

## Synthesis, characterization and cytotoxicity of Chitosan/Polyvinyl Alcohol/Bioactive Glass hybrid scaffolds obtained by lyophilization

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### ABSTRACT

One of the important research topics in tissue engineering is the development of optimum three-dimensional scaffolds for regeneration and growth of bone tissue. The scaffold developed should promote an initial bio-mechanical support, provide the formation, deposition and organization of the new organic matrix generated, and degrade proportionally to the growth of the new tissue. In this study hybrid scaffolds based on the blend Chitosan (CHI)/Poly (vinyl alcohol) (PVA), with two different CHI:PVA molar ratios (1:1, and 3:1), and Bioactive Glass as the inorganic phase, were developed by a sol-gel route, followed by lyophilization. The materials were cross-linked with Glutaraldehyde. The obtained porous scaffolds were characterized by SEM, FTIR, porosity measurements by Archimedes method, and compression test. The in vitro degradation was studied by immersion in simulated body fluid for several time periods and evaluation of mass loss. Cytotoxicity analysis was carried out on samples as prepared and after immersion in PBS solution for 24hrs, using human fibroblast cells and MTT method to evaluate cell viability. The matrices obtained showed promising results, presenting about 96% porosity, pore size varying in the range 20-300  $\mu\text{m}$ , and interconnected pores. The mass loss presented by the matrices with CHI/PVA ratios 3:1 and 1:1 during the degradation test in vitro, was around 10% after a week of testing, with macroscopic preservation of their physical structure. Cytotoxicity tests showed that the samples were toxic as produced and not toxic after treatment with PBS, showing this approach was suitable as a final preparation step of these samples.

**Keywords:** Chitosan, PVA, bioactive glass, lyophilization, porosity, scaffolds

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### 1. INTRODUCTION

Synthetic three-dimensional matrices are currently widely studied for application in the regeneration of bone tissue by presenting architecture similar to bone extracellular matrix and providing a suitable microenvironment for cell adhesion, proliferation and differentiation, ensuring tissue growth inside [1,2]. Among other properties, these scaffolds should exhibit biocompatibility with the damaged tissue, interconnected network of pores, pore size range from 100 to 300  $\mu\text{m}$ , mechanical strength similar to bone tissue, and biodegradability at the rate which the tissue regenerates [3,4]. The production of nanocomposites based on biodegradable polymers and bioactive glasses has been the focus of extensive studies in the literature [5, 6], aiming at obtaining scaffolds with the described characteristics.

Bioactive glasses in the system  $\text{SiO}_2\text{-CaO-P}_2\text{O}_5$  have been investigated as biomaterials to be introduced into the organic phase due to their osteoconductive and osteoinductive properties [7, 8]. When implanted in the human body, the formation of a layer of carbonated hydroxyapatite (HCA) occurs on the surface of the bioactive glass, responsible for the interfacial bonding of the implant to the tissue. However, its fragility and low mechanical strength limit its clinical applications. Mechanical properties of porous scaffolds are crucial, especially with regard to the regeneration of hard tissue such as bone, which must support a load and meet specific mechanical needs while stimulating bone regeneration. Pure bioactive glass scaffolds, with

high porosity around 90%, although inducing a biological response that stimulates bone regeneration [9], are fragile and have such a low resistance that makes it difficult even to handle the material. An alternative approach is the production of composites and hybrid systems [10-13].

Chitosan has been widely studied for bone tissue engineering because it is biocompatible, biodegradable, and also favors osteoconduction [14, 15]. However this natural polymer presents relatively low strength and low flexibility. Despite its tremendous promise in bone tissue engineering application, the poor mechanical properties of chitosan limits its clinical application in weight bearing bones, which has been addressed by the addition of bioceramics in chitosan scaffolds [16-19]. The mechanical properties of the organic matrix can be improved further by mixing Chitosan with another polymer and by crosslinking [18-19].

In previous work, LEMOS et al [20] investigated the production of Bioactive Glass/Chitosan hybrid films, with various contents of the inorganic phase, using a sol-gel route. The hybrid films obtained exhibited high tensile strength, high bioactivity and cell viability. It was shown that the optimal concentration of added bioactive glass was 20% (w/w). This study investigates Chitosan/PVA hybrids containing 20% of bioactive glass (w/w), with two different Chitosan:PVA mass ratios (3:1 and 1:1). The use of a polymeric blend is proposed in this work, based on the results obtained by Costa-Junior et al [21-23], which showed that the flexibility of chitosan is increased by addition of PVA. The materials were synthesized by the sol-gel route, crosslinked with glutaraldehyde, and dried by lyophilization to produce a porous scaffold. This technique, also known as freeze-drying, is widely used to create biomaterials with high porosity and complex hierarchical architecture [24, 25].

