Comparison of chemical treatments on the chain dynamics and thermal stability of bovine pericardium collagen

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Abstract: A new approach for the replacement of heart valves consists of obtaining an acellular matrix from animal aortic valves that performs mechanically, is nonantigenic, and is free from calcification and fibroblast proliferation. Novel biochemical treatments must be developed for this purpose. In this work, we focus on the characterization of collagen in acellular bovine cardiovascular tissues, fresh or glutaraldehyde treated, and stored in different solutions [phosphate-buffered saline (PBS), ethanol, octanol, and glutaraldehyde], to determine whether the resulting fibrous material is structurally preserved. The preservation of the triple helical structure of collagen is checked by differential scanning calorimetry (DSC), which is a well suited technique to analyze thermal transitions in proteins, such as denaturation. To get insight into the molecular dynamics of collagen in the nanometric range, we used thermally stimulated currents, a dielectric technique running at low frequency, that measure the dipolar reorientations in proteins submitted to a static electrical field. The combined use of these two techniques allowed us to evaluate the physical structure and conformation of collagen after the different chemical treatments. We have found that the glutaraldehyde treatment followed by octanol storage preserves the triple helical conformation of the polypeptidic chains of collagen, contrary to the ethanol and PBS storage that induce drastic changes in the thermal and dielectric behavior of the protein. Moreover, this particular chemical treatment stabilizes the collagen structure (shift toward high temperature of the collagen denaturation and stiffening of the chains by a cross-linking action) when compared to the control sample, and so could provide interesting fibrous material for the conception of bioprosthetic heart valve. © 2002 Wiley Periodicals, Inc., J Biomed Mater Res 64A: 330–338, 2003

Key words: bovine pericardium; differential scanning calorimetry; thermally stimulated currents; collagen denaturation

INTRODUCTION

Calcification of bioprosthetic heart valves is typically considered to be a pathologic process. If the focus of the study of calcification is changed from the study of the pathology to that of normal physiology, then we can ask—What is different when comparing a tissue that calcifies and one that does not? In one case, bone calcifies as part of normal physiology. When bone no longer calcifies, it is a pathological process. Along the same lines, a person's heart valve will function normally for many years before calcifying. Pericardium exists in its normal condition without calcifying, yet upon fabrication into a bioprosthetic heart valve, it calcifies. Allografts, when harvested, are not calcified, yet when implanted, they calcify in 10–15 years. At some point in time, something changes to begin the pathologic process of calcification.

The purpose of this work is to determine whether proteins change as a function of chemical treatment. In all of the cases described above, there is a need to know whether an inflammatory response results in the presentation to the implanted tissue the cellular components that lead to calcification, or whether a change in the protein structure causes the initiation of the calcification process. This work does not address which comes first, but instead investigates whether there is a difference between the proteins of a normal tissue compared to a calcified tissue.

The tissues evaluated, in this case, were bovine pericardium, which was used because it is a relatively simple case compared to a heart valve leaflet, either porcine or human. It also provides more relatively uniform material that can be compared.

Several different tissue treatment processes were evaluated to see if changes in protein structure could

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be identified, including fresh, glutaraldehyde-treated, glutaraldehyde-treated/ethanol-stored, and glutaraldehyde/long-chain alcohol storage solution. The analytic methods used for the initial studies were developed to study conformational changes in polymers. They include differential scanning calorimetry (DSC) and thermally stimulated currents analysis (TSC).

EXPERIMENTAL

Materials

We prepared six types of tissues, with different treatments and storage conditions detailed as follows:

1. *Control samples of fresh tissue stored in phosphate-buffered saline (PBS):* Bovine pericardium was obtained fresh from the abattoir and placed in chilled PBS (pH 7.4) until the time of analysis or implantation. Extraneous fat or muscle was removed, and sections with heavy vasculature or attached ligaments were discarded. These samples are referred to as control or **/PBS**.
2. *Fresh tissue stored in 80% ethanol:* Fresh bovine pericardium stored in chilled PBS was transferred to 80% ethanol-buffered *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (10m*M*) solution for 3 days prior to analysis. These samples are referred as **/Eth**.
3. *Glutaraldehyde-treated, PBS-stored:* Fresh bovine pericardium stored in PBS was transferred to a buffered solution of 0.25% glutaraldehyde for 1 week. The tissue was transferred to chilled PBS for 3 days prior to analysis. These samples are referred as **Glu/PBS**.
4. *Glutaraldehyde-treated, stored in 80% ethanol:* Glutaraldehyde-treated bovine pericardium was transferred to 80% ethanol-buffered HEPES (10m*M*) solution for 3 days prior to analysis. These samples are referred as **Glu/Eth**.
5. *Glutaraldehyde-treated, stored in octanol:* Glutaraldehyde-treated bovine pericardium was transferred to 5% octanol/40% ethanol solution for 3 days prior to analysis. These samples are referred as **Glu/Oct**.
6. *Glutaraldehyde-treated, stored in glutaraldehyde:* Fresh bovine pericardium stored in PBS was transferred to a buffered solution of 0.25% glutaraldehyde for 1 week prior to analysis. These samples are referred as **Glu/Glu**.

