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Inhibition of calcification of bovine pericardium after treatment with biopolymers, E-beam irradiation and *in vitro* endothelization

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ABSTRACT

This work has investigated the *in vitro* calcification of bovine pericardium (BP) treated with chitosan (C), silk fibroin (SF) and electron beam irradiation after its endothelization *in vitro*. For this purpose, freeze-dried BP membranes treated with mixtures of C and SF (1:3, 1:1 and 3:1) and then irradiated by electron beam irradiation were seeded with human umbilical vein endothelial cells (HUVEC) *in vitro*. After 3 weeks of cultivation these membranes were submitted to *in vitro* calcification tests using simulated body fluid as the calcifying agent. Control membranes were also studied (without endothelial cells exposure). The results have shown that the membrane compatibility with HUVECs *in vitro* prevent such biomaterial from calcifying, showing a potential application in biomaterial area, such as cardiac valves and repair patches.

1. Introduction

Bovine pericardium (BP) has been widely used in the manufacture of bioprostheses for more than 30 years. BP is an anisotropic material composed mainly of collagen fibers and elastin embedded in an amorphous matrix, which constituted of proteoglycans and hyaluronic acid. Collagen fibers are arranged in layers, with different alignment direction on each layer, giving rise to interesting mechanical properties, including the ability to undergo large deformation during the execution of its physiological functions [1]. The advantage of using this tissue is its high content of collagen, in which modifications can be performed in amino (—NH2), carboxyl (—COOH) and hydroxyl (—OH) groups. However, the same groups are responsible for the degradation process once the BP membrane is isolated. This fact then requires a treatment of this membrane by some chemical or physical process for ex-vivo uses.

The usual treatment used to stabilize and cross-link the tissue is its treatment with glutaraldehyde (GA) [2]. Besides GA treatment present various advantages [3] it also leaves cytotoxic residues and it is considered one of the main reasons to the calcification of the bioprosthesis [4,5]. The calcification, formation of calcium phosphate or other calcium-derived compounds, is the main cause of failure in pericardial bioprosthesis. The calcification leads to valve failure and the need of prosthesis replacement [6,7]. For this purpose, several works have been developed as alternatives to the use of GA as a crosslinking agent [8–11] or to its detoxification [12–15]. The freeze-drying of glutaraldehyde-treated tissue also appears to be very useful for decreasing the toxicity [16].

The use of biopolymers and their blends for construction and modification of biomaterials has been increasing in order to achieve materials that mimic human tissue and to better understand biomimetic processes (e.g. biomineralization) [17–20]. Biopolymers such as chitosan (C) and silk fibroin (SF) have been extensively studied in biomaterials filed [20,21] and showed promising results on BP treatments concerning the decrease on calcification on this tissue [22]. Chitosan, a natural polysaccharide, presents excellent biocompatibility, biodegradability, affinity to biomolecules, and wound-healing activity. These features allow the employment of this biopolymer as wound dressing material, drug delivery vehicle and in tissue engineering field [23–25]. Silk fibroin is a protein obtained from the silkworm *Bombyx mori*. SF is widely studied as a material for scaffolds in tissue regeneration and cell therapy, due to its excellent mechanical properties and thermal stability, low toxicity, low degradation, wide pore-size distribution among other features [26–28]. Furthermore, some

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researchers investigated the interaction of silk fibroin with fibroblasts [29], keratinocytes [30] and endothelial cells [31]. The ability of implanted material to adhere and grow endothelial cells are desired characteristics of cardiovascular materials, since the growth of a cell layer on the material surface can improve their characteristics, such as mechanical strength [32]. Blends of C and SF have also been studied for tissue engineering (e.g. scaffolds) [21,33].

This work was inspired by previous results in which treated BP membranes with C and SF followed by irradiation, showed great interaction with HUVECs [34]. The biopolymers chitosan and silk fibroin were chosen as substitutes to the GA treatment and other chemicals in order to reduce toxic residues, reduce calcification and keep tissue biocompatibility. Therefore, the objective of this work was to investigate the *in vitro* calcification of these BP membranes after treatments and endothelization.

