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Thermal and spectrophotometric studies of new crosslinking method for collagen matrix with glutaraldehyde acetals

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Abstract Despite the many existing crosslinking procedures, glutaraldehyde (GA) is still the method of choice used in the manufacture of bioprosthesis. The major problems with GA are: (a) uncontrolled reactivity due to the chemical complexity or GA solutions; (b) toxicity due to the release of GA from polymeric crosslinks; and (c) tissue impermeabilization due to polymeric and heterogeneous crosslinks formation, partially responsible for the undesirable calcification of the bioprosthesis. A new method of crosslinking glutaraldehyde acetals has been developed with GA in acid ethanolic solution, and after the distribution inside de matrix, GA is released to crosslinking. Concentrations of hydrochloride acid in ethanolic solutions between 0.1 and 0.001 mol/L with GA concentration between 0.1 and 1.0% were measured in an ultraviolet spectrophotometer to verify the presence of free aldehyde groups and polymeric compounds of GA. After these measurements, the solutions were used to crosslink bovine pericardium. The spectrophotometric results showed that GA was better protected in acetal forms for acid ethanolic solution with HCl at 0.003 mol/L and GA 1.0%(v/v). The shrinkage temperature results of bovine pericardium crosslinked with acetal solutions showed values near 85 °C after the exposure to triethylamine vapors.

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Introduction

Glutaraldehyde is largely used in the crosslinking of collagen matrices, such as bovine pericardium or porcine valves, to increase their mechanical and biological properties for the application to cardiovascular area [1, 2]. However, its reaction is very complex, as the GA aqueous solution forms very heterogeneous compounds, like the single hydrated GA and polymeric forms [3], which are present in this solution. The reaction with free amine groups is very fast, resulting in a heterogeneous material with superficial impermeabilization [2, 3]. These problems are frequently associated with calcification process [4–10], cytotoxicity [11] and deterioration of mechanical properties after bioprosthesis implant of heart valves [12].

Despite several problems associated with bioprosthesis of heart valves, mainly degeneration and calcification process [4–10], the GA used is still the best method for crosslinking [13]. The GA action under BP in the manufacture of bioprosthesis valves increases both their biological durability and mechanical properties of tissues by the crosslinking formation through Schiff base, which is a product of the reaction between ε-amine groups of Lysine and hydroxylysine residues near tropocollagen molecules. However, this crosslinking should occur between one mol of GA and two groups of amine present in the protein [7].

The present paper uses a GA alternative method, through which aldehyde groups may react with alcohol to form acetals or hemiacetals in acid medium [14]. They are stable forms, which do not react with amine groups (they are also protected in their protonated form) to form Schiff base, without the formation of α and β-unsaturated polymers. Thus, these chemically protected forms of aldehyde and amine groups could be rapidly activated by the

addition of organic base [15], permitting more homogenous reagent diffusion inside the tissue (Fig. 1).

The biomaterials with very well chemically defined crosslinking and more homogeneous distribution (Fig. 1), i. e., one GA mol for two mols of free amine groups ($-NH_2$), mainly inside the materials, minimize the impermeabilization. This process could reduce the effects with a probable reduction of cytotoxic effects and calcification in function of the employed conditions for GA hemicetals and acetals formation. Such distribution does not occur in aqueous buffer solution (conventional method) [13].

Materials and methods

All reagents and solvents were PA grade, except GA (25% from Union Carbide, Charleston, WV, USA), which was purified before use by activated charcoal. BP was kindly purchased from Braile Biomedica S.A., S. J. Rio Preto, SP, Brazil.

Preparation of GA acetals

The preparations of GA acetals (GAAs) or diacetals (GADA) were accomplished by the addition of purified GA (to make a 0.1, 0.5 and 1.0% GA solution) into absolute alcohol (EtOH) with HCl concentrations between 0.001 and 0.1 mol L^{-1}(M) After 24 h, the formation of these acetals were followed by ultraviolet (UV) spectroscopy. The spectra were compared with solutions of GA with similar concentrations in phosphate buffer solution (PBS, pH 7.40, 0.13 M), EtOH and aqueous solution.

GADA crosslinking of bovine pericardium

Bovine pericardium fragments (9 cm^2), collected and treated by conventional procedures as those described for valve manufacture, except without GA treatment, were equilibrated from aqueous to absolute EtOH by successive increasing steps of 20% in EtOH for 30 min, and the final solution was acidified between 0.001 and 0.01 M by the addition of concentrated HCl. After an equilibrium period of 24 h, BP fragments were transferred to the GADA solutions for periods between 24 and 96 h, followed by exposure to triethylamine (Et_3N) vapors for 12 and 24 h. They were finally equilibrated in PBS for 24 h.

Conventional GA crosslinking of bovine pericardium

This procedure was performed with BP fragments (9 cm^2) with 0.05% GA in PBS for periods of 24, 48 and 312 h [13].

