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Bovine pericardium coated with biopolymeric films as an alternative to prevent calcification: *In vitro* calcification and cytotoxicity results

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ABSTRACT

Bovine pericardium, for cardiac valve fabrication, was coated with either chitosan or silk fibroin film. *In vitro* calcification tests of coated and non coated bovine pericardium were performed in simulated body fluid solution in order to investigate potential alternatives to minimize calcification on implanted heart valves. Complementary, morphology was assessed by scanning electron microscopy — SEM; X-ray diffraction (XRD) and infrared spectroscopy (FTIR-ATR) were performed for structural characterization of coatings and biocompatibility of chitosan. Silk fibroin films were assayed by *in vitro* cytotoxicity and endothelial cell growth tests. Bovine pericardium coated with silk fibroin or chitosan did not present calcification during *in vitro* calcification tests, indicating that these biopolymeric coatings do not induce bovine pericardium calcification. Chitosan and silk fibroin films were characterized as non cytotoxic and silk fibroin films presented high affinity to endothelial cells. The results indicate that bovine pericardium coated with silk fibroin is a potential candidate for cardiac valve fabrication, since the affinity of silk fibroin to endothelial cells can be explored to induce the tissue endothelization and therefore, increase valve durability by increasing their mechanical resistance and protecting them against calcification.

1. Introduction

Pathological calcification is one of the major problems related to bioprosthetic heart valve failure or deterioration [1]. Biological materials, such as bovine and porcine pericardia, are preferred to fabricate cardiac valves due to their low tendency to thrombus formation and superior hemodynamic properties. However, natural bovine pericardium is not suitable for valve fabrication due to its ability to be biodegraded. Furthermore, carboxyl, hydroxyl and amino groups from collagen present antigenic properties that could lead to rejection of the implanted material. For cardiac valve fabrication, bovine pericardium is crosslinked in order to stabilize its structure and neutralize its immunogenicity. The most common commercial crosslinker used in this situation is glutaraldehyde. Glutaraldehyde-treated bovine pericardium presents high stability, resistance to thrombus formation and low immunogenicity. However, the durability of these valves is low due to its tendency to calcify [2].

Several researchers have studied alternatives to replace or complement the crosslinking with glutaraldehyde in order to prevent pathological calcification on implanted bovine pericardium cardiac valves. Some of the alternative treatments are lyophilization [3], coating with biocompatible polymers [4], decellularization [5,6] or crosslinking with epoxy compounds [7]. The potential of these new materials to calcify was studied by *in vitro* or *in vivo* calcification tests.

Characteristics of the material surface considerably influence the calcification process, either due to its chemical composition or due to its roughness [8,9]. Surface chemistry influences the calcification process by complexation of chemical groups on the material surface with calcium or phosphorus ions leading to the nucleation of calcium phosphates crystals that, depending on the medium conditions, will grow and spread on the material surface. Even if a material does not present reactive groups on their surface, it can still be prone to calcification by ion anchorage in their pores or roughness, initiating the depositing process to mineral phase formation [7,10,11].

Both chemical and physical factors can be involved on bovine pericardium calcification. Glutaraldehyde treatment, essential for stabilize its structure and decrease its immunogenicity, induces the tissue calcification through chemical factors. On the other hand, the high roughness of bovine pericardium surface can promote the anchorage of calcium ions and cells that would act as nucleation sites for mineral deposition [1,5,6].

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In order to minimize bovine pericardium calcification, we have studied the feasibility of coating lyophilized samples with either chitosan or silk fibroin. Chitosan and silk fibroin are potential candidates for biomaterial fabrication or coating due to its compatibility to several cell types and suitable mechanical and thermal stability [12–15]. The potential of coated bovine pericardium to calcify was investigated by *in vitro* calcification tests in SBF solution. Calcification results for coated bovine pericardium samples were compared to results obtained for non coated samples. Complementary, the biocompatibility of chitosan and silk fibroin films was assayed by *in vitro* cytotoxicity and endothelial cell growth tests.

2. Materials and methods

2.1. Bovine pericardium samples

Bovine pericardium was extracted from a commercial patch (SN: 201731 — Braile Biomédica, Brazil). This sample was pre-treated in 0.5% glutaraldehyde solution and stored in a sterile glass tube containing 4% formaldehyde solution. The patch was rinsed with 0.9% NaCl solution to remove formaldehyde residues from the storage recipient. After rinsing, the patch was cut into 4 cm^2 pieces and then frozen in liquid nitrogen and freeze-dried for 24 h at −50 °C and −760 mm Hg in Liobrás — L101 equipment.

2.2. Biopolymer solutions

Silk fibers, supplied by Bratac — Brazil (BR08B), were washed for three times in 0.5% Na_2CO_3 at 85 °C and then in distilled water to remove sericin. Purified silk fibroin fibers were dried at room temperature and then dissolved in $CaCl_2$–CH_3CH_2OH–H_2O (1:2:8 molar) ternary solvent to a concentration of 10% at 85 °C [16]. Silk fibroin solution was dialyzed against water for 3 days at approximately 10 °C in a cellulose membrane (Viscofan, 22 EU — 20 USA) to remove salts. Dialyzed silk fibroin solution was diluted to a concentration of 1%.

