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Synthesis, neutralization and blocking procedures of organic/inorganic hybrid scaffolds for bone tissue engineering applications

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Abstract Bioactive glasses (BaG) can bind to human bone tissues and have been used in many biomedical applications for the last 30 years. However they usually are weak and brittle. On the other hand, composites that combine polymers and BaG are of particular interest, since they often show an excellent balance between stiffness and toughness. Bioactive glass-poly(vinyl alcohol) foams to be used in tissue engineering applications were previously developed by our group, using the sol–gel route. Since bioactive glass-polymer composite derived from the sol–gel process cannot be submitted to thermal treatments at high temperatures (above 400°C), they usually have unreacted species that can cause cytotoxicity. This work reports a technique for stabilizing the sol–gel derived bioactive glass/poly(vinyl alcohol) hybrids by using glutaraldehyde (GA), NH_4OH solutions and a blocking solution containing bovine serum albumin. PVA/BaG/GA hybrids were characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM/EDX) analyses. Moreover, MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) biocompatibility and cytotoxicity assays were also conducted. The hybrids exhibited pore size varying from 80 to 820 μm. After treatments, no major changes in the pore structure were observed and high levels of cell viability were obtained.

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1 Introduction

Tissue engineering combines the principles of engineering and biology to design and fabricate constructs to enable the repair and regeneration of damaged tissue [1]. One of the scientific and technological challenges in the tissue engineering is associated with the development of suitable scaffold materials, usually porous, that can act as templates for cell adhesion, growth and proliferation [2–4].

Wide varieties of both natural and synthetic materials, and a combination of them, are being investigated in the design of scaffold for tissue engineering [2, 5–7]. Among the several choices of bioresorbable synthetic polymers available, poly(vinyl alcohol) (PVA) have attracted increasing attention for medical applications, since this polymer displays high hydrophilicity, good film forming ability and processability [8, 9]. Furthermore, results presented by Lin et al. [10] indicated that no significant differences were found in quantitative or in qualitative cytotoxicity evaluations of PVA when compared to TCPS (tissue culture polystyrene) [11]. It was also shown that the amount of PVA accumulated in organs was too small to affect biological processes, which suggests that PVA is excreted to the same extent as for example poly(ethylene glycol), supporting the safety of PVA. It has been considered to be biocompatible [12] when used with molecular weight less than around 10,000 g/mol.

PVA has been employed in several biomedical applications including artificial cartilage [4, 13], corneal

implants [14] and substitutes or tissue engineering scaffolds for skin [15].

Considering the inorganic materials, some silicate compounds have been extensively studied and used to repair and reconstruct damaged bone tissues. The so-called bioactive glasses (BaG) [4] are partially soluble and can promote apatite deposition when exposed to the body environment that ensures a stable attachment of the implant [16]. However, bioactive glasses have low mechanical properties, particularly in a porous form, compared to cortical and cancellous bone [13].

One approach to enhance the mechanical properties of materials is the production of inorganic–organic hybrids because they often show an excellent balance between stiffness and toughness and usually improved characteristics compared to their individual components [17].

Hybrid organic–inorganic materials can be obtained by the sol–gel method [18]. When PVA is used as the polymer component of the hybrid, during the sol–gel reaction, its water solubility and polar nature facilitate the formation of hydrogen bonds and eventual covalent bonds derived from condensation reactions between silanol groups formed by hydrolysis of the silicon alkoxides [14]. Since bioactive glass-polymer composite derived from the sol–gel process cannot be submitted to thermal treatments at high temperatures (above 400°C—since they would lead to polymer thermal degradation), they usually have unreacted species that can cause cytotoxicity [19].

In this work, unreacted and potentially toxic species [20] within sol–gel derived hybrids were deactivated by submitting them to a NH_4OH solution followed by attaching proteins, such albumin. However, since sol–gel derived bioactive glass/poly(vinyl alcohol) hybrids show fast dissolution in aqueous media, a versatile method that involves crosslinking the hybrid with glutaraldehyde was used in this work in order to enhance the chemical stability. Crosslinks in hybrids can reduce degradation enough to maintain the structure during and after the treatments with NH_4OH solution and improve the cell viabilty level. The PVA/BaG/GA samples were characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM/EDX) analyses. Moreover, MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) biocompatibility and cytotoxicity assays were also conducted.

