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1,4-Dioxane enhances properties and biocompatibility of polyanionic collagen for tissue engineering applications

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Abstract Polyanionic collagen obtained from bovine pericardial tissue submitted to alkaline hydrolysis is an acellular matrix with strong potential in tissue engineering. However, increasing the carboxyl content reduces fibril formation and thermal stability compared to the native tissues. In the present work, we propose a chemical protocol based on the association of alkaline hydrolysis with 1,4-dioxane treatment to either attenuate or revert the drastic structural modifications promoted by alkaline treatments. For the characterization of the polyanionic membranes treated with 1,4-dioxane, we found that (1) scanning electron microscopy (SEM) shows a stronger reorientation and aggregation of collagen microfibrils; (2) histological evaluation reveals recovering of the alignment of collagen fibers and reassociation with elastic fibers; (3) differential scanning calorimetry (DSC) shows an increase in thermal stability; and (4) in biocompatibility assays there is a normal attachment, morphology and proliferation associated with high survival of the mouse fibroblast cell line NIH3T3 in reconstituted membranes, which behave as native membranes. Our conclusions reinforce the ability of 1,4-dioxane to enhance the properties of negatively charged polyanionic collagen associated with its potential use as biomaterials for grafting, cationic drug- or cell-delivery systems and for the coating of cardiovascular devices.

Keywords 1,4-Dioxane organic solvent · Bovine pericardium · Cellular adhesion and proliferation · Polyanionic collagen matrix · Physicochemical and biological properties

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1 Introduction

The most perfect biomaterials for tissue engineering are those with the ability to interact in vitro with cells or with living tissue and to induce repair, regeneration, remodeling, replacement and maintenance or enhancement of a physiological function [1]. Usually, these materials are produced from synthetic and natural polymers or in combination with bioinorganic compounds; they are ideally designed in such a way that cells are constantly stimulated by biological, mechanical, electrical, structural and chemical signals [2]. Consequently, tissue engineered devices should maximally enhance the cell/material interactions for specific purposes, which are controlled by the material properties, including the morphology, crystallinity, and biochemical composition [3, 4]. Collagen, a well-known natural polymer, is a major mammalian structural protein comprising approximately 90% of the bone tissue's organic fraction, and it is one of the best natural raw materials for the biomaterial manufacture and development of biomedical devices [1, 5].

The wide use of collagen in the field of biomaterials is associated with natural properties that include low immune response, even from heterologous sources; low toxicity; the ability to promote cellular growth and attachment; homeostasis and the ability of collagen solutions to reconstitute in vitro the microfibrillar structure found in

natural tissues [5–7]. Collagen is used in a variety of physical forms, such as sheets, sponges, gels, powder, strings and threads. Among the materials synthesized from collagen, matrices are particularly interesting and advantageous because their properties can be altered by chemical modifications that might improve the characteristics of collagen in terms of the stimulation of osteogenesis and the piezoelectric properties of collagen [8–10].

An important modification in the structure of these matrices is the removal of carboxyamide groups from the asparagine and glutamine residues present in tropocollagen α-chains by alkaline hydrolysis and the consequent formation of carboxyl groups [11]. These modifications do not alter the triple helix structure of the collagen molecule but change the self-assembled pattern of its microfibrils and its dielectric properties. In addition, the isoelectric point of collagen is reduced to 4.6–5.0 compared to native collagen, which has an isoelectric point ranging from 6.7 to 7.1. Thus, these matrices are more negatively charged at physiological pH compared to the preexisting negative charge domains of the native collagen [12, 13]. The advantage of using polyanionic matrices is that when they are implanted into experimental animals, these matrices do not induce an inflammatory response and show good biocompatibility, indicating that the use of these substrates as bone implants might be a viable alternative [14–16].

Different chemical treatments for modifying collagenic matrices that employ organic solvents have been used to improve biological and physicochemical properties and to remove the lipid fraction and cells present in native tissues [11, 17, 18]. However, most of these organic treatments were developed as reaction media for cross-linking procedures [5, 19]. Recently, 1,4-dioxane was shown to induce conformational changes, leading to aggregation and change in the orientation of collagen fibers present in native bovine pericardium (NBP) matrix. This solvent, known to be helix-forming due to its reduced dielectric constant, increases the thermal stability of BP and reduces collagenase degradability in in vitro biological assays [20].

Here, we report the thermal analysis and extensive microscopic evaluation of BP submitted to two sequential chemical treatments: first, by the selective alkaline hydrolysis of asparagine (Asn) and glutamine (Gln) side chains present in collagen molecules that acquire negative charges [11] and second, by increasing weak noncovalent bonds of intra- and intercollagen microfibers using 1,4-dioxane [20]. The doubly chemically treated materials were also tested by the ability to attach mouse fibroblast cells and to allow proliferation in a biocompatibility assay. The obtained data show that 1,4-dioxane recovers the morphological organization of collagen fibers and collagen:elastin association inside the pericardial matrix, leading to increased thermal stability, and that cells behaved similarly in the presence of the material obtained from native tissues with normal adhesion and growth in addition to reduced cell death. This protocol extends the applicability set of polyanionic collagen in the biomaterial field, as well as highlight the use of 1,4-dioxane for other polymeric matrices.

2 Materials and methods

2.1 NBP storage and pre-equilibration

NBP was originally stored in a 70% ethanol solution at 4°C. NBP pieces were pre-equilibrated with a 0.13 M phosphate buffer at pH 7.4 to charge and select reactive groups over the protein surface through the different chemical treatments.

2.2 Polyanionic collagen-enriched bovine pericardium (BP)

NBP was treated at 25°C for periods of 48 or 148 h with an alkaline solution (3 ml of solution/g of tissue) containing 6% (v/v) dimethylsulfoxide, salts (chlorides and sulfate) and bases of alkaline (K^+, 1.19 M and Na^+, 1.74 M) and alkaline earth metals (Ca^{2+}, 0.86 M). The resulting materials were equilibrated with a solution containing Na_2SO_4, NaCl, KCl, and $CaSO_4$ (6 ml of solution/g of tissue) for a period of 12 h, and the excess salts were removed by washing with the following solutions: 3% (w/w) boric acid solution (3× for 2 h each, 250 ml), deionized water (3× for 6 h each, 250 ml), 0.3% (w/w) EDTA solution (3× for 2 h each, 150 ml) at pH 11.0, followed by washing with deionized water (6× for 2 h each, 250 ml) [11].

