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Increased response of Vero cells to PHBV matrices treated by plasma

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Abstract The copolymers poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) are being intensely studied as a tissue engineering substrate. It is known that poly 3-hydroxybutyric acids (PHBs) and their copolymers are quite hydrophobic polyesters. Plasma-surface modification is an effective and economical surface treatment technique for many materials and of growing interest in biomedical engineering. In this study we investigate the advantages of oxygen and nitrogen plasma treatment to modify the PHBV surface to enable the acceleration of Vero cell adhesion and proliferation. PHBV was dissolved in methylene chloride at room temperature. The PHBV membranes were modified by oxygen or nitrogen-plasma treatments using a plasma generator. The membranes were sterilized by UV irradiation for 30 min and placed in 96-well plates. Vero cells were seeded onto the membranes and their proliferation onto the matrices was also determined by cytotoxicity and cell adhesion assay. After 2, 24, 48 and 120 h of incubation, growth of fibroblasts on matrices was observed by scanning electron microscopy (SEM). The analyses of the membranes indicated that the plasma treatment decreased the contact angle and increased the surface roughness; it also changed surface morphology, and consequently, enhanced the hydrophilic behavior of PHBV polymers. SEM analysis of Vero cells adhered to PHBV treated by plasma showed that the modified surface had allowed better cell attachment, spreading and growth than the untreated membrane. This combination of surface treatment and polymer chemistry is a valuable guide to prepare an appropriate surface for tissue engineering application.

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Introduction

Development of novel biomaterials for medical applications has attracted much interest in recent years. Biodegradable polyesters are probably the group of polymers most widely studied in this application field [1]. Given the importance of strategies for acceleration of tissue healing following surgical interventions, research groups have been performing several experiments for in vitro testing of biomaterials as a prerequisite for in vivo evaluation [2–13].

PHBV are now being studied in tissue engineering as substrates for the growth of bone tissue [14–16], however it is known that they are quite hydrophobic polyesters [17]. Among biomaterials, the copolymer poly [3-hydroxybutirate-co-hidroxyvalerate (PHBV)], a polyester, has been attracting increasing attention because of its variety of applications in medicine and industry [18], as the construction of many biomedical products such as heart valves [19], blood vessels, pericardial substitutes, orthopedic applications [20] and drug release systems [21]. Recently, it was demonstrated that untreated samples showed good biocompatibility for fibroblast in vitro and no adverse

events, such as inflammation, necrosis, calcification of fibrous capsule or in vivo malignant tumor formation [1, 22]. More recently, studies with PHBV matrices showed that surface treatment enhanced its water absorbing capacity [17] and bone cell adhesion [23].

Establishment of a stable interface between a biomaterial and the host environment is driven by both the material surface properties and the type and state of the biological tissue [24]. Surface characteristics of materials such as topography, wettability, chemistry or surface energy are important because they affect the biological interactions on the material [17, 24] and they are also an important feature for in vitro biocompatibility and cytotoxicity studies. The control of cell adhesion to the polymer substrate is one of the most important issues in tissue engineering, which assures the ability to guide proliferation, migration and differentiation of the cells to construct an equivalent tissue [14].

The cell–biomaterial interaction must provide conditions for cell adhesion as well as its spreading, and many studies have shown a clear distinction between materials supporting or not cellular adhesion. The inability of certain materials to support cell adhesion/spreading enhances the possibility to observe changes in cell adhesion induced by, e.g. surface modification or surface coating. As a consequence, it can be used to acquire knowledge of the surface properties that might stimulate cell adhesion, and its subsequent spreading and growth. The drawback of this approach is that differences in cellular responses to both groups are contrasted in a black-and-white pattern, as a result of strong differences in the morphologies of spreading and non-spreading cells [25].

Surface properties can be altered to suit a specific biomedical application [26]. Plasma treatments offer a method of altering the surface characteristics of materials without affecting their bulk properties [27]. A number of studies have investigated plasma treatments for the modification of PHBV, and have demonstrated that plasma treatment lead to the creation of a more hydrophilic structure in comparison to the untreated material [14, 17, 23]. Plasma-surface modification is an effective and economical surface treatment technique for many materials and of growing interest in biomedical engineering [28].

The use of cell cultures to assess the bioactivity and cytotoxicity of biomaterials has overcome some of the limitations of in vivo studies. The aim of this study was to investigate the advantages of oxygen and nitrogen plasma treatment to modify the PHBV surface in cultured Vero cells. We chose to study growth and differentiation of these cells, as well as their interactions with biomaterials [10], since they are recommended for such investigations in standard protocols [29–31].