Prior to DSC and TSC experiments, each of the tissues was flash frozen in liquid nitrogen and freeze-dried in a Freezone® 4.5 freeze-dry system (Labconco Corp., Kansas City, MO).

Methods

Differential scanning calorimetry (DSC)

The phase transition thermograms were recorded with a Perkin Elmer DSC7 differential scanning calorimeter. The temperature and energy scales were calibrated via the manufacturer's instructions, with indium and tin as standards. Samples 5–10 mg in weight were sealed in aluminum pans. Empty pans were used as references. Investigations were performed between 30 and 250°C with 10 or 20°C/min heating rates. Determination of transition parameters was performed with Origin software.

Thermally stimulated currents (TSC)

This technique was first used to characterize metallic divalent impurities in ionic crystals and was then applied to polymers, because it allows scanning dielectric relaxation processes at a lower frequency than classical dielectric spectroscopy.[1] Furthermore, the relaxations observed by TSC are largely due to the reorientation of polar groups or segments of the macromolecules.

TSC measurements were carried out with a dielectric apparatus developed in our laboratory and previously described.[2] Samples of 20–40 mg were compressed (2×10^3 kg/cm^2 for 2 min), resulting in disks 1 mm thick, with 8 mm diameter. These samples were placed between two stainless steel plate electrodes. Before experiments, the sample cell was flushed and filled with dry He, to ensure good thermal exchange. To record complex spectra, the sample was polarized with a static electric field (E_p) at a given polarization temperature (T_p) for a sufficient time (t_p), so that the polarization reached equilibrium. Then the sample was quenched by a cooling process to $T_0 < T_p$, allowing the orientation polarization [$P(T_p)$] to be frozen in. Finally, the electric field was cut off, and the sample was short-circuited for a sufficient time t_{cc} to remove fast-relaxing surface charges and to stabilize the sample temperature. The capacitor was then connected to a very sensitive electrometer (Keithley 642, 10^{-16} A accuracy). The depolarization current [$I(T)$] induced by the linear increase of temperature ($T = qt + T_0$) was subsequently recorded versus temperature, giving the relaxation spectrum of the sample. The peaks, which are associated with dipolar relaxations, must obey different criteria: Their intensity must be proportional to the applied field, and the temperature maximum must be below T_p and invariant with different T_p values. In this study, the polarization conditions resulting in reproducible dipolar relaxations

were as follows: t_p = 2 min, E_p = 400V/mm, t_{cc} = 2 min, and q = 7°C/min.

RESULTS

Thermal transitions—DSC analyses

Loss of bound water

A first DSC measurement was performed between 30 and 150°C for all the lyophilized samples (3 measures for each kind of sample). We have presented in Figure 1 the characteristic first-scan thermogram of each sample from a given series. In all cases, a broad and endothermic peak is observed between 50 and 150°C. By analogy with previous studies on lyophilized proteins,[3,4] this endothermic peak has been essentially attributed to the evaporation and vaporization of bound water. In fact, lyophilized samples are able to absorb water when exposed to the ambient atmosphere.

The characteristic parameters of this endothermic peak (ΔH, T_{max} and the half-width ΔT, which is a measure of the broadness of the peak) were computed for all the samples. The enthalpy ΔH that corresponds to the required energy to evaporate and to vaporize the water bound to the collagen is between 150 and 275 J/g, values that are in good agreement with literature data for the departure of bound water.[4,5] No significant differences are reported between the various treatments.

The temperature location of the peak T_{max} is between 96 and 116°C. The maximum mean T_{max} is found for the samples **/PBS**, but with a quite important variability; no significant results are found at the 0.05 level.

The large values found for the broadness of the peak, without significant differences between the treatments (45°C < ΔT < 60°C), can be explained by the complexity of the transition that includes disruption of protein/water interactions, evaporation, and vaporization.

Finally, it is important to note that the thermograms of samples stored in ethanol possess a shoulder between 80 and 120°C can be indicative of bound ethanol departure. This feature could be related to the existence of distinct adsorption sites of water and ethanol in connective tissues proteins.