2.3 Swollen polyanionic collagen or positively charged pericardium

To swell and acidify the tissues, BP materials treated with alkalis, as described above, were suspended in deionized water, and the pH was adjusted to 3.5 with pure acetic acid. Pericardium samples were then soaked in 0.13 M phosphate buffer at pH 7.4 until the complete removal of acetic acid was achieved, as monitored by conductimetry [25].

2.4 1,4-Dioxane treatment

Pieces of BP containing polyanionic collagen, prepared as described above, were extensively washed with water to remove the buffer and were transferred to water/1,4-dioxane solutions with increasing 1,4-dioxane concentrations up to 100% (20, 40, 60, 80%) for 0.5 h each and maintained in pure 1,4-dioxane for 40 h. Then, the BP pieces were transferred to 80, 60, 40, and 20% 1,4-dioxane

solutions and finally to pure water for 0.5 h each to completely remove the organic solvent from the collagen matrix, as the collagen structural modifications caused by 1,4-dioxane cannot be reversed by rehydration. Afterward, the BP samples were stored in 0.13 M phosphate buffer at pH 7.4 at 5°C until analysis. Measurements of the percentage transmittance of the solvent solutions from the washings were monitored by spectrophotometry for the complete removal of 1,4-dioxane from the BP pieces [20].

2.5 Native collagen gels

Native collagen gels were prepared by treating 50 g of wet BP at 20°C with 300 ml of 6% (w/v) dimethylsulfoxide solution and adjusting to pH 2.5 with acetic acid with occasional stirring for 1 week. The suspension was homogenized, and gels were dialyzed against an aqueous solution of acetic acid at pH 3.5. The complete removal of dimethylsulfoxide was confirmed by conductimetry. The final gel concentration was adjusted to 0.7% (w/w) by a hydroxyproline assay [15].

2.6 Polyanionic collagen gels and membranes (PACMs)

To hydrolyze the amide groups selectively, 50 g of BP in the wet state were treated at 20°C for periods of 48 or 144 h with an alkaline treatment according to the protocol described above. After the equilibration and removal of excess salts, the resulting materials were suspended in deionized water, the pH adjusted to 3.5 with pure acetic acid and the mixture homogenized in a blender. The concentration of polyanionic collagen gels were adjusted to 0.7% w/w, as determined by the hydroxyproline assay. The membranes were prepared by the addition of 7 ml of a 0.7% (w/w) native or polyanionic collagen gel into six-well multiplates, 3.5 cm in diameter. Before casting, the gels were equilibrated in 0.13 M phosphate buffer at pH 7.4. Under these conditions, the PACMs were approximately 3.0 mg/cm^2 and were characterized by increments in carboxyl groups/collagen molecules from 0 (NBP), 87 $\pm$ 17 and 134 $\pm$ 12, corresponding to the materials submitted to 48 and 144 h, respectively, under alkaline conditions [11, 12, 15]. The PACMs attached to the culture plates were then subjected to 1,4-dioxane treatment for precisely 40 h, as previously described [20].

2.7 Thermal analysis

A computer-interfaced differential scanning calorimeter (Model 910) and a thermal analyzer (Model 9900) from DuPont Instruments performed differential scanning calorimetry (DSC) on NBP samples of approximately 30 mg on sealed aluminum pans. The samples were scanned at a constant heating rate of 5°C/min from 25 to 180°C under a nitrogen atmosphere, and the results are all representative of three independent experiments.

2.8 Scanning electron microscopy (SEM)

After the treatments described above, the samples of BP were equilibrated with a 0.13 M phosphate buffer at pH 7.4, followed by three washes with deionized water to remove the excess salt. Samples previously lyophilized and coated with gold and photomicrographs were obtained using a Zeiss DSM 960 (Zeiss, Jena, Germany) SEM operating with a 10 keV electron beam.

2.9 Histological evaluation by light microscopy

Longitudinal segments (1 cm wide) of BP submitted to the treatments described were cut and fixed in 10% buffered formalin. Subsequently, the specimens were embedded in paraffin using standard procedures, and 5 mm sections were stained by routine histochemical procedures with hematoxylin and eosin (for visualization of the cell removal and collagen fiber morphology), Gomori's trichromic stain (for visualization of the collagen fiber morphology, orientation and distribution) and Weigert's resorcin–fuchsin stain (for visualization of the elastic fiber morphology) [14, 21]. Sets of microscopic slides and photomicrographs were comparatively examined by histological analysis classically based on a 5+/5 scale corresponding to the total number of events present in the native material compared to that present after the proposed chemical treatments. The absence of events was indicated by zero (0).

2.10 Biocompatibility assay as a measure of cellular proliferation and adhesion

The biocompatibility of BP membranes in the presence of cells was monitored using reconstituted membranes from the solubilized collagen gels submitted to the described alkaline and dioxane treatments [15]. Frozen stocks of mouse cells from the NIH3T3 fibroblast cell line were kept in liquid nitrogen, routinely thawed and grown in Dulbecco's modified Eagle's medium (DME) with 10% heat inactivated fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Native and PACMs treated or not treated with 1,4-dioxane were used to cover the bottom of six-well multiplates with 35 mm diameter, and a fibroblast suspension containing $\sim 4 \times 10^5$ cells was seeded into each well. The adhesion viability was determined by counting the number of unattached or floating cells in a sample of the medium after 3 h of plating. After

this time, the culture medium was changed, and the cellular proliferation was determined by counting the number of live cells after 48 h from the plating in a Neubauer chamber. Adherent and proliferating cells were visualized by light microscopy. Cells considered viable were those usually presenting normal and flat morphology, attached to the membrane and in replicative state. Floating cells, which may be those that did not adhere because of material cytotoxicity and/or because of the unfavorable but biocompatible tissue surface, were examined by re-plating them in 12-well multiplates for additional 24 h of incubation. After the second platting, remaining floating cells were considered suffered anoikis (apoptosis dependent on unattachment or deattachment) [23].