Characterization of materials

(a) Shrinkage temperature

Shrinkage temperatures (T_s), which are the average of 3 independent determinations, were obtained in strips of 0.2 × 2.0 cm of native and crosslinked BP previously equilibrated in PBS in melting pointing equipment adapted for T_s determinations.

(b) Wettability of bovine pericardium in ethanol solution

The percentage of wettability (*W*%) was determined in BP pieces with diameter of 1.0 cm subjected to the treatment

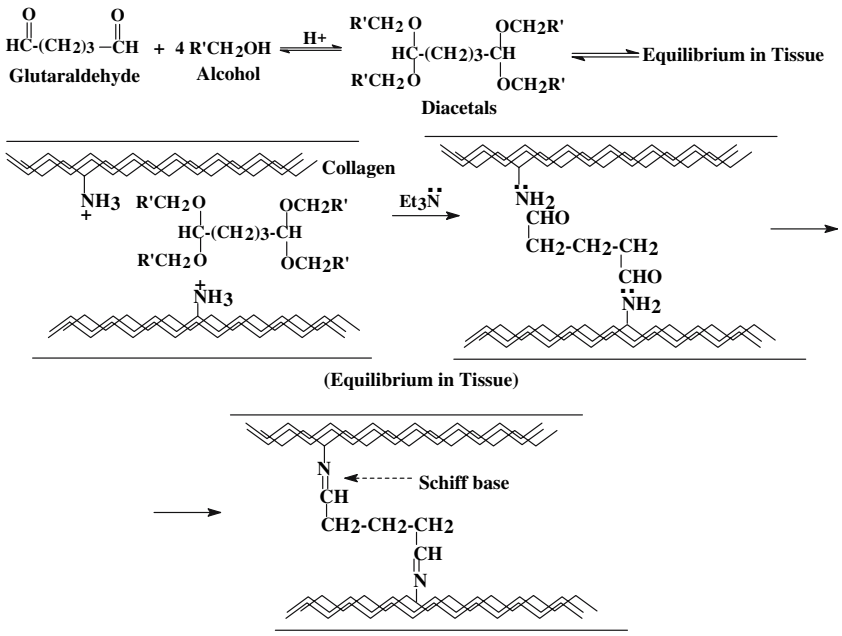

Fig. 1 Scheme for the crosslinking of collagen matrices by glutaraldehyde acetals way (a) formation of acetals and (b) crosslinking induction of matrix with triethylamine and Schiff base formation

with increasing concentrations of 0, 30, 70 and 100% in absolute EtOH. These measurements were taken after the gradual reduction of EtOH until the balance of all the pieces in TF for 24 h. After exhausting washings with water, its excess was removed and the pieces were weighed.

Each piece of BP was then dried by freezing-dry methods (Edwards freezing-dryer, Modulyo model) until reaching a constant weight. The obtained results of wettability correspond to an average of three independent determinations and were established by the following formula:

$$W_{\%} = \frac{(m_{wet} - m_{dry})}{m_{dry}}.100 \quad (1)$$

where m_{wet} is the mass of the wet BP and m_{dry} is the mass of the dry BP.

Results and discussions

Formation of glutaraldehyde acetals in acidified ethanol by spectroscopy

A way to assess the presence of polymeric and aldehydic forms of GA is taking a spectrophotometric measurement of its aqueous solution with about 0.05% (v/v) GA [16].

Figure 2 corresponds to the spectra in the UV region between 200 and 350 nm of the GA solutions (1.0%) in both water (Fig. 2aI and 2bI) and buffer phosphate solution, pH 7.40, 0.13 M (PBS) (Fig. 2II 2bII), after 1 h and 22 h of their preparations. A small peak was initially observed at 235 nm and the large one was obtained at 280 nm, corresponding to unsaturated polymeric and free forms of GA, respectively, in both solutions after 1 h of preparation. After 22 h of preparation, the GA solution in water did not present any significant variation in the absorbances in the UV region. On the other hand, in PBS it revealed a significant increase at 235 nm, showing that the unsaturated polymer formation of the GA in PBS, as used in the conventional treatment, may produce an uneven crosslinking in biological tissues.

Figure 2 also shows that under the conditions of conventional use for crosslinking process, i.e., in PBS, the great absorbance variation at 235 nm must be the high value of molar absortivity (ε) of the polymeric forms, since their formations do not affect the concentration of the GA monomeric form significantly. Such polymeric forms are one of the products responsible for the superficial impermeabilization of biological tissues during the reaction in this solution, which they are formed by –C=C–C=O structure, due α and β polymers formation throught a several GA molecules (mers), like CHEUNG et al. [7, 9] had found.

