BIOCOMPATIBILITY STUDY ON SUBSTRATES FABRICATED FOR NERVE GUIDES USING SCANNING ELECTRON MICROSCOPY AND COMPARING TWO DRYING SAMPLE METHODS

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ABSTRACT
Currently, peripheral nerve regeneration can be achieved by applying nerve guides after a lesion. These guides should be fabricated from permeable, biodegradable and biocompatible materials that stimulate, orient and preserve the integrity of the axonal fibers. In this investigation, the objective was to evaluate the interaction of Vero cells with the surface of the substrates employed to construct a nerve guide, designed for enhancing nerve regeneration. The analyzed cell seeded material consisted of collagen matrices crosslinked with genipin, porous poly (ε-caprolactone) (PCL) membranes and polyblends of different range of composition of poly (butylene succinate) and PCL. Using scanning electron microscopy (SEM), the growth and morphology of Vero cells cultivated on these substrates were evaluated. For sample preparation, the traditional method of carbon dioxide (CO_2) critical point drying (CPD) was applied, and compared with an alternative method, in which liquids from the sample are removed by evaporation of hexamethyldisilazane (HMDS) at room temperature. When evaluating the interaction of cells with these materials, it was observed that the porosity of the biopolymer induces a favorable effect in the adhesion process. Also, it was found that drying samples with HMDS was a simple method, in which the cellular structure was preserved, obtaining comparable results, and in some cases, better than those obtained with CPD.

Keywords: Biopolymers; critical point drying method; hexamethyldisilazane; nerve guides; SEM.

ESTUDIO DE BIOCOMPATIBILIDAD SOBRE SUSTRATOS FABRICADOS PARA GUÍAS NERVIOSAS EMPLEANDO MICROSCOPIA ELECTRONICA DE BARRIDO Y COMPARANDO DOS METODOS DE SECADO

RESUMEN
Actualmente se puede lograr la regeneración en nervios lesionados utilizando guías nerviosas. Estas guías deben ser fabricadas con matrices permeables, biodegradables y biocompatibles que estimulen, orienten y preserven la integridad de las fibras axonales. El objetivo de esta investigación, fue evaluar la interacción celular con la superficie de los sustratos que componen una guía nerviosa, diseñada para amplificar la regeneración de nervios seccionados. En este sentido, se analizó mediante microscopia electrónica de barrido (MEB), el crecimiento y la morfología de células Vero cultivadas sobre estos componentes, los cuales consisten en matrices de colágeno entrecruzadas con genipina, láminas de poli (ε-caprolactona) (PCL) porosas y polimezclas de diferentes rangos de composición de poli (butilsuccinato) (PBSucc) y PCL. Para la preparación de las muestras, se comparó el método de secado tradicional por Punto Crítico (MPC) empleando dióxido de carbono (CO_2), con un método alternativo, donde el secado se realiza por evaporación del hexametildisilazano (HMDS) a temperatura ambiente. Al evaluar la interacción de las células sobre los sustratos, se observó que la porosidad en el biopolímero induce un efecto favorable para el proceso de adhesión. Además, se encontró que el método de secado con HMDS es sencillo y capaz de preservar la monocapa celular, obteniendo resultados comparables, y en algunos casos superiores a los conseguidos por el MPC.

Palabras claves: Biopolímeros; guías nerviosas; hexametildisilazano; MEB; método de secado por punto crítico.

