Cellular Interaction with PLA Biomaterial: Scanning Electron Microscopy Analysis

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Abstract

Polymeric biomaterials are used in tissue repair due to characteristics as biocompatibility and cell interaction. In the category of synthetic polymers, poly lactic acid (PLA) is especially used in several clinical applications. This biomaterial can be prepared in the form of dense and porous membranes, in order to characterize the interaction with Vero cells in culture, using scanning electron microscopy (SEM). For this purpose, dense and porous PLA membranes were previously prepared, sterilized by ultraviolet light (UV) and used as a substrate for cultivation of Vero cells, for periods of 2 h and 7 days. To observe the cell interaction with the membranes, different methodologies for processing the samples for observation by SEM were considered, and a comparative analysis of the results was performed. There was no significant difference in cell morphology comparing samples with and without osmium tetroxide, however samples that underwent drying at a critical point had better preservation of cell morphology compared to samples in chemical drying. Osmium tetroxide is not a major factor in the processing of cells for SEM, and that the type of drying is the factor that most affects cell morphology in this process. It was possible to observe cell adhesion after 2 h of culture, with morphological changes such as cytoplasmic transition to a more flat aspect being more characteristic for 7 days of culture.

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1 Introduction

Tissue engineering is a multidisciplinary science that aims to maintain, repair or improve tissue function. In this concept, three elements are fundamental: biomaterials (such as scaffolds, supports or membranes), cells and a specific microenvironment [1, 2]. In this context, polymers are biomaterials widely used as scaffolds due to their characteristics, mainly of biocompatibility [3, 4].

Polymeric biomaterials, such as poly (α-hydroxy acids), the poly (lactic acid) (PLA) [5, 6], are used for the repair of soft tissue functions, such as tendon and cartilage, and of hard tissues, such as bones [3, 7]. The ease of synthesis, the possibility to control the properties of the biomaterial and the improvement of the mechanical and degradation properties are advantages of synthetic polymers, when compared with natural polymers [3, 8].

The clinical application of a biomaterial must attend the safety and efficacy of its use. The interaction of these biomaterials with living tissues must allow an adequate biological response [9]. In addition to biocompatibility, bioreabsorption and mechanical resistance, the interaction with cells that make up living tissues with biomaterials is modulated by the biomaterial surface characteristics [10, 11]. The presence of pores, electrical charges and hydrophilicity may influence contact with cells, both in vitro experimentation and in vivo applications [11–14].

The cell culture technique is applied as an initial step to assess the biomaterials biocompatibility, and may include cytotoxicity tests, cell interaction and tissue engineering techniques. The Vero cell line, isolated from the epithelium of the African green monkey kidney, is used for the mentioned purposes. They are anchorage-dependent cells, with effective cell adhesion and spreading on favorable surfaces, and with a high mitotic index [15].

The interaction between cells and biomaterials can be evaluated by different techniques. Microscopy techniques are widely used, mainly for qualitative analysis of this

interaction [13, 14, 16]. Phase contrast light microscopy is used to accompany cells in culture during experiments on two-dimensional substrates, and some three-dimensional substrates, especially translucent ones. Electron microscopy, both scanning and transmission, are complementary tools for analyzing cell-substrate interaction, as they allow the analysis of substrates of different shapes and thicknesses, with higher resolution. However, the processing of biomaterials, specially containing cells on their surface (from in vitro experimentation or even in contact with living tissue) can lead to changes, artifacts, which affect the quality and objectivity of the results [17].

The objective of this project is to characterize the interaction of Vero cells in culture on PLA membranes by scanning electron microscopy (SEM), evaluating cell interaction and the sample preparation protocol, thus providing a modified protocol for this kind of analysis that can optimize time, and diminish material usage and costs, in other experiments.