A method to minimize both the reactivity effect of the aldehydic groups with the tissue and the formation of the GA polymeric forms is the protection of the aldehydic groups in the non-reactive group forms to the tissue, so that these groups can be uniformly distributed inside the tissue before crosslinking (Fig. 1). This method does not produce polymeric forms of GA, therefore this solution does not have absorbance at 235 nm.

Neither GA acetal nor hemiacetal forms possess groups that can absorb radiation in the UV spectrum region (Fig. 1), in contrast to the GA in the unsaturated polymeric or free forms, which occur in two very well specific regions, i.e., at 235 and 280 nm (Fig. 2). These bands

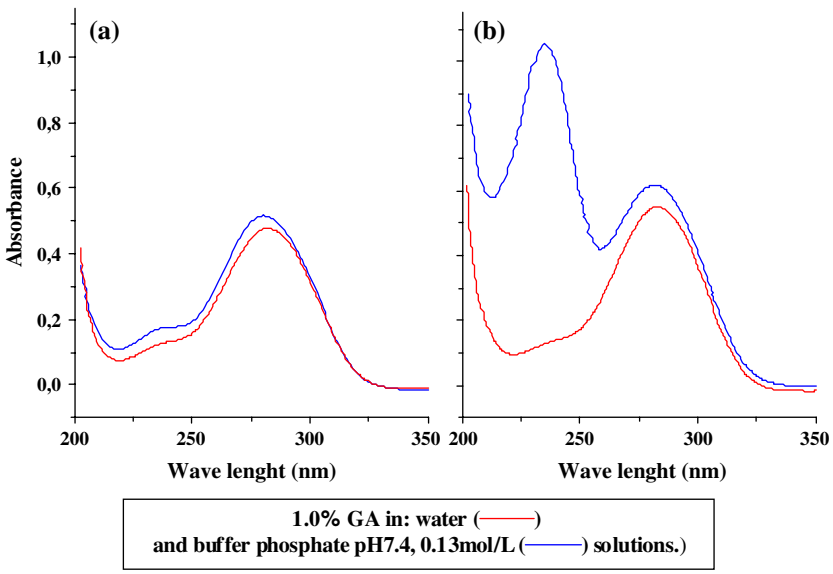

Fig. 2 Spectrophotometric curve in ultraviolet for glutaraldehyde solution at 1.00% in aqueous and phosphate buffer solution, pH7.4, 0.13 M, after 1 h (a) and 22 h(b) of preparations at 25 °C

correspond to the groups of the double carbon–carbon (C=C) and carbonyl (C=O) links [7, 9, 16], therefore the conditions to obtain a more stable solution of GA with the minimum presence of the unsaturated polymeric form of GA in acidified EtOH (Figs. 3, 4, and 5) have been evaluated in a way to protect the aldehyde groups. Thus, the GA distribution can occur in a more homogeneous way in the tissue without the superficial impermebilization.

Comparative studies in the UV spectra were also performed with GA solutions in EtOH in the presence of variable concentrations of both HCl (between 0.010 and 0.001 M), and GA (between 0.05 and 1.0%, v/v) in an interval between 2 and 96 h (Figs. 3–5) to observe either the reduction or the absence of GA in the monomeric and polymeric forms in the UV region studied. The time interval used in this experiment was the necessary time for the formation and homogeneous distribution of GA in its protected form (acetals) inside, which presents a thickness of approximately 0.3 mm (wet tissue).

In the most acidified EtOH solutions of GA (Figs. 3–5), it is possible to observe the presence of peaks at 235 nm and 280 nm, which correspond to free aldehydic and polymeric forms, respectively. Some of the solutions present negative absorbances, probably due to the formation of water molecules after the reaction between alcohol and aldehyde groups, which have smaller absortivity than alcohol. The displacements of the absorbance peaks must be due to the probable solvent and/or the formation of hemiacetal and acetal in the solution.

Therefore, Figs. 3–5 showed that, except for the GA solution in EtOH with concentration of HCl 0.003 M and GA 1.0%, the spectrophotometric behaviors in the UV (200–350 nm) of the GA solutions in EtOH in HCl concentrations between 0.01 and 0.001 M and in GA between 0.05% and 1.0%, in time intervals between 2 and 96 h of preparations were very variable. The solutions present free aldehydic groups and unsaturated polymers of GA, which makes the crosslinking of collagenic matrices inadvisable.

Only one solution presented the most stable spectrum, probably with the formation of hemiacetals or acetals (solution of GA in EtOH with 0.003 M HCl and 1.0% GA (Fig. 4). There was no variation in its spectrum even after 96 h of its preparation. The formation of acetals under this condition is fast and stable and does not form other compounds, like polymeric forms.

This solution was compared with the spectra in the UV of the GA (1%) solutions in PBS, water, EtOH and EtOH with 0.003 M HCl, after 1 h and 22 h of their preparations at 25 °C, and 22 h followed by 1 h at 40 °C (Fig. 6).