2. Materials and methods

2.1. Bovine pericardium preparation

BP was collected at butchery, and its fat content was removed by using a scalpel. Afterwards, it was washed with saline solution (NaCl 0.9% w/v), and stored under glycerol (89% v/v) for preservation. Before being used, BP was washed with saline solution (NaCl 0.9% w/v) to remove glycerol excess. The samples were freeze-dried in a FTS Systems (TDS-00209-A) [35].

The treatment with chitosan and silk fibroin was performed as previously described [34]. Briefly, pieces of freeze-dried BP (6×11 cm) were immersed in a hybrid solution of SF 2% mixed with C 2% in three different proportions (1:3, 1:1 and 3:1) at a ratio of 1 mL× cm^2 at room temperature for 12 hours. The samples were immersed in 1.0 M sodium hydroxide solution (NaOH) and ethanol 70% at a ratio of 50:50 (v / v) for 1 hour [36]. Finally, the membranes were exhaustively washed with ultrapure water to remove the excess of the solution and then, they were freeze-dried. Half part of the membranes was exposed to irradiation at a dose of 25 kGy, at a dose rate of 4.67 kGy/s under vacuum. The irradiation was performed in the electron beam accelerator Dynamitron® with a 0.550 MeV radiation energy.

2.2. Endothelial adhesion on BP membranes

The endothelial cultivation was performed with the human vein endothelial cells (HUVEC-CRL 1730 ATCC). Cells were cultured in Ham's F12K (Gibco, cat. no. 21700075) with sodium bicarbonate at a concentration of 1.5 g/L (Sigma, cat. no. S5761), supplemented with 2 mM of L-glutamine (Gibco, cat. no. 25030-081), antibiotic and antimycotic solution (Gibco, cat. no. 15240062) (100 units/mL penicillin, 100 μg/mL streptomycin and 0.025 μg/mL amphotericin), 0.1 mg/mL heparin (Merck), 0.05 mg/mL ECGs (endothelial cell growth supplement) (Sigma, cat. no. E2759) and 10% fetal bovine serum (Cultilab, Brazil). Incubation was at 37 °C with 5% CO_2. When cells were grown to approximately 80% on the bottle culture, they were detached by enzymatic action of trypsin 0.05% (Sigma, cat. no. T4799)/0.02% EDTA (Sigma, cat. no. E 6758) solution and subcultured at a ratio of 1:5. For endothelial cells cultivation on the pericardium, BP membranes were sterilized by exposing them to an ultraviolet lamp, rehydrated with saline solution for 24 hours and stored in a 6-well culture plate. Over each BP membrane was placed a stainless steel ring of 16 mm internal diameter to define the area of cultivation. The membranes were left with F12K culture medium in an incubator at 37 °C for one night. The endothelial cells were removed from the culture by using trypsin/EDTA solution and 12,000 cells seeded in the inner area of each ring lying on top of the membranes with the same culture medium used for culturing. After 24 hours, the rings were removed and held, the first change of culture medium was done using Ham's F12K with sodium bicarbonate at a concentration of 1.5 g/L, supplemented with 2 mM of L-glutamine, antibiotic and antimycotic (100 units/mL penicillin, 100 μg/mL streptomycin and 0.025 μg/mL amphotericin), 0.1 mg/mL heparin, and 10% fetal bovine serum. The membranes with cells were kept in the incubator for 3 weeks, and the culture medium was changed every 3 days. After incubation, cells were then fixed in methanol and subjected to reaction with 1:10 monoclonal anti-human factor VIII (Santa Cruz, cat. no. SC-80782), which was developed with a secondary antibody labeled with fluorescein (Vector, cat. no. FI-2000) in order to identify the endothelial cells functional activity. Finally, the nuclei were stained with ethidium bromide (Sigma, cat. no. E 7637) solution. The samples were then analyzed in the confocal microscope LSM 510 META-ZEISS, at wavelengths of 488 and 514 nm [22,34].