## 2. MATERIALS AND METHODS

### 2.1 Synthesis of three-dimensional scaffolds

#### 2.1.1 Preparation of Polymer and Bioactive Glass Precursor Solutions

All reagents were supplied by Aldrich Chemical.

Poly (vinyl alcohol) (PVA) solution 5.0% (w/v) was prepared by dissolving PVA (80% degree of hydrolysis) in deionized water (100 mL) under mechanical stirring at 70°C ( $\pm 2^\circ\text{C}$ ) for 45 minutes.

A solution of Chitosan (CHI) with high molecular weight and degree of deacetylation (DD) > 75% (1% w/v) was prepared by dissolving 1 g of commercial powder in 100 ml of deionized water. 2 ml of acetic acid was added to the solution, and then subjected to mechanical stirring for 24 hours.

Bioactive glass 60S precursor solution (BG) was obtained by acid hydrolysis and polycondensation of Tetraethylorthosilicate (TEOS -  $(\text{Si}(\text{OC}_2\text{H}_5)_4)$ ), alkoxide precursor of  $\text{SiO}_2$ , and Triethylphosphate (TEP -  $((\text{C}_2\text{H}_5\text{O})_3\text{PO})$ ), alkoxide precursor  $\text{P}_2\text{O}_5$ . Hydrolysis occurred by adding deionized water and nitric acid as catalyst reagent. Calcium Nitrate ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ) was then added as a precursor of CaO. The nominal composition of the bioactive glass was: 60%  $\text{SiO}_2$ , 36% CaO; 4%  $\text{P}_2\text{O}_5$ .

Glutaraldehyde solution (2.0% w/v) was prepared by diluting 25% glutaraldehyde 2ml in 23mL of deionized water.

#### 2.1.2 Scaffolds Production

The scaffolds were fabricated by mixing PVA solution with Chitosan solution in the ratios of CHI:PVA 3:1 and 1:1 in accordance with Table 1, and mixing under agitation for 30 minutes. The precursor solution of bioactive glass (20% of the total weight of the scaffold) was added and mechanical stirring was continued for 45 minutes. Finally, the solution of glutaraldehyde 2% (3% of polymer mass) was added and mechanical stirring remained for 15 minutes.

**Table 1:** Composition of scaffolds.

Scaffold CHI:PVA ratio	Composition (%)			
	Chitosan	PVA	VB	Glutaraldehyde*
3:1	60	20	20	3
1:1	40	40	20	3

\*In relation to polymers mass

The resulting solution was poured into vials of 7mL with a syringe and kept at room temperature for 72 hours, the time required for gelation to occur. The vials were kept closed during gelation. The vials were then frozen for 72 hours in a refrigerator at -20°C.

The frozen vials were immersed in liquid nitrogen for 20 minutes and then placed on the lyophilizer (Model: K105 - Company Liotop – SP/Brazil) for 48h with -98°C condenser temperature and -4°C sample collector temperature. The pressure in the collector was 30mmHg.

## 2.2 Characterization of three-dimensional scaffolds

### 2.2.1 Scanning Electron Microscopy

The scaffolds were characterized by Scanning Electron Microscopy (SEM) FEI-Inspect-S50/Czech Republic. The samples were immersed in liquid nitrogen and fractured to obtain the internal fracture surface for analysis, which was then coated with gold.

### 2.2.2 Fourier Transform Infrared Spectroscopy-ATR

The scaffolds were characterized by Fourier Transform Infrared Spectroscopy (FTIR) by ATR mode (attenuated total reflectance) the wave number range 4000-500 cm<sup>-1</sup> at a resolution of 1cm<sup>-1</sup> with an average of 64 scans, the equipment used was the Nicolet 380 ThermoScientific.