2 Materials and methods

2.1 Hybrids preparation

The Fig. 1 illustrates the experimental procedure and describes all steps conducted to prepare PVA/BaG/GA hybrids.

2.1.1 Preparation of PVA solution

The poly(vinyl alcohol) (PVA) selected for use was from Aldrich-Sigma, degree of hydrolysis 80%, molar mass: 9,000–10,000 g/mol. PVA aqueous solution was prepared with concentration 28 wt.% by dissolving the PVA powder in water at 80°C, under constant stirring, for 2 h. The pH of the solution was adjusted to 2.0 by hydrochloride acid (HF) solution 2 N.

2.1.2 Preparation of the starting bioactive glass solution

The starting solution with designed composition 58 wt.% SiO_2–33 wt.% CaO–9 wt.% P_2O_5 was synthesized by mixing tetraethoxysilane (TEOS), D.I. water, triethylphosphate (TEP), and calcium chloride in presence of hydrochloride acid solution 2 N. The H_2O/TEOS molar ratio used was 12.

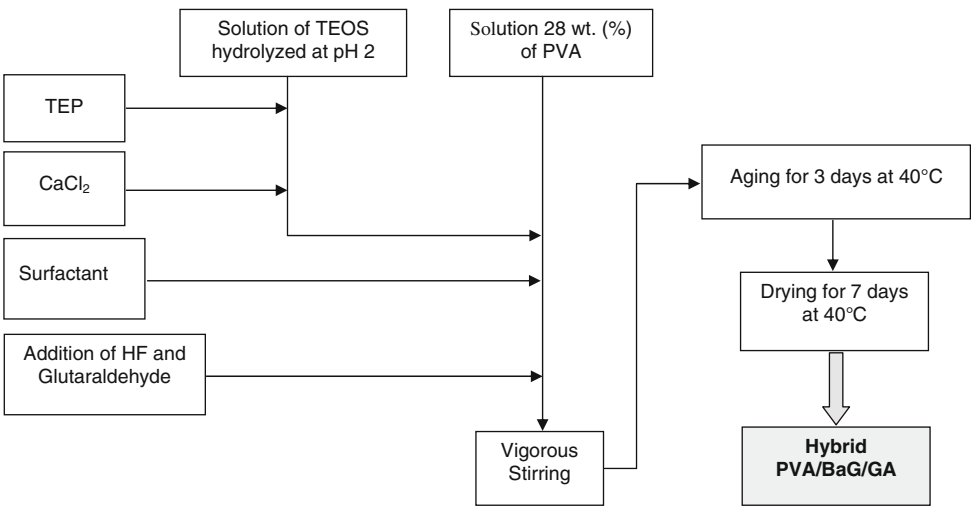

Fig. 1 Flow chart of the procedure to obtain PVA/BaG/GA

2.1.3 Preparation of the PVA-bioactive glass hybrid foams

The hybrid composition was 30 wt.% glass and 70 wt.% polymer. The hybrids were obtained using a procedure similar to the one described in our previous work [20, 21]. An appropriate amount of the starting glass solution was added to the PVA solution and stirred for 1 min. To this resulting solution were added the surfactant, sodium laureate sulfate (SLS, Oxiteno 27%v/v) as a foaming agent, HF 10%v/v solution as a gelling agent and the glutaraldehyde 25 wt.%/v as crosslinking agent. The mixture was foamed by vigorous agitation. The foams were cast just before gelation in plastic containers and sealed. The samples were, aged at 40°C for 3 days and then dried at 40°C for 7 days. The crosslink agent was introduced to improve chemical stability since the PVA/BaG hybrids display high hydrophilicity and degrade in aqueous media. The reaction between PVA and glutaraldehyde involves the hydroxyl groups of PVA and the aldehyde to form an acetal bridge, as show in Fig. 2. The reaction is simple to conduct and the resultant product is stable [22].