All the spectra in Fig. 6 were characterized by the presence of two maximums of absorptions at 280 and 235 nm, corresponding to the absorptions of free and polymeric GA, respectively [7, 9, 16].

The results indicate that a characteristic band for GA in EtOH or PB solution at 280 nm, corresponding to the free aldehyde group, is, in some cases, no longer observed in acidified EtOH. It is due to the formation of acetals or hemiacetals [14], particularly after 24 h of reaction in acidified EtOH solution with 1.0% GA and 0.003 M HCl. In PB solution, it is possible to observe the formation of a polymeric group corresponding to the band at 245 nm, besides an aldehyde group band.

The spectra in the UV (Fig. 6) show alterations in the absorbance curves of GA solutions for all the cases.

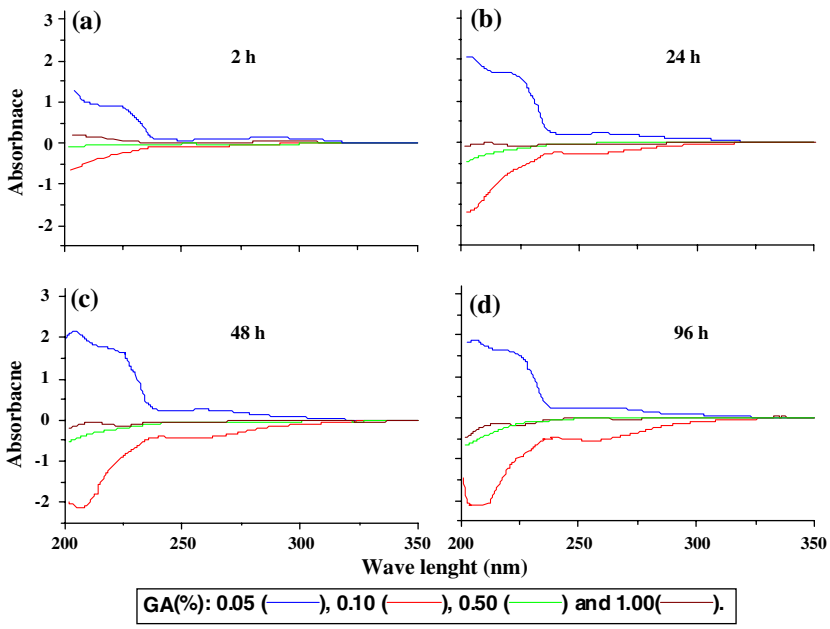

Fig. 3 Spectra in the ultraviolet of the solutions of glutaraldehyde in ethanol with concentration of HCl 0.001 M glutaraldehyde in the presence of: (I) 1.00%, (II) 0.50%, (III) 0.10 % and (IV) 0.05% between 2 and 96 h at 25 °C

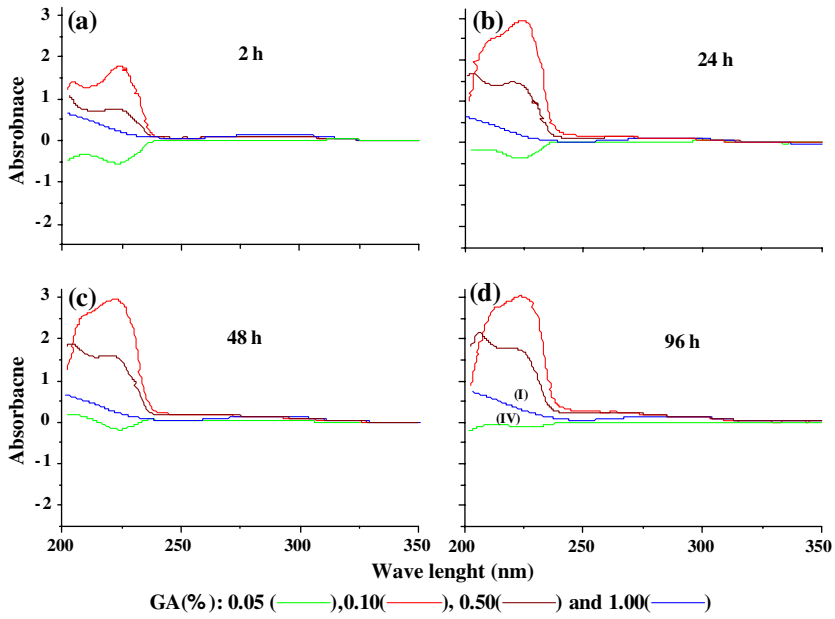

Fig. 4 Spectra in the ultraviolet of the solutions of glutaraldehyde in ethanol with concentration of HCl 0.003 M glutaraldehyde in presence of: (I) 1.00%, (II) 0.50%, (III) 0.10 % and (IV) 0.05% between 2 and 96 h at 25 °C