### 2.2.3 Porosity - Method of Archimedes

The mass of dried samples ( $m_{dr}$ ), saturated with fluid ( $m_{sa}$ ) and suspended in the fluid ( $m_{fl}$ ) were measured six times. Five hybrid samples of each type with 18 mm x 9 mm were used. Deionized water (density of 0.9982g/cm<sup>3</sup>) was used as fluid. The volume density ( $\rho_{vol}$ ) was calculated using Equation 1, and the true density ( $\rho_{tr}$ ) was estimated through Equation 2. Apparent porosity was calculated by Equation 3 and finally the total porosity was obtained from Equation 4.

$$\rho_{vol} = \frac{m_{dr} \times \rho}{m_{sa} - m_{fl}} \quad (1)$$

$$\rho_{tr} = (\% \text{ Chi} \times \rho \text{ Chi}) + (\% \text{ PVA} \times \rho \text{ PVA}) + (\% \text{ BG} \times \rho \text{ BG}) \quad (2)$$

$$\text{Porosity}_{\text{apparent}} (\%) = \frac{m_{sa} - m_{dr} \times 100}{m_{sa} - m_{fl}} \quad (3)$$

$$\text{Total porosity} = \frac{\rho_{tr} - \rho_{vol} \times 100}{\rho_{tr}} \quad (4)$$

### 2.2.4 Mechanical test

The evaluation of scaffolds mechanical behavior was performed using the compression test equipment Universal Instron 5882 machine and a load cell of 5 kN and test speed of 0.5 mm/min at 22°C and according to ASTM D 695 (Standard Test Method for Compressive Properties of Rigid Plastics). The samples were in cylindrical shape with 18 mm diameter and 9 mm in height.

### 2.2.5 Degradation test

The degradation index (DI) was performed with samples in triplicate. The samples were dried by lyophilization and kept in vacuum desiccator for 48 hours to weight stabilization. The samples were weighed on an analytical balance and placed in containers containing simulated body fluid (SBF), according to the relationship between the surface area and the volume of solution equal to 0.1cm<sup>-1</sup>.

The SBF was prepared according to ISO/FDIS 23317: 2007 (E), by dissolving NaCl (7.996 g), NHCO<sub>3</sub> (0.350 g), KCl (0.224 g), K<sub>2</sub>HPO<sub>4</sub>.3H<sub>2</sub>O (0.228 g), MgCl<sub>2</sub>.6H<sub>2</sub>O (0.305 g), CaCl<sub>2</sub> (0.228 g) and Na<sub>2</sub>SO<sub>4</sub> (0.071 g), all supplied by Synth Brazil, in 1L of distilled water. The solution was buffered at pH 7.4 adjusting the volume of trihydroxymethylaminomethane and HCl (Merck) at a temperature of 37°C (± 1°C). The containers with the samples were kept in a bath at 37°C (± 1°C). The degradation test was carried out in five periods (0.5, 2, 12, 24 and 168 hours). After that the samples were removed from the containers, rinsed thoroughly with deionized water and dried by lyophilization. Before being weighed samples remained in a

vacuum desiccator for 48 hours. The DI was calculated according to Equation 5 where IDW is the initial dry weight and FSW is the final sample weight.