2 Materials and Methods

2.1 Biomaterial Preparation and Sterilization

The PLA membranes (NatureWorks®, Cargill) were obtained by dissolving the PLA polymer in chloroform (Synth), at a concentration of 10% (w/v) by weight of the polymer in relation to the solvent volume, at room temperature. The solution was poured into glass Petri dishes until the solvent evaporated, in an exhaust hood, at room temperature. The porous membranes were obtained with the addition of NaCl (Synth) to the polymer solution, 5 g of NaCl for 10 mL of polymeric solution. The dense and porous samples of PLA were sterilized by ultraviolet (UV) light for 30 min each face [3, 18].

2.2 Vero Cell Culture

The Vero cell culture (Instituto Adolfo Lutz/SP, CCIAL 057) was performed in HAM F10 medium, with 10% fetal calf serum (FCS), and 100 µg/ml of penicillin/streptomycin, and was maintained at 37 °C with 5% CO_2. The cultures were monitored by phase microscopy, using an inverted light microscope (Axiovert A1/Zeiss). This cell line is considered a standard for cell cytotoxicity tests, and is also used as a standard for studies of cell-biomaterials interaction [15, 19].

2.3 Cell Interaction—Scanning Electron Microscopy

Vero cells were inoculated on sterile PLA membranes, cut into squares of approximately 0.25 cm^2, by direct deposition of a suspension containing 2×10^5 cells/mL, and cultures were maintained for periods of 2 h and 7 days in a 24-well cell culture plate. After the culture period, different sample preparation protocols were performed. As a basic protocol, the samples were fixed with 2.5% glutaraldehyde, in phosphate buffer (PBS, pH 7.4, 0.1 M), for 1 h, washed in PBS, for 1 h, followed by washing in distilled water (30 min) and dehydration in an increasing series of ethanol (30, 50, 70, 90, 96 and 100%), followed by critical point drying (EM CPD300, Leica).

As an alternative to this protocol, modifications related to post-fixation and dehydration were included. In the post-fixation stage, samples of biomaterials with cultured cells were treated with 1% osmium tetroxide for 1 h. In the dehydration stage, critical point drying was replaced by chemical drying, with evaporation of the dehydrating agent, ethanol.

The PLA samples with cultured cells were then covered with 15 nm gold in sputtering (AC200, Leica) and observed with a scanning electron microscope (Quanta 250, FEI). The images were analysed for cellular morphology, and the cell perimeter and area were measured with software Image J (NHI, Bethesda, MD, USA).

3 Results and Discussion

During the culture, Vero cells were observed under a light microscope, with phase contrast (Fig. 1) [19]. The cells showed rounded morphology, without spreading, in both dense and porous PLA. The opacity and the thickness of the biomaterial samples leads to difficulty of visualizing the cells on the biomaterial, impairing the proper characterization of cellular interaction, a fact that motivated the use of the SEM for more accurate analyzes.

After 24 h of cell culture, the samples were observed under a light microscope, both on the surface of the biomaterials (Fig. 1) and on the bottom of the culture plate (Fig. 2). The observation of the cells growing on the culture plate near biomaterial samples can be used to verify a possible cytotoxic cellular response, the PLA samples were considered non-cytotoxic, once the cells showed typical morphology [15, 16, 20] (Fig. 2).