2.1.4 Stabilization and blocking procedure of the remaining cytotoxicity groups

A stabilizing step was introduced to reduce the acidity of the hybrids that can lead to less cytotoxic materials. Dried samples were immersed, for periods of time that varied from 1 to 10 h at room temperature, in NH_4OH solutions which concentrations were varied from 0.075 to 0.750 M. The amount of stabilizing solution used was 100 ml for each gram of dried hybrids. Samples of hybrids after the stabilizing process were immersed in a bovine serum albumin (BSA) solution for 16 h at room temperature to promote the adsorption of protein on the hybrid surface. The adsorbed BSA can block the cytotoxic groups derived from the sol–gel process and prevent the adsorption of proteins dispersed in the culture medium, that could affect the cellular metabolism and influence the cellular viability results. The block solution was prepared by dissolving 1.0 g of BSA powder in 1 l of phosphate buffered saline (PBS) solution, under magnetic stirring, at temperature of 25°C ± 2°C.

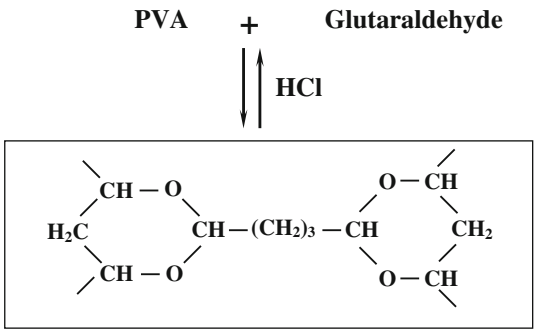

Fig. 2 The crosslinking reaction between PVA and glutahaldehyde showing acetal ring group

2.2 Characterization of PVA/bioactive glass hybrids

2.2.1 Scanning electron microscopy and energy dispersive spectroscopy (SEM/EDS)

SEM images were taken from organic–inorganic hybrids with a JSM 6360LV (JEOL/NORAN) microscope. SEM photomicrographs were used for the evaluation of hybrid foam microstructure. Chemical composition was analyzed by Energy Dispersive X-ray analysis (EDS) with the apparatus Quest Spectrometer coupled to SEM. Prior to SEM examination, samples were coated with a thin gold film by sputtering. Images of secondary electrons (SE) were obtained using an accelerating voltage of 10–15 kV.

2.2.2 Chemical characterization by FTIR spectroscopy

Fourier transform infrared spectroscopy (FTIR) was used to characterize the presence of specific chemical groups in the PVA hybrid networks. FTIR spectra were obtained within the range between 4,000 and 600 cm^{-1} (Perkin–Elmer, Paragon 1000), using the attenuated total reflectance spectroscopy method (ATR–FTIR).

2.3 Cytotoxicity and cellular viability/activity by MTT assay

2.3.1 Samples preparation for cytotoxity assays

Composite scaffolds with 5 mm in diameter × 2 mm in thickness for in vitro cell study were sterilized by exposure to saturated steam of ethylene oxide. The cell viability assay was performed in 4 replicates for each stabilizing process. Samples were placed in 96-well plates, soaked in 250 μl/well minimum essential medium eagle (MEM) and were maintained in incubator at 37°C with 5% CO_2 for 24 h. Then the resultant solutions were removed and the pH was measured.

2.3.2 Culture of cell-scaffold

The VERO cells (cell culture isolated from kidney epithelial cells extracted from African green monkey) were seeded at concentration of 5×10^5 cells/ml in 96 well polystyrene plate and also to the microplatewell used as the reference (control). A MEM solution, together with 2 mM L-glutamine and 10% fetal bovine serum (FBS), was then

added in the wells. The system was then incubated at 37°C with 5% CO_2 for 24 h.

2.3.3 Cellular viability/activity by MTT assay

After 24 h post-immobilization, the supenatant of wells was removed and replaced with fresh MEM supplemented with L-glutamine and 10% FBS. Following, 30 µl of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 tetrazolium) (MTT)was added to each well. The plate was then re-incubated for 4 h with 5% CO_2 at 37°C and protected from light. After 2 h of post-immobilization, qualitative images were acquired using Olympus IX70 Microscope and, after 4 h, 35 µl/well of sodium dodecyl sulfate (SDS) with 10% HCl was added. Subsequently, the solution was carefully homogenized and re-incubated for 14–16 h with 5% CO_2 at 37°C and protected from light. After that, 100 µl/well was transferred to a 96-well plate and absorbance was measured at 595 nm. The background (control with no cells) was subtracted from all samples.