Chitosan (Sigma Aldrich) was dissolved in 3% acetic acid solution to a concentration of 1%. No pH adjustment was performed.

2.3. Biopolymer films

20 mL of chitosan or silk fibroin solution was casted on polystyrene Petri dishes and the solvents were evaporated at 30 °C. Silk fibroin films were treated in 70% ethanol to induce film stability in aqueous environment by inducing β-sheet structure formation. Chitosan films were neutralized in 1 M NaOH solution for 1 h to avoid film solubility in aqueous medium.

2.4. Bovine pericardium coating

Lyophilized bovine pericardium (BP) samples were immersed in either silk fibroin or chitosan solution for 20 min to promote a film deposition. The samples were removed from biopolymer solutions and dried at room temperature. Afterwards, samples were treated to promote film stability in aqueous environment: BP samples coated with chitosan were treated in 1 M NaOH solution and samples coated with silk fibroin were treated in 70% ethanol solution. After 1 h of treatment, the samples underwent calcification *in vitro* tests.

2.5. Structural characterization of coatings

2.5.1. Morphology

Coated and uncoated bovine pericardium were frozen in liquid nitrogen, fractured and then freeze-dried (Liobras, L101, Brazil) for 24 h. The surface microstructure of samples was observed by scanning electron microscope (LEO 440i, Leica, USA), with a filament voltage of 15 kV. Lyophilized samples were mounted on double-sided carbon tapes attached to aluminum stubs and coated with gold with a sputter coater (SCD 050, Bal-Tec, Switzerland).

2.5.2. Chemical composition

Energy dispersive X-ray spectroscopy (EDS) was performed, using a Leica microscope (LEO 440i, USA), to observe and characterize the chemical elements present on samples. They were mounted on double-sided carbon tapes attached to aluminum stubs and coated with carbon with a sputter coater.

2.5.3. Crystallinity

X-ray diffraction was performed on lyophilized samples with a Philips Analytical X'Pert PW 3050 diffractometer with Cu-Kα radiation. The X-ray source was operated at 40 kV and 40 mA. Diffraction intensity was measured in reflection mode at scanning rate of 0.6°/min for $2\theta = 5–35°$.

2.5.4. Molecular structure and conformation

Fourier transform infrared spectroscopy with attenuated total reflection apparatus (FTIR-ATR) (MB 102, Bomem, USA) was used to determine the structure of coatings on bovine pericardia sample. Horizontal ATR technique was used with ZnSe crystal to assure that we would mostly have information about the surface region. The analysis was performed within the range of 650–4000 cm^{-1}, with a resolution of 4 cm^{-1}, and 256 scannings.

2.6. In vitro calcification tests

In vitro calcification tests are usually performed using a simulated body fluid (SBF) solution. SBF solution contains ionic concentration nearly equal to the body plasma fluid. Ion concentration of conventional SBF is (in mM): 142.0 Na^+, 5.0 K^+, 2.5 Ca^{2+}, 1.5 Mg^{2+}, 148.8 Cl^-, 4.2 HCO_3^-, 1.0 HPO_4^{2-}, and 0.5 SO_4^{2-}, using "Tris" buffer [17,18]. To accelerate the *in vitro* calcification, a modified SBF solution with ionic concentration 50% higher than the conventional SBF (1.5 × SBF) was used.

Bovine pericardium samples of 4 cm^2 were soaked in 50 mL of 1.5 × SBF solution at 36.5 °C. Temperature was controlled by a thermostatic bath under agitation for 7 days. SBF solution was changed every 48 h and solution pH was measured to verify whether the solutions were contaminated. After 7 days in SBF solution, the samples were carefully rinsed with deionized water to remove excess of salts on their surface and then frozen in liquid nitrogen and lyophilized at −50 °C and −760 mm Hg for SEM and EDS analyzes. Duplicates of each sample were tested.

X-ray absorption fine structure spectroscopy (EXAFS) analyses of samples that were soaked in SBF for 7 days were performed at the D04B-XAS beamline, at the Synchrotron Light National Laboratory (LNLS, Campinas, Brazil). Spectra of samples were collected around the K-edge absorption of Ca (4038 eV). The LNLS ring energy was 1.37 GeV, and the current used was up to 175 mA. The X-ray energy incident on the sample was defined using a double-crystal Si(111) monochromator. The instrument was evacuated to ~10–8 Pa of pressure in order to reduce X-ray losses due to attenuation in the air [19]. The samples were analyzed in fluorescence mode, using a Ge energy-dispersive detector mounted in the horizontal plane perpendicular to the beam, minimizing the contribution of elastic scattering [20]. Each sample was analyzed three times. The raw EXAFS spectra were treated with the ATHENA software, from the IFFEFIT package (http://cars9.uchicago.edu/~ravel/software/). Spectra from each sample were averaged, subtracted from the pre-edge background and then normalized. The normalization procedure corrects effects resulting from different sample thickness, and allows comparison of samples with different absorber contents [21].

2.7. Cytotoxicity test on biopolymer films

In vitro cytotoxicity of silk fibroin and chitosan films was carried out by metabolic effect as biological endpoint, following the ISO 10993-5: *Biological evaluation of medical devices* — Part5: Tests for cytotoxicity: *in vitro* methods.