3 Results

3.1 Thermal analysis

The polyanionic type I collagen was obtained by submitting the NBP to alkaline treatment for 48 or 148 h to produce collagen molecules with two different contents of negatively charged carboxyl groups, which are known to reduce the thermal stability by increasing the repulsive charges in the α-tropocollagen molecules inside the matrix (Fig. 1; Table 1) [5]. 1,4-Dioxane has promoted ~10°C increments in the NBP thermal stability without producing covalent cross-links but by increasing weak noncovalent bonds with intra- and intercollagen microfibers. Therefore, the samples of anionic BP were then treated with pure 1,4-dioxane for 40 h to achieve the highest thermal improvements already shown [20]. The results from Table 1 show a 6.5°C decrease in the thermal stability of native pericardium after alkaline treatment. The increase in the number of negative

Table 1 DSC results of BP submitted to different chemical treatments with alkaline hydrolysis and/or organic solvent 1,4-dioxane

Treatments	Denaturation temperature (°C)[a]	Enthalpy (J/g)[a]
BP pH 7.4	61.93 ± 0.1	2.585 ± 0.17
BP + 1,4-dioxane 40 h	72.25 ± 0.1***	22.175 ± 1.90*
BP + alkaline 148 h	55.53 ± 0.2*	3.45 ± 0.33***
BP + alkaline 148 h + dioxane 40 h	56.91 ± 0.3**	2.100 ± 0.53*
BP + alkaline 48 h	58.03 ± 0.2*	3.159 ± 0.76**
BP + alkaline 48 h + dioxane 40 h	62.14 ± 0.4***	2.731 ± 0.54*

[a] Values are the mean ± standard deviation of three independent measurements; statistic significance by the *F*-test or ANOVA

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

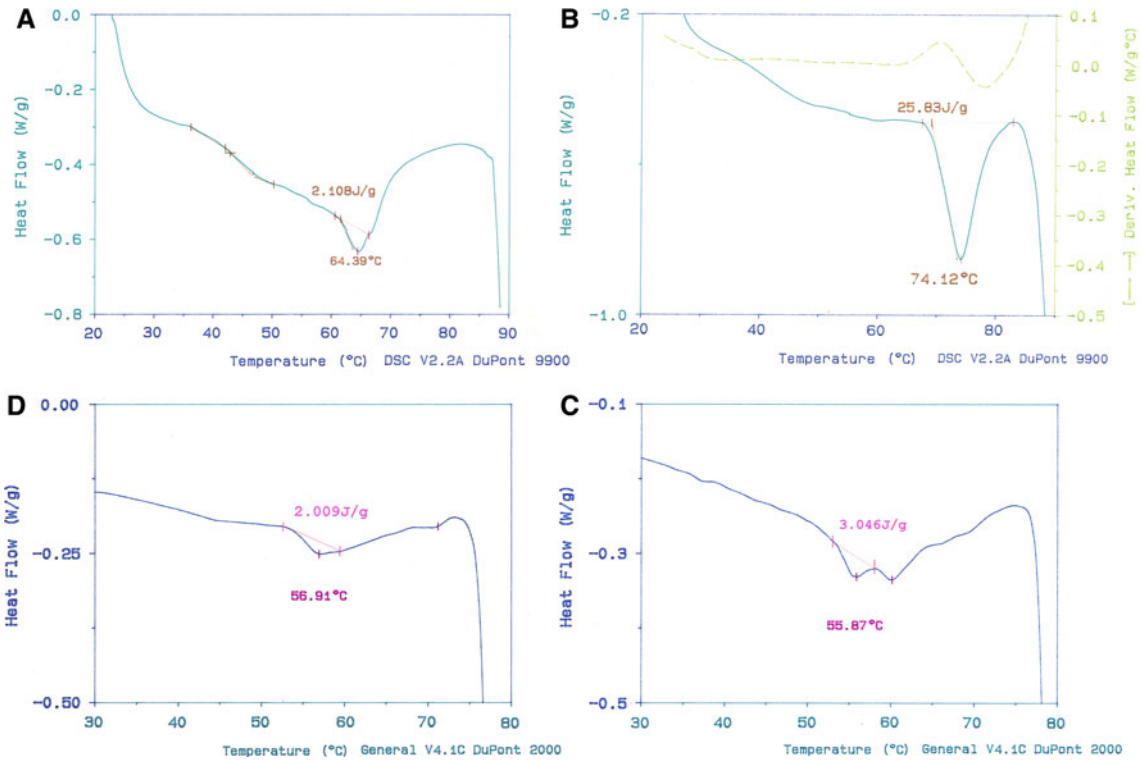

Fig. 1 Representative DSC thermograms from analysis of BP submitted to different treatments showed in Table 1: **a** BP pH 7.4, **b** BP +1,4-dioxane 40 h, **c** BP + alkaline 148 h, **d** BP + alkaline148 h + 1,4-dioxane 40 h

charges on the collagen molecules promoted by 148 h of alkaline hydrolysis of carboxyamide groups of Asn and Gln seems to strongly reduce endothermic transitions inside the collagenic matrix, which was even so slightly elevated by ~2°C after 40 h of treatment with 1,4-dioxane, a high dielectric-constant solvent with the ability to induce hydrophobic interactions for inter-collagen chains. However, because the alkaline hydrolysis can be controlled, the negative charge content of anionic collagen was reduced by the alkaline treatment from 148 to 48 h. This treatment per se resulted in a 2.5°C elevation of the thermal stability of anionic BP and allowed a more pronounced effect of the 1,4-dioxane leveling endothermic transitions than that found in native materials (Fig. 1; Table 1).

3.2 SEM and histological analysis

Regardless of 1,4-dioxane's ability to improve the thermal stability of polyanionic collagen, an evaluation of its actions on collagen fiber organization by SEM shows important morphological changes promoted by this solvent even after the actions promoted by the alkaline treatment over the matrix (Fig. 2). As previously shown, dioxane actions on the collagen fibrils are strong, irreversible and essential in the context of practical applications [20]. Drastic defibrillation properties are associated with the alkaline treatments of collagen matrices, as observed in Fig. 2c compared to Fig. 2a. Despite this polyanionic character of the pericardium, 1,4-dioxane is still able to induce aggregation and gap formation inside the matrix, as clearly evidenced by comparing Figs. 2b to a and 2d to c.