Collagen denaturation

It is important to remember that hydrogen bonds that maintain the secondary and tertiary structure of collagen are disrupted with an increase of temperature, inducing the uncoiling of the triple helix in α chains of random conformation, individually or covalently linked depending on the degree of heating.[6,7]

The denaturation phenomenon, distinct from degradation, implies that the rupture of peptide bonds leads to the formation of an amorphous polymer, namely, gelatin. It can be used as a test to study the thermal stability of collagen.[8] Previous calorimetric measurements on different collagens[9] have shown

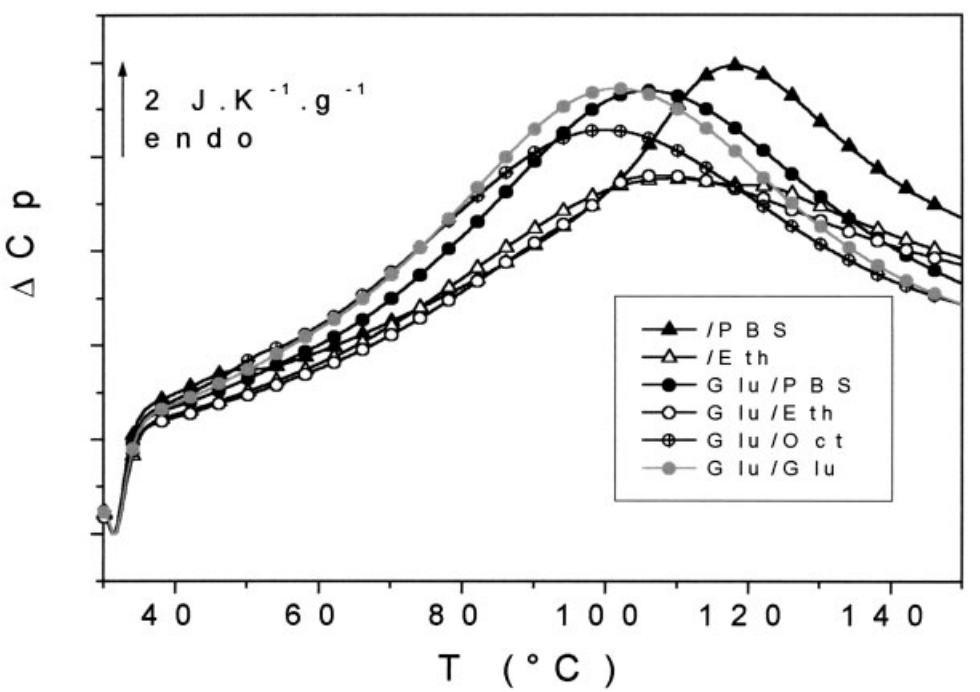

Figure 1. DSC first scans (from 30 to 150°C, 20°C/min) of bovine pericardium samples.

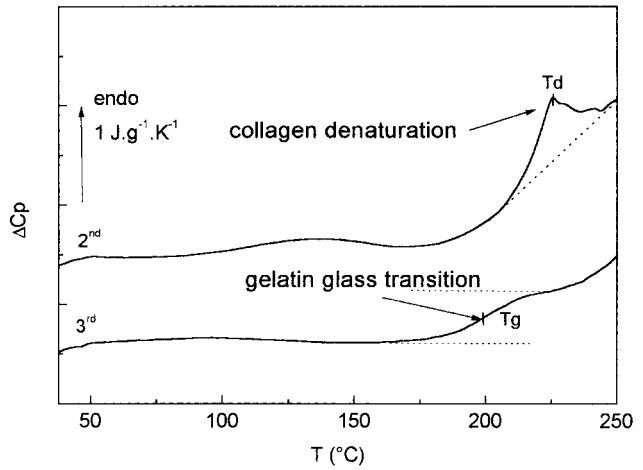

Figure 2. Second and third DSC thermograms of pure collagen (type I, from bovine Achilles tendon) at 20°C/min.

that the denaturation of the dry protein occurs in the 180–250°C temperature range as an endothermic peak (Fig. 2). This endothermic peak, which can be assigned as a first-order transition, is not reversible on successive scans in the dry state: In this case, we just observe a jump of the specific heat around 200°C, attributed to the glass transition of denatured collagen (i.e., gelatin), characteristic of its amorphous nature. The loss of triple helical conformation drives to a random network without long-range order.

Figure 3 shows the DSC second thermograms of the differently treated samples, recorded at 20°C/min from 30 to 250 or 280°C.

In this study, we focused on the thermal events occurring in the 180–280°C zone. The denaturation peak of the control sample stored in PBS is quite similar to the endothermic denaturation of pure collagen studied in a previous work.[4] It is noteworthy that the glutaraldehyde-treated sample stored in octanol possesses a well-defined endothermic denaturation that can be compared, for the general shape, with the control sample stored in PBS. We have reported in Table I the corresponding denaturation temperatures Td computed from a statistical study: The denaturation temperature is shifted toward high temperature with glutaraldehyde. As for the other samples, we did not detect the well-shaped denaturation peak expected in this zone of temperature.