Materials and methods

Preparation of PHBV membranes

PHBV was dissolved in methylene chloride (5%W/V) and the solution was slowly poured onto a glass plate to avoid air bubbles. The solvent was evaporated at room temperature for about 24 h, inside a solvent-saturated glass container. The obtained membranes were stored under vacuum until they were plasma-treated. The plasma treatment was performed using a radio frequency Anatech LTD Ashing plasma apparatus. The plasma conditions employed in the treatment was (PHBV 0—untreated); (PHBV O_2 1—100 W, 80 Pa and 600 s); (PHBV O_2 2—100 W, 40 Pa and 600 s), (PHBV N_2 3—100 W, 80 Pa and 600 s); (PHBV N_2 4—50 W, 20 Pa and 600 s). The values of power, pressure, and time were chosen based on preliminary studies; and the (2 and 3) plasma for each polymer refers to data reported by Tezcaner et al. [14] as the best treatment parameters for PHBV. The membranes were sterilized by UV irradiation for 30 min and placed in 96-well plates before cell culture [25].

Contact angle

The contact angles were measured in air using a NRL CA goniometer of Ramé-Hart Imaging System, model 100–00, and the images were obtained by using the Ramé-Hart Imaging 2001 software. The measurements were done using 10 μL of demineralized water as probing liquid in order to obtain the contact angles of the surfaces.

Atomic force microscopy (AFM)

The AFM analyses were performed using a Digital Instruments NanoScope IIIa Scanning Probe Microscope Controller. It was employed a Si cantilever with constant forces of 13–70 N/m. The images were obtained in the taping mode at room temperature, in the fundamental resonance frequency of Si of about 300 kHz. The scanning rate was 1 Hz, and the maximum scale for the scanning heads was 10×10 μm. All images were obtained with directional amplitude of A0 $\approx$ 500 nm.

Cell culture

Vero cells, a fibroblastic cell line established from the kidney of the African green monkey (*Cercopithecus aethiops*) that was used in this work, were obtained from Adolfo Lutz Institute, Sao Paulo, Brazil. These cells were cultured in Ham's F10 medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal calf serum

(FCS, by Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37 °C. Vero cells lineage is recommended for studies of cytotoxicity and cell-substratum interactions in biomaterials research [28, 30, 31].

Cell adhesion and direct cell cytotoxicity assays

Identification of the effects of plasma treatments on early cell adhesion and late cytotoxicity were carried out by performing the MTT assay, a modification of Mosmann's [32] method, which was used for both Cell adhesion and direct cell cytotoxicity assays [8]. Previously, the sterilized PHBV membranes were placed in a 96-well plate (Corning) with 100 μL of culture medium and incubated at 37 °C for 24 h, according to Santos Jr, et al. (2005) [3]. After incubation, 2×10^5 cells/mL in 100 μL F-10 Ham medium supplemented with 10% FCS were added to the wells containing the membranes. The cells were cultured for 2 h and 24 h to allow cell adhesion and to conduct direct cell cytotoxicity assays, respectively. After the cells were washed twice with 0.1 M phosphate-buffered saline (PBS), pH 7.4, at 37 °C and incubated with 100 μL F-10 Ham medium, an MTT assay mixture [10 μL per well, containing 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide—MTT, Sigma] was added to each well and incubated for 4 h at 37 °C. After 4 h, 100 μL of Dimethyl sulphoxide (DMSO, Sigma) and 25 μL of Glycine/Sorensen buffered solution replaced the assay mixture in each well to dissolve the formazan crystals. Absorbance was quantified spectrophotometrically at 540 nm, using a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). MTT is a colorless tetrazolium salt that forms a dark compound when oxidized by mitochondria, which is detected by spectrophotometry. For cytotoxicity assay, latex membranes and the culture plate itself (polystyrene) were used as positive control, and the culture plate itself was used as negative control. For cell adhesion teflon dishes were used as negative control and the culture plate itself was used as positive control.

Absorbance of all experimental conditions (PHBV 0–4, negative and positive controls) was also read. Comparison of continuous variables for all groups was done with ANOVA. When a significant difference was found ($p < 0.05$), the groups were compared using Tukey's test.