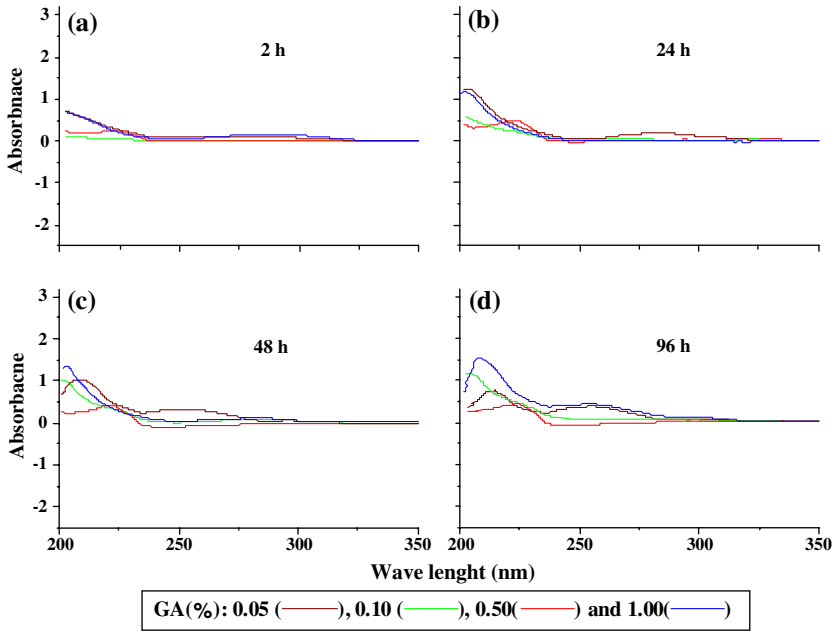

Fig. 5 Spectra in the ultraviolet of the solutions of glutaraldehyde in ethanol with concentration of HCl 0.001 M glutaraldehyde in the presence of: (I) 1.00%, (II) 0.50%, (III) 0.10 % and (IV) 0.05% between 2 and 96 h at 25 °C

However the most significant variations were observed only for the solutions of GA in PBS (Fig. 6b), whose characteristic was a significant increase in the absorbance at 235 nm. For the GA solution in EtOH acidified with HCl of 0.003 M, in which there was a drastic reduction, the peak corresponding to the band at 280 nm practically disappeared (Fig. 6a, b). This band relative to the free GA corresponds to the double link of the carbonyl group (–C=O) of the aldehydic function.

As the 280/235 ratio is a measurement of the relative concentration variation of the free GA in comparison to the polymeric forms [15], these variations of these relations in function of both time and solution conditions were also evaluated (Table 1).

For aqueous solutions of GA, the values observed for the relation of 280/235 nm after 1 h, 22 h and 22 h of reaction followed by 1 h at 40 °C (Fig. 6 and Table 1) were, respectively, 3.8, 5.2 and 4.2, suggesting that, under room temperature, the GA solutions have their balance dislocated towards the free form of GA. This is probably due to the forms of polymeric hemiacetals already existent in the GA solution formed by the free GA. However, this balance is

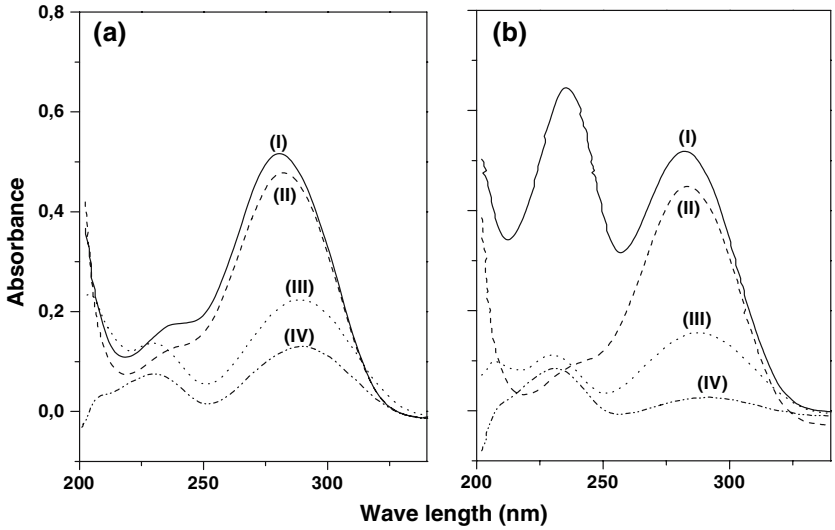

Fig. 6 UV spectra of 1% glutaraldehyde solution in 0.13 Mol L^{-1} phosphate buffer, pH 7.4 (I), water (II), absolute ethanol (III) and 0.003 Mol L^{-1} in absolute ethanol (IV) after 1 h (a) and 24 h (b) of its preparation