Fig. 1 Vero cells cultured on PLA biomaterial. **A**, **B**: 30 min, **C**, **D**: 24 h; **E**, **F**: 7 days. **A**, **C**, **E**: dense PLA; **B**, **D**, **F**: porous PLA. The arrows indicate the presence of the cells. Magnification: 200×
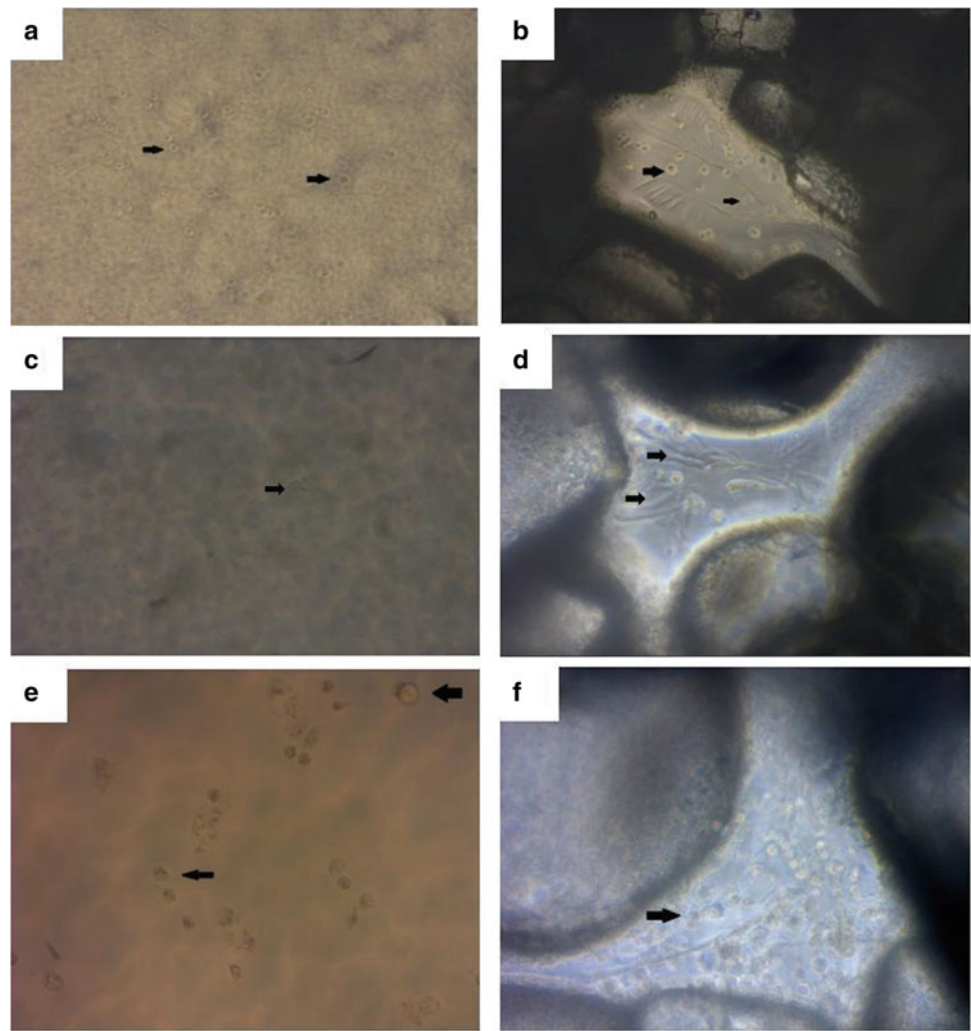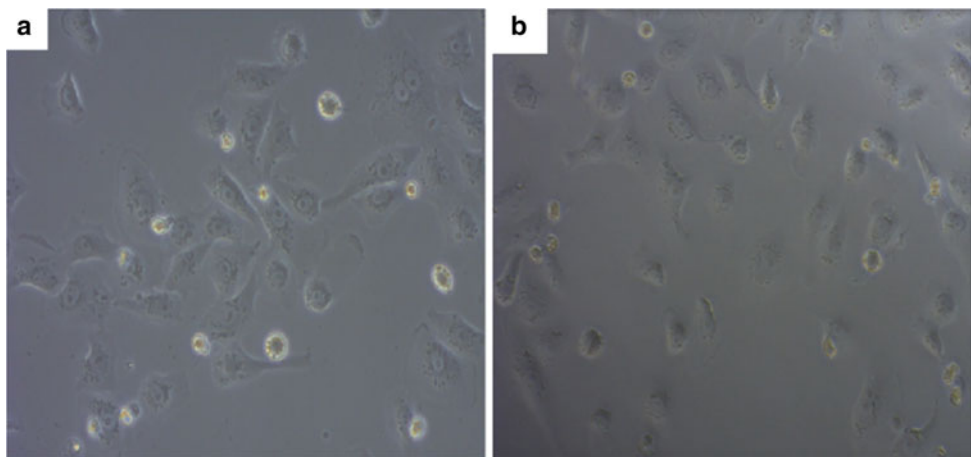