2.3. Calcification tests

The *in vitro* calcification test was performed by leaving the samples in contact with simulated body fluid (SBF) [37]. To perform these test solutions 1× SBF and 1.5× SBF were prepared, that have approximately one and one and half times the plasma ion concentration, respectively. A 1.5×SBF solution was used in order to accelerate calcification process *in vitro*, because it shows ionic concentration 50% higher than the conventional SBF. Samples of 4 cm^2 were soaked in 40 mL of SBF solution at 36.5 °C. The temperature was controlled by a thermostatic bath, under stirring, for 7 days. The SBF solution was changed every 48 hours (1×, 1.5× and 1.5× SBF) and pH was measured to verify whether the solutions were contaminated. After 7 days in SBF solution, the samples were washed thoroughly with deionized water to remove the excess of salts on the surface and then freeze-dried. The presence of calcium phosphate deposits was observed by Scanning Electron Microscopy (SEM) and confirmed by energy dispersive X-ray (EDX). Duplicates of each sample were tested.

2.4. Raman spectroscopy

The secondary structure of native and treated BP membranes were determined in a FRA106 Raman spectrometer (Bruker Optics) coupled to a NIR (model IFS 28/N), which has a wide spectral range of analysis (0000–4000 cm^{-1}). All samples were analyzed via off-line at room temperature (20 °C) using an analytical resolution of 4 cm^{-1}, laser power of 510 mW and an average of 512 scans. Data acquisition and spectra processing were performed on OPUS program. To each sample an average of three scans was acquired.

3. Results and discussion

3.1. Endothelization of the membranes

As previously mentioned, BP tissue has been widely employed as cardiac valve substitutes to human calcified heart valves. The use of this natural membrane as heart valves and patches requires the stabilization of the collagen fibers by the chemical cross-link with GA. However, these valves usually present failure because of calcific degeneration [38].

The physiological valve calcification is an active process that involves the activation of the mediators and pro-inflammatory pathways. The ECs play an important role to the valve function, which involves the regulation of the blood and the underlying tissue. For this reason, previous ECs adhesion onto the grafts can enhance the tissue patency and prevent it from thrombosis [39,40].

Endothelized biomaterials can be achieved by seeding ECs prior to implantation or by relying on the infiltration of the host's vasculature into the material. Due to this fact, the application of materials that present high affinity to the ECs is important. It is known that collagen [40–42] and silk fibroin [43–45] are among the natural materials that present favorable properties regarding to cell attachment and behavior.

Recent work developed by our group revealed that the treatment of freeze-dried BP membranes with C, SF followed by ionizing irradiation, at a sterilizing dose, is not cytotoxic and that these news membranes favored the endothelial cell adhesion and growth within the membranes [34]. These results suggest their employment in the biomaterial field. However, as mentioned before, the calcification test is an important assessment to be done before the use of these biomaterials as vascular grafts or cardiac valves. Taking into account evidence from studies showing that the endothelial cell layer can prevent pathological calcification [46], we decided to investigate whether this deposited layer of endothelial cells in the tissue could have an inhibitory effect on calcium phosphate deposition on BP modified with C and SF.

Fig. 1 shows HUVECs adhered to BP membranes without any treatment (pristine), BP treated with GA and BP treated with biopolymers followed by irradiation. It is possible to notice the presence of endothelial cells, which appears as lighter points on the images, spread all over the membrane. Looking at the transversal slides it is possible