$$DI = \frac{IDW - FSW \times 100}{IDW} \quad (5)$$

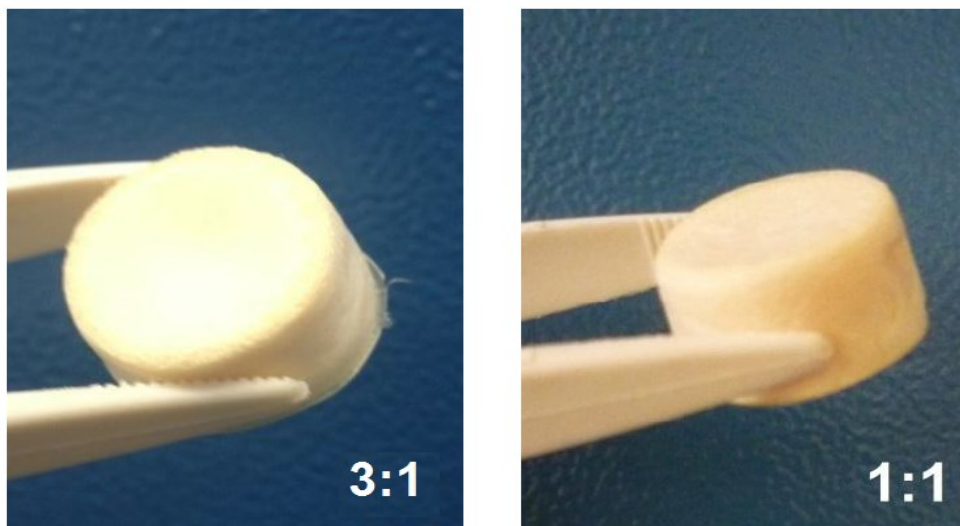
### 2.3 Cytotoxicity Assay

The cytotoxicity was measured by MTT assay [26]. Formazan crystals were solubilized and the optical density was determined by a spectrophotometer at 595 nm. Primary culture of human fibroblasts cells at the fourth passage were plated in 24-well plates at a density of  $1 \times 10^4$  cells/well. The cell populations were normalized with DMEM for 24 hours, after which time the medium was changed and the samples were placed in respective wells. The cylindrical samples were cut into four equal parts and were sterilized by irradiation at 15kGy for 30 minutes, half of the samples were soaked in saline solution (PBS) for 24 hours. DMEM, was used as experiment positive control and PBS 10x as a negative control. All assays were performed in triplicate ( $n = 3$ ). The cells were incubated at 37°C, humidified atmosphere of 5% CO<sub>2</sub> for 72 hours. At the end of this incubation period, the culture medium was removed and discarded and 210 µL/well of DMEM was added. Then 170 µL/well of MTT solution (Invitrogen) (5 mg/ml) was added and the plate was incubated at 37°C, humidified atmosphere of 5% CO<sub>2</sub> for 2 hours. The cells were observed under an optical microscope (MO) for displaying the formazan crystals that were solubilized by the addition of 210 µL/well of a solution of SDS 10%-HCl (0.01M hydrochloric acid - 10% of sulphate sodium dodecyl water) followed by incubation at 37°C, humidified atmosphere of 5% CO<sub>2</sub> for 18 hours. 100 µL was transferred from each well to a 96 well plate, in triplicate, and the optical density was measured in a spectrophotometer at 595 nm. All the steps were performed in minimal lighting conditions. The results were analyzed by One-way ANOVA followed by Bonferroni test and expressed as mean ± SEM (standard error mean).

## 3. RESULTS AND DISCUSSION

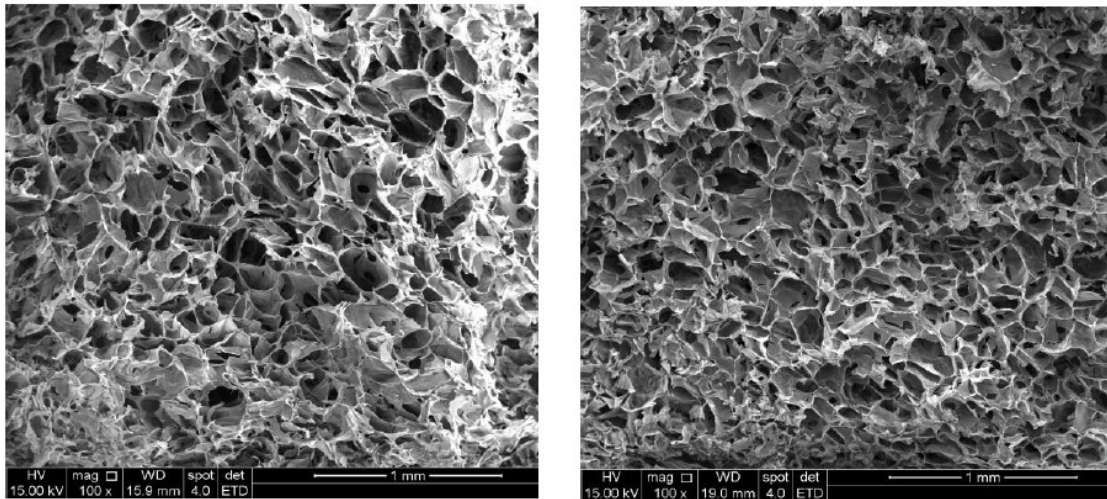
### 3.1 Morphology and pore structure

The Chitosan/PVA/BG scaffolds showed final dimensions of 18x10mm, spongy consistency and homogeneous physical structure (Figure 1). The scaffolds pore morphology was analyzed by SEM and is shown in Figure 2, where it can be noted a network of well-defined, open and interconnected pores with thin walls. Overall, the pores located on the periphery of the scaffold showed a slight flattening on its surface, possibly caused by sample handling during the fracture to obtain the specimen, although fracture was conducted immersed in liquid nitrogen.