The samples were sterilized by gamma irradiation (25 kGy) and cut to 1 cm^2/mL RPMI 1640 culture medium extract in sterile conditions. The samples were let at 37 °C for 48 h. Afterwards, the extract was filtered in sterile filter syringe with 0.45 μm cellulose membrane (Corning) and dilutions from 100% to 6.25 % were prepared with RPMI 1640 culture medium. Positive and negative controls were 0.4% phenol solution and high density polyethylene (HDPE), respectively.

For the cytotoxicity test, a colorimetric method with tetrazolum compound MTS was employed to determine the number of viable cells in proliferation (Cory 1991). Microplates of 96 wells were prepared with 50 μL of extract diluted from 100 to 6.25% in RPMI medium in quadruplicates. A suspension of CHO-k1 with 6×10^4 cell/mL was prepared and 50 μl/well was pipetted into the microplates. Then, they were incubated for 72 h at 37 °C in a humidified 5% CO_2 atmosphere. The cell viability was measured by adding 20 μL of MTS/PMS (20:1) solution followed by incubation for 2 h at 37 °C in the humidified 5% CO_2 incubator. The microplates were read in a spectrophotometer reader at 490 nm. The cytotoxicity index at which 50% of the cell population were killed (CI_{50}) was evaluated.

2.8. Adhesion and growth of endothelial cells on biopolymers films

For this study human umbilical vein endothelial cells from ATCC (CRL 1730) were used. They were maintained in F12 medium (Gibco) supplemented with antibiotic and antimicotic solution (Gibco — final concentration: 100 units/mL penicillin, 100 μg/mL streptomycin and 0.025 μg/mL amphotericin), 2 mM glutamine (Gibco), 20 μg/mL endothelial cell growth supplement (ECGS, prepared in our own laboratory), 90 μg/mL heparin (Liquemine — Merck) and 10% calf serum (Gibco), at 37 °C in a humidified 5% CO_2 atmosphere until they reached 80% confluence. For subculturing and for experiments, cells were harvested using 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline, pH 7.4.

Sterile silk fibroin and chitosan films were placed on the bottom of a 6 multiwell plate (Corning). A stain steel ring with 16 mm internal diameter was placed on each membrane and the cell suspension of 12,000 cells was poured into. Two wells without membrane were used as control. The cells growing were accompanied daily in light inverted microscopy with phase filter (Nikon) and the medium culture was changed every three days. Digital photographs were taken after 3, 7 and 14 days of culture, and the number of cells was counted using the program UTHSCSA ImageTool V 3.0.

3. Results

3.1. Structural characterization of coatings

Scanning electron micrographs of samples before calcification (Fig. 1) indicate that the bovine pericardium after coating presents different morphology of the pristine one. A qualitative decrease in rugosity can be observed when pictures 1-b and 1-c are compared to 1-a indicating that the surface of bovine pericardium is very irregular. The micrographs of coated samples indicate that silk fibroin and chitosan formed superficial layers with lower micro-rugosity that recall the structures that can be formed when dealing with these compounds alone [22,23]. 1-a presents surface irregularities of about *c.a* 5 μm whilst 1-b and 1-c present pores of about *c.a* 1–3 μm of diameter. This fact would indicate that chitosan and silk fibroin would probably fill the surface defects of pristine pericardium and produce a porous, but a denser and smoother surface than that found in the original biomaterial.

FTIR-ATR (Fig. 2) spectrum of pericardium before coating was similar to the ones reported in the literature [24]. The bovine pericardium covered by fibroin presented tiny changes in the fingerprinting section. As known, silk fibroin exhibits peaks of amide I at 1660 cm^{-1} correspondent to silk I conformation or at 1630 cm^{-1} for silk II; peak of amide II (1543 cm^{-1} for silk I and 1515 cm^{-1} for silk II) [25]. In Fig. 2, the peaks of amide I and II are more directed to 1630 and 1515 cm^{-1}, indicating the presence of silk fibroin possibly with conformation of silk II. This result would be expected mainly because of the treatment with alcohol performed after coatings. Silk II is a more chemically stable structure.

The sample coated with chitosan presented more intense peaks (in comparison to the other samples) in the region of 1070 to 1110 cm^{-1}, indicating the presence of aliphatic amines, characteristic of chitosan [26].

XRD results are depicted in Fig. 3. The diffractograms show that there are not visible differences observed among the samples. They are amorphous phases and probably could not indicate particularities of silk fibroin and chitosan because of the low amount of these materials that are present in samples in comparison to the amount of structures of bovine pericardium (mainly collagen). As XRD is a technique that would analyze a significant amount of material (X-ray penetrates about 10 μm depending on the density of sample), we would say that the observed diffractograms reflect mainly the structures of collagen and other constituents of bovine pericardium. This result would still reflect that the coatings are really thin superficial structures.

Other techniques such as XANES (X-ray absorption near edge structure) analysis and AFM were considered for improving the coating structural characterization of samples before calcification. However, the particular characteristics of pericardia samples did not allow the feasibility of such analyses. In the first technique, the low signal of light elements (such as carbon) and, in the second technique, the irregularity of pericardia surface (even after coating) were barriers to perform such analyses.