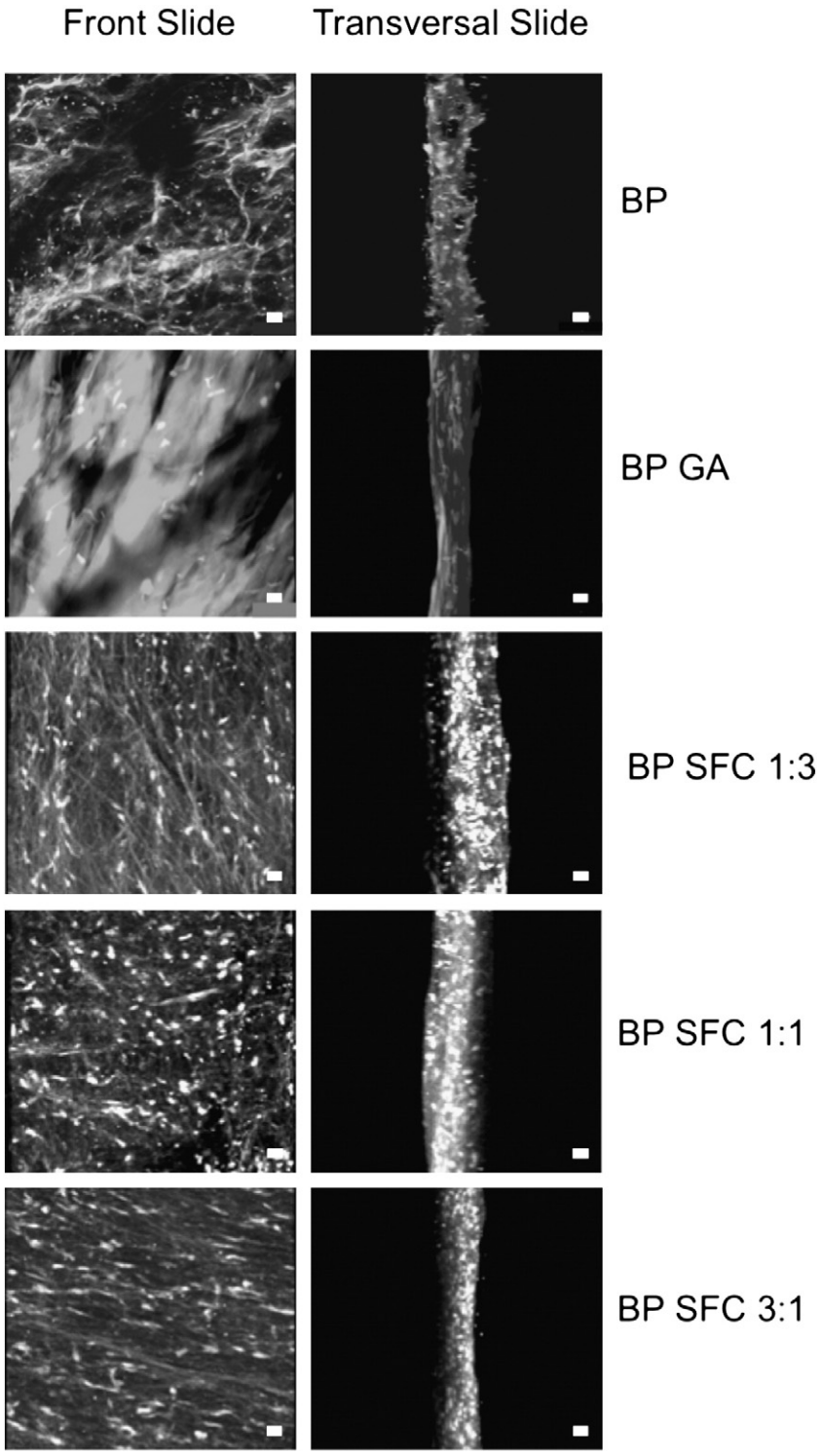

Fig. 1. Confocal images of endothelized BP membranes of: BP, BP GA, BP treated with silk fibroin (SF), chitosan (C) at three different proportions (1:3, 1:1 and 3:1) and irradiated. All scale bars are 10 μm.

to note that the HUVECs are within the membranes and not only at their surfaces.