Scanning electron microscopy (SEM)

The membranes were sterilized by UV irradiation for 30 min and placed in 96-well plates. Approximately 2×10^5 cells/mL Vero cells in Ham's F10 medium supplemented with 10% FCS were seeded in each well, containing the treated PHBV membranes and cultured at 37 °C. After 2, 24, 48 and 120 h, the samples were fixed in a fixative solution prepared by dissolving 2.5% paraformaldehyde, 2.5% glutaraldehyde, 0.03% picric acid, 1% tannic acid in 0.1 M cacodylate buffer and the same volume of Ham F-10 medium for 1 h at room temperature (RT), washed in 0.1 M cacodylate buffer, post-fixed in 1% osmium tetroxide in water for 1 h at RT in the dark, washed in water, dehydrated with ethanol, critical point dried [Balzers CDT 030] and coated with gold in a sputter coater [Blazers CDT 050]. The coated specimens were observed with a JEOL 5800 scanning electron microscope.

Results and discussion

The hydrophilicity of plasma treated PHBV samples

Exposure to oxygen or nitrogen plasma caused a reduction in the contact angle (Table 1). The significantly lower contact angle at room temperature indicates that the plasma film has become more hydrophilic. The observed increased in the hydrophilicity is related to the plasma treatment conditions (gas, power, pressure, and time). In the PHBV plasma treated membranes, the contact angle diminished to one half of its initial value even at low values of pressure, power, and treatment time. Treatment of PHBV with oxygen plasma seemed to be more effective than the one performed with nitrogen plasma. This decrease in the contact angles and consequent increase in the hydrophilicity suggest an increase in the polar groups formed by the plasma treatment [33].

Qualitative and quantitative analyses of PHBV surface after treatment with O_2 and N_2 plasmas

The Atomic Force Microscopy (AFM) gives a detailed analysis of the substrate surfaces. Nano-roughness pattern of PHBV films were shown as laterally ordered conical or ellipsoid structures perpendicular to the substrate plane (Fig. 1). The average roughness of the structures is showed in Table 2. The AFM showed that the films kept their nano pattern at roughness after plasma treatment, but it is clear that plasma composition can affect the film morphology. It was possible to observe that the surface morphologies were

Table 1 Contact angle measurement of samples of untreated PHBV and PHBV treated with Nitrogen (N_2) and Oxygen (O_2) plasma at different gas pressures, power and time

Samples	Angles (degree)	Standard deviation
PHBV (0)	79.6	±12
PHBV O_2 (1)	22.5	±1.5
PHBV O_2 (2)	37.9	±0.8
PHBV N_2 (3)	37.0	±5.0
PHBV N_2 (4)	43.3	±8.2