Table 1 Variations of the absorbances and values of the 280/235 nm relation for GA solutions (1%) in water, buffer phosphate (pH 7,4) and ethanol acidified or not, before and after the heating of 40 °C

λ (nm)	Absorbances in function of reaction time[a]											
	Buffer (I)			H_2O (II)			EtOH (III)			EtOH/HCl (IV)		
	1 h	22 h	23 h[b]	1 h	22 h	23 h[b]	1 h	22 h	23 h[b]	1 h	22 h	23 h[b]
280	0.51	0.51	0.61	0.47	0.46	0.54	0.21	0.14	0.14	0.11	0.02	0.03
235	0.16	0.64	1.05	0.12	0.08	0.12	0.12	0.10	0.08	0.07	0.07	0.07
280 235	3.00	0.80	0.60	3.80	5.20	4.20	1.60	1.30	1.70	1.60	0.20	0.40

[a] These data refer to the spectra in Fig. 6

[b] Reaction time of 22 h followed by 1 h at 40 °C

unfavored by the heating of the solution, as shown in the decrease in the 280/235 ratio (Table 1) for a value of 4.2.

For the case of the buffered solutions of GA, the values observed for the 280/235 ratio for equilibrium times of 1 h, 22 h and 22 h of reaction followed by 1 h at 40 °C (Fig. 6) were 3.0, 0.8 and 0.6, respectively (Table 1). These reductions in the ratios suggest that, differently from the aqueous solutions of GA in buffered solutions, the concentration of the polymeric structures increases constantly in function of the time and the conditions used in the reaction [8, 9]. These conditions can cause a superficial impermebilization and the cytotoxic effect after implants of cardiac valves bioprostheses compromises their mechanical and biological properties [11].

However, the GA solutions in EtOH showed a differentiated behavior when compared with the aqueous or buffered solutions of GA. In the two cases, the solutions of GA in EtOH and EtOH/HCl were characterized by the presence of two well-defined absorption bands at 235 and 280 nm with a common characteristic, which was the significant absorbance reduction at 280 nm, indicating a reduction in the concentrations of free GA in the two cases. This effect was much sharper for the acid solution, whose absorbance values for the time interval of 22 h were practically zero, probably in function of the catalysis promoted by the presence of H^+ species, as showed in the project of Fig. 1. The 280/235 ratio for the EtOH and EtOH/HCl solutions after 1 h and 22 h at 25 °C, and 22 h followed by 1 h at 40 °C were, respectively, 1.66, 1.33, 1.75 and 1.67, 0.26 and 0.41.

Despite the addition of the 235/280 ratio to the solution of ETOH/HCl for 22 h and 1 h at 40 °C, the values of the polymeric form (235 nm) continued constant and equal to 0.07, as well as the values at 280, which were only 0.02 and 0.03. This probably showed that a small amount of the polymeric form was still steady, even in EtOH/HCl solution.

Differently from the profiles of the spectra in the UV observed for water and buffered solutions of GA, the absorbance reductions observed at 280 nm were not

followed by simultaneous increases in the absorbances at 235 nm, suggesting that the disappearance of the free GA in the solutions of EtOH was not associated with the formation of polymeric species. Under these conditions, due to a high concentration of EtOH, which does not favor the formation of hemiacetals (GAHA), the acetals (GADA) must be the predominant chemical form for GA (Fig. 1). In these two cases, the increasing temperature favored the free GA form, as suggested by the increases observed in the 280/235 ratios.

In both spectrophotometric studies, there was a significant difference of absorbance peaks at 235 and 280 nm for the GA solutions, as they were stock solutions of GA with different time intervals after purification.

Thermal stability and wettability of bovine pericardium in EtOH

One of the concerns about the introduction of the BP crosslinking by means of GA acetals is associated with the long exposure of the material to ethanol, therefore the reaction is not only performed in alcoholic medium, but also in its storage. The results of Table 2 show the behavior of the thermal stability for BPs subjected to these conditions. While for the native BP equilibrated in PBS, the T_s value was 63.6 ± 0.5 °C, for the BP kept in ethanol concentrations of 30, 70 and 100%, the T_s values were, respectively, 64.5 ± 1.2, 65.5 ± 0.4 and 65.3 ± 0.5 °C, indicating that, from the point of view of thermal stability, the structural variations were not significant.

Possible structural alterations can be also detected by wettability in view of the dehydrating capacity of the EtOH. The results of Table 2 show the values of wettability of native BP and samples of BP kept initially in EtOH for 24 h, followed by the equilibrium in PBS for 24 h. While the value of wettability was 76.6 ± 1.0% for the native BP, for the materials previously balanced in EtOH 30, 70 and 100% these values were, respectively, 79.2 ± 1.4, 78.5 ± 2.1 and 78.5 ± 1.0%. These results confirmed the thermal stability, indicating that the gradual increase in water solutions for absolute EtOH did not significantly modify the structure of the BP, showing that it is possible to perform the crosslinking.