INTRODUCTION

Peripheral nerve lesion often interrupts the continuity of a nerve fiber. If the gap generated by the lesion between the sectioned nerve stumps, has a critical distance, the regeneration of the axonal fibers will not take place [1]. For neurotissue engineering to be successful in repairing severed nerves, it is necessary to implement strategies that guide and induce nerve growth, allowing the restoration of connectivity among axons. To achieve this goal, nerve guides, defined as tubular structures implemented to support the growth of axonal fibers, are used as bridging technique [2-4]. For this purpose, the implant material should be biodegradable, biocompatible, permeable to nutrients, and must promote cell adhesion, proliferation and differentiation [5, 6]. Several authors have demonstrated that porous and biodegradable guides, exhibit better regenerative results compared with those elaborated from nonreabsorbable inert polymers that are impermeable to diffusion [7-9]. Nerve guides manufactured from polyesters, such as poly (ε-caprolactone) (PCL), offer the advantage of degrading in a physiological environment. Therefore, they do not generate secondary effects on the patient by compressing the nerve once it has regenerated [9, 10]. Also, blending PCL with other polymers like poly-butylene succinate (PBSucc) can modulate the resorption rate of the guide, adjusting the degradation process to the time required for nerve continuity to be restored [11, 12]. Numerous works have revealed that devices, manufactured for tissue engineering from natural materials, such as collagen, combined with porous synthetic scaffolds, exhibit a stimulating surface for cell migration and proliferation [13].

Cell cultures constitute an important tool in order to evaluate *in vitro* cellular response to the biomaterial. This fast and reliable method for screening the toxicology of an implant allows preselecting the best samples for *in vivo* analysis [14, 15]. One of the most frequently used cell lines in cell culture is the Vero cell line. These are recommended for cytotoxicity, adhesion and proliferation evaluations, as well as to assess the interactions between cell and substrate [16]. In relation to this, scanning electron microscopy (SEM) is a widely used technique to qualitatively examine the influence of the the surface characteristics on cell morphology [17, 18]. However, drying the sample is an essential step of sample preparation in conventional SEM analysis. A traditional method used, is carbon dioxide (CO_2) critical point drying (CPD), in which the liquid is drawn from the biomaterial by adjusting the temperature and pressure, so that the gas and liquid phases remain in equilibrium [19-21]. Other drying procedures are based on the evaporation of chemical agents, like hexamethyldisilazane (HMDS) from the sample. This method has been reported to be carried out in a very short time and does not require specialized equipment [20, 22]. In this work, we aimed to evaluate by SEM the morphology and interaction of Vero cells seeded on different substrates that will be used for the construction of a nerve guide. These materials consists of collagen matrices crosslinked with genipin, an external scaffold made from porous and nonporous PCL films and polyblends of different range of composition of PBSucc/PCL, while comparing two drying methods of sample preparation for SEM analysis, traditional CO_2-CPD with HMDS.

MATERIALS AND METHODS

Fabrication of porous and nonporous external scaffolds of nerve guides

Poly (ε-caprolactone) (PCL, PM = 56,000grs/mol, Tone 767, Union Carbide) and poly (butylene succinate) (PBSucc, PM= 23,000grs/mol, Showa Highpolymer Co. Ltd.)/PCL were used to manufacture porous and

nonporous films according to a protocol previously described by Kokai *et al*. [4]. Briefly, membranes were prepared using a solvent casting and particulate leaching technique in which polymers were dissolved in chloroform (3% w/v) at 60 ºC. Immediately, salt crystals of NaCl that were sieved using a mesh 400 were added to the polymer solutions in order to obtain pores with diameters of 38 µm or less in the biomaterial. However, salt crystals precipitated forming clusters that were dissolved and dispersed applying ultrasound sessions to the solutions for 20 min with a sonicator (Sonic Dismembrator 60F, Fisher Scientific Industries). The polymer mixtures were then poured onto glass plates and placed under ventilation at 25 °C to allow solvent evaporation. Finally, the obtained films were cut into units of 2 cm^2. Concentrations of polymer/salt selected to manufacture porous scaffolds were 100/0 (nonporous), 80/20, 70/30 and 50/50 w/w. Polyblends, also fabricated through the same protocol, were made using 20/80 w/w and 10/90 w/w proportions of PBSucc/PCL respectively.