2.3.4 Statistical analysis

All data were presented as average ± standard deviation (SD). To test the significance of the observed differences between the study groups, a statistical evaluation was carried out using a one-way ANOVA. A value of $P < 0.05$ was considered to be statistically significant.

2.3.5 Cell morphology

Cell spreading was assessed by electron microscopy examination of the specimens after culturing for 24 h. For electron microscopy, specimens were fixed with 2% glutaraldehyde for 16 h and dehydrated by passing through a series of alcohols (ethanol) before they were dried in nitrogen flowing reactor for 4 h and outgassed in vacuum desiccator for 12 h. Samples were coated with a thin layer of sputtered Au to examine the cell morphology using a SEM.

3 Results and discussion

Figure 3a and b show MEV micrographs of hybrids PVA/BaG/GA, before and after the blocking treatment with NH_4OH for 1 h. Results indicate a reduction in the pore size from 120–340 µm to 90–250 µm.

Differences were also observed in a high level of magnification shown in Fig. 3c and d, associated with the surface of samples before and after stabilizing process respectively. The pore surface that appeared non-uniform before treatment, showed a more regular structure with some spherical elements after the process, suggesting that coarsening and Ostwald ripening may have occurred during the contact of the material with the NH_4OH solution. In Ostwald ripening, many small crystals initially form in a system but slowly disappear except for a few that grow larger, at the expense of the small crystals [23].

The EDS spectra presented in the Fig. 3e and f, show similar features before and after the stabilization process, but with significant reduction of the chlorine peak, indicating the effectiveness of the NH_4OH solution in reacting with Cl^- to form NH_4Cl salt. In addition, the EDS results showed a decrease in the calcium and phosphate peaks for the stabilized samples. The reduction of the chlorine peak is responsible for increasing the proportion of the other elements in the semi-quantitative analysis shown in Table 1. Moreover, the increase of the silicon concentration led to a slight reduction of the ratio of calcium and phosphorous peaks, when compared with the silicon peak. The significant aspect is that, even with all changes promoted by using of stabilizing solution, the final concentration of elements was similar to that usually associated with glasses that display bioactivity.

Figure 4 shows the FTIR spectrum of the PVA/BaG/GA sample crosslinked with 5 wt.% of glutaraldehyde followed by the stabilizing treatment with NH_4OH that was compared to the spectrum of the sample that was not submitted to stabilizing process. From these results, a broad absorption band from 3,550–3,200 cm^{-1} and a typical adsorption band at 1,640 cm^{-1} that may be assigned to O–H group of adsorbed water can be observed. In the region (I) it was noted, for samples treated with NH_4OH aqueous solution, a considerable decrease in the intensity of the OH band, which suggests that part of PVA in hybrids has been removed of the surface, probably due to the high pH of the solution or due to further polycondensation of silanol groups.

Other typical peaks presented and which can be associated with PVA are located at 2,937 and 2,870 cm^{-1} referent to νCH, the peak at 831 cm^{-1} of ν(C–C); 1,461–1,417 cm^{-1} assigned to $\delta(CH)CH_2$; 1,093 cm^{-1} of group ν(C–O)–C–OH and 1,333 cm^{-1} due to δ(OH)–C–OH [14].

IR absorption bands at 980 cm^{-1}, 1,029 cm^{-1} and 1,080 cm^{-1} related to Si–O–Si [20, 24] are more evident in the spectra of the samples subjected to the stabilizing process.

Comparing the spectra before and after stabilizing procedure, it is possible to note the behavior of bands located at 1,461–1,417 cm^{-1} and 1,333 cm^{-1} in the spectrum "a" shifted toward higher wavenumber and showing a slight increase on the intensity in the "b"and "c" spectra.