To confirm these results in the materials obtained by the treatments (alkaline + dioxane) described in the Sect. 2, histological preparations of BP samples were first stained using the hematoxylin–eosin method (Fig. 3) followed by Gomori's trichromic staining method (not shown, but accounted for in Table 2). Again, a much lower content of cells and ground substances can be observed in Fig. 3b, d

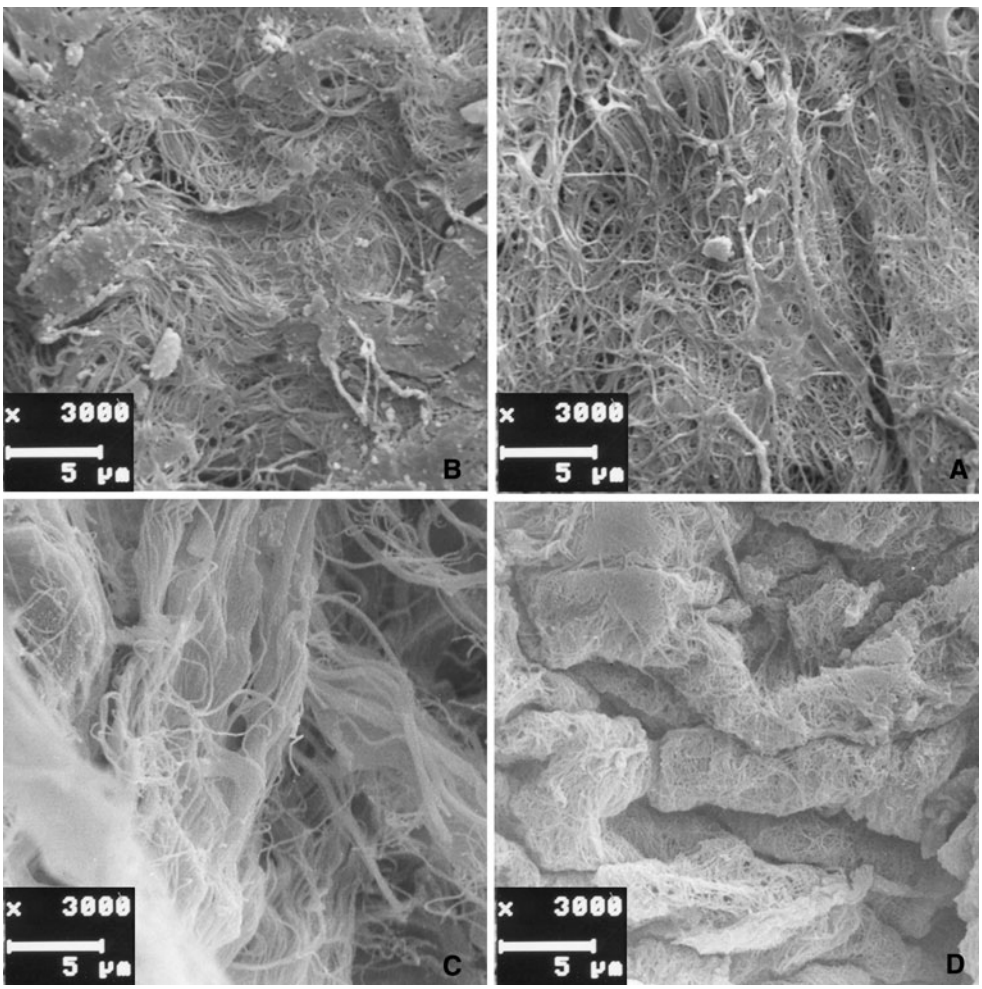

Fig. 2 SEM micrographs of NBP at pH 7.4 (**a**) and sequentially submitted to 1,4-dioxane treatment for 40 h (**b**), to alkaline treatment for 148 h (**c**) or associated with alkaline 148 h + 1,4-dioxane 40 h treatments (**d**)

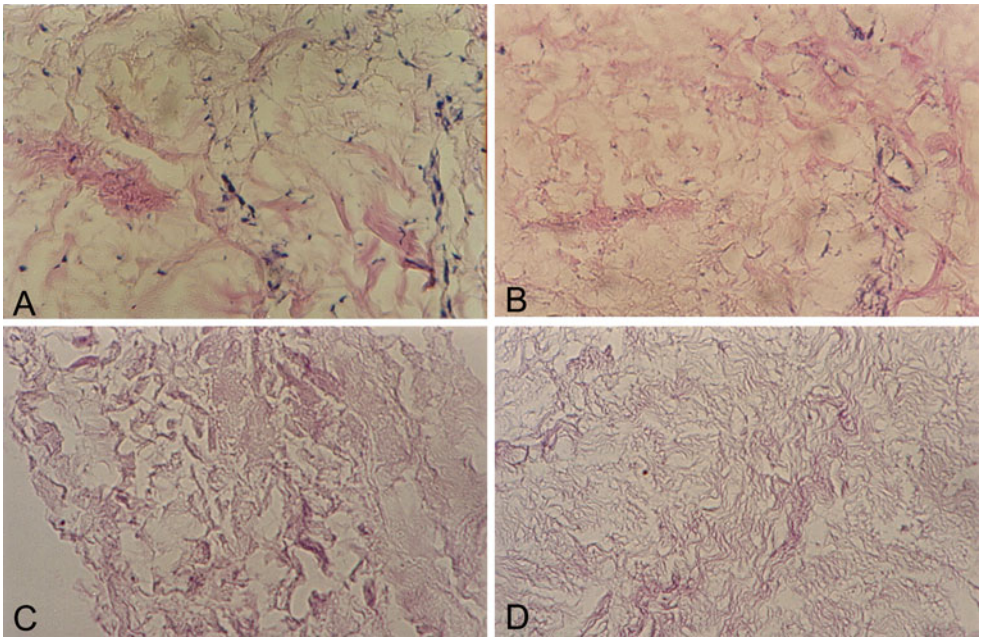

Fig. 3 Histological photomicrographs of the following: **a** NBP, **b** NBP + 1,4-dioxane treatment, **c** NBP + alkaline treatment for 148 h, and **d** NBP + alkaline + 1,4-dioxane treatments, after staining with hematoxylin–eosin for the collagen fiber and cell detection. Magnification = 450×

Table 2 Histological characteristics of native or treated BP showing the different effects of alkaline and/or 1,4-dioxane treatments over the tissue morphology

	NBP samples[a]			
	pH 7.4 equilibrated	1,4-Dioxane treatment 40 h	Alkaline treatment 148 h	Alkaline + 1,4-dioxane
Cells presence	5+/5	1+/5	0	0
Collagen fibers				
Wavy	2+/5	2+/5	1+/5	2+/5
Fragmentation	0	1+/5	3+/5	1+/5
Gaps/furrows	1+/5	3+/5	4+/5	3+/5
Aggregation	2+/5	3+/5	1+/5	3+/5
Elastic fibers				
Amount	5+/5	3+/5	2+/5	2+/5
Fragmentation	0	1+/5	2+/5	2+/5
Aggregation	2+/5	3+/5	1+/5	3+/5

[a] Quantitative analysis based on a 5+/5 scale corresponding to the absence (0) or presence (1+/5 to 5+/5) of tissue components from NBP samples. Values are average of three independent measurements

compared to Fig. 3a, c, respectively. In addition, a much more homogeneous collagen matrix, exhibiting mostly laterally organized collagen fibers, can be seen in 1,4-dioxane-treated materials (Fig. 3b, d). It is important to notice in Fig. 3c that the structural disorganization promoted by the alkaline treatments, including tissue rupture with gaps and disordered alignment of fibers, can be fully recovered by the organic solvent (Fig. 3d) resembling more natural tissues (Fig. 3a, b).