Molecular mobility—TSC analyses

Hydrated state

For measurements in the semihydrated state, the samples were evacuated at 1.3×10^{-4} Pa for 5 min at

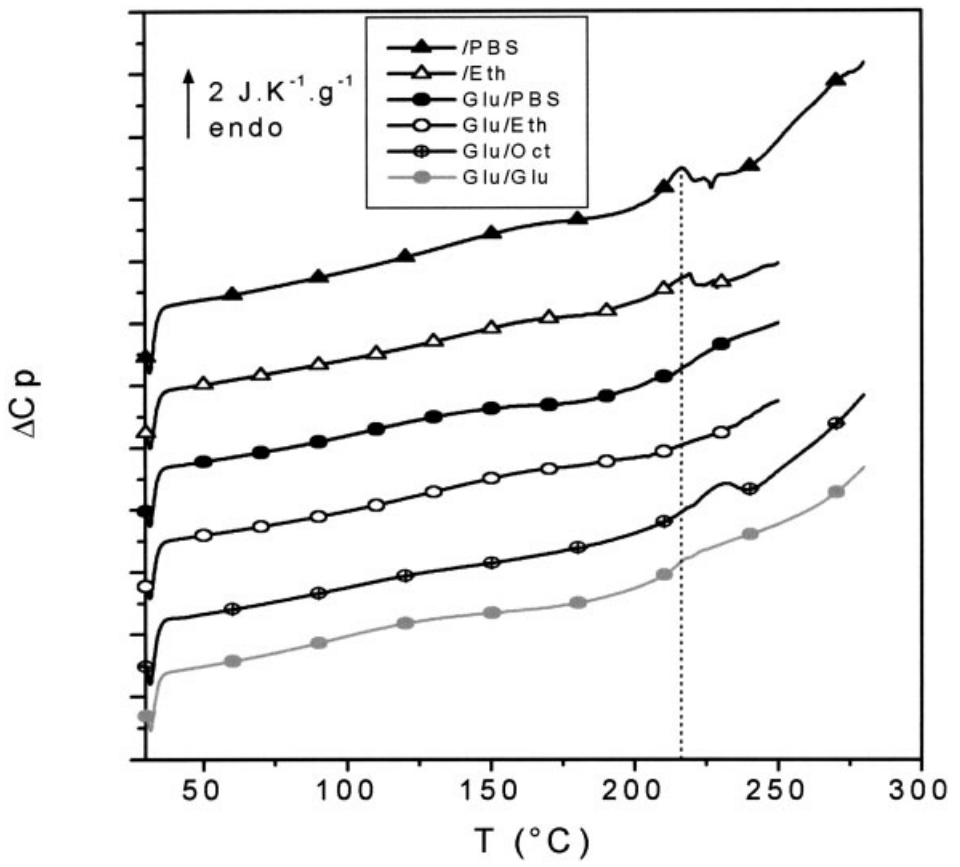

Figure 3. DSC second scans (20°C/min) of bovine pericardium samples.

TABLE I
Denaturation Temperatures of the Bovine Pericardium Samples Possessing a Well-Defined Denaturation Peak

	Td (°C)	ΔHd (J/g)
\PBS	214.8 ± 0.8	12.3 ± 2.4
Glu\Oct	230.0 ± 0.6	7.8 ± 0.7

Significant difference on Td mean values: $p = 1.13.10^{-4}$
Significant difference on ΔHd values: $p = 4.23.10^{-9}$

20°C, and then the cell was flushed with dry He. All the samples were subjected to a static electric field E_p (400V/mm) at 0°C, and the spectra recorded between −180 and 20°C.

We reported in Figure 4 the depolarization current recorded after a polarization at 0°C (black curve) and 20°C (gray curve) of the control sample, stored in PBS buffer.

When the sample is polarized at 0°C, two main relaxation modes are brought to the fore in the −180/0°C range: The first one, labeled β mode, is located at −110°C, with an intensity of 1.2×10^{-13} A (see enlarged zone). Previous work on collagen[5,10] has allowed us to associate this mode with localized motions of the complex bound water/polar sequences of the backbone (few atomic bonds). This large class of proteins possesses a similar β mode.[11] The second one, labeled β′ mode, is located at −7.5°C and is 15 times as intense as the first mode. Its origin is more uncertain, but TSC studies have shown that this mode could be related telopeptide motion[12] plasticized by water.

A second measurement was performed with a poling temperature T_p = 20°C, in order to check the intrinsic and reproducible character of these two relaxations (gray curves). We observed that the two peaks remain unchanged; the β mode is visible as a shoulder because of the partial depolarization of a mode located at higher temperature that was not studied in the hydrated state because of the possible water evaporation.

Figure 5 presents the superimposition of the TSC spectra of the six samples, recorded after a poling temperature T_p = 0°C. In all cases, the β and β′ modes are observed. The characteristic parameters of the β and β′ modes, namely, the location T_{max} and the maximum intensity I_{max}, were computed from a statistical analysis and reported in Figures 6 (β mode) and 7 (β′ mode).

Concerning T_{max} for the β mode, the most significant differences are found between the samples **/Eth** and **Glu/Oct** (p = 0.01324), samples **/Eth** and **Glu/Glu** (p = 0.0202), samples **Glu/Eth** and **Glu/Oct** (p = 0.02368), samples **Glu/Eth** and **Glu/Glu** (p = 0.02938).

The most significant differences in the intensities of the β mode are observed between samples **Glu/PBS** and **Glu/Oct** (p = 0.02471).