3.2. In vitro calcification tests

SEM micrographs of bovine pericardium samples after *in vitro* calcification tests are exhibited in Fig. 4. Bovine pericardium without biopolymer coating presented deposits of calcium phosphates confirmed by EDS analysis. However, samples coated with chitosan or silk fibroin did not present calcium phosphate deposits even after 7 days exposed to high concentrated calcification solution.

In vitro calcification tests carried out with simulated body fluid (SBF) or alternative solutions are fast and inexpensive methods to simulate the interactions between the material surface and the ions presented in the human blood plasma [27]. However, this method does not simulate cellular and enzymatic interactions or any other biological process that could occur in an organism after implantation. If the new material calcifies during *in vitro* calcification tests, it is not adequate for cardiac valves fabrication or any other application where calcification is not desirable. Whether the new material does not calcify during *in vitro* tests, it can be submitted to more complex tests, such as dynamic *in vitro* calcification tests and *in vivo* calcification tests [28,29].

EXAFS data acquisition was very difficult for pericardium covered by biopolymers because of the low quantity of calcium present in these samples. Fig. 5 depicts the Fourier transformed results of EXAFS data from samples that were soaked in SBF for seven days. The direct comparison of atomic distance values found in standard salts of calcium could not indicate any specific binding of calcium atoms in pericardium samples. However, it is still possible to compare the distances of vicinal atoms for calcium: the peaks of coated samples would be located almost at the same distances obtained for pristine sample, however still indicating a tendency for higher distances in the coated samples. If there was a significant amount of calcium in all

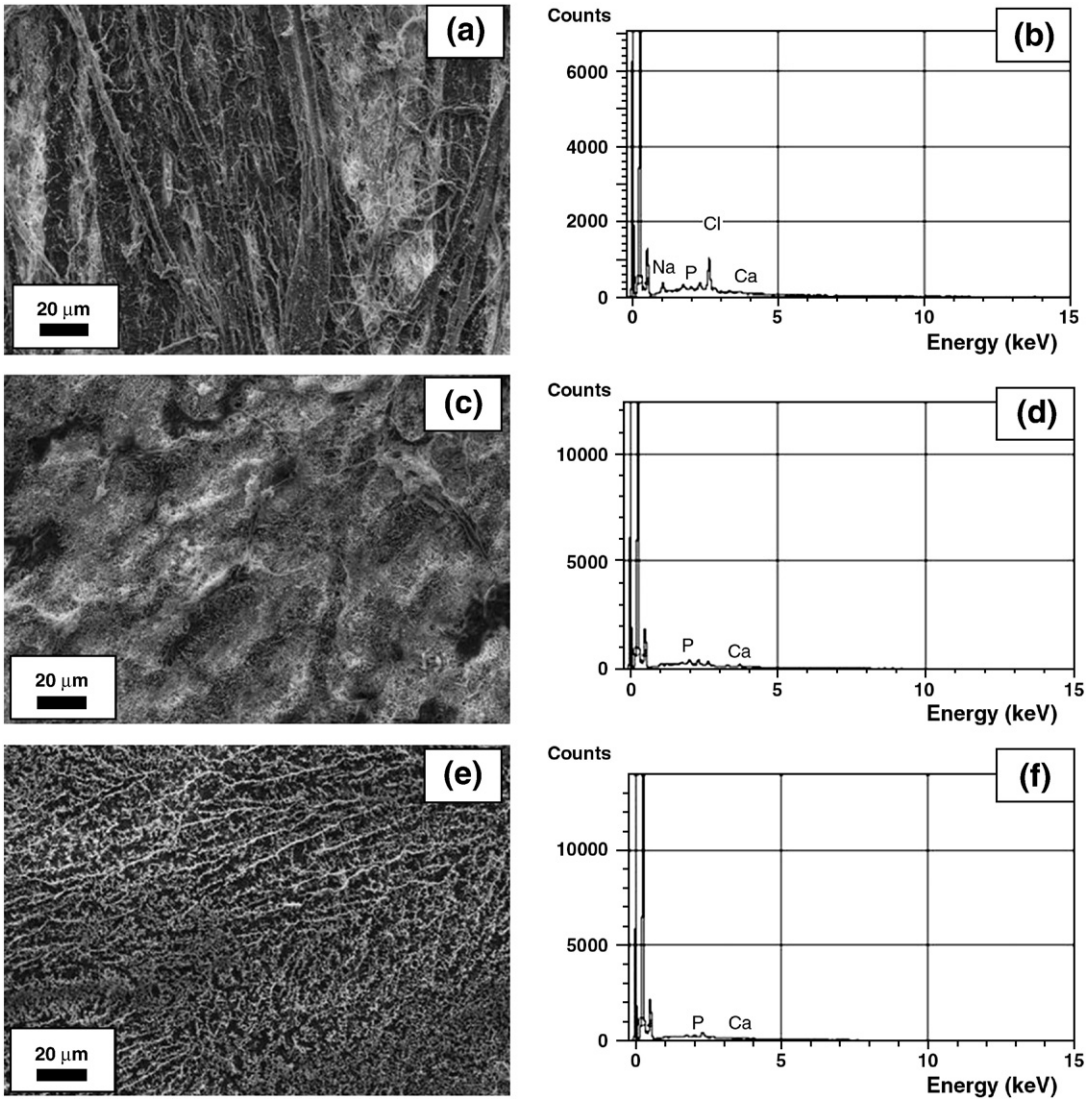

Fig. 1. Scanning electron micrographs of (a) bovine pericardium surface, (c) surface of bovine pericardium coated with silk fibroin and (e) surface of bovine pericardium coated with chitosan. (b),(d),(f) are the elemental EDS spectra of samples (a),(c) and (e), respectively.