To gain more insight into the structural effects of 1,4-dioxane and the alkaline treatments, another histological evaluation was performed in the polyanionic collagen matrices using Weigert's resorcin/fuchsin, specifically staining for elastic fibers (Fig. 4). In Fig. 4b, d, showing 1,4-dioxane-treated pericardium, the elastic fibers are more equally distributed all over the tissue and co-localized with the collagen fibers, thus turning the doubly-treated collagen matrix into a more structurally homogeneous material with discrete gains in thermal stability. By comparing Fig. 4c, d with native pericardium (Fig. 4a), it is possible to see the lower elastic fiber content and more reminiscent fragmented elastic fibers in alkali-treated tissues, which most

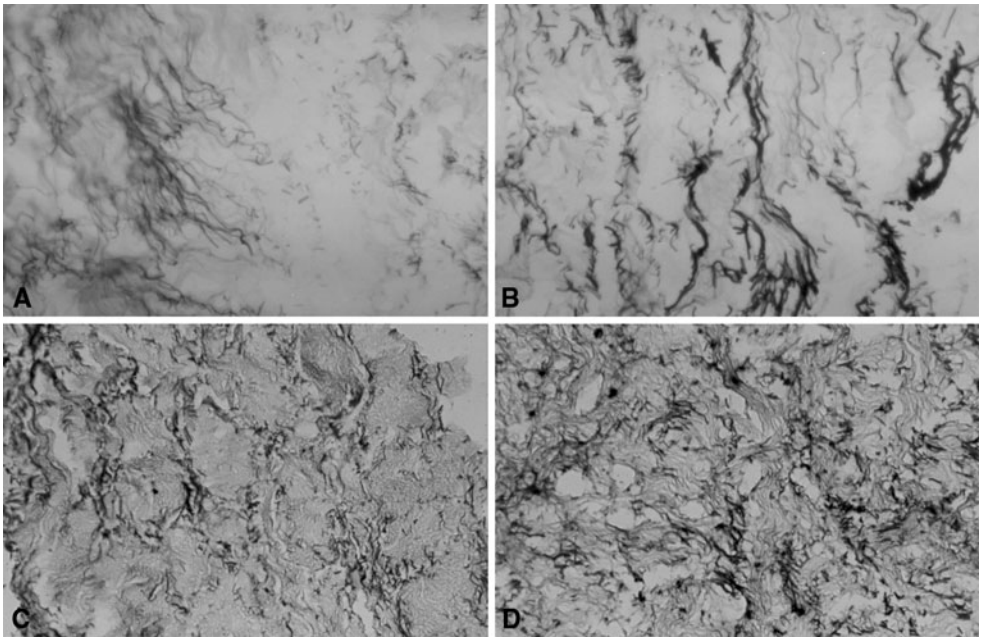

Fig. 4 Histological photomicrographs of the following: **a** NBP, **b** NBP + 1,4-dioxane treatment, **c** NBP + alkaline treatment for 148 h, and **d** NBP + alkaline + 1,4-dioxane treatments, after staining with Weigert's resorcin/fuchsin for the elastic fiber evaluation. Magnification = 450×

likely reflects the reduced mechanical properties, as suggested [9, 13, 27, 28]. However, 1,4-dioxane is less drastic for the fragmentation and removal of elastic fibers, as it is able to organize and aggregate the fragmented fibers with collagen lattices (Fig. 4b), as seen in the filamentous dark structures. This behavior of elastic fibers inside the collagenic matrix to 1,4-dioxane treatment reflects major collagen–elastin interactions, which in turn cause higher thermal stability (Table 1). Table 2 summarizes all the histological aspects that distinguish alkaline effects from 1,4-dioxane effects plus the associated treatment on pericardium's collagenic matrix; it also includes comparisons with the native material. More importantly, we observed remarkable morphological improvements in the anionic collagen promoted by 1,4-dioxane after the drastic structural changes imposed on that material by the alkaline treatments (Figs. 2, 3, 4; Table 2).

Because alkaline treatment of collagenic matrices is associated with an increase in the distribution of negative charges along the collagen fibers and because these chemical, electrostatic and structural characteristics are affected by 1,4-dioxane effects (this work), we decided to test native positively charged BP materials (Fig. 5). Native pericardium submitted to consecutive alkaline and acidic treatments, with or without subsequent treatment of 1,4-dioxane for 40 h, was evaluated by SEM and DSC. The resulting material is an amorphous collagen matrix with low thermal stability unable to reorganize to form micro- and macrofibrils and characteristics of denatured (gelatinized) collagen, which usually has few applications as a biomaterial (Fig. 5). The thermal and morphological results show that 1,4-dioxane cannot recover the collagen matrices exhibiting high contents of positive electric charges, which seems to strongly reduce the hydrophobic-inducing effects promoted by this solvent.

3.3 Cellular biocompatibility assays

A different strategy was employed to access the cytotoxic effects associated with the alkaline and 1,4-dioxane treatments to cellular survival, adhesion and proliferation in in vitro assays [15]. To circumvent the experimental challenges, six-well culture plates (35 mm diameter) were covered by native and PACMs, treated or not treated with 1,4-dioxane, as described in Sect. 2. Our analysis focused on microscopic observations of the cell attachment, proliferation and death by manually counting cells after 48 h of plating [23]. In Table 3 and Fig. 6, it is possible to observe, both numerically and morphologically, that cell adhesion and proliferation were comparatively the same as the native collagen membranes in dioxane-treated membranes. However, alkaline-treated membranes impair adhesion and proliferation, reducing the number of surviving cells in proportion to the increasing time of the alkaline hydrolysis and negative charges of the collagen molecules. The more important effect from these results is