In some cases, we found a special feature for sample **Glu/Oct** (very broad β mode constituted by two relaxations).

In a manner similar to the β mode, the β′ mode is largely influenced by the storage conditions and glutaraldehyde treatment. The characteristic parameters (T_{max}, I_{max}) of the β′ mode are reported in Figure 7.

The most important feature concerns the temperature location of the β′ mode. In fact, for the samples **Glu/Oct** and **Glu/PBS**, the maximum of the peak is

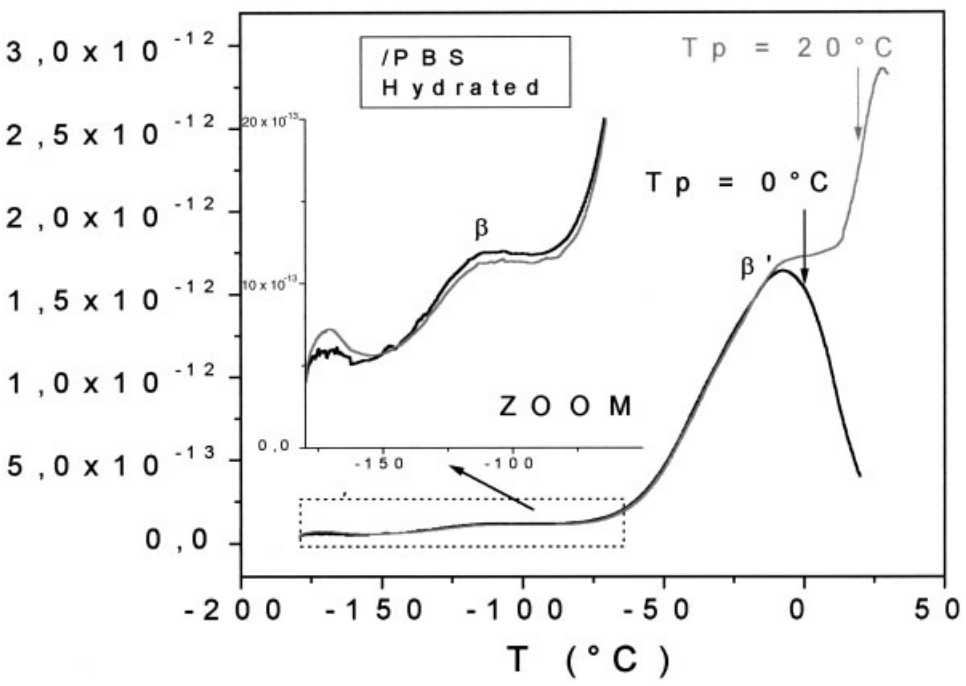

Figure 4. TSC spectra of control bovine pericardium sample (low-temperature study, hydrated state).

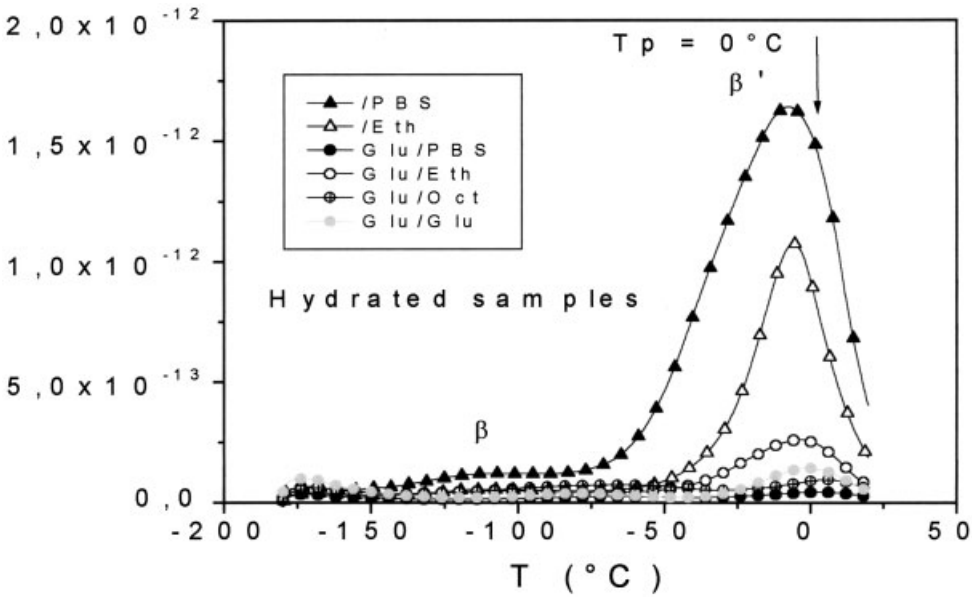

Figure 5. TSC spectra of bovine pericardium samples (low-temperature study, hydrated state).

located above 0°C: These modes cannot be considered intrinsic; they are not resolved in the hydrated state. In this case, we observe an important diminution of the intensity of the peak. In the other cases, the β′ mode is located below 0°C, and we cannot observe significant differences between the values of T_{max}. Despite an important variability of the corresponding values, the classification of the mean values of I_{max} for the intrinsic β′ modes is as follows:

/PBS > /Eth > Glu/Eth > Glu/Glu

Glu/Oct and **Glu/PBS** are not classified, because they do not possess an intrinsic β′ mode in the explored temperature range.