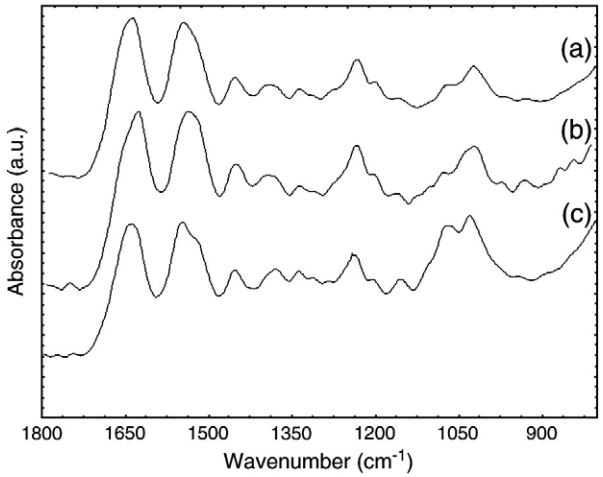

Fig. 2. FTIR-ATR spectra of (a) bovine pericardium, (b) bovine pericardium coated with silk fibroin and (c) bovine pericardium coated with chitosan.

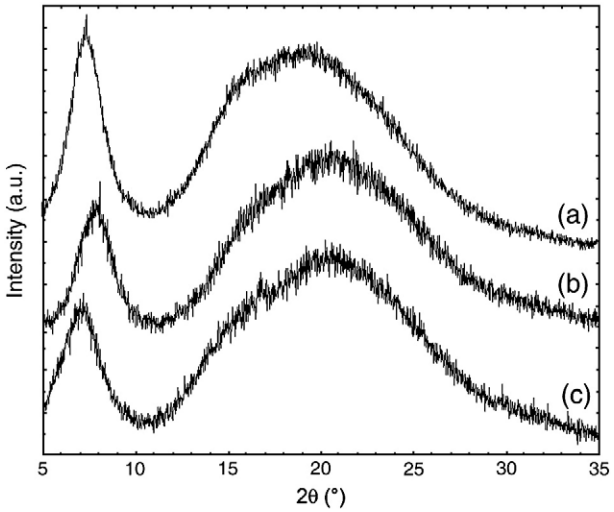

Fig. 3. X-ray diffractograms of (a) bovine pericardium, (b) bovine pericardium coated with silk fibroin and (c) bovine pericardium coated with chitosan.