Fig. 2 Vero cells cultured on PLA biomaterials after 24 h, a representative result of the non-cytotoxicity of dense **A** and porous **B** samples. Magnification: 200×

For electron microscopy, biomaterials, and biological materials, are exposed to an electron beam as a source to form the image, allowing a high resolution image. In scanning electron microscopy (SEM) this electron beam interacts with the surface of the sample, in a high or low vacuum environment. The samples are exposed to high acceleration voltage (kV), and must be able to interact with the beam of electrons generating backscatter or secondary electrons, for example [21].

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The preparation of samples of biomaterials containing cells is necessary in order to preserve the structures to be observed with a SEM. The preparation includes fixation, post-fixation, washing, dehydration, drying and metallization steps [16, 20, 22].

The chemical fixation process aims at the preservation of the cellular structure, creating crosslinking and chemical bonds that allow the stabilization of the cell morphology as close to its natural and functional state [23]. Glutaric aldehyde, or glutaraldehyde, is generally used as a primary fixative, which acts on the fixation of proteins by divalent bonds with amino groups. Other fixatives, such as paraformaldehyde, can be used in association with glutaraldehyde (the Karnovsky's Mixture).

The post-fixation step with osmium tetroxide (OsO_4) can be used to preserve lipid structures, such as cell membranes. However, OsO_4 has a strong oxidizing effect, in addition to its toxic, carcinogenic, mutagenic and teratogenic effects, as well as it is a high-cost reagent [24].

After the fixation, the washing is important to eliminate fixatives, mainly if they may form precipitations in contact with the dehydration solution. The dehydration step is made in increasing concentrations of ethanol or acetone, with the function of replacing the water present in the biological samples, as the observation by SEM usually occurs in a vacuum condition [22]. The final drying can be done in air, allowing the agent used in the dehydration to dry naturally. However, during this process it is common to observe morphological changes in biological structures, such as shrinking, destruction of surface structures, and even collapse of the sample, due to the surface tension of the chemical drying agent, creating artifacts that prevent the correct morphological observation of the sample. It can alternatively be used as the technique of critical point drying, which establishes a condition of low temperature and high

pressure, to replace the dehydration agent with liquid CO_2. The transition from the liquid to the gaseous phase occurs with a minimum of surface tension [25], preserving biological samples [26].

All the described processing aims at the ultrastructural preservation of biological structures, cells, tissues or organs, with the least possible deformation (or artifact), and the proper interaction with the electron beam [27, 28]. For this study, the standard mentioned protocol was altered in the post-fixation and dehydration phases, to observe the impact that the absence of a post-fixator and different dehydration agents can bring to the analysis.

In this study the cells cultured on PLA samples for the period of 2 h showed cell adhesion, with few cells widely spread among themselves, with rounded morphology (Fig. 3). In some areas it was possible to observe cell spreading, with cytoplasmic expansions contact with the biomaterial (Fig. 3B). The cells were well preserved with glutaraldehyde fixation, and the post-fixation with osmium tetroxide did not result in improved cell morphological preservation (Fig. 3C, D, G, H). However the use of a critical point for dehydration results in better cytoplasmic preservation of expansions in areas of cell adhesion (Fig. 3 B, D, F, H), which was expected for this kind of technique, as the liquid CO_2 transitions to gas and it occurs with a minimum of surface tension, bringing less harm to the biological samples [25, 26].