Dehydrated state

For the measurements in the dehydrated state, the samples were annealed at 110°C for 30 min under an inert atmosphere (pure helium) and then evacuated at 1.3×10^{-4} Pa for 1 h. All the samples were subjected to a static electric field E_p (400V/mm) at 110 or 130°C, and the spectra were recorded between −160 and 130 or 150°C.

We have reported in Figure 8 the depolarization current of the different samples recorded after a polarization temperature varying from 110 to 130°C; the polarization temperature was chosen to record an intrinsic peak (below T_p).

A main relaxation mode is observed for all the

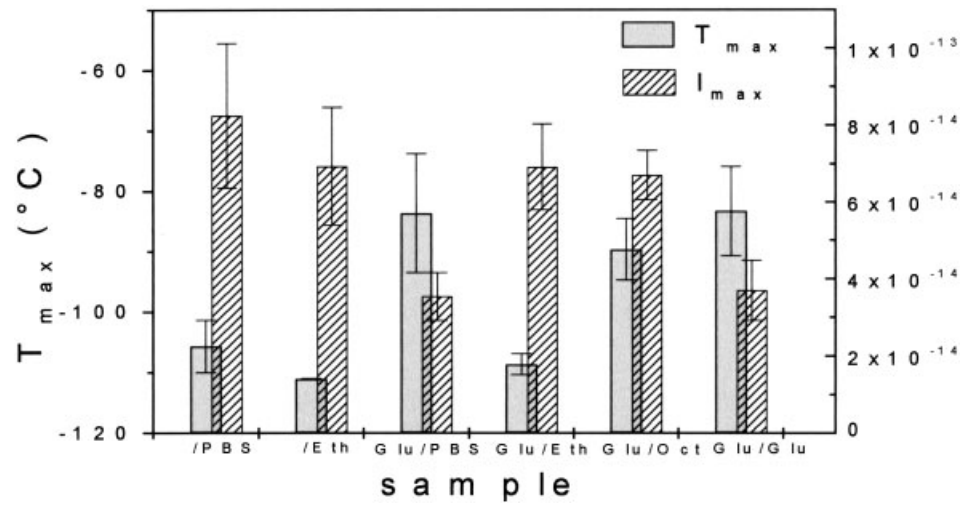

Figure 6. Parameters of the β mode for bovine pericardium samples (low-temperature study, hydrated state).

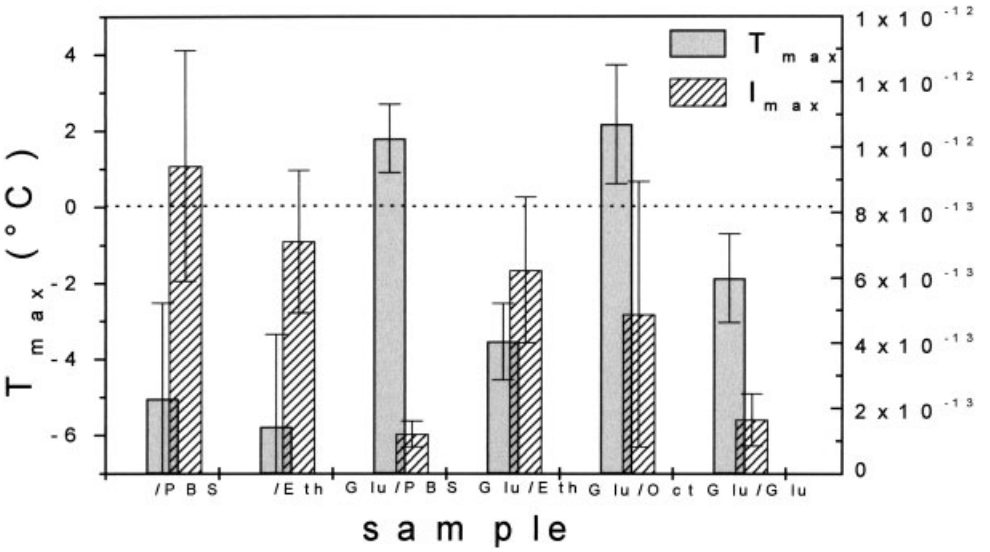

Figure 7. Parameters of the β′ mode for the bovine pericardium samples (low-temperature study, hydrated state).

samples in this temperature range; this mode was previously studied in pure collagen or in collagenic tissues[13] and was attributed to more delocalized motions of the backbone (some nanometers) leading to the glass transition of collagen. DSC studies are unable to bring to the fore this type of glass transition in the ordered collagen because of its extreme stiffness. The feature is compatible with the concept of "strong" liquid, developed by Angell[14] concerning the amorphous phase of very ordered proteins that cannot be detected by the classical DSC technique.