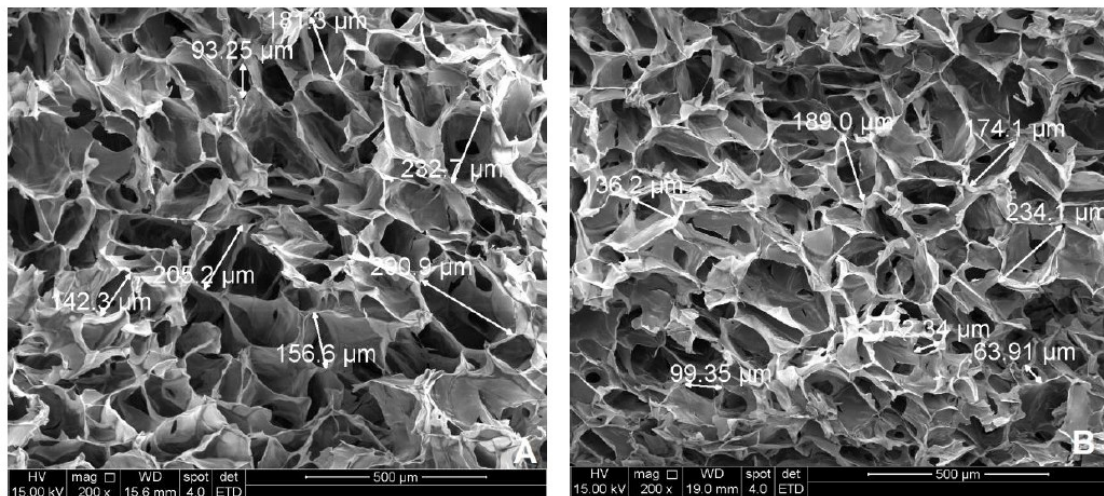


**Figure 1:** Structure of 3:1 and 1:1 scaffold successfully obtained with homogeneous and regular physical structure without cracks.

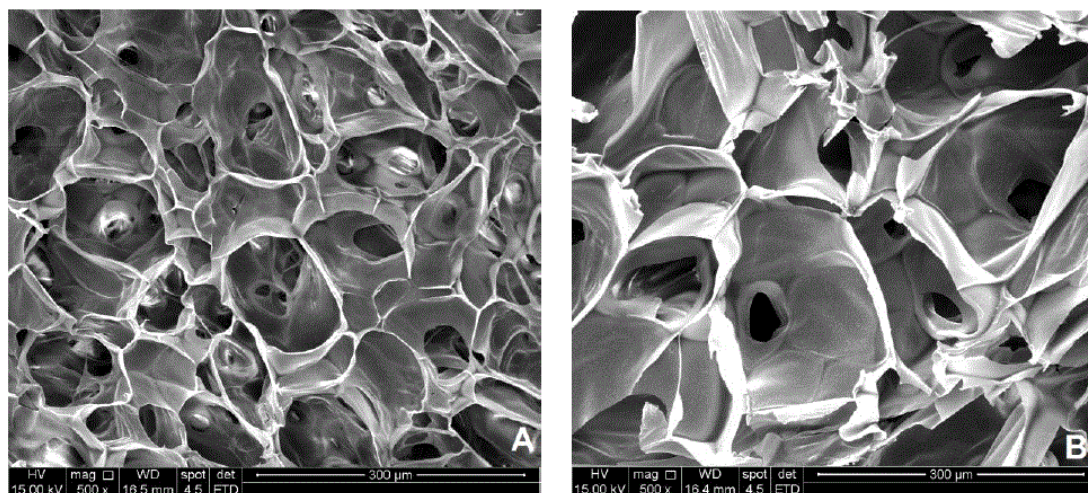
It was observed homogeneous and organized porosity similar for the 1:1 and 3:1 scaffolds, although the first presented more uniform pores (Figure 3). The pore size in the regions analyzed ranged from 64 to 234  $\mu\text{m}$  in 1:1 scaffold, and 93 to 360  $\mu\text{m}$  in 3:1 scaffold. Both scaffolds presented satisfactory pore size range, and opened, interconnected pores (Figure 4), essential for tissue ingrowth according to the literature [27]. The presence of pores of different sizes is very important since bone tissue grows through interconnected pores in the range from 100 to 200  $\mu\text{m}$ , while cell adhesion and vascular formation occurs in less than 100  $\mu\text{m}$  pores.