Leaching polymer films

Salt crystals were extracted from polymer films using distilled water to induce porosity in the external scaffolds. To attain this, films were constantly stirred and medium was changed every 24 hrs. Two methodologies were applied to determine when the scaffolds were leached out completely. The first one was a qualitative procedure, in which a dye was added to the salt, so the complete removal of NaCl from the substrate was determined when the aqueous medium became colorless after each change. In the second technique, a quantitative determination was carried out as previously described by Reignier *et. al.* [23]. In this case, voltage (mV) was measured in the wash solution using a voltmeter (pH11meter, Oakton instruments / Eutech Instruments Pte Ltd) and compared to the potential difference of pure distilled water. The procedure was considered complete, when the leaching solution presented a voltage similar to the one obtained in pure distilled water (82 – 86 mV).

Collagen extraction and gel fabrication

Type I collagen was obtained from adult *Sprague - Dawley* rats tail tendon fibers, and processed for gel fabrication using the Francis & MacMillan method [24]. Briefly, 5-6 rat tails were washed with graded ethanol, then fractured in each intervertebral space and flayed. Next, tendons were pulled from the tail, weighted on an analytical balance (HR - 200 A y D Company, d= 0.1mg) and stirred in acetic acid (3% v/v) in a proportion of 1grs of tendon per 200 ml of acid solution for 24 hrs at 8 °C. The resulting solubilized collagen was later filtered and centrifuged 30 min at 4 °C, 3,000 rpm in order to eliminate insoluble residue. For gel preparation, soluble collagen was neutralized with NaOH (1N), which was added by titration to achieve a neutral pH in the mixture, in which a collagen gel is formed. Removal of the salts derived from the gelification process gels were washed with 10 mM Tris-HCl buffer, pH 7.4 and stored at 4 °C.

Preparation of genipin crosslinked gelatin films

Crosslinking gelatin films with genipin (Challenge Bioproducts Co, LTD) was done using a modified protocol previously described by Yao et al. [25] and Chen et al. [26]. Briefly, gels fabricated at a theoretical concentration of 3.65 mg/ml were immersed in a 0.05%, 1% and 2% w/w genipin solution for 60 hrs at 8 °C respectively. Genipin crosslinking solutions were made from a stock suspension of genipin in ethanol which was then diluted with 10 mM Tris-HCl buffer, pH 7.4 to the specific concentrations. Afterwards, samples were repeatedly washed with distilled water, submerged in ethanol, and placed on thin mica films, where they were

irradiated for 3 min with a UV lamp (365 nm, Cole-Parmer Instruments Co.) in sterile conditions.

Cell culture on substrates fabricated for the construction of nerve guides

Evaluation of cell biocompatibility on the substrates analyzed in this study was done seeding 1.24 x 10^4 Vero cells/cm^2 (Instituto de Higiene Rafael Rangel, Caracas) into collagen matrices and 1.00 x10^4 Vero cells/cm^2 on to PCL and PBSucc/PCL scaffolds. These cultures were maintained in DMEM supplemented with 8% v/v fetal bovine serum (FBS) for 72 hrs at 37 °C, 5% CO_2. Cells seeded on mica films and culture plate (polypropylene) where used as positive control.

Scanning Electron Microscopy

For SEM analysis, samples were fixed with 2.5% w/v glutaraldehyde in phosphate buffered saline (PBS) for 1 hr, then postfixed with 1% w/v OsO_4 in PBS for 1 hr and rinsed in distilled water. Next, for dehydration the material was taken through a graded series of ethanol 20%, 50%, 70%, 80% and 100% v/v. In order to compare both HMDS and CPD methods, samples were separated into two groups. In one group, CPD with CO_2 was applied using a HCP – 2 Critical Point Dryer (Hitachi), in the second group the samples were dried with the alternative method in which the materials were submerged in HMDS (Sigma) for 2 min, and left at room temperature for about 3 min. Then, the samples were covered with gold/palladium using a sputter coater (EMS 950 Turbo evaporator, Electron Microscopy Science) and observed with a field emission scanning electron microscope (S-4500, Hitachi), operated at 5 kV.

Determination of pore diameters in the porous external scaffolds of nerve guides

Using the scale bar in SEM micrographs the average diameters of the pores were estimated in the external surface of each scaffold prepared with different proportions of polymer/salt ratio.