Previous study developed by our group showed that BP membranes treated by C or SF, as well as their mixtures, followed by E-beam treatment (or not) were not able to prevent from calcification (unpublished data). Also, the non-irradiated BP SFC did not favor the endothelization process *in vitro*. Therefore we can suggest that the deposition of HUVECs in the BP SFC irradiated membranes must be favored by four associated factors to be considered: a) the non-cytotoxic behavior presented by these membranes after our treatment protocol [34]; b) the pores left by the freeze-drying process favors the endothelial cells deposition in the matrix [47,48]; c) the cell receptors (e.g. integrins) are free to interact [49,50]; d) and the assumption that the E-beam irradiation promotes partial scission of collagen and fibroin chains producing some peptide fragments that can promote adhesion and generates beneficial matrix cues [51–54].

3.2. Calcification tests

Fig. 2 (A1–C1) shows the calcification tests for the BP SFC irradiated membranes performed without HUVECs adhesion (as a control). The results revealed spherical deposits at all membranes surface, showing that the treatments with the biopolymers and irradiation can no prevent from calcification. The spherical deposits of calcium phosphate seem to be regular and homogeneous among the BP samples, suggesting similar mechanisms of deposition. The nature of the deposits was confirmed by EDX, confirming the presence of Ca and P deposits. The molar ratio (Ca/P) can be seen in Table 1. This atomic relationship allows us to obtain evidences about the type of deposit. Normally, the main type of calcification deposits investigated is hydroxyapatite (HA), whose molar ratio Ca/P is 1.67 [55]. However, the samples showed a Ca/P ratio smaller than 1.67 indicating that other type of calcification is being formed but not HA.

For the endothelized membranes SEM images, depicted in Fig. 2 (A2–C2), revealed a considerable inhibition in the calcium phosphate deposits, suggesting that the endothelial layer can help to avoid the deposition. Due to the scarce points of the calcification deposits it was not possible to perform EDX analysis with high reliability. This inhibition can occur due to the mimicry of the vein and artery tissues, achieved by the endothelization process. The endothelization process is desired in an attempt to recreate the innate properties, by producing natural biochemicals [49,50]. Innate roles of the ECs include the creation of a nonthrombogenic surface through the expression of anti-coagulant and fibrinolytic agents. The endothelial cells in general play an important role as a regulatory interface between the blood and the underlying tissue by being able to respond to their mechanical and humoral environment. The endothelium releases a variety of biologically active molecules. In general, the valve endothelium is associated with a protective effect against valve calcification [39,40]. Therefore, a plausible explanation for the calcification inhibition in regards to the surface charges of the membranes is that the resulting charges of the blends were possibly covered by organic compounds produced naturally by endothelial cells and released on the matrix.

Although the classical treatment with GA also permits the HUVECs attachment one of the major problems of this material is its cytotoxicity residue, which seems to be related with its calcification induction [4,5]. Thus, besides the endothelization process, the used treatments to fix the BP tissue are also an important point to be considered.

3.3. Raman spectroscopy

For further information on the BP secondary structure Raman spectroscopy was applied to investigate the sensibility to modifications made on BP with biopolymers and the adhesion of ECs on the irradiated membranes. Raman spectroscopy is an easy technique to be used to characterize and understand the secondary structure as