**Figure 2:** Chitosan/PVA/BG scaffolds SEM images (A) 3:1 and (B) 1:1 ratios.



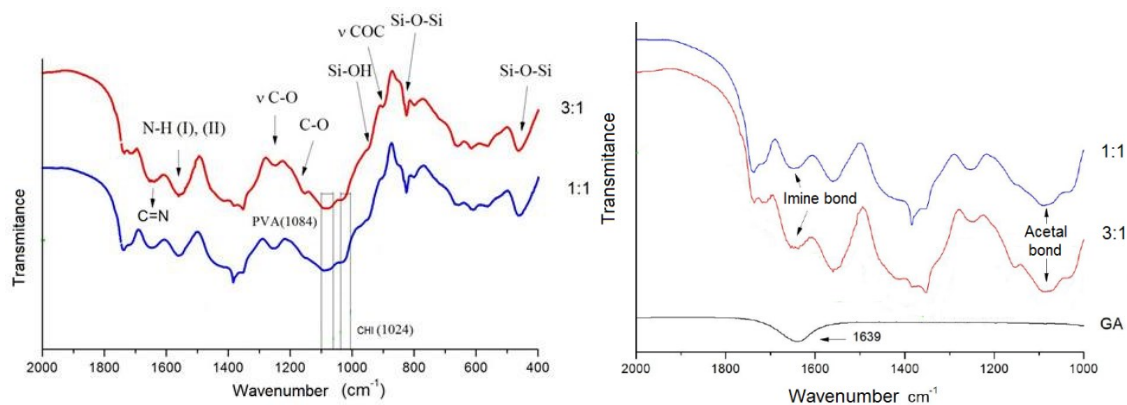
**Figure 3:** SEM images showing interconnected network pores with thin walls and uniform cavities. Chitosan/PVA /BG scaffolds (A) 3:1 and (B) 1:1 ratios.



**Figure 4:** SEM images showing the pores architecture and interconnectivity of the 3:1 (A) and 1:1 (B) scaffolds.

### 3.2 Fourier Transform Infrared Spectroscopy

Figure 5 presents the FTIR spectra of the scaffolds normalized at the  $1450\text{cm}^{-1}$  band. The typical bands corresponding to both polymers in the blend are identified. It was observed by FTIR spectra characteristic peaks of amino group binding of Chitosan and broad and superimposed peaks associated with stretching vibration of Si-O-Si group on PVA ( $1084\text{ cm}^{-1}$ ) and Chitosan ( $1029\text{ cm}^{-1}$ ) chemical groups. The band at  $966\text{ cm}^{-1}$  corresponds to Si-OH stretching. The shape of  $1648\text{ cm}^{-1}$  band, associated with the imine bond ( $\text{C}=\text{N}$ ), and  $1558\text{ cm}^{-1}$  band, associated with the amino group ( $\text{NH}_2$ ) are similar to that found by Costa-Jr. et al [21], and Monteiro Jr. [28]. The band at  $1720\text{ cm}^{-1}$ , related to free aldehyde groups ( $-\text{COH}$ ) appears in the spectra of 3:1 scaffold. However, the spectrum on the 1:1 scaffold, this band does not appear, indicating a possible crosslinking with the amino group, forming the  $\text{C}=\text{O}$  connection [22,29]. Dias et al. [30], identified in the FTIR spectrum of his work, the characteristic bands of PVA ( $1084\text{ cm}^{-1}$ ) and Chitosan ( $1024\text{ cm}^{-1}$ ) intensified by the presence of BG and the band  $1644\text{ cm}^{-1}$ , associated with the links of the  $\text{C}=\text{N}$  imine (Schiff base) formed by the amino group of Chitosan with aldehyde group of glutaraldehyde. The presence of glutaraldehyde causes an increase in the intensity of band  $1562\text{ cm}^{-1}$  associated with ethylenic bonds; and  $2922\text{ cm}^{-1}$  frequency related to the stretching of CH groups. This fact can be attributed to increased contributions of glutaraldehyde molecule in quitosana-glutaraldehyde reaction that promotes the increase in crosslinking of the chain. The increasing intensity of the bands relating to the imine bond also suggests that GA crosslinking preferably occurs via Schiff base at carbon 2 glycosidic ring over the link with the hydroxyl groups on carbons 3 and 6 [22]. Ma et al. [31] reported that the potent cytotoxicity of glutaraldehyde can be reduced with the presence of Chitosan due to the large number of amino groups in its molecular chain, serving as a bridge, increasing the efficiency of crosslinking by glutaraldehyde. The band  $1100\text{ cm}^{-1}$  is related to the glutaraldehyde crosslinking of the PVA, forming bridges acetals. The  $1650\text{ cm}^{-1}$  and  $1638\text{ cm}^{-1}$  bands are associated with the formation of the imine from the amine group of Chitosan during crosslinking by glutaraldehyde [32].