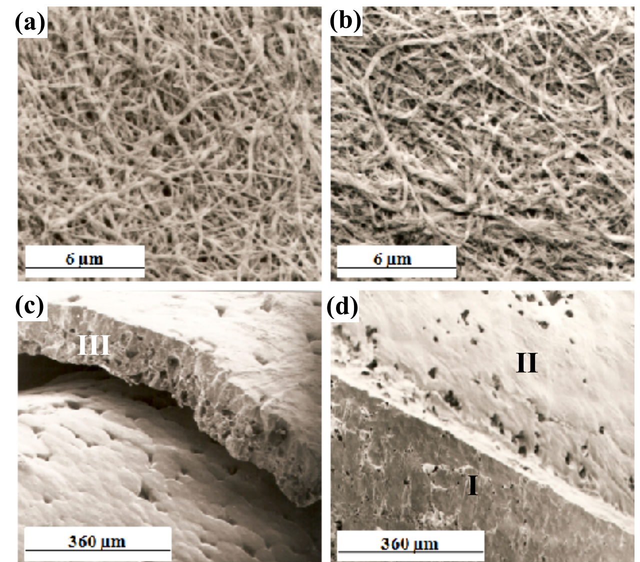

Fig. 1. SEM micrographs of collagen matrix crosslinked with 1% w/w of genipin submitted to CPD (a) collagen matrix crosslinked with 0.5% w/w of genipin dried with HMDS (b) porous PCL substrate without drying treatment (c) and immersed in HMDS 48 hrs (d). Polymer/salt ratios of the PCL substrates were: 70/30 w/w. Internal surface (I) External surface (II) and cross section of the wall (III) of porous scaffolds.

RESULTS AND DISCUSSION

The surface properties of an implant used in tissue engineering will determine the degree of interaction between the cells and the material and therefore the success of the regenerative therapy applied. The device implanted should not only provide mechanical support for cell attachment but also stimulate the adhesion, proliferation and cell differentiation process. For analysis of the interface between the cell and the substrate, scanning electron microscopy has been an essential tool [14, 27]. In that matter, to obtain the maximum information from the material analyzed, the selection of the proper procedure for sample preparation is fundamental, especially when the specimen has to be

dried. CO_2-CPD has often been employed to dry samples. However, several studies have reported alterations in cell morphology and adhesion to the sample carrier due to the conditions of temperature and depressurization implemented in this technique [20, 28, 29]. For this reason, some researchers have implemented less aggressive means for drying the material, such as chemical reagents Peldri II and HMDS [10, 20-22, 28, 29].

One of the objectives of this investigation was to evaluate the effect of CPD and HMDS on substrates manufactured for the construction of a nerve guide. While comparing the complexity of each of the drying procedures applied. Figure 1 shows gels treated with CPD (figure 1a) and HMDS (figure 1b), next to PCL films without drying treatment (figure 1c) and immersed in HMDS for 48 hrs (figure 1d), strongly suggesting that the different drying procedures did not generate any obvious alterations in this set of samples.

On the other hand, different results were observed when comparing SEM images of Vero cells seeded on thin mica films and submitted to each drying technique. It was found that samples treated with CPD presented ruptures on various areas of the cells (figure 2b, arrows), in contrast with the images of Vero cells seeded on the same type of material and dried with HMDS, in which usual cell morphology was observed (figure 2a). With regard to the technical difficulties of each drying method, CPD resulted to be more complex than HMDS. This is because CPD is a time consuming technique that required a critical point dryer, an expensive instrument necessary for the application of a transitional fluid, in this case CO_2. In contrast, drying process with HMDS was simple and took only a few minutes.