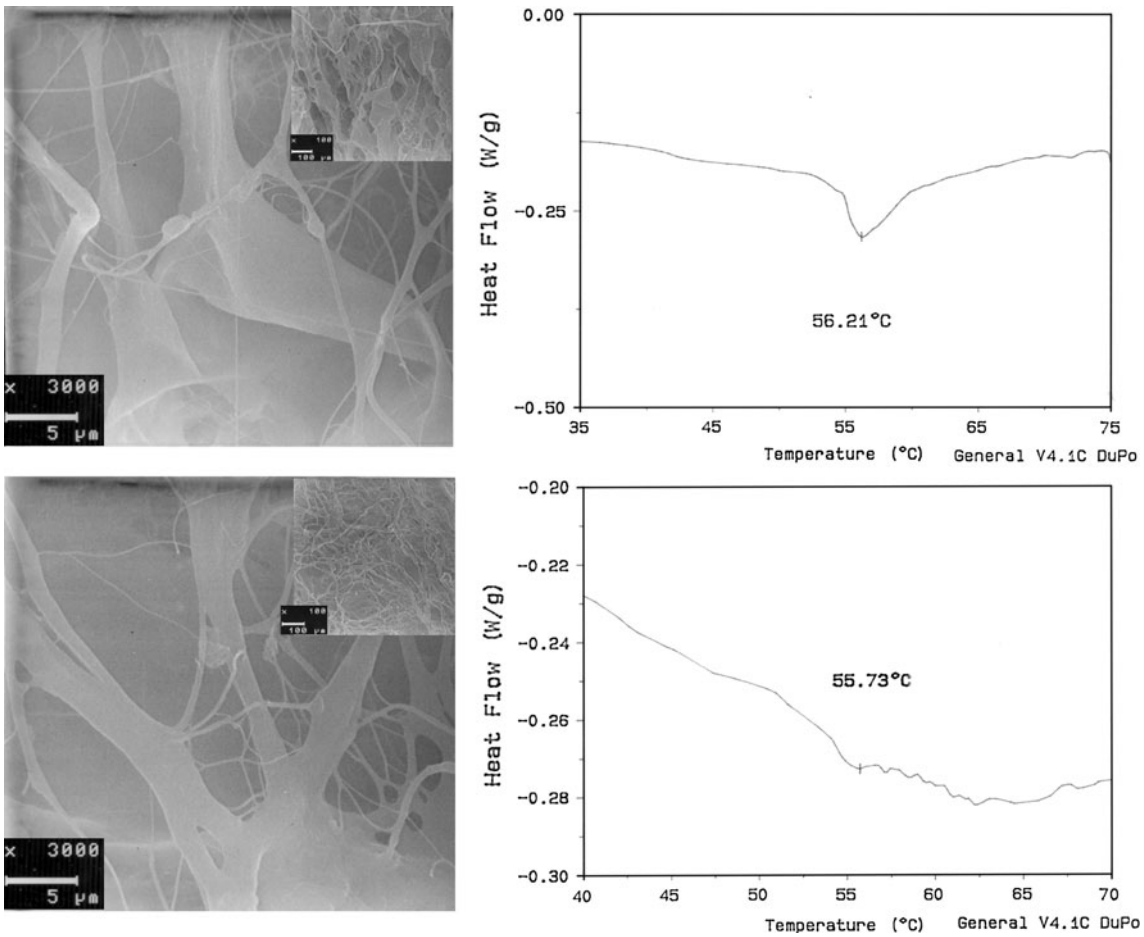

Fig. 5 SEM micrographs and DSC thermograms of the swollen pericardial collagen in two different magnifications (100 and 3,000×) submitted to acidic treatment at pH 3.5 alone (*upper*) and with additional 1,4-dioxane treatment (*lower*)

Table 3 Number of attached and proliferating NIH3T3 fibroblast cells on plates coated with collagen membranes submitted to the different chemical treatments after 48 h of culture

Membrane coating culture plates	Alive cells/plate (×1,000)[a]	Floating cells/plate (% of plated cells)[a]
Control (no membrane)	1,188 ± 13	0
Native collagen	1,045 ± 24***	4.4 ± 0.8**
Native collagen + dioxane 40 h	1,029 ± 22*	5.6 ± 0.4***
Polyanionic collagen (48 h)	597 ± 12**	27.7 ± 3.1**
Polyanionic collagen (48 h) + dioxane (40 h)	911 ± 29*	10.1 ± 1.3**
Polyanionic collagen (148 h)	327 ± 12*	48.6 ± 2.9*
Polyanionic collagen (148 h) + dioxane (40 h)	611 ± 19***	23.9 ± 4.1*

The number of unattached and dead cells after 3 h of plating was counted in the medium

Cells were counted in a Neubauer chamber

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

[a] Values are the mean of three independent plates ± standard deviation; statistic significance by the *F*-test or ANOVA

the recovering ability of the 1,4-dioxane treatment in the doubly-treated membranes, independent of the time of hydrolysis, resulting in an increase of adherent and proliferative cells by ~43 and 37%, respectively, for 48 and 148 h of alkaline treatment. Encouraging results with 1,4-dioxane treatments after, respectively, 48 and 148 h of