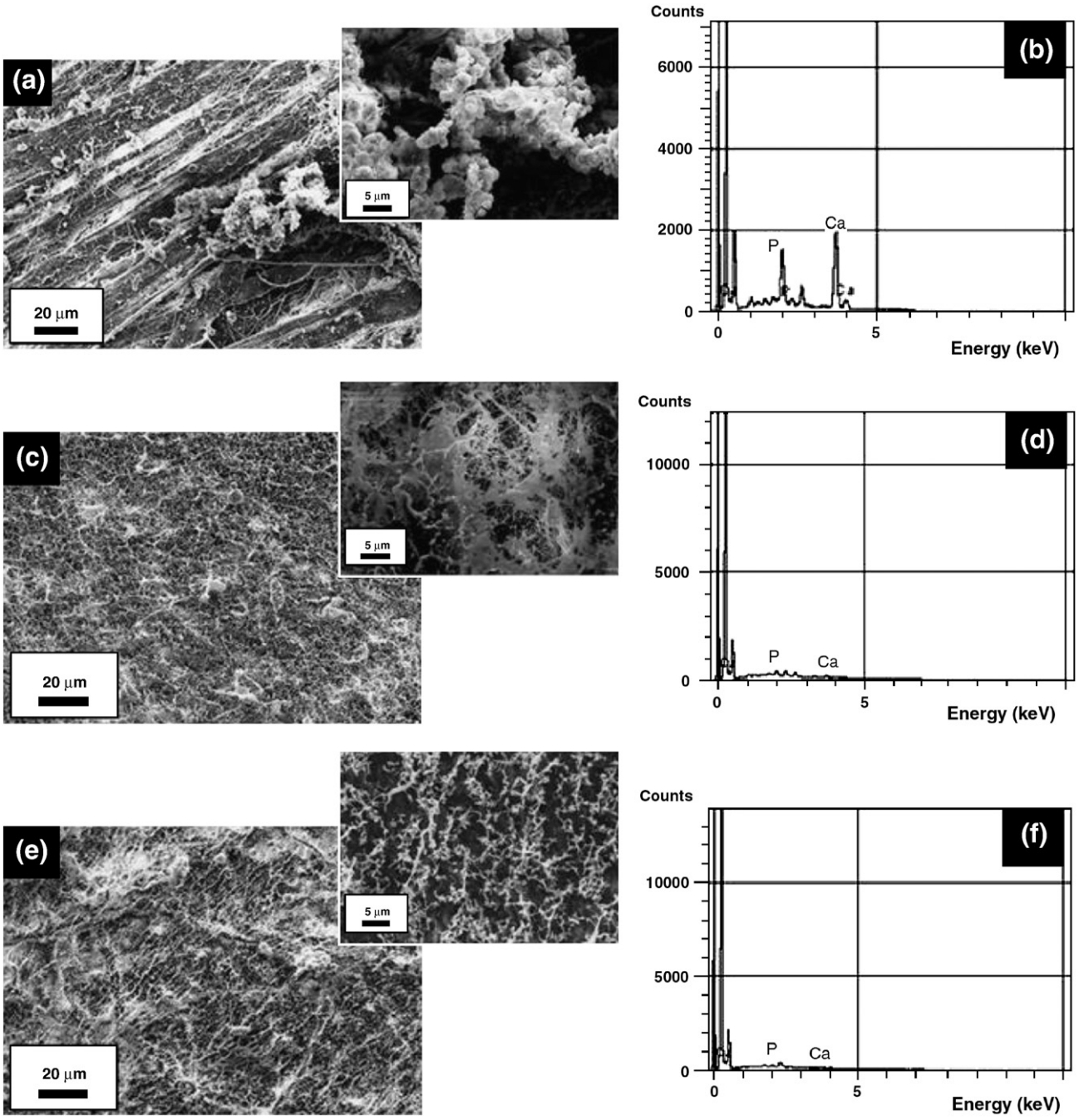

Fig. 4. Scanning electron micrographs of (a) non coated bovine pericardium, (c) chitosan coated bovine pericardium and (e) silk fibroin coated bovine pericardium after *in vitro* calcification tests and (b, d, f) respective EDS analysis highlighting the presence of phosphorus and calcium on the sample surface.

samples, this result would indicate that the coated samples would involve calcium bindings that are more loosely done (higher values).

Calcification of bovine pericardium can be induced by several parameters, such as residual cells on its tissue, extension of glutaraldehyde treatment or physical factors, such as surface roughness [1,2,4–6]. Therefore, the control of all the parameters that can influence its calcification is complex and sometimes not feasible. However, the study of *in vitro* calcification is useful to test new materials or modifications of existed materials, as we present for bovine pericardium coated with biopolymers. In the present study, as evidenced by Fig. **1**, the coating can be playing the role of a filler of bovine pericardium superficial defects. The decrease on these irregularities would then provide less receptive surface for calcification anchorage. In this sense, the fact that covered samples would be less prone to *in vitro* calcification could be more a result of surface micromechanics rather than the chemistry of surface [30].

Aimoli et al. [7] showed that non-lyophilized bovine pericardium samples presented calcium phosphate deposits after *in vitro* calcification tests, whilst bovine pericardium coated with chitosan or silk fibroin did not present any indication of calcification after being tested under the same conditions tested by Aimoli. Since no deposition of calcium phosphate was observed on coated bovine pericardium, this modified material could be a potential alternative to fabricate cardiac valves. Besides, biopolymers coating could present the advantage of incorporating drugs, such as anticoagulants and calcification inhibitors in the films, which could be controlled released when implanted.

It is well known that silk fibroin has high affinity to calcium ions. This characteristic could lead silk fibroin derived materials prone to