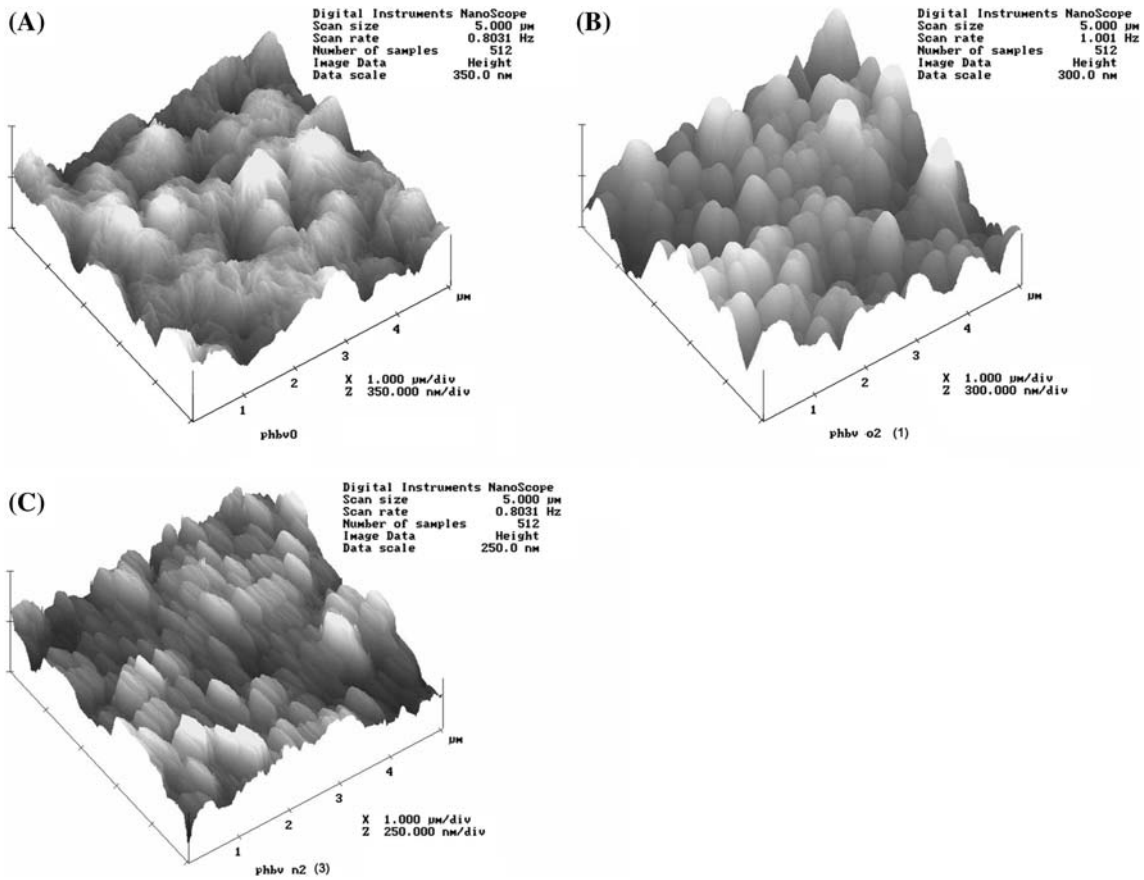

Fig. 1 Atomic force microscopy for the PHBV membranes with or without plasma treatment. (**A**) PHBV without treatment, (**B**) PHBV (1) treated with O_2, (**C**) PHBV (3) treated with N_2

Table 2 Rugosity measurement Ra (nm) of PHBV membranes treated with N_2 and O_2 plasma with different parameters, obtained through the analysis of atomic force microscopy with different scanning

Samples	Rugosity Ra (nm)
PHBV (0)	52.0
PHBV O_2 (1)	63.9
PHBV O_2 (2)	37.6
PHBV N_2 (3)	81.8
PHBV N_2 (4)	66.9

clearly modified by both treatments with O_2 and N_2 plasmas. PHBV presented a rough surface even before the plasma treatment. The O_2 plasma treatment showed morphology of peaks (Fig. 1C), whereas the N_2 plasma treatment presented a morphology consisting of a set of ellipsoids (Fig. 1B). PHBV treated with O_2 plasma presented the highest roughness values. It is supposed that the plasma etches preferentially the amorphous regions, leaving the crystalline regions practically with no alteration as it was shown by Riccardi et al. 2003 [34]. The modification of the amorphous regions results in raised surfaces with novel physical and chemical properties [34]. Also, according to Yang et al. [35], the increase in the roughness is one of the reasons for the enhancement of the hydrophilicity in the plasma treated samples, since the surface wettability is a consequence of the surface roughness.

In summary, we can modify completely the physical and chemical properties of polymer surfaces by using O_2 and N_2 plasma treatments. Not only chemical changes of the surface, but also topographic changes can contribute independently for modifications in the hydrophilicity of the PHBV polymer. Despite the increase in the roughness that causes an enhancement in the hydrophilicity of the polymer, this is not the only factor, as mentioned by Yang et al. [35].

Vero Cell behavior—direct cell cytotoxicity and cell adhesion assays

Analyses of PHBV cytotoxicity—O_2 and N_2 plasma treatments

The results obtained by direct cytotoxicity assay showed that all samples studied did not present cytotoxic effects on Vero cells. Similar results were obtained for untreated PHBV

when compared to PHBV O_2 (2) and PHBV N_2 (3), whereas untreated PHBV showed better results than PHBV O_2 (1) and PHBV N_2 (4) ($p < 0.05$). No difference was noticed between PHBV O_2 (1) and PHBV O_2 (2) ($p > 0.05$), although there were differences between PHBV O_2 (1) and PHBV N_2 (3) ($p < 0.05$) and between PHBV N_2 (3) and PHBV N_2 (4) ($p < 0.05$). The membranes, independently of the treatment, showed better results than positive (latex + phenol) and negative controls (polystyrene) ($p < 0.05$).

MTT assay revealed that all the materials in the study did not affect mitochondrial activity of Vero cells (Fig. 2). However, toxic substances do not act at one specific level but affect several cellular functions [36]. In our opinion, the early determination of the mitochondrial activity in terms of cell sensitivity would be enough to evaluate cell viability, despite the late cell proliferation. The absence of cytotoxicity does not confer information about the biocompatibility of the biomaterial. However, the determination of potential cytotoxicity is an important aspect of biomaterial when testing standards such as ISO and ASTM [29, 30], but MTT by itself cannot be used to make a strong statement about the proliferative status of a cell population as suggested in some strategies for cell proliferation [36, 37]. A great challenge in the development of novel biomaterials is to support the interpretation of the cytotoxicity results by the characteristics of the materials. In particular, degradable polymers display variable behavior in biological systems depending on various properties such as molecular weight, hydrophobicity, distribution of charge, residual monomer and pH of degradation products. Therefore, these factors, combined with the degradation kinetics, are important in determining the toxicity of promising biomaterials [36].