Table 2 Values of embebition and shrinkage temperature for bovine pericardium subjected to different concentrations of EtOH for 24 h

Property	EtOH (%)			
	0	30	70	100
Embebition (%)	76.6 ± 1.0	79.2 ± 1.4	78.5 ± 2.1	78.5 ± 1.0
T_s (°C)[a]	63.6 ± 0.5	64.5 ± 1.2	65.5 ± 0.4	65.3 ± 0.4

[a] Values obtained after equilibrium in PBS for 24 h

GA Acetal crosslinking of bovine pericardium

The results in Fig. 7 correspond to the values of the thermal stability determined as T_s of the BP pieces equilibrated for 48 h in a GA solution in EtOH, with GA concentration of 1.0% and HCl of 0.003 M (GADA). The measurements were taken after the pieces had been exposed to TEA vapors in time intervals between 0 and 72 h.

These results showed the gradual increases in the thermal stability, which varied from 63.6 ± 0.5 °C for the native pericardium to 85.8 ± 0.3 °C after 24 h. After this time, the T_s values remained constant and the values for 48 and 72 h were, respectively, 86.8 ± 0.5 and 86.8 ± 0.5 °C. On the other hand, such results showed that the idea of protection of the GA as hemiacetals or acetals (Fig. 1) works effectively in practice. Therefore the T_s values obtained are inside what is accepted for those BP treated by conventional process and must be higher than 83 °C. This is observed in Fig. 7, which also shows that the reaction of GA desprotection occurred practically at 24 h, since until that time, the increases in T_s values were not significant.

Although the results of UV showed that the best conditions to obtain the homogeneous solutions in acetals or hemicetals forms were with GA 1.0% and HCl 0.003 M (GADA), so the crosslinking reaction was performed in BP to prove that the thermal stabilities of BP treated in EtOH solutions with variable concentrations of GA between 0.5 and 2.0%, and HCl between 0.1 and 0.001 M were higher. The time varied from 0 to 72 h and the BPs were exposed to TEA vapors for 24 h (Fig. 7). This experiment was performed to confirm the efficiency of the process of GADA formation and GA free delivery through the analyses of Fig. 8.

It was observed that for GA concentration of 0.5% (Fig. 8a), the minimum stability of the BP obtained after 96 h was the one treated in the presence of HCl 0.100 M,

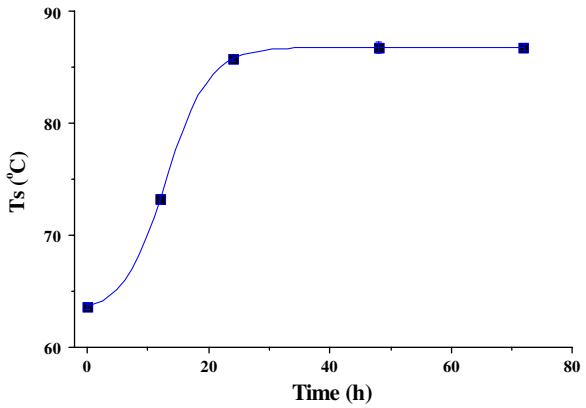

Fig. 7 Shrinkage temperature of the bovine pericardium fragments treated with acetals with GA 1.0% and HCl 0.003 M for 48 h after exposition to TEA vapors in different time intervals

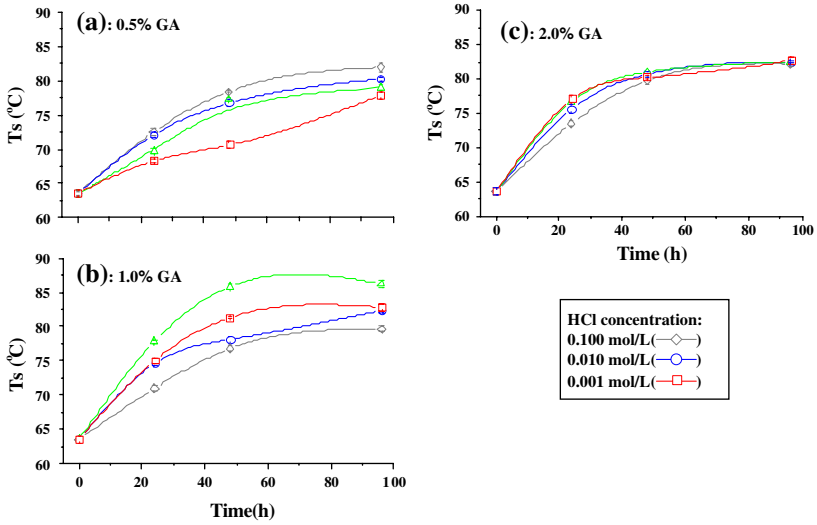

Fig. 8 Behavior of shrinkage temperature of bovine pericardium treated with several concentrations of glutaraldehyde acetals and HCl, after exposition to triethylamine vapor for 24 h

whose value was 81.8 ± 0.5 °C. In the treatments with HCl concentrations of 0.010, 0.003 and 0.001 M, the T_s values were, respectively, 80.2 ± 0.3, 79.0 ± 0.3 and 77.8 ± 0.5 °C. However none of them reached a constant temperature in the time interval studied.