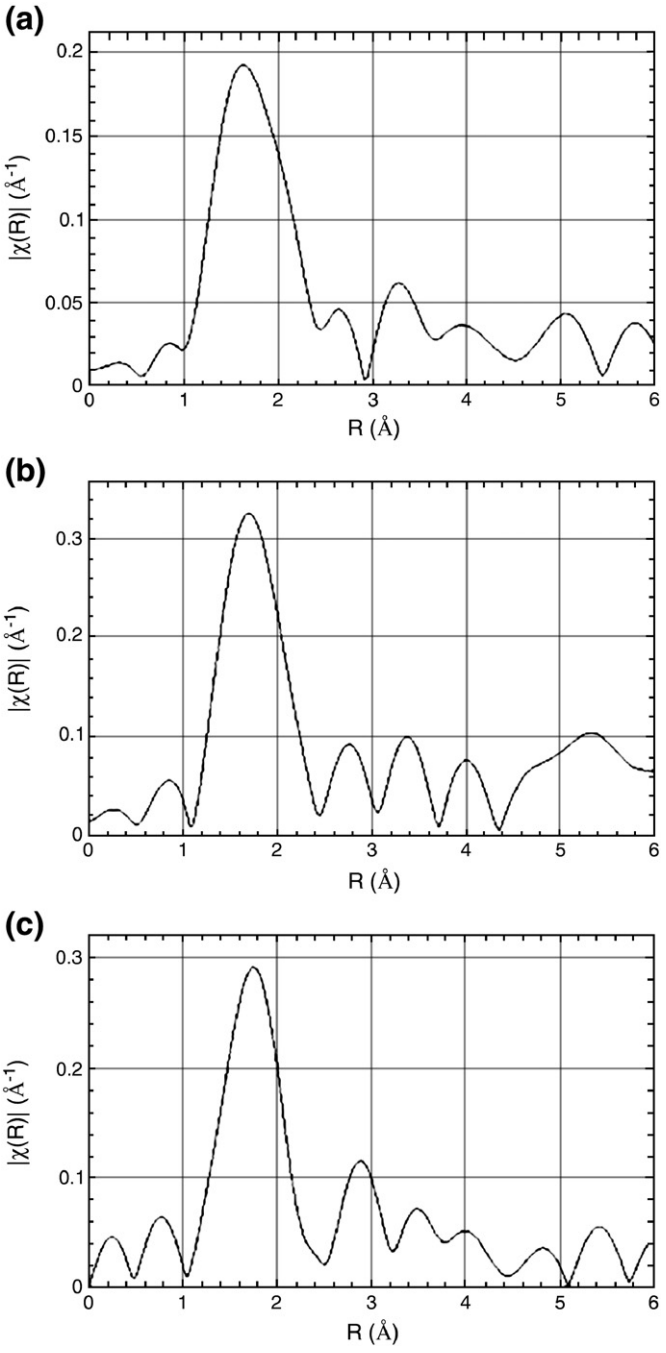

Fig. 5. EXAFS Fourier transformed data of (a) bovine pericardium, (b) bovine pericardium coated with silk fibroin and (c) bovine pericardium coated with chitosan after 7 days of contact with SBF.

calcify *in vitro* or *in vivo* [22,31–35] what would be undesirable for cardiac valves implants. Even after silk fibroin coating, bovine pericardium presented high surface roughness, which could act as ions anchorage site. The presented results, however, indicate that silk fibroin modifies the surface of bovine pericardium avoiding its calcification during *in vitro* calcification tests even in high concentrated calcification solution (1.5 × SBF).

3.3. Cytotoxicity and endothelial cell growth on silk fibroin and chitosan films

Besides investigating the potential of coated bovine pericardium to calcify, it is important to check the biocompatibility of the biopolymer films coating its surface. An initial approach to assay the biocompatibility of new materials is to test its cytotoxicity *in vitro*. Fig. 6 exhibits the results for the cytotoxicity test on silk fibroin and chitosan films. Both films did not present cytotoxicity to any extract concentration analyzed, indicating that both biopolymer films might be biocompatible *in vivo*.

Endothelial cell growth was assayed to both chitosan and silk fibroin films and the results are presented in Fig. 7. Endothelial cells adhered and spread on silk fibroin film surface. The number of cells was counted after 3, 7 and 14 days of culture, and the results are presented in Fig. 8.The number of cells was lower on silk fibroin films. This difference may be attributed to non uniformity of the silk fibroin films compared to the flat surface of the positive control, preventing the seeded cells to homogeneously adhere on the film surface. Nevertheless, the growth rate of these cells was the same for both positive control and silk fibroin films (~1.8) indicating that silk fibroin is indeed compatible to endothelial cells.

Chitosan films did not present compatibility to endothelial cells as silk fibroin film did. We did not observe adhesion and proliferation of endothelial cells on chitosan films, which indicates that silk fibroin films present more affinity to this type of cells than chitosan films. These results highlight the high potential of using silk fibroin films for biomaterial coating.

Compatibility of silk fibroin and chitosan to several cell types, including biocompatibility of silk fibroin with endothelial cells, has been reported in the literature [36–39]. This property can be useful for applying these materials as biomaterials including in the cardiovascular field. Silk fibroin affinity to endothelial cells could be explored, for instance, to favor the endothelization of bovine pericardium cardiac valves *in vitro* or *in vivo*. This endothelial cell layer on the valve surface could help to keep its integrity and furthermore, improve its mechanical properties and even avoid its pathological calcification [40,41].

One could be concerned about the fact that silk fibroin films are biodegradable and can, in long term, calcify *in vivo* leading to valve deterioration or malfunction. However, the high affinity of silk fibroin films to endothelial cells can be explored as an alternative to overcome these problems. Cell growth on bovine pericardium coated with silk fibroin has been studied by our group in order to investigate whether silk fibroin film keeps its affinity to endothelial cells when coating bovine pericardium surface. We expect to have an endothelized valve that would be more resistant to pathological calcification and fatigue failure, whether the cells grow and spread on the valve

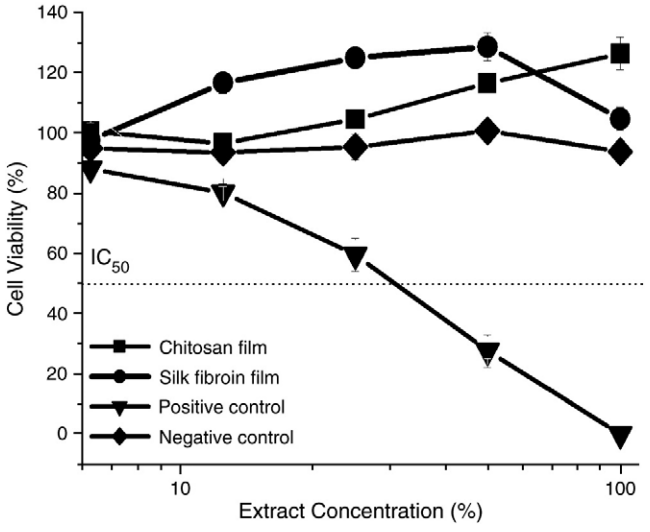

Fig. 6. Cell viability of chitosan and silk fibroin films assayed by cytotoxicity tests.