The biological tests, assessed by the MTT assay, performed in samples blocked and stabilized showed that, for

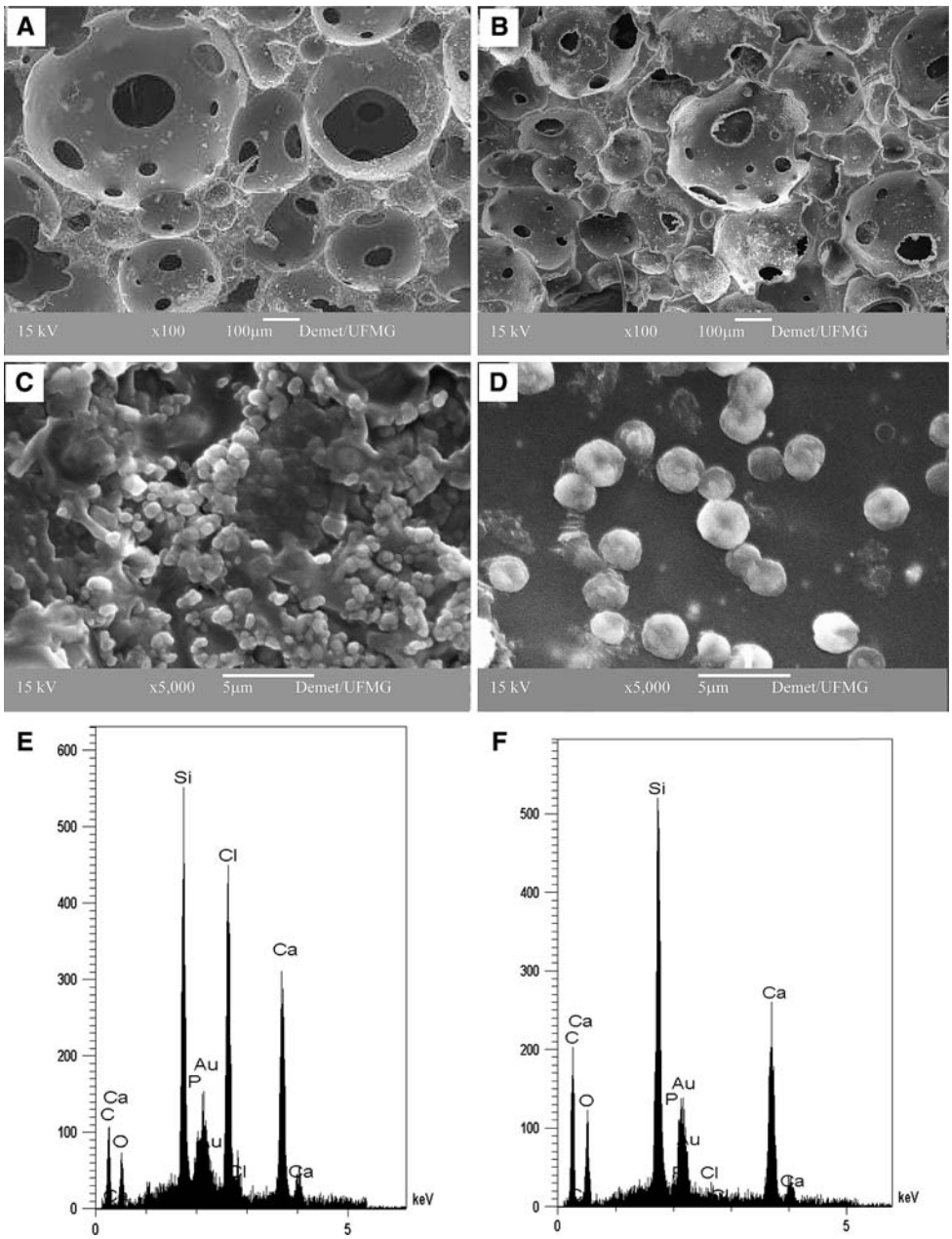

Fig. 3 SEM photomicrographs of hybrids PVA/BaG/GA (**a**) before and (**b**) after stabilization with NH_4OH solution of 0.750 M for 1 h, (100×). (**c**) and (**d**) show the surface of pore walls magnifications (5,000×). (**e**) and (**f**) show EDS analyses of the region indicated in "c" and "d", respectively

Table 1 Ratio of the P, Cl and Ca elements as function of silicon (in atomic%)

	Ratio (at.%)		
	Non-stabilized	Stabilized	Reduction (%)
Cl/Si	1.17	0.04	97
P/Si	0.30	0.27	9
Ca/Si	1.10	0.73	34

all periods of time used in the stabilization step, more favorable results were observed for samples treated with NH_4OH in concentration of 0.300 M, as indicated in Fig. 5. These results may be related to the effectiveness of the solution to remove part of PVA from the hybrid surface, as suggested by the SEM images of Fig. 3d, which may expose BaG sites, that present more bioactive features.

