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

Roberta Polak [a], Andrea C.D. Rodas [b], Dennis L. Chicoma [c], Reinaldo Giudici [c], Marisa M. Beppu [d], Olga Z. Higa [b], Ronaldo N.M. Pitombo [a,*]

[a] Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo, USP, São Paulo, SP, Brazil
[b] Biotechnology Center, Energy and Nuclear Research Institute, IPEN-CNEN/SP, São Paulo, SP, Brazil
[c] Department of Chemical Engineering of Polytechnic School, University of São Paulo, SP, Brazil
[d] School of Chemical Engineering, University of Campinas, UNICAMP, Campinas, SP, Brazil

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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.

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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

* Corresponding author at: Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo, Av. Professor Lineu Prestes, 580, 05508-900, São Paulo, SP, Brazil. Tel.: +55 11 30912379; fax: +55 11 38156380.
E-mail address: pitombo@usp.br (R.N.M. Pitombo).

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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.

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