Regarding the biomaterial, the processing allowed a good preservation, without structural modifications. In porous PLA samples, it can be observed that the cell deposition inside the pores, where they showed rounded morphology, with focal contact with the biomaterial, indicative of the beginning of the cell adhesion process, the morphology is distinct from cells in dense samples (Fig. 3).

The observation of cells cultured on PLA biomaterials at 7 days allowed the verification of their proliferation pattern (Fig. 4). The cells cultured on samples of dense PLA after 7 days of cultivation showed very spread morphology, with well-preserved cytoplasmic expansions, and few scattered cells were observed, indicating little cell proliferation (Fig. 4 B–D).

For the porous samples, small cell clusters were observed, two or few cells, isolated from each other, preferably in areas of depression or biomaterial surface (Fig. 4A). The cells maintain a rounded shape, little scattered, indicating little interaction with the biomaterial.

The presence of isolated cells, or small groups of these, demonstrates that proliferation is not favored. The rounded morphology of the cells, without cytoplasmic breaks or interruptions, also reveals that they are in good morphological preservation conditions (Fig. 4).

The samples fixed only with glutaraldehyde showed cells with very visible cytoplasmic expansions, with good

Fig. 3 Vero cells cultured on PLA biomaterials after 2 h, fixed with glutaraldehyde. Arrows indicate the presence of cells. **A–D**: dense samples; **E–H**: porous samples; **C**, **D**, **G**, **H**: post-fixed in osmium tetroxide; **A**, **C**, **E**, **G**: chemically dehydrated; **B**, **D**, **F**, **H**: critical point drying
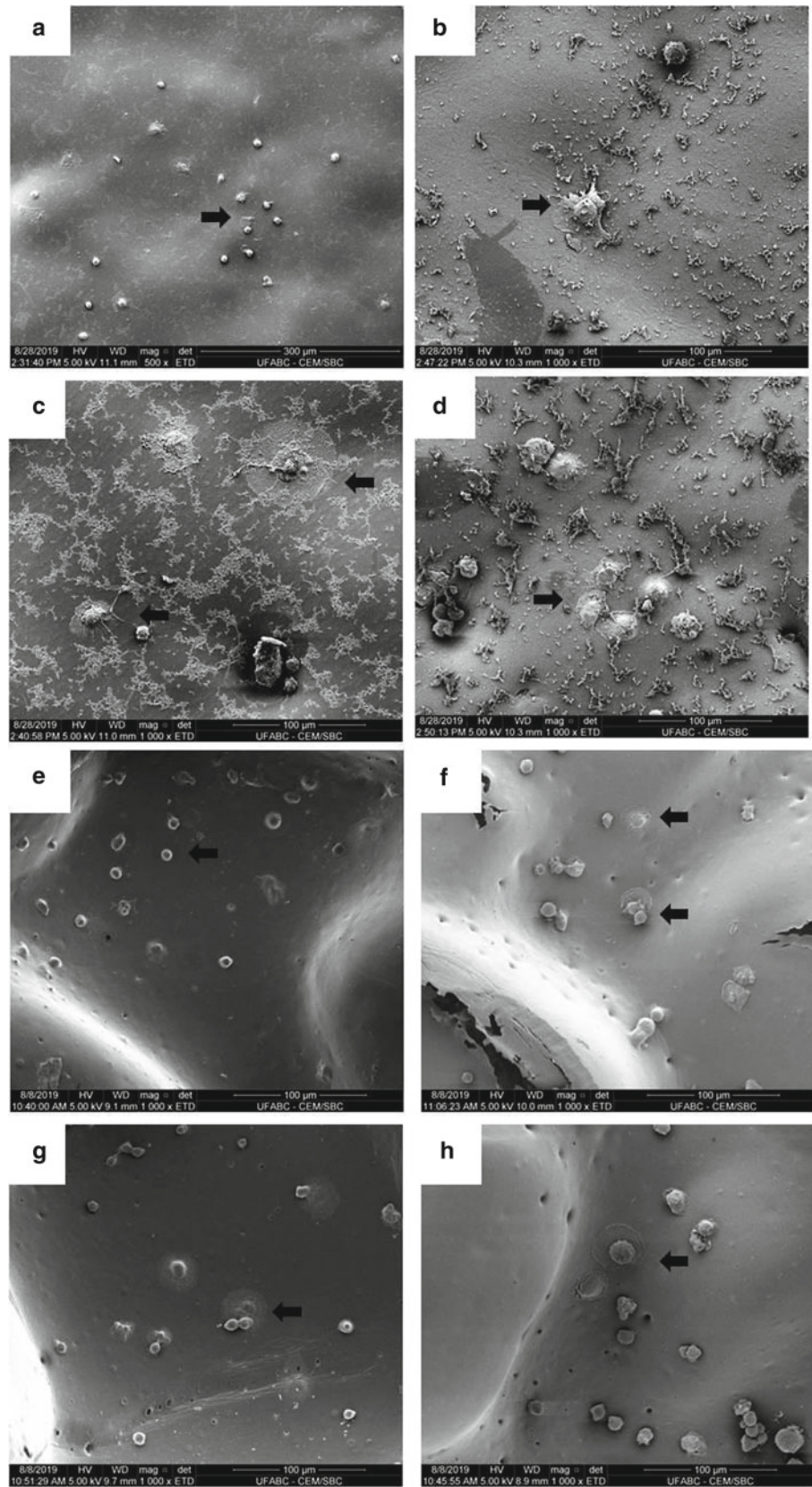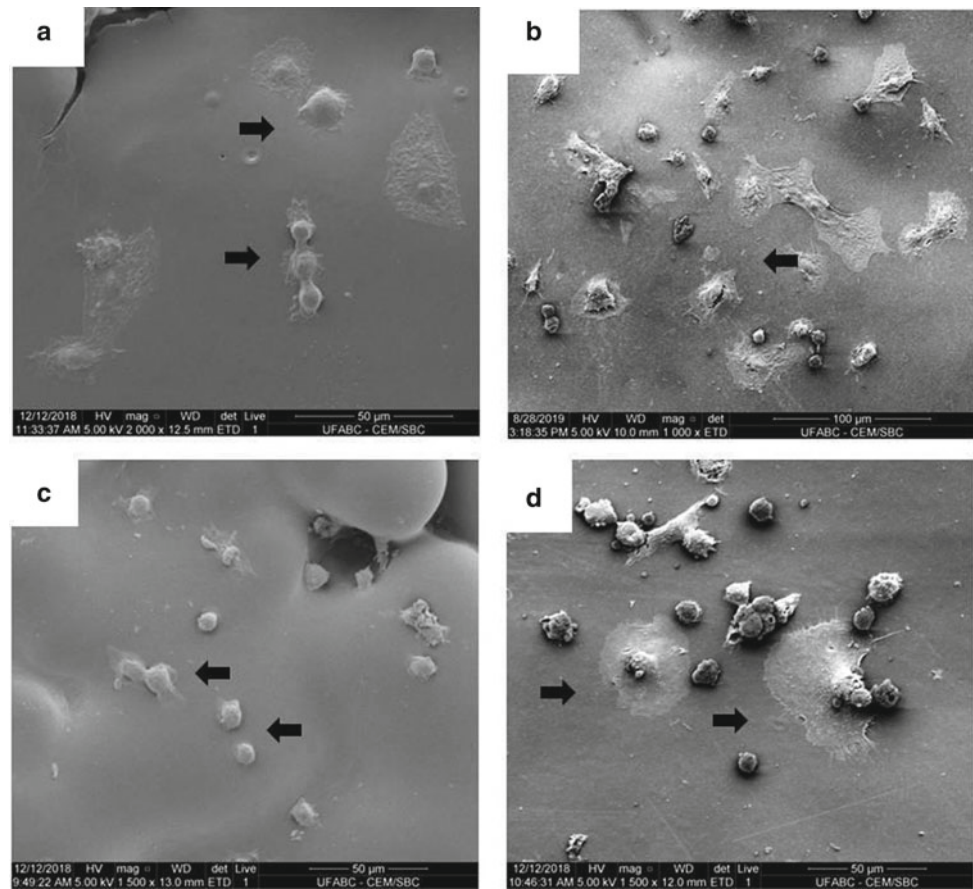
Fig. 4 Vero cells cultured on PLA biomaterials after 7 days, fixed with glutaraldehyde. Arrows indicate the presence of cells. **A**: porous sample, flattened pore surface; **B–D**: dense samples; **C**, **D**: post-fixed in osmium tetroxide; **A**, **C**: chemically dehydrated; **B**, **D**: critical point drying