In Fig. 5 it can be realized that the most viable cultures were observed in samples stabilized for 10 h in both concentrations of 0.300 or 0.075, which values were about 50% of those of the cell control. These results may indicate that the period of 10 h of treatment promotes a more well succeeded environment for cell growth, in which chlorine ions were removed as indicated in EDS analyses in Fig. 3 and Table 1. In addition, since the samples were obtained through the sol–gel process by using solutions with pH values about 2, it is expected that an increase in pH (that was obtained by chemically treating the hybrids with

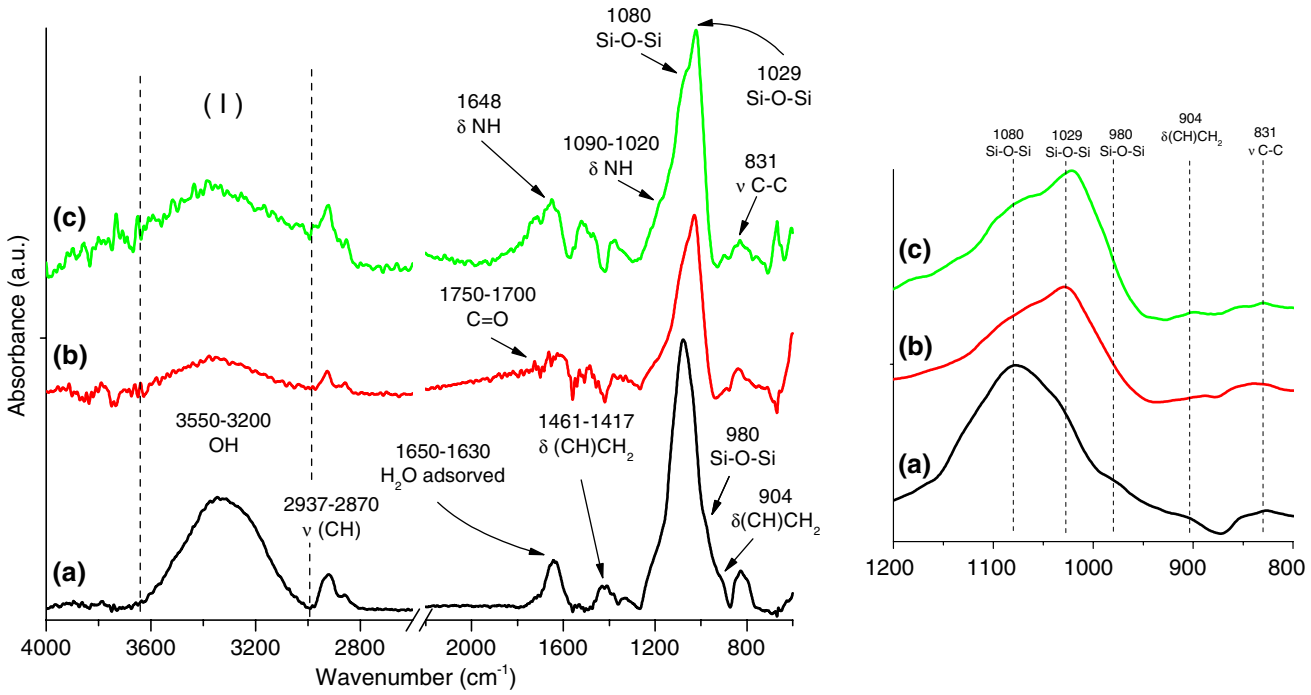

Fig. 4 FTIR spectra of samples PVA/BaG/GA, crosslinked with 5 wt.% of glutaraldehyde: (a) non-treated samples, (b) samples treated for 10 h with NH_4OH solution of 0.075 M and (c) 0.300 M. Region of spectra between 1,200 and 800 cm^{-1} shown on the right