Our results are similar to those of other authors who showed both PHB and PHBV biocompatibility [5, 37] and that O_2 and N_2 plasma do not induce cytotoxicity, but there is increasing evidence that changes in scaffold surface chemistry and topography alter cellular activity [23]. Currently, gaseous plasma treatment is an effective and widely used as a method to modify the surface of a material for cell adhesion even under shear stress field [35].

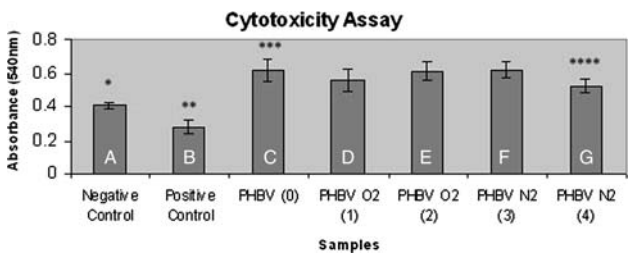

Fig. 2 Cytotoxicity assay results obtained after 24 h of cell culture: (A × B–G), *$p < 0.0005$; (B × C–G), **$p < 0.00000005$; (C × D,G), ***$p < 0.05$ and (G × E, F), ****$p < 0.005$

Analysis of cell adhesion PHBV samples—treated by plasma

The one-way ANOVA results showed that there were significant differences among adhesion absorbance in the different analyzed samples ($p < 0.05$). Tukey's test was used to compare the samples and showed that cell adhesion on all PHBV membranes was significantly different when compared to negative controls (Teflon dishes)(Fig. 3). Untreated PHBV showed a result that was lower than the positive control (polystyrene) ($p < 0.05$). PHBV N_2 (4) presented the greatest cell adhesion, which was also greater than the positive control ($p < 0.05$). There was no significant difference between PHBV treated with oxygen plasma or PHBV N_2 (3) membrane and the positive control ($p < 0.05$).

Our results showed that the cell adhesion capacity on PHBV N_2 (4) membrane was better than the positive control (polystyrene) (Fig. 3). The values observed for the control were similar to the ones observed for untreated PHBV and higher than the adhesion values presented by either mesenchymal [38] or Vero cells [6]. The quantification of cell adhesion on plasma treated surfaces showed considerable differences between the oxygen and nitrogen treatments. This result is in accordance with the ones in which the surface chemical composition and/or topography affect the interaction force that acts between the biomaterial and biological medium, for instance, water and ion sorption, protein adsorption, adhesion, cellular expansion and proliferation [6]. Many studies showed that cells adhere, spread, and grow more easily on substrates with moderate hydrophilicity than on hydrophobic or highly hydrophilic substrates [14, 32], because hydrophilic surfaces permit the adsorption of serum proteins with labile and reversible bond. It is believed that this mechanism is to be slower on extremely hydrophobic or hydrophilic surfaces, which is probably one reason that may contribute for cells to adhere and proliferate poorly on them [34, 35]. It is known that adhesion and spreading are the morphologic phenomena of

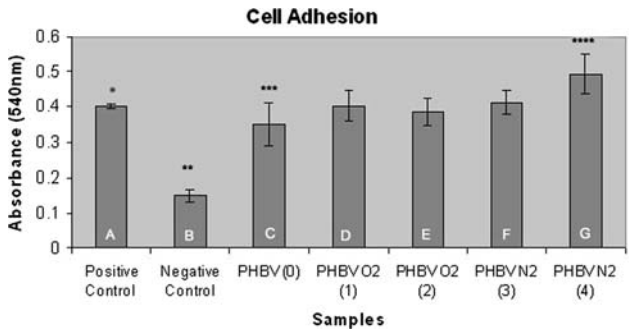

Fig. 3 Cell adhesion assay results obtained after 2 h of culture, (A × C), *$p < 0.05$; (B × A, C–G), **$p < 0.0000005$; (C × F, G), ***$p < 0.005$ and (A × G), ****$p < 0.0005$

a series of molecular events occurring in and around the cell, mediated by trans-membrane receptors, the integrins and extracellular matrix proteins [25]. However, other authors [39] suggest that some chemical groups have a more significant role in cell adhesion than the general surface properties. Both studies are in accordance with our results, which show that cells present better adhesion on treated PHBV surfaces. It is clear that specific surface properties have pivotal role on cell adhesion behavior. The data generated by this battery of assays allow a response on the cytotoxic potential of materials or devices with a higher grade of certainty. Therefore, direct test allowed the determination of a non significant induction of cytotoxicity and presented a good surface for adhesion and proliferation of Vero cells [35].