morphological preservation (Figs. 3 and 4). This finding corroborates data presented in the literature. In general, porous substrates, even with larger pores, favor the differentiated state of cells, and little proliferative activity [13]. It was possible to evaluate that despite a slight improvement in the preservation of cell membranes, the presence of osmium tetroxide did not cause a significant improvement in cell morphology preservation. Due to the extreme toxicity and the high course, we do not consider it necessary to use this post-fixative. Other reports also consider the possibility of dispensing this reagent in the processing of biological samples for SEM [24, 29].

However, the preservation of samples with critical point drying was shown to be effectively superior than chemical drying (Fig. 5) to avoid technical artifacts, in concordance with the literature [30–32]. The samples dehydrated by ethanol, we can observe less cellular flattening and adhesion when compared with the ones dehydrated with critical point, as can be seen comparing Fig. 5C, D, for example, which indicates that the latter is more efficient in preserving cellular structure. This can be observed in Fig. 3, comparing Fig. 3 E, F, for example, it can be observed that cells dehydrated by ethanol present a slightly more polygonal morphology and are more scattered, while in the ones dehydrated by critical

point present more round and clustered cells, which indicates better preservation of cellular morphology and interaction with the biomaterial.

The results shown in Figs. 4A, D, and 5B, E were used to evaluate cell perimeter and area (Table 1). It was observed that cell area was 45,45% larger in the preparations submitted to critical point drying. This result corroborates with the morphological description for the figures, showing a better preserved morphology with this protocol.

4   Conclusion

Analyzing the results we concluded that the most impacting factor for the good preservation of both the biomaterial and the cells was the dehydration protocol. The use of critical point drying for both types of PLA samples, dense and porous, resulted in fewer biomaterial failures and better preservation of cell morphology. As for the use of post-fixative osmium tetroxide, there was no significant improvement in the morphological preservation of the cells. There were also no alterations in the PLA samples used for cell cultures. Therefore, it can be proposed that there is no need for post-fixation PLA samples with cultured cells, thus