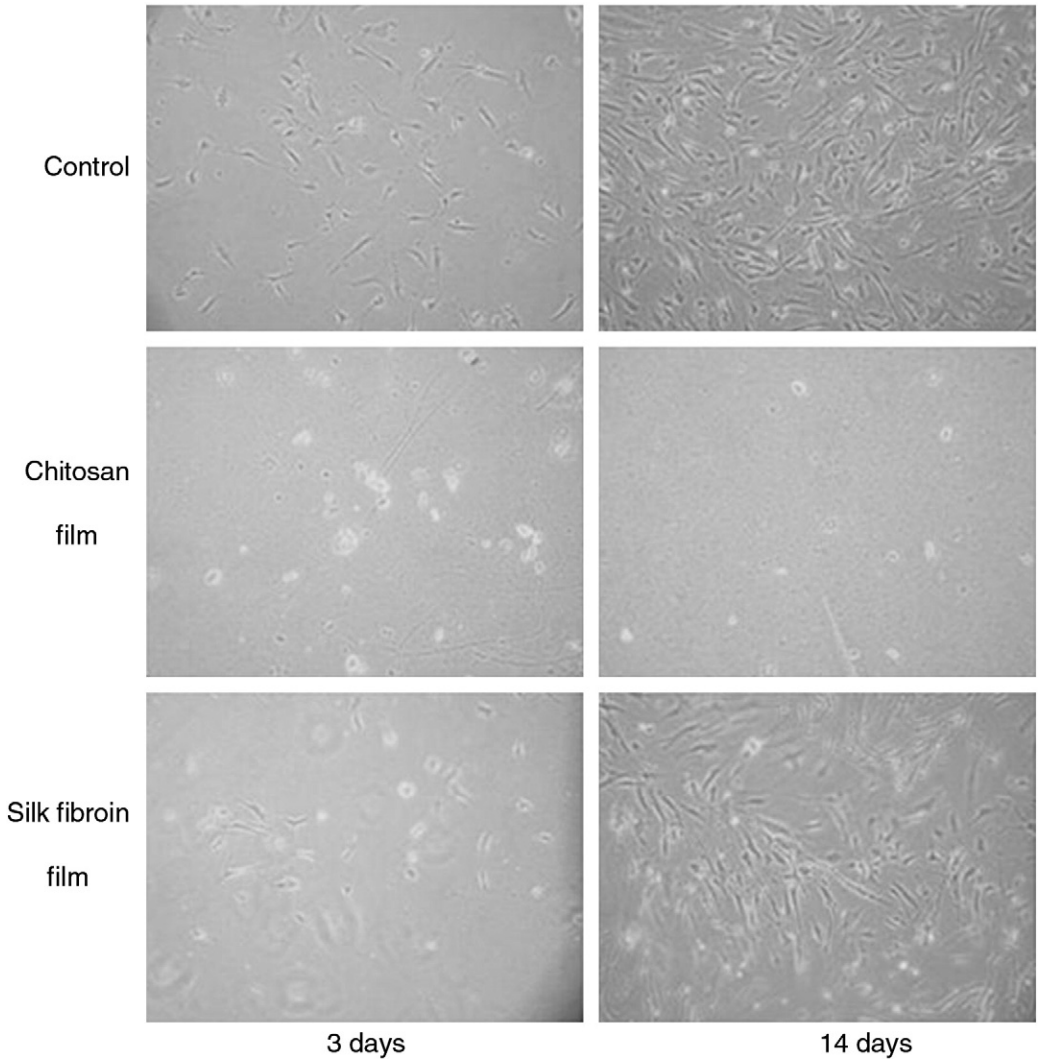

Fig. 7. Endothelial cell growth on culture plate (control) and on either silk fibroin or chitosan films.

surface before silk fibroin film either degrades or induces calcium phosphate deposition.

4. Conclusion

Silk fibroin coatings would be a promising surface treatment for bovine pericardium heart valves as they did not undergo calcification in *in vitro* tests. These coatings are also compatible to endothelial cells and can possibly be explored to induce the bovine pericardium valves endothelization.

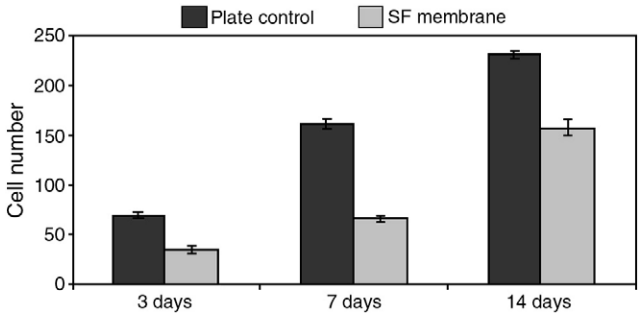

Fig. 8. Number of endothelial cells counted on silk fibroin films and positive control after 3, 7 and 14 days of culture.

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