Ultrastructural analysis of cell adhesion

With the use of scanning electron microscopy we were able to observe cell growth in all studied treatments, suggesting that the material was innocuous to the cells. Early cell growth on untreated PHBV showed these cells also appear to have more elongated, flattened structures and not just the round morphology attached on biomaterial surfaces by thin cytoplasmatic filaments (Fig 4A), such behavior was observed previously in Vero cells seeded on untreated scaffolds by our group [3, 6, 9, 10, 12]. After 2 h of cultivation of Vero cells on PHBV treated with plasma, it is noticed a greater density of adhered cells (Fig. 4B–E). The morphologies of the cells on oxygen treated membranes and mainly nitrogen were quite different. The cells on the oxygen treated membranes sometimes showed cells with a round profile (Fig. 4B–C) that were still moving, while nitrogen treatment showed a larger number of cells with phillopodes and large lamellipods on their edges (Fig. 4D–E), as it was observed on the surfaces where cells will probably complete their movement and adhere tightly, forming focal contacts within a shorter time of cultivation [40]. Surface charge of substratum is a very important factor for the adherence of Vero cells [3, 6, 7, 41]. We have successfully induced surface modifications to improve cell adhesion. The results of Vero cells cultured in vitro showed that the creation of polar functionalities on PHBV surface was favorable for the adherence and spreading of Vero cells due to the enhancement of the interaction between membrane polar groups and the surface cells.

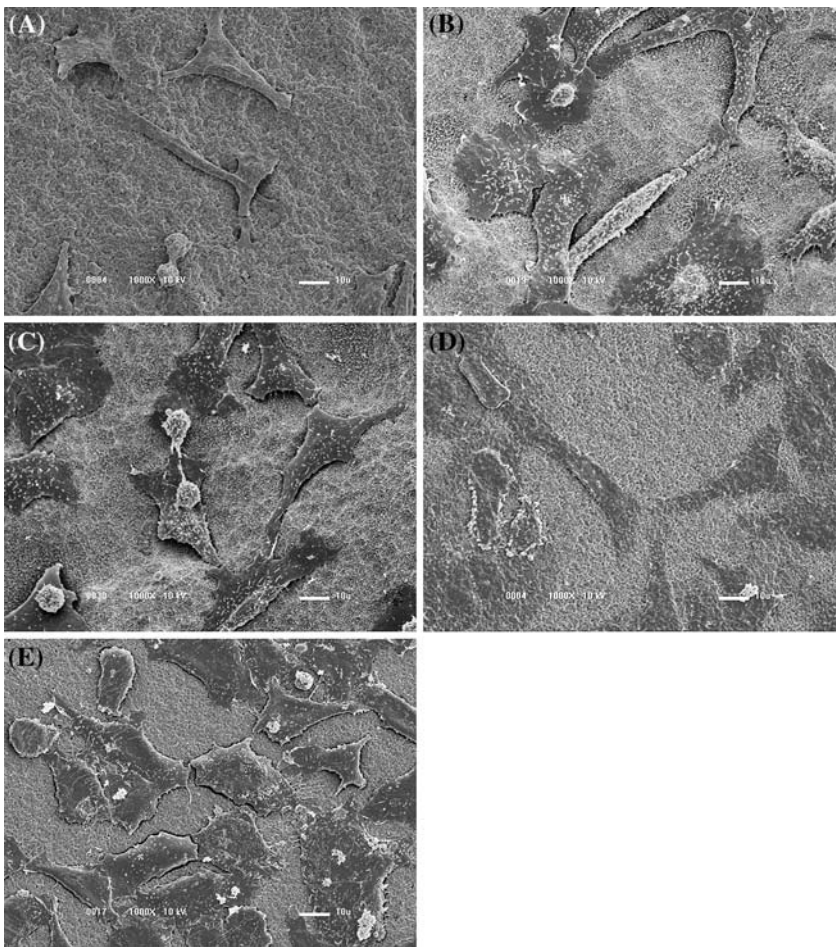

Fig. 4 Ultrastuctural view (SEM) of Vero cells on PHBV membranes after 2 h. (**A**) Untreated PHBV, showed few adhered cells; (**B**) PHBV O_2 (1) showed round cells with thicker filopodia, lamellipodia and vesicles; (**C**) PHBV O_2 (2) showed features similar to those of PHBV O_2 (1); (**D**) Notice flat cells stretched on PHBV N_2 (3) and PHBV N_2 (**E**). Bar: 10 μm