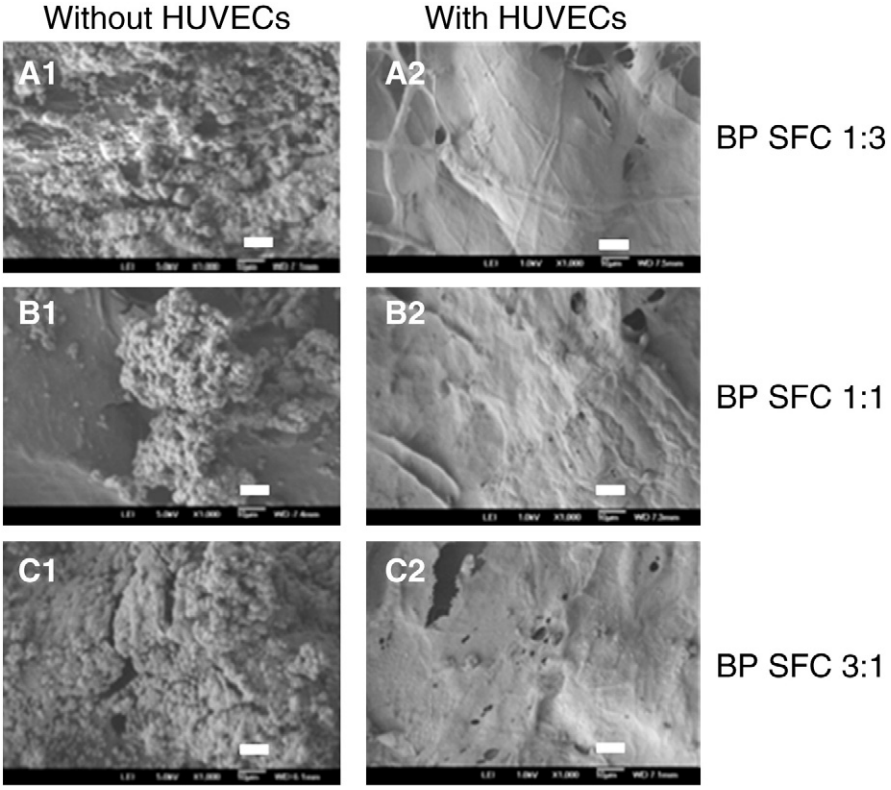

Fig. 2. Images of the surface of BP treated with biopolymers and irradiation after calcification test, obtained by SEM (A1–C1) and SEM images of BP endothelized membranes after calcification test (A2–C2). All scale bars are 10 μm.

Table 1
Values of the Ca/P obtained by EDX before and after endothelization with the HUVECs.

Sample	Mean* (Ca/P)	
	Without HUVECs (control)	With HUVECs
BP SFC 3:1	1.38 ± 0.07	–†
BP SFC 1:1	1.04 ± 0.11	–†
BP SFC 1:3	1.24 ± 0.20	–†
BP SFC 3:1 irradiated	1.29 ± 0.07	Not detected
BP SFC 1:1 irradiated	1.33 ± 0.11	Not detected
BP SFC 1:3 irradiated	1.31 ± 0.04	Not detected

*$n = 5$; †Data not obtained, since the HUVEC cells showed no adhesion to these membranes.

well as the determination of biochemicals on various human tissues, therefore its application is increasing in biomedical field [56,57].

Fig. 3 shows the Raman spectra for the treatments made on BP native with chitosan, silk fibroin (at three different proportions 1:3, 1:1 and 3:1) and E-beam irradiation. As noted, the spectral shape of BP is largely preserved, recognizing for example, collagen characteristic peaks regions of the amide III (1246 cm^{-1}), the molecular vibration mode deformation to NH (approximately 1450 cm^{-1}) and the amide I region (about 1665 cm^{-1}) [58–60]. The preservation of these bands indicates that the collagen structure is preserved. However, with the incorporation of C and SF variations were detected in the peak intensities. These variations are recorded in approximately 936 cm^{-1} for the incorporation of C (product of the symmetric stretching of C—O—C and deformation of the NH_2 wagging, out of phase mode) [61,62], and peaks at 874 cm^{-1} and 1060 cm^{-1} product of the incorporation of SF assigned to the peaks at 885 cm^{-1} (C—C) and 1087 cm^{-1} (C—N) [63].