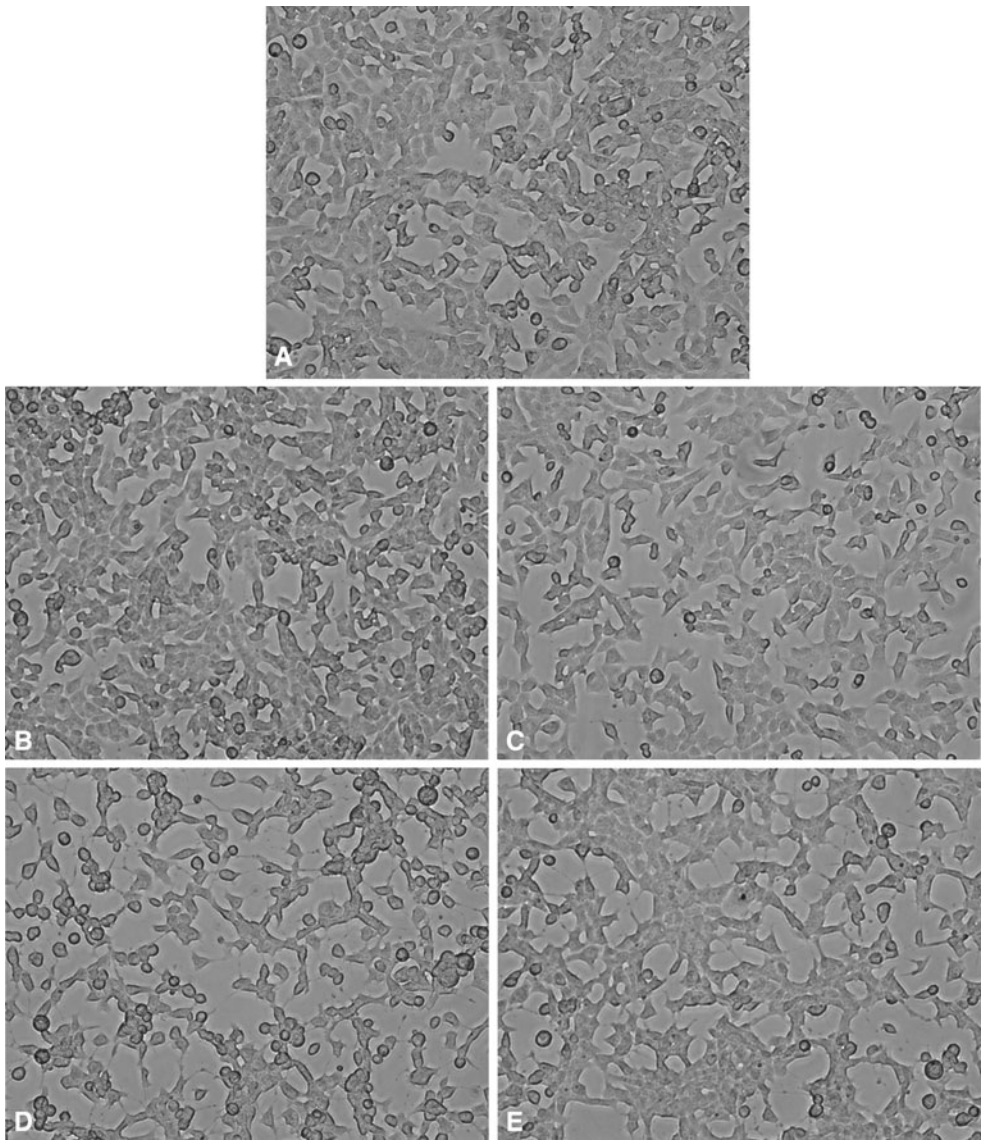

Fig. 6 Transmitted light micrographs of NIH3T3 cells growing in culture plates (**a**) covered by native collagen membranes (**b**) and treated by 1,4-dioxane (**c**); polyanionic collagen membranes from 148 h of alkaline treatment (**d**), subsequently submitted to 40 h of 1,4-dioxane (**e**) treatment. Magnification = 100×

alkaline treatment were reinforced by preliminary experiments using intermediate time-points (24, 72, 96, 120, and 172 h) in the cell proliferation kinetics, and the results were well-correlated to those in Table 3, showing same tendency of action to dioxane treatment (not shown).

Furthermore, the cellular adhesion viability was measured as the number of floating cells 3 h after plating 4×10^5 cells (Table 3). The results show that adhesion of cells to the substrate (chemically treated membranes) within 3 h of contact is dependent on the type of chemical and structural modifications occurring inside the collagenic matrix, being more impaired in the alkaline-treated membranes. Numerically, the percentage of unattached cells has subtractive effects in the final number of surviving cells, and both results present a proportional agreement (Table 3). In addition, only those cells attached along 48 h can divide and proliferate, whereas unattached and dead cells were observed daily during the experiment. Finally, alkaline-treated membranes followed by the 1,4-dioxane treatment are presented as better substrates for the adhesion of fibroblast cells after plating with reduced non-adherent and floating cells, which is likely due to the reorganization of the collagenic matrix caused by this solvent. On the other hand, the remaining cells very likely did not adhere either because of the material cytotoxicity (less evident) or because of the unfavorable but biocompatible tissue surface. To access viability of floating cells they were observed 24 h after re-plating them in culture dishes and the remaining floating

cells were considered in the anoikis state (apoptosis induced by substrate unattachment or detachment) [24].

4 Discussion

The use of biomaterials in the medical field has steadily grown over the past 15 years due to the further increasingly development of specific materials to different clinical needs. This is due to a bigger variation of the chemical composition of materials, allied to a better characterization of physical and mechanical properties of them. These factors combined with a controlled surface modification of materials have resulted in cellular responses decidedly better after implantation [25]. The commitment of tissue engineering to develop replacement and filling materials for seriously injured regions can always count on collagen-derived biomaterials from different sources, including BP [6, 26]. Numerous chemical treatments tested before and only used in synthetic polymer matrices, can now be used in different natural tissue matrices in order to improve the biodegradability and biocompatibility [18, 27]. In the context of derivatization arrays focused on applicability, this paper employs two chemical treatments and a biopolymer matrix previously characterized to innovatively provide a biomaterial with biological properties quite acceptable, although mechanically limited [11, 20, 28]. The alkaline chemical treatment of various tissues has great potential in generating soluble injectable matrices to modify the exact distribution of negative charges on the surface of fibrous proteins [29]. However, for BP membrane, this treatment strongly reduced the thermo-mechanical characteristics, directly reflecting on applicability that depend on them [12, 15]. The use of organic solvent 1,4-dioxane, compared to other organic solvents and chemical crosslinking promoters, proved to be a promising alternative for the improvement of thermal properties and biodegradability of this material in the native state [20]. Thus, our results of thermal analysis show that the 1,4-dioxane is still able to increase the thermal stability of BP treated with alkaline salts bringing it close to the native material. This increase is consistent with the amount and distribution of negative charges in the molecules of tropocollagen and probably present in elastin tissue, but prevented by the accumulation of positive charges in the material [14]. Thus, the possibility of using the 1,4-dioxane treatment for improving the physicochemical and/or morphological properties of BP was performed on collagen matrices obtained after strong acidic treatments at pH 3.5. This procedure is used in soluble collagens extracted from native tissues by alkaline treatments to equilibrate electric charges and pH, resulting in a material known as "swollen" collagen, which has very high levels of positive charges (suggesting higher solvation of tropocollagen molecules) and is more structurally rigid [12, 29]. Our calorimetric and microscopic analysis revealed that the positively charged matrix is much less sensitive to hydrophobic actions of 1,4-dioxane resistant to collagen fibril aggregation commonly induced by this solvent. Hence, these effects of dioxane could be used in other natural or synthetic polymer matrices in order to improve or maintain some properties of practical interest and applications.