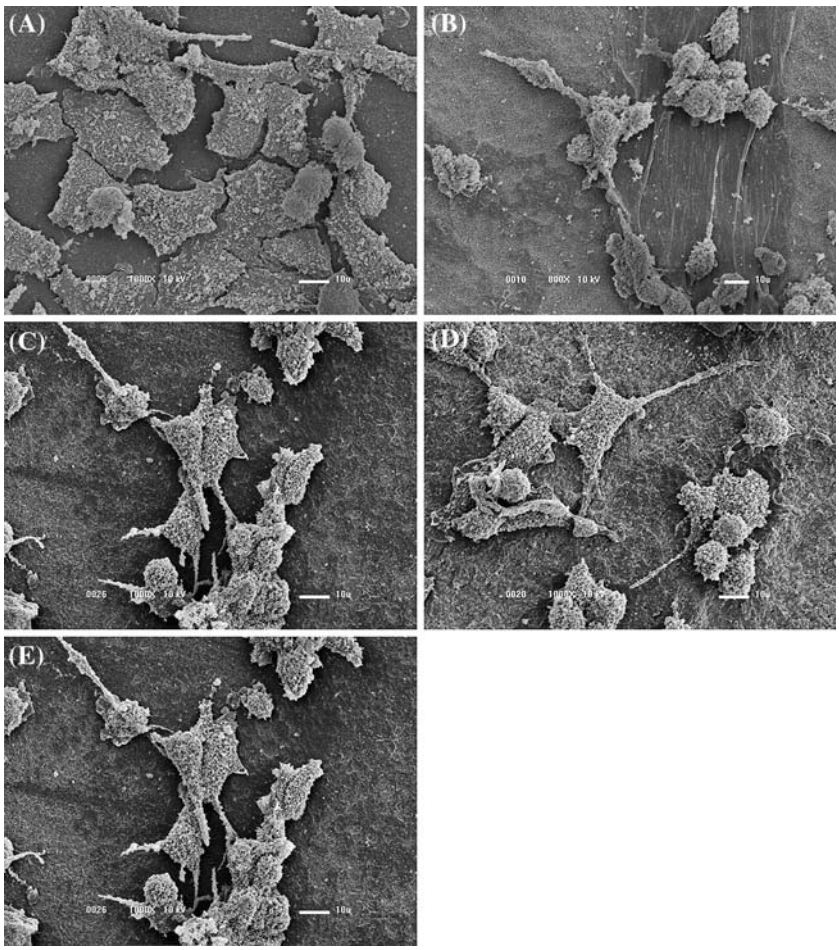

Fig. 5 Ultrastructural view (SEM) of Vero cells on PHBV after 24 h of cultivation. (**A**) Untreated PHBV has lamellipodia outlines similar to those observed on membranes treated with PHBV (**B**–**E**); (B) PHBV O_2 (1) showed more round cells as also observed on PHBV O_2 (2) (C); (D) PHBV N_2 (3); (E) PHBV N2 (4) showed more lamellipodia than PHBV O_2. Bar: 10 μm

Consequently, the cells could grow and proliferate naturally in a short lapse of time. Nitrogen plasma enhances rapidly (2 h) the interaction between Vero cells and PHBV membrane by providing an anchor for the substrate and promoting spreading, thus the proliferation ratio observed is higher than the proliferation ratio on oxygen-treated and untreated membranes. Therefore, it can be concluded that the charge of the substrate surface would greatly influence the growth of Vero cells in adhesive experiments [42].

After surface attachment, the high number of round cells observed on all plasma treated membranes at 24 h (Fig. 5) could be associated with cell proliferation during this time, demonstrating that plasma treatment not only support adhesion but proper division and growth. A previous study demonstrated that very low cell proliferation ratio are due to a lower attachment ratio on negative charged membrane which results in a negative proliferation ratio, indicating that many cells die within a short period [43].

After 48 h in culture, all seeded cells were anchored on the membranes treated by plasma and displayed flattened and spreading morphology, mainly on N_2 treated PHBV (Fig. 6), as the result of a good arrangement of (COOH and NH) polar groups on the treated substrate that improves its cytocompatibility with Vero cells. Cell number reached confluence on all samples, as it was observed in long-term culture, and also displayed a behavior of aligned fibroblastic cells that probably increased their adherence to surface, as compared to cells seeded on polished surfaces [40, 44].