The characteristic parameters (T_{max}, I_{max}) of this peak are reported in Figure 9. The maximum mean value of the intensity is found for the control samples stored in PBS. Storage in ethanol or glutaraldehyde treatment makes this mode decrease. The most important feature concerns the temperature location of the main mode of the different pericardium tissues. As a matter of fact, significant differences are found between the T_{max} values for samples **/PBS** and **Glu/Oct** (p = 0.00277), samples **/Eth** and **Glu/Oct** (p = 0.0003812), samples **Glu/PBS** and **Glu/Oct** (p = 0.03491), samples **Glu/Eth** and **Glu/Oct** (p = 0.01483), and samples **Glu/Glu** and **Glu/Oct** (p = 0.000802).

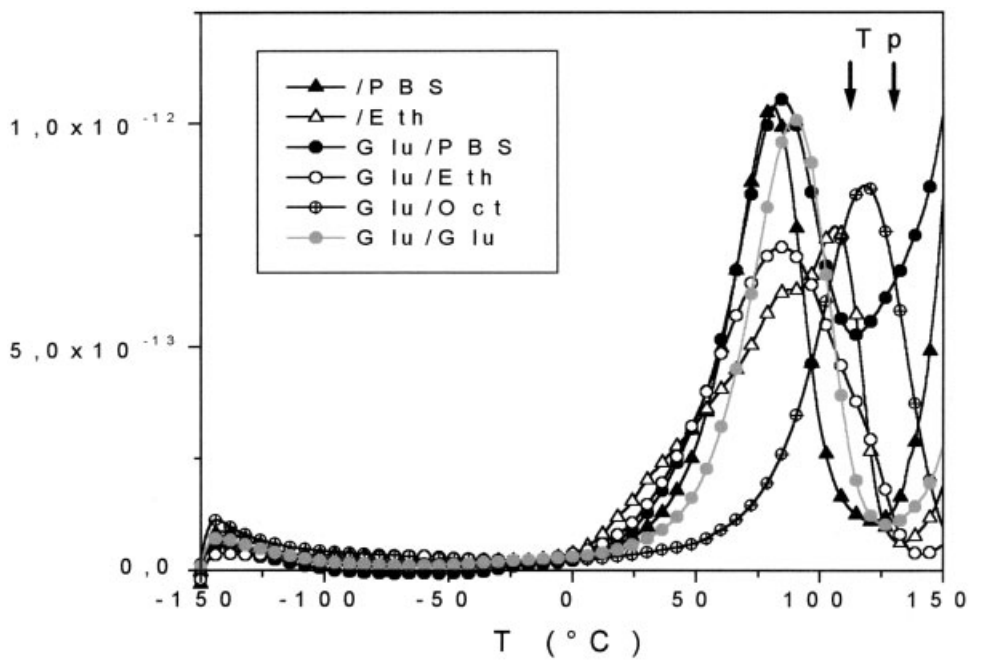

Figure 8. TSC spectra of the bovine pericardium samples (high-temperature study, dehydrated state).

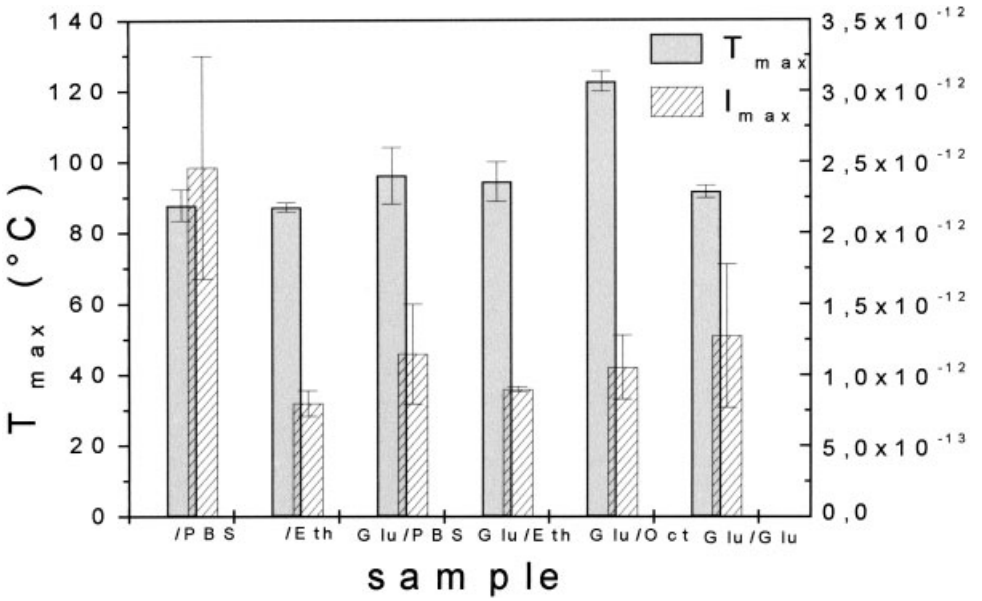

Figure 9. Parameters of the α mode for the bovine pericardium samples (high-temperature study, dehydrated state).