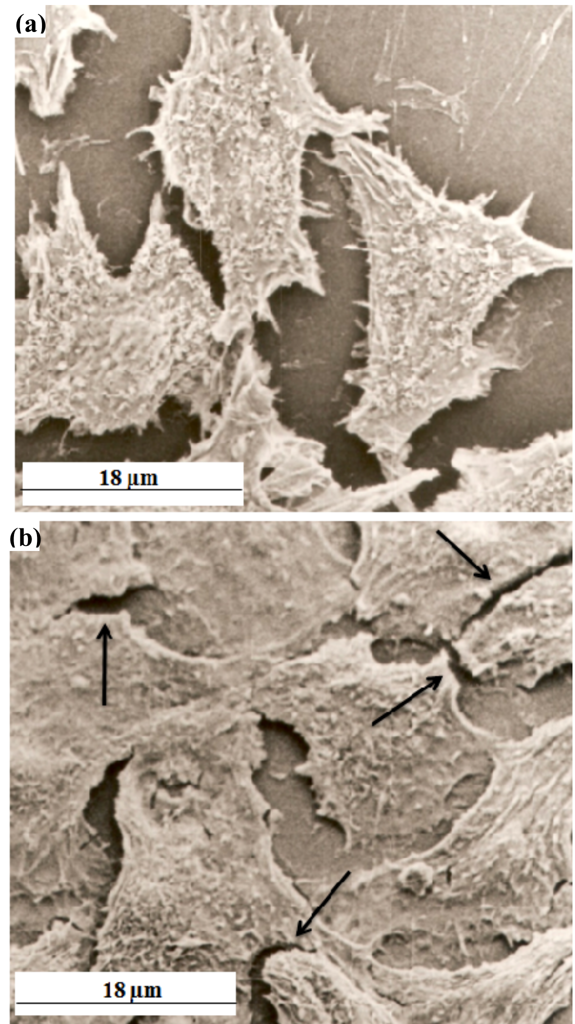

Fig. 2. SEM images of Vero cells grown on thin mica films. Samples were dried using HMDS (a) and CPD (b). Arrows indicate ruptures present in cells dried with CPD; these are not seen in cells dried with HMDS.

With regard to porosity, in the external scaffolds pore sizes of samples prepared with different polymer/salt ratios were determined using SEM images (figure 3). Scaffolds fabricated with a 100/0 w/w polymer/salt proportion are shown in figures 3a and 3b. Smooth areas and the absence of pores can be observed in the micrographs of the cross sections (figure 3a) and external surfaces (figure 3b) of the 100/0 w/w samples confirming that the porous structures present in the 80/20, 70/30 and 50/50 w/w polymer/salt scaffolds were due to the solvent

casting and salt particulate leaching process and not to other fabrication factors.