It is well known that roughness is an important parameter that can affect the enhancement of surface wettability. However, in this case the improvement of surface hydrophilicity can not be only attributed to the roughness changes but also to the chemical modifications on the surface of the material [45]. It is speculated that the increased roughness, as obtained in the present study on NaOH-treated PLGA scaffolds, may be an important parameter that promoted the chondrocyte function. Nevertheless, it is clear that chemical changes occurred due to NaOH treatment of PLGA [46].

Conclusion

Our results showed that plasma treatment of PHBV increases the nano-roughness pattern of PHBV films and consequently, resulted in a moderate hydrophilicity. This physicochemical change modified the behavior of Vero

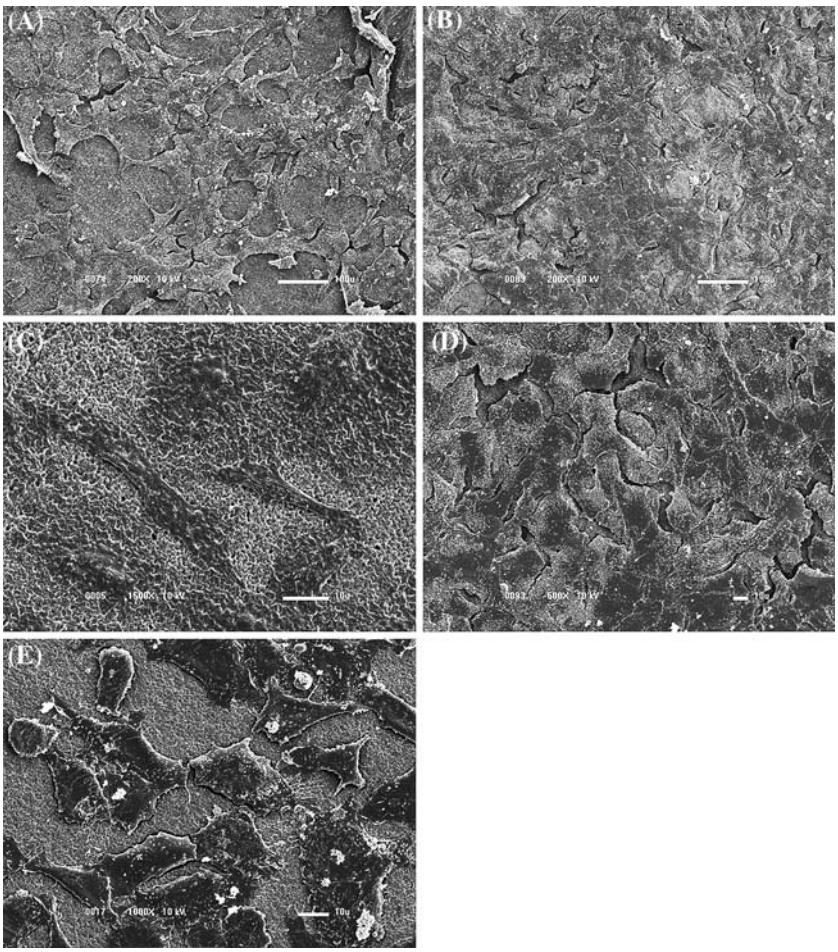

Fig. 6 Ultrastructural view (SEM) of Vero cells on PHBV membranes after 48 h of cultivation. (**A**) Untraeted PHBV; (**B**) PHBV O_2 (1); (**C**) PHBV O_2 (2); (**D**) PHBV N_2 (3); (**E**) PHBV N_2 (4). Cell number reached confluence in all samples, as it can be observed in long-term culture, and displayed flattened and spreading morphology. A, B, E scale bar: 100 μm; C–D, bar: 10 μm

cells stimulating cell adhesion, cell growth and spreading. At a very early time of cultivation, it was shown that cell size on untreated PHBV was smaller than the size of the cell growing on oxygen treated membrane. More flattened cells were observed on the nitrogen treated PHBV membranes. The qualitative and quantitative results indicate that cells were better spread on nitrogen treated surface. Our results are similar to those found elsewhere in the literature concerning cell growth on biomembranes after plasma treatments. This study has also shown that the best parameters for adhesion of cells cultivated on biomaterials are the intermediate levels of exposure to plasma pressure and potency [PHBV (2) and (4)], which is in accordance with the literature. Nevertheless, adherence and growth were not as flattened as it was observed in cells cultivated on PHBV that had been exposed to high levels of plasma pressure and potency [(1) and (3) plasma]. In these PHBV treatments (2 and 4), cells also presented a great amount of small microvilli on their surfaces. It was also shown in the current study that the cells adhere, spread, and grow in an easier manner on substrates with moderate hydrophilicity.

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