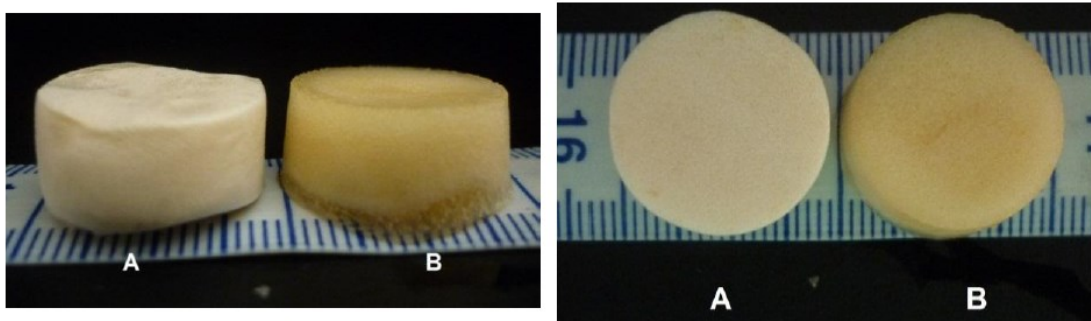
**Figure 5:** Normalized FTIR spectra of 1:1 and 3:1 scaffolds and glutaraldehyde (GA).

### 3.3 Porosity evaluation

The results of porosity and density are presented in Table 2. The matrices obtained showed high porosity as expected, both samples showing 97% total porosity. During the execution of the method, the matrix began to swell slightly as shown in Figure 6. To decrease the swelling effect on the measurements, the time of immersion in water was fixed as 20 minutes.

**Table 2:** Results obtained for porosity and density of scaffolds.

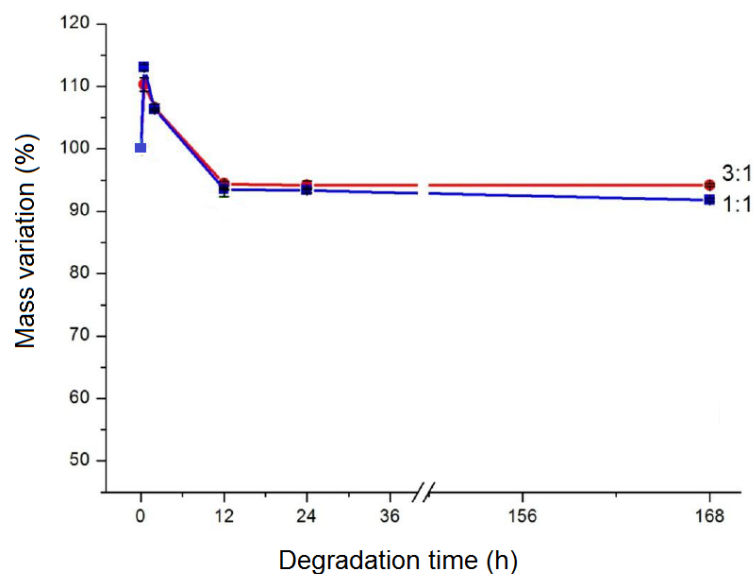
Scaffold	$P_{\text{apparent}}$ (%)	$P_{\text{total}}$ (%)	$\rho_{\text{vol}}$ (g/cm <sup>3</sup> )	$\rho_{\text{tr}}$ (g/cm <sup>3</sup> )
3:1	50 ±5	97.5±0.3	0.04 ±0.02	1.6
1:1	56 ±3	97.2 ±0.5	0.04 ±0.01	1.5



**Figure 6:** Representation of the swelling presented by 3:1 and 1:1 scaffolds before immersion in deionized water (A) and after 20 minutes immersion in deionized water (B) for the test of apparent porosity.

### 3.4 Degradation test

The hybrid matrices designed for tissue regeneration are expected to degrade at the same proportion that the regeneration of damaged tissue occurs. The most common signs that degradation is occurring are the mass loss and deterioration of the mechanical properties of the material [33]. Figure 7 shows the mass loss that occurred with time during the degradation test. Both scaffolds gained mass in the first 0.5 hour (3:1 gained 10.4% and 1:1 gained 14.0%), possibly due to the formation of a carbonated hydroxyapatite layer on the surface of the scaffold.