Figure 8b also shows a gradual increase in the majority of HCl concentrations after 48 h of treatment, with the T_s value of 85.8 ± 0.5 °C, reaching the maximum value of 86.2 ± 0.5 °C for the BP treated with 0.003 M HCl and 1.0% GA (Fig. 8b).

In excessive concentrations of HCl, the T_s values had a gradual increase after 48 h, but with lower values than 83.0 °C (Fig. 8b). On the other hand Fig. 8c, in which the GA concentration is 2.0%, shows a similar gradual increase in all HCl concentrations (between 0.010 and 0.001 M), becoming almost constant until 48 h of treatment, with T_s average values lower than 83.0 °C after 96 h of treatment.

The results above show that, for GA concentration higher than 1% (Fig. 8a), the HCl concentration inside the tissue was so high that either the posterior exposure to TEA vapors did not neutralize all the HCl, or the GA concentration was so diluted that it did not present a uniform crosslinking, obtaining lower T_s values than 83.0 °C (Fig. 8).

In the case of GA concentration higher than 1.0%, the BP obtained did not increase considerably in the thermal stabilities after the exposure to TEA vapors. This material had a GA excess (2.0%), forming an almost impermeable surface, which hindered the entrance of TEA vapors to the interior (Fig. 1). It has native collagen and presents lower T_s values than 85 °C.

According to these results, the treatment condition of BP in ethanolic solution, which had the highest value of thermal stability of BP obtained, was the one that had HCl concentrations of 0.003 M and GA 1.0%(v/v), where the T_s value reached around 85.8 ± 0.5 °C after 48 h of equilibrium in GADA solution and 86.2 ± 0.5 °C after 96 h, followed, of course, by exposure to TEA vapors for 24 h.

The UV spectrophotometric results previously obtained in the EtOH solutions containing strong acid and GA (Figs. 3–5) showed that, spectrophotometrically, the most stable solution also liberated more easily the aldehyde group protected in the form of acetal after exposure to TEA vapors (Fig. 7).

The results above show that, in acidified EtOH, the GA has its aldehydic groups effectively protected, probably forming diacetals (GADA), which are not able to react with the free amino groups of protein for the formation of the Schiff base (–CH = NH–). This link was efficiently formed, when compared with the conventional methods, after the exposure of the material to TEA vapors (Table 2).

The protection efficiency of GA as acetals was confirmed by experiments and comparisons shown in Table 2, which refers to the thermal stability values of the BP. BP is subjected to several routines established for crosslinking by means of GADA, and in comparison with the conventional procedure.

The T_s values for BP in EtOH solution with only HCl 0.003 M were not significantly different from those obtained for native BP (63.6 ± 0.5 °C) or balanced in absolute EtOH (65.3 ± 0.5 °C). These results also suggest that sequential exposures of BP to solutions of EtOH/HCl and TEA vapors, for over 24 h did not cause alterations in the structure of the matrix. No denaturation of the tissue was observed when TEA was added.

Besides the spectrophotometric data of glutaraldehyde protection, the efficiency of the GA reaction in the form of diacetals (GADA) was demonstrated, when effectively comparing the volumes and concentrations used for the

reaction with BP to the conventional procedure. Considering that the wettability volumes for BP in EtOH were similar to those determined for BP (Table 2), the effective volume of the reagent available for the reaction for BP mass (gBP) would be approximately 0.77 mL. This volume would correspond to effective concentrations of about 108 mmoles GA/gBP for GA in the GADA form, in relation to 271 mmoles/gBP for the conventional method with GA solution 0.05% (total volume of 5 mL). As the total number of mmoles of amine groups is 30.8 residues of lysines/gBP and considering the similarity of the T_s values obtained in the two cases, the molar GA concentration in the GADA form was about 2.5 times more efficient than the conventional method with GA. This result can also be confirmed by biostability tests with collagenase, which have been published in another paper [15].

Conclusions

The results above show that this new process is more efficient to protect free aldehydic reactive groups and minimize a polymeric formation of GA, reducing superficial impermebilization with GA crosslinking on collagen matrices. The crosslinking reaction occurs after exposure to TEA vapors of collagen matrix.

The data already published have showed that collagen matrices crosslinked with GA acetals are 3 times more stable to biodegradation with collagenase [15] than the conventional methods of GA.

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