The thermal stability of synthetic or natural polymeric matrices has been shown to reflect the morphological status, depending on type and frequency of the covalent and non-covalent bonds between molecules [2, 5]. The potential of 1,4-dioxane as a thermal stability-promoting agent resides in its power to aggregate and orient collagen fibers inside the pericardium matrix due not only to the properties of the organic solvent already discussed, but also to the exclusion of the solvation water layer recovering the tropocollagen molecules [5, 27]. As previously shown, dioxane actions on the collagen fibrils are strong, irreversible and essential in the context of practical applications [20]. 1,4-Dioxane is likely causing the organization of collagen fibers and leading to the formation of bundles, generating thicker and more porous matrices that will be applicable in tissue repair and grafts. Anionic or 1,4-dioxane-treated pericardium presents a cell/lipid-free matrix when compared to native pericardium, which results in very low cytotoxic and calcification effects in in vitro applications [30, 31]. Alkali treatments of connective tissues were shown to strongly reduce general lipids content including acylglycerols, free fatty acids, glycerophospholipids, and cholesterol-derived lipids [11]. Additionally previous analysis by thin-layer chromatography (TLC) with specific staining also showed removal of lipids from the pericardial tissue after dioxane treatments, and found phosphatidylcholine, phosphatidylserine, lysophosphatidic acid, free-cholesterol, and cholesterol-esters in the organic extracts (Forti FL & Plepis AMG, unpublished results).

Enhancement of biological properties of alkaline ± dioxane-treated BP membranes were investigated in cellular biocompatibility assays using collagenic reconstituted membranes in the presence of mouse NIH3T3 fibroblast cells [15, 22, 23]. However, evaluation of cell behavior in the presence of doubly-treated BP membranes, as a biocompatibility measure, is a difficult assay to perform because of the many intrinsic factors of the material characteristics, including (1) the high thickness of the BP; (2) the inability of BP to attach to the bottom of culture plates; and (3) the high porosity of the natural membrane, which allows the cells to penetrate through the tissue, impairing its detection, visualization and quantitation.

Our morphological and quantitative analysis focused on microscopic observations of (1) cell attachment over the assembled collagenic membrane, (2) cellular proliferation by manually counting cells after an established time of

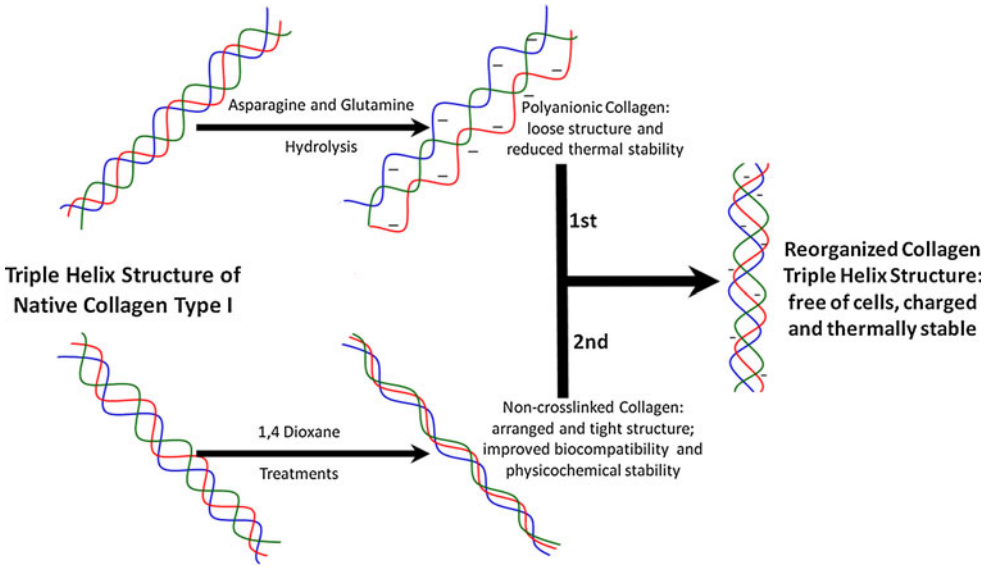

Fig. 7 Suggestive, illustrative scheme depicting the structural modifications promoted in the collagen matrix submitted to single or associated (alkaline + 1,4-dioxane) chemical treatments proposed in this study

plating, and (3) cell death by re-plating floating cells for additional 24 h, after the previous time of adhesion and proliferation. Cells considered viable were those usually presenting normal and flat morphology, attached to the membrane and in replicative state. After the second plating the remaining floating cells, which may be those that did not adhere because of material cytotoxicity and/or because of the unfavorable but biocompatible tissue surface, were considered apoptotic because their survival is dependent on attachment (anoikis state) [24].

According to Forti et al. [23], alkaline-treated collagen membranes present negative charge distribution that impaired adhesion and proliferation [15]. However, the more important effect brought by this work is the ability of the 1,4-dioxane to recover the doubly-treated membranes, resulting in an increase of adherent and proliferative cell number, and showing to be more favorable to cell survival. Preliminary results fixing the time of alkaline treatment to 148 h and extending the proliferation kinetics time-points to 5 more days (120 h) resulted in slight but proportional increase of surviving cells and decrease of dead cells. Furthermore the high cellular adhesion viability of cells in the double-treated membranes is exclusive to their surface, while in the pericardial membranes fibroblast cells can migrate within the tissue [22]. These findings suggest the doubly-treated materials (alkaline + dioxane) as potential support for cells in tissue engineering applications. That is reinforced by preliminary in vivo experiments performed in rabbits, where small pieces of BP treated by alkalis plus 1,4-dioxane (this work) were implanted into hypoderm of the animals and followed by histological analysis (Fazal Z et al., unfinished and unpublished results). According to those studies, samples of membranes rescued from rabbits after 4 weeks of implantation presented connective and adipose cells inside the matrix, which behaved as an adequate support to cells with an apparent low biodegradability (Goissis G, personal communication).

5 Conclusions

In this work, we explored the 1,4-dioxane treatment over the polyanionic collagen matrices of pericardial tissues with hydrolyzed Asn and Gln generated by dimethylsulfoxide solutions in the presence of salts and bases of alkaline and alkaline earth metals. We found that 1,4-dioxane can revert the disorganized structural status caused by the increased levels of negative charges along the collagen molecules due to a powerful ability to promote (1) reorganization, (2) aggregation, and (3) orientation of anionic collagen fibers and elastic fibers. It can also subtly increase the thermal stability of anionic collagen after the alkaline treatments (Fig. 7), but not after the acidic treatments that elevate the positive charge content and block all the dioxane effects. Either the isolated or the proposed combined alkali + dioxane treatments compromised mechanical and porosity properties of BP, needed for manufacture of cardiac valves, arteries and other bioprosthesis. However, the properties of BP treated by alkali + dioxane found in this study, associated to preliminary in vivo tests using small pieces of this material implanted into hypoderm of rabbits, suggest that this biomaterial has potential applications as soft tissue filler since it presented low content of lipid and cells inside the matrix as well as the adequate cellular biocompatibility, which did not promote cell death but rather stimulated adhesion and proliferation of fibroblast cells wanted for the genesis and development of new connective and vascular tissues.

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