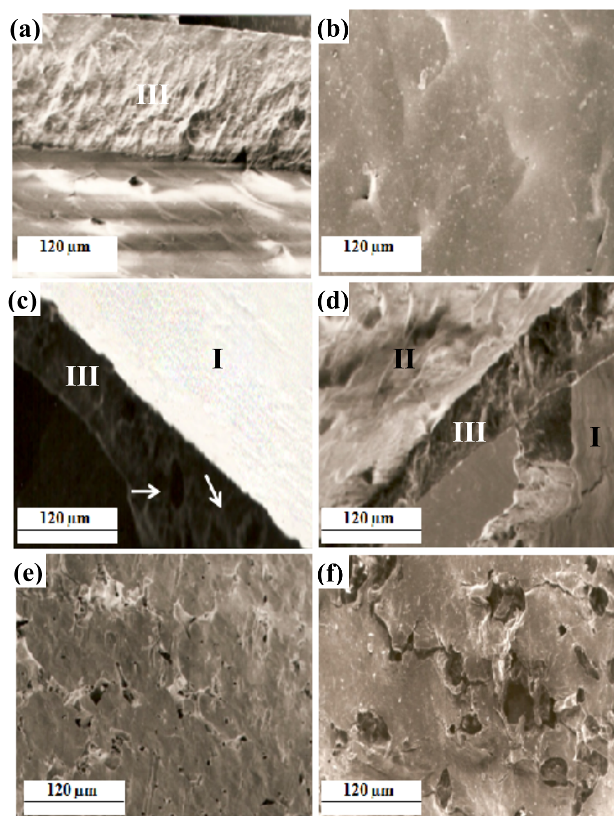

Fig. 3. Micrographs obtained by SEM of the surfaces of porous substrates made from PCL and different polymer/salt ratio: 100/0 w/w (without pores) (a, b), 80/20 w/w (c, d), 70/30 w/w (e) and 50/50 w/w (f). Internal surface (I) external surface (II) and cross section of the scaffolds wall (III). The arrows indicate the hollow spaces immersed in the walls of PCL scaffolds with 80/20 w/w a polymer/salt ratio

To that effect, films fabricated with a proportion of polymer/salt 80/20 w/w had an approximate pore diameter average of 21.1 $\pm$ 9.5 µm (figures 3c and 3d), while the opening size of the pores, obtained in films 70/30 w/w were of 23.0 $\pm$ 11.9 µm (figure 3e) and in the 50/50 w/w scaffolds, pores of 46.0 $\pm$ 9.6 µm were estimated (figure 3f). It is noteworthy, that all the substrates had been fabricated with NaCl particles of about 38 µm. So, larger openings in films with a higher polymer/salt ratio were probably obtained by salt aggregates in the polymer matrix. This formation of NaCl clusters were most likely favored by the higher concentrations of the porogenic agent, that left open spaces once the salt crystals were washed out during the leaching procedure.

The porosity distribution in the synthetic scaffolds is shown in the micrographs of figures 1c, 1d and figure 3. It can be observed that pores presented different arrangements based on the polymer/salt relation. In this way, in figure 1 it is shown that the samples 70/30 w/w, have a randomly arranged porosity across the outer (figure 1d II) and internal (figure 1d I) surface. In the same figure, a cross section of the 70/30 w/w support obtained by cryogenic fracture can be observed (figure 1c III); there a highly rugged area is evidenced by the presence of interconnected pores. These holes, that penetrate the wall of the material, communicate the internal and external environment of the scaffold. In contrast, films fabricated with a polymer/salt ratio of 80/20 w/w (figures 3c and 3d) presented a very different porosity distribution when comparing it to the one observed in the 70/30 w/w samples; even though the size of the pores measured in both proportion was similar. Figure 3c shows that the scaffold fabricated with a ratio of PCL/salt 80/20 w/w, exhibit a large inner plane of the support without holes, much like the surface of the nonporous films, made with a polymer/salt relation of 100/0 (figure 3a).

In addition, in samples 80/20 w/w, empty cavities embedded in the walls of the material were observed probably due to the accumulation of NaCl crystals in this area (figure 3c III, arrows). Some of these hollow spaces can be seen projecting to the outer surface of the support (figure 3d III). Also, when comparing micrographs 3e and 3f, it is apparent that substrates made of lower proportions of polymer/salt, show a more uniform surface. This is detailed when comparing the samples of PCL/salt 70/30 w/w to the surface of the 50/50 w/w supports (figure 3f).