T_{max} for **Glu/Oct** was significantly higher than all the other groups, indicating that this particular treatment has a stabilizing effect on the collagen chains.

As for the sample treated with glutaraldehyde and conserved in the glutaraldehyde solution (**Glu/Glu**), no shift toward high temperature was noticed.

DISCUSSION

Our study of the thermal denaturation of collagen in differently treated bovine pericardium samples yields interesting information on the effects of treatment on the triple helical structure and stability of this protein.

The control sample possesses a well-defined endothermic denaturation at 215°C, as is generally expected for lyophilized collagenic material. Another tissue presents a well-shaped denaturation, namely, the glutaraldehyde sample stored in octanol. In this case, the denaturation temperature is shifted toward high values, allowing us to conclude that the triple helical domains of collagen are preserved and, above all, stabilized by glutaraldehyde and preserved by octanol storage. This result indicates good agreement with several studies that showed the stabilizing effect of glutaraldehyde on biological tissues.[15,16] The cross-linking action of this product can explain this peculiar behavior.[15,17,18] This is the reason that glutaraldehyde is used in many graft procedures, stabilizing and preventing protein degradation.

All the other treatments have a harmful influence on the preservation of triple helical structure.

Storage of the untreated sample in ethanol seems to change the triple helical structure of collagen, increasing the heterogeneity (multiple peaks) and facilitating the uncoiling of the protein (weak denaturation enthalpy). This assumption must be associated with the texture of the air-dried sample (strong rigidity) that does not have the features of the freeze-dried sample stored in PBS; all the samples treated with this procedure have this peculiar feature. The denaturation of the sample treated with glutaraldehyde and stored in PBS is very weak and broad, and the denaturation, hardly detectable, is shifted toward high temperature. We can conclude that the denaturation phenomenon is impaired by the combination of glutaraldehyde treatment and storage in PBS buffer.

The combination of glutaraldehyde treatment and ethanol storage involves drastic alterations of the helical structure of collagen. The fraction of denatured collagen is certainly important. This aspect of the air-dried sample (modification of color and rigidity, from all the series) is also indicative of profound chemical and/or structural modifications.

As for the sample treated with glutaraldehyde and stored in the glutaraldehyde solution, the denaturation phenomenon is not well defined; it reveals a narrow or a very weak peak. Some triple helical domains must subsist, differently cross-linked. The heterogeneity and the fraction of denatured collagen are important.

Complementary information results from the study of molecular mobility through the dielectric relaxations. The shift of the β mode toward high temperatures for the samples **Glu/PBS**, **Glu/Oct**, and **Glu/Eth** means that the localized motions on the polypeptidic chains require more energy to occur in these cases. The associated treatments can induce a variation of the environment, inducing constraints on the proteins.

The cleavage of the β mode observed in some cases can be associated with a new phase in the material (new cross-links or residual dipolar compound). So we have to be cautious with the conclusions: The glutaraldehyde treatment, following the octanol solution, can induce a new population of dipoles, but it also depends on the variability of the tissue. Concerning the β′ mode associated with telopeptide motions, ethanol storage induces a decrease of this mode, and glutaraldehyde treatment induces another important decrease. These treatments are associated with a loss of mobility of the whole collagen triple helix.

Finally, important differences are observed for the delocalized motion (α mode). An important decrease of molecular mobility is observed with ethanol storage: The presence of tightened and short cross-links could explain this phenomenon; the mobile sequences of the chains tend to disappear, because this particular type of physical or chemical cross-link is made inside the mobile units. The glutaraldehyde treatment makes the magnitude of the main mode decrease, too. This lack of mobility is certainly due to the stabilizing effect of glutaraldehyde by cross-linking the collagen chains. Moreover, the significant shift of the main mode toward high temperature for the samples treated with glutaraldehyde and stored in octanol can be interpreted as an important stabilization of collagen chains with this novel treatment. This stabilization (shift toward high temperature of the α mode) is not found for the glutaraldehyde-treated sample conserved in the glutaraldehyde solution. It could appear quite paradoxical when compared with the behavior of the glutaraldehyde samples stored in octanol. Nevertheless, in this case, an excess of glutaraldehyde can induce a plasticizing effect (if some molecules of glutaraldehyde remain inside the collagen fibers without being cross-linked), or an excess of reticulation creates hanging groups that increase the free volume around the chains.

CONCLUSIONS

This work on bovine pericardium samples has allowed us to propose a classification of different chemical treatments and storage conditions. The best conditions for the preservation of collagen structure and the enhancement of triple helical stabilization are the glutaraldehyde treatment followed by octanol storage; this procedure must be now applied to the conception of bioprosthetic heart valve, in order to increase the allograft durability *in vivo*.

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