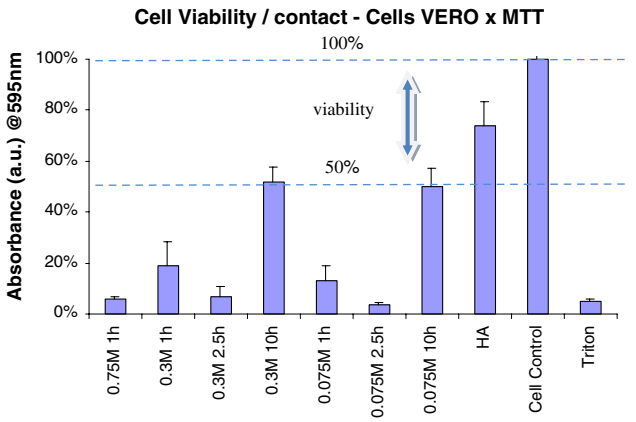

Fig. 5 Relative cell viability of VERO cells cultured, in direct contact, with hybrids of PVA/BaG/GA treated in NH_4OH solution at concentrations from 0.075 M to 0.750 M, for periods of time from 1 to 10 h, followed by blocking procedure with BSA solution

NH_4OH for longer periods of time) would be necessary to allow the stabilization of an environment much more comparable to the natural one familiar to cells."

On the other hand, the stabilizing procedures as well as in vitro tests require chemical, mechanical and dimensional stabilities to be successfully performed, since the hybrids present high degradation rates in aqueous media. Therefore, a crosslink agent was also incorporated during the synthesis to promote higher levels of stability for the hybrids.

Previous works [20, 21, 24] had been conducted to evaluate cell viability using extracts of hybrids foams without stabilization and blocking treatments, namely the indirect contact method. These results showed cell viability similar as the control. However for in vitro tests employed with samples immersed into the culture medium, the so called direct contact method, hybrids with comparable compositions that were used in this work, showed high levels of cytotoxicity.

The results presented in this work demonstrated a significant level of viability obtained for PVA/BaG/GA samples stabilized and blocked, since pronounced cell growth in the direct contact cell culture assay was observed.

The cell viability results obtained using the MTT assay was also confirmed by the SEM micrographs in Fig. 6. Figure 6a shows cells attached to the PVA/BaG/GA hybrid surface stabilized for 10 h and blocked, while in Fig. 6b, an image of dead cells cultured close to the same hybrids but stabilized for only 1 h is shown. In addition, Fig. 6a shows the adhesion and spreading of cells throughout the surface by protruding lamellipodia, that indicate that viable cells are well adapted to the substrate.

4 Conclusions

A macroporous scaffold composed of PVA/BaG/GA stabilized with NH_4OH solution and blocked with bovine serum albumin showed much lower levels of toxicity than the original sol–gel derived hybrids. Moreover, no major changes on the morphological structure of the scaffolds

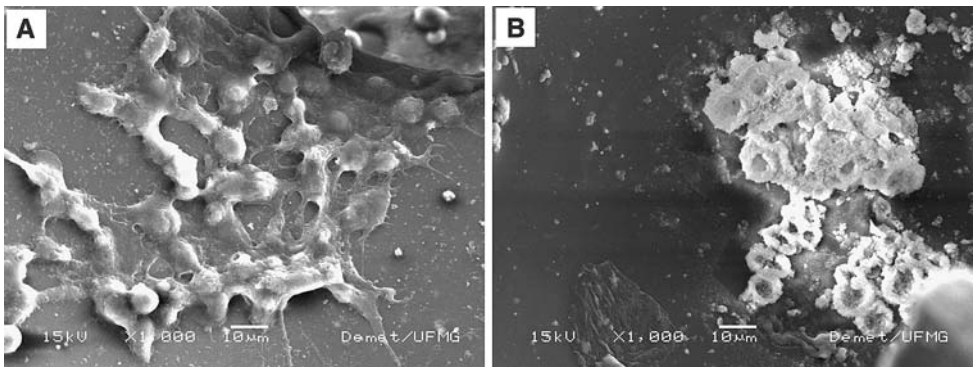

Fig. 6 SEM micrograph of cell spreading and attachment on PVA/BaG/GA hybrids treated for (**a**) 10 h and (**b**) 1 h in NH_4OH solution at concentrations of 0.300 M

were observed after the chemical treatments that are important for cell adhesion and growth. The MTT assay confirmed the advantage of the treatment with stabilizing and blocking solutions for the period of time about 10 h.

The designed and tested treatments are an interesting strategy to control citotoxicity associated with the sol–gel process, specially in biomedical hybrids, where the minimization of harmful species is critical.

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