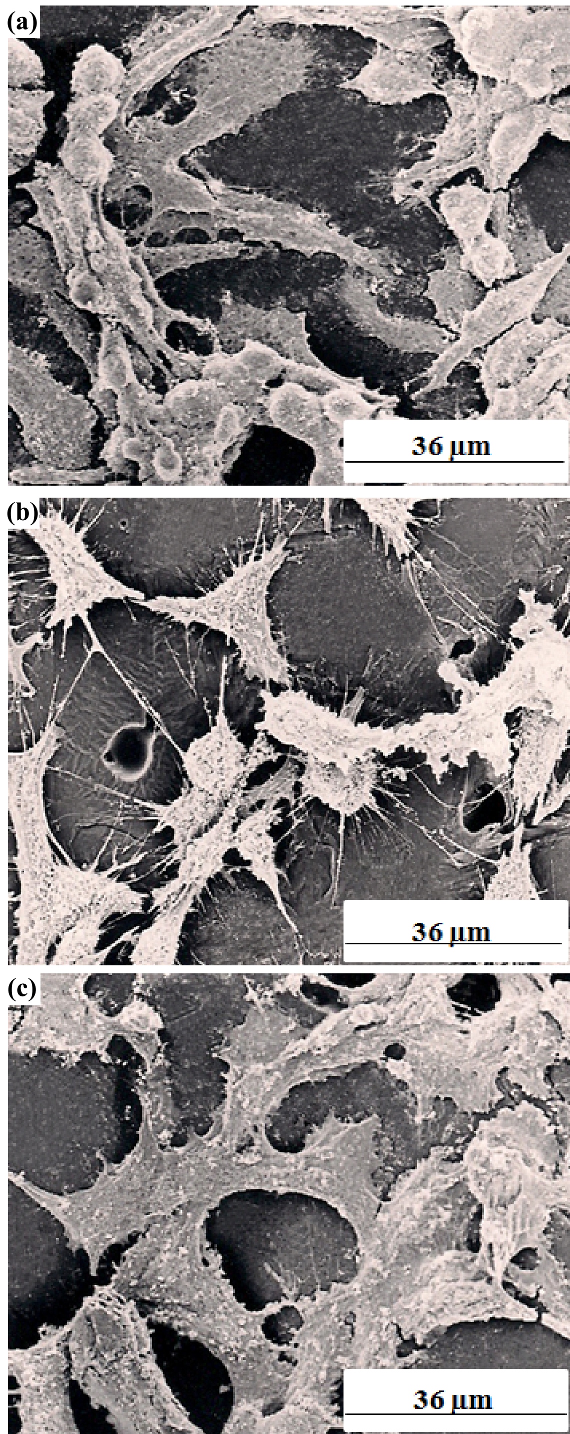

Fig. 4. SEM micrographs showing adherent cells on a nonporous surface of PBSucc/PCL (a) and on PCL samples with varying degrees of porosity and pore size, determined by the polymer/salt ratio: 80/20 w/w (b) and 50/50 w/w (c). Samples were dried with HMDS.

With this last proportion, the scaffolds constructed presented a large number of grooves and ridges on the surface, generated by aggregation of NaCl particles. These results indicate that the method of solvent casting and particulate leaching, used to produce porous matrices does not allow to control precisely the size and distribution of the NaCl aggregates, resulting in high variability of the diameters and arrangement of the pores obtained for each polymer/salt ratio. However, it is a simple and inexpensive procedure that allows manufacturing of polymer membranes with an interconnected porous structure. As for this research rather than obtaining pores with exact diameters it was more important the functionality of the porosity and the cellular response it generated, which will be next to be discussed.

Analysis of cell – substrate interaction was also done using SEM micrographs (figures 4 and 5), determining the affinity between Vero cells and the materials tested for the construction of a nerve guide. Cells in figure 4a are observed mostly rounded, with few microvilli adhered to the surface and showing little interaction with the nonporous polymer. In contrast, figure 4b shows a PCL film constructed with a polymer/salt proportion of 80/20 w/w, in which flattened and spherical cells are seen, projecting abundant cellular extensions to the surface of the material. With similar characteristics in figure 4c supports with greater pore size can be observed (50/50 w/w polymer/salt ratio); in which, the cells adhered exhibited an irregular morphology, with a flattened and elongated structure, suggestive of better cell adhesion to the porous scaffolds.

