that the treatment of GA crosslinked BP with Glu in neutral pH has no effect on the reduction of calcification levels[19]. Under this condition the α-amino group of Glu is almost 100% in the form of the conjugated acid and not available to function as a nucleophyle.

With respect to BP surface morphology micrographs of Figure 3a and b showed that independent from the exposure to Glu/borate buffer the surface of material crosslinked with 0.5% GA were characterized by the presence of a pore like structure associated with the exposition of collagen fibers.

On the other hand BP materials previously crosslinked with 0.05% were characterized by a more homogeneous type of structure (Figure 3c and d). These results suggest that while the exposure of crosslinked BP to Glu/borate buffer is an important procedure to prepare BP materials with improved mechanical properties, the previous crosslinking of BP with lower concentration may be an important step for the preservation of the integrity BP surface as a result of a more homogenous crosslink formation with respect to BP native structure.

4. Conclusions

The results showed that while the treatment of 0.5% GA crosslinked BP with borate/glutamic acid significantly improves the mechanical properties, it had no visible effect on surface morphology. Better surface preservation was only achieved for BP pre-treated with a lower GA concentration followed by the conventional treatment (0.5% GA). Improvements in mechanical properties probably arises from structural changes as shown by collagenase results and probably



(a) (b)

(c) (d)

Figure 3. Scanning electron microscopy of bovine pericardium after crosslinking with glutaraldehyde (GA): a) 0.5% GA for 10 days; b) 0.5% GA for 10 days + glutamic acid/borate buffer; c) Pretreatment with GA 0.05% + 0.5% GA for 10 days; and d) Pre-treatment with GA 0.05% + 0.5% GA for 10 days + glutamic acid/borate buffer.

involves the depolymerization of polymeric GA crosslinks and an increase electrostatic interaction due to covalent binding of glutamic acid to free carbonyl groups (Schiff base). These results indicate that the processing of BP as described in this work may be of potential use not only for the manufacture of BHV but also to other collagen biomaterials with high demand in mechanical properties.

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