**Figure 7:** Mass loss upon immersion in SBF (simulated body fluid).

The ratio of weight loss was very similar for the matrices 3:1 and 1:1. The mass loss presented by the matrices during the degradation test *in vitro* was around 10% after a week of testing, with macroscopic preservation of their physical structure. The mass loss was more intense for both scaffolds up to 12 hours. After this period, degradation of the matrices was quite slow. The degradation occurs probably by the solvation of ionic groups and by depolymerization of the polymer chains [23]. Degradation of the 3:1 scaffold was slightly lower than that of the 1:1; which can be explained by the preference of glutaraldehyde cross-linking of the amino group of Chitosan with the formation of the group C=N, rather than forming acetal bridge with PVA [23].

### 3.5 Mechanical test

The stress strain compression curves obtained from the compression test of 3:1 and 1:1 scaffolds are presented in Figure 8. A strain limit (around 63%) was established to avoid damage of the equipment. The average elastic modulus resulted in values of 2.2 and 0.9 MPa for 3:1 and 1:1 scaffolds respectively. Maximum stress supported at the strain limit established was 0.18 and 0.26 MPa, for 3:1 and 1:1 scaffolds respectively. The 3:1 scaffold showed higher modulus of elasticity and lower maximum stress in relation to 1:1, that is, the higher PVA content in the sample 1:1 compared to sample 3:1 affected differently these two properties. It should be pointed out that the toughness, as measured by the area under the stress-strain curve, is markedly higher for sample 1:1.

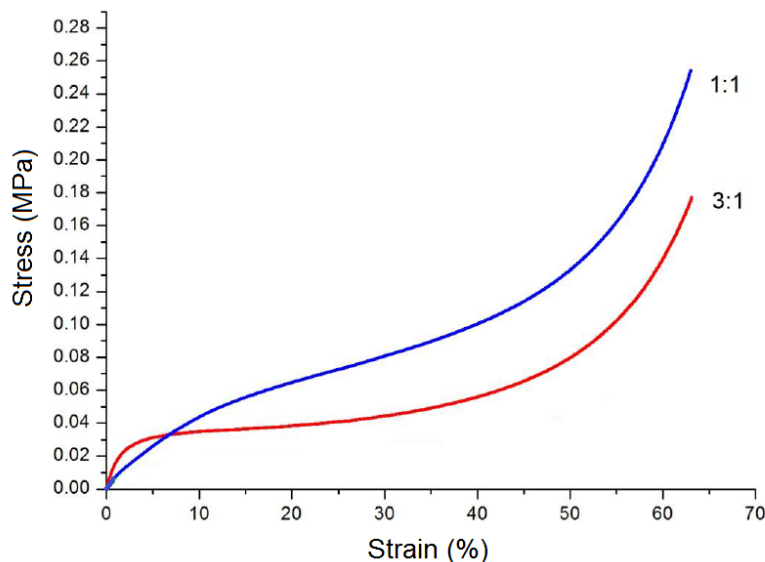


Figure 9: Stress strain compression curves for dried samples.

### 3.6 Cytotoxicity

The cytotoxicity assay aims to detect the potential of a material or device to produce lethal or sub lethal effects in biological systems at the cell level. This assay should be applied to all categories of biomaterials. The release of toxic sub products of the biomaterial can damage the cells or reduce the rate of cell culture growth. Compared to the positive control (human fibroblasts in culture medium) the cells cultured directly in presence of 1:1 and 3:1 scaffolds showed about 20% and 15% of viability respectively. This low viability is probably due to residual products inside the scaffolds pores. A biomaterial can be considered toxic to be used in biological systems under 50% of cell viability. However, when both scaffolds were soaked in PBS for 24 hours, the cells presented more than 90% of viability. The assay showed that both samples were toxic as produced and not toxic after treatment with PBS, showing this approach was suitable for neutralization of these samples.

## 4. CONCLUSIONS

The scaffolds obtained through the lyophilization route showed adequate pore structure, presenting about 97% porosity, pore size varying in the range 20-300  $\mu\text{m}$ , and interconnected pores. The mass loss during the degradation test *in vitro*, was around 10% after a week of testing, with preservation of their macroscopic physical structure. The scaffolds obtained showed good deformation capacity but low compression strength. Cytotoxicity tests showed that the samples presented low cell viability before immersion in PBS. After im-



mersion in PBS cell viability increased and was similar to control for both samples 1:1 and 3:1. Therefore, the 1:1 and 3:1 hybrids Chi/PVA with bioactive glass 20% (w/w) cross-linked with glutaraldehyde and dried by lyophilization showed characteristics suitable for application in tissue engineering.

## 5. ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of CNPq, Capes and Fapemig.

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