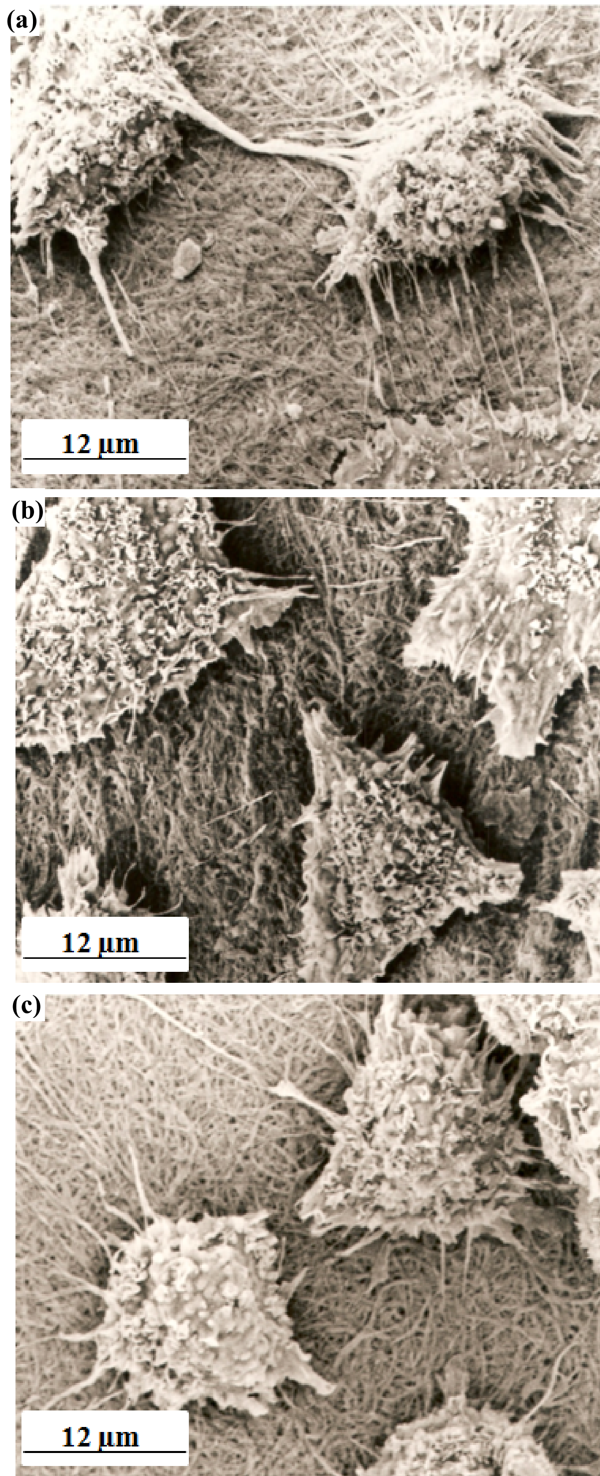

Fig. 5. SEM images of Vero cells cultivated on collagen matrices crosslinked with various concentrations of genipin: 0.05% w/w (a), 1% w/w (b) and 2% w/w (c). Samples were dried with HMDS.

From these images can be interpreted that the porous structure and pore size in mixtures of synthetic polymers may generate differences in cell morphology cultured on the substrate. That would be in accordance to previous reports that indicate that a porous architecture has greater capacity to stimulate cellular adhesion and proliferation, compared to a flat two-dimensional setting. It is believed that the presence of pores ensures a greater contact surface and facilitates cell communication and interaction [30]. As for the adhesion and proliferation of Vero cells on collagen membranes with different degrees of crosslinking, figures 5a, 5b and 5c show morphologically similar cells, distributed throughout the biological matrix without a specific pattern of adhesion.

CONCLUSIONS

In summary, HMDS was a simple and rapid method which preserved the monolayer of cells adhered to the substrate surface, obtaining results comparable and, in some cases, better than those obtained by CPD. Additionally, SEM images revealed interconnected pores of different sizes and distributions, formed after dispersion of the salt particles in a polymer solution, according to the proportion of the polymer/salt ratio of the scaffold. Furthermore, the porosity seems to induce a favorable effect in cell adhesion on the material while the different crosslinking degrees of collagen membrane with genipin, did not appear to induce significant variations on the morphology of Vero cells seeded on this substrate. In conclusion, the polymers tested show appropriate surfaces for the construction of nerve guides that may well amplify the regeneration of injured nerves; although further studies should be made.

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