Fig. 5 Vero cells cultured on PLA biomaterials after 2 h, fixed with glutaraldehyde. **A–D**: dense samples; **E**, **F**: porous samples; **A**, **C**, **E**: dehydrated in ethanol; **B**, **D**, **F**: dehydrated at a critical point
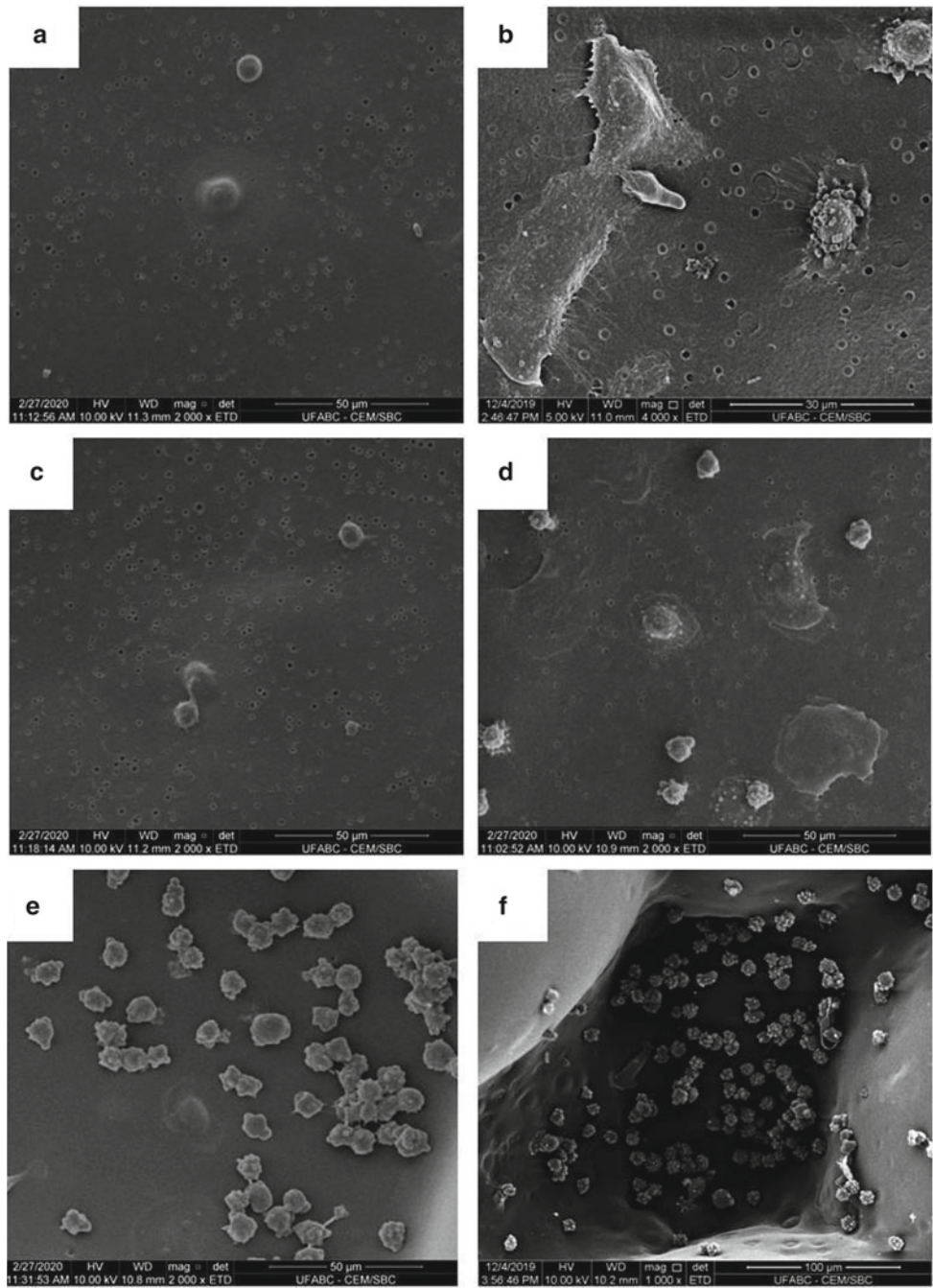

Table 1 Image J measures on Figs. 4 and 5. Perimeter μm; area μm^2. SD: standard-deviation; E: ethanol; CP: critical point

Figure	N	Perimeter	Area	Mean perimeter	Mean area
4A-E	9	66,554	287,581	E: 53,808	E: 194,157
4D-CP	9	76,835	487,370	CP: 71,530	CP: 355,952
5E-E	5	41,061	100,732	Difference	Difference
5B-CP	5	66,224	224,533	24,78%	45,45%
SD		15,224			

reducing costs and possible risks from handling them, but critical point drying is recommended.

Conflict of Interest
The authors declare that they have no conflict of interest.

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