Presented in Fig. 4 are the spectra of the irradiated samples with cellular deposition (endothelialization process), after calcification test. According with the spectra it observed a significant change in intensity of the peaks, mainly in the range of 1200–1400 cm^{-1}. The wide variation in the intensity in this region suggests that it can be related as interaction between endothelial cells with BP tissue, since those are the representative peaks related with the preservation of the collagen. Furthermore, the Raman spectra of treated BP membranes exhibited variations in the intensity between 950 cm^{-1} and 1100 cm^{-1} region (mainly peaks in 961 cm^{-1} and 1093 cm^{-1}, approximately) as a product of the calcification test. These results we refer to Otero et al. [64] research that evaluated calcified cardiac human valves and showed that the major bands at 962 and

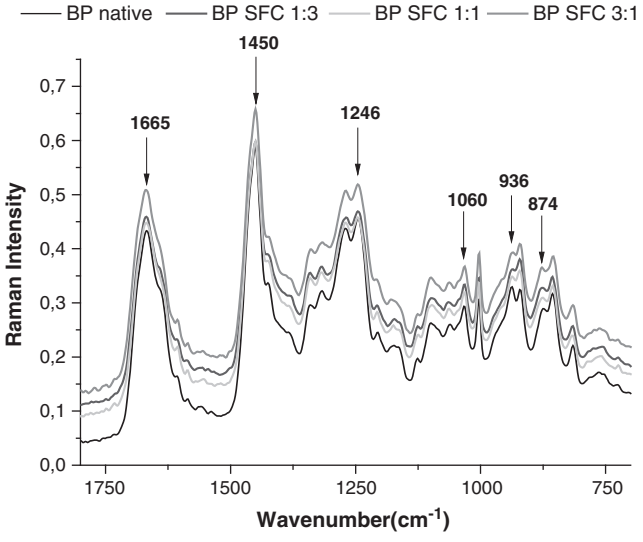

Fig. 3. Normalized Raman spectra of native bovine pericardium (BP) and BP treated with silk fibroin (SF), chitosan (C) at three different proportions (1:3, 1:1 and 3:1) and irradiation.

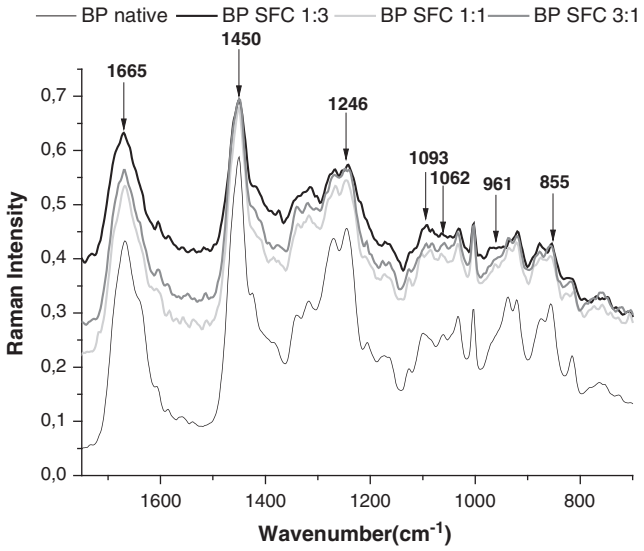

Fig. 4. Normalized Raman spectra of native bovine pericardium (BP) and endothelized BP treated with silk fibroin (SF), chitosan (C) at three different proportions (1:3, 1:1 and 3:1) and irradiated. Treated samples were submitted to accelerate calcification tests.

1073 cm^{-1} are ascribed to phosphate and carbonate symmetric stretching vibrations of accumulated calcium hydroxyapatite and of carbonate apatites. In our analysis small shoulders appeared at those regions (Fig. 4), and could be related to the scarce points of calcification, as encountered in SEM analysis (Fig. 2).

The treatments employed with biopolymers do not consume functional groups that are important for cell adhesion and proliferation. A possible explanation for the endothelial cell adhesion on irradiated membranes is probably not a chemical alteration on secondary structure (according to Raman spectra) but that the biopolymers protected the signaling sites that favor the adhesion and growth of cells.

4. Conclusion

In summary, this work showed that the previous *in vitro* endothelization to BP modified with chitosan, silk fibroin followed by irradiation appears to be an excellent anticalcifying treatment.

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