Cell Culture on PCL/PLGA Blends

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ABSTRACT: Bioresorbable polymers have been studied as support for cell culture in the tissue engineering area. Osteoblastic cells were cultivated on poly(ε-caprolactone), poly(lactic acid-*co*-glycolic acid), and (70/30), (50/50), and (30/70) blends. Cytotoxicity and cell adhesion assays and scanning electronic microscopy studies were described. The cells presented significant growth on the blends, showing no cytotoxic response. Results indicated that these blends are promising as devices for bone tissue applications. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 2609–2615, 2010

Key words: biomaterials; poly(ε-caprolactone); poly(L-lactic acid-*co*-glycolic acid); cell adhesion; osteoblast

INTRODUCTION

The treatment of bone defects remains a critical challenge in reconstructive surgery. Currently, autograft bone provides the best available solution for repairing bone defects caused by tumors and serious trauma, but even this approach has limitations. Patients frequently suffer from donor-site morbidity, and there is only limited supply of bone available for grafting. Bone tissue engineering has the potential to solve many of the current clinical challenges, which require large amounts of bone in specific sizes and shapes.[1] Besides, tissue engineering of bone requires cellular components, extracellular matrices, and scaffolds and growth and differentiation factors.

Osteoconductive matrices used as scaffold should satisfy certain requirements. They should be designed to allow diffusion of the nutrients of the transplanted cells and guide cell organization, attachment, and migration.[2] They are prepared from biodegradable materials of natural origin like collagen, gelatin, hyaluronic acid, and biodegradable polymers such as poly(hydroxybutyric-*co*-hydroxyvaleric acid) (PHBV) and also from synthetic polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic acid-*co*-glycolic acid) (PLGA), etc.[2,3]

As all materials implanted in the organism, the polymers destined for bone regeneration should be biocompatible, molded, or polymerized *in situ* to provide good adjustment to the defect area. As essential characteristics, they should support adhesion, growth, and cellular differentiation, and allow fast diffusion of metabolites and generated residues.[4]

The *in vitro* assays with polymers are fundamental strategies for the development of the tissue engineering field.[5] In this regard, two fields have progressed: the association of osteoconductive factors with the implanted material and the interaction of sources of osteogenic cells with these materials. In both fields, the understanding of the cellular adhesion phenomena and the expressions of proteins involved in osteoblast adhesion to biomaterials is of crucial importance in the maintenance of tissue structure, cicatrization, immune response, and also in the interaction of tissue and biomaterials.[6]

The cells interaction with biomaterials is an important feature of *in vitro* biocompatibility and cytotoxicity studies. The main parameters of cell–biomaterial interaction are cell adhesion and spreading, and in many studies, a clear distinction is made between materials supporting or hindering adhesion.[7]

Bioresorbable polymers have been receiving special attention as supports for cell culture being a possible alternative for treatment of lesions and tissue losses.[8]

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Among polymers, PLGA is one of the synthetic polymers approved for clinical use in humans beings. This polymer can be processed easily, and their physical properties, chemistry, mechanics, and degradability can be manipulated to assist a specific need.

PLGA has been known as a biocompatible and biodegradable polymer that undergoes degradation through simple hydrolysis into lactic acid and glycolic acid, which are eliminated by the body through normal metabolic processes.[9] Its degradation rate can vary from some weeks up to years, by simply varying the percentage of copolymers,[10] whereas studies demonstrated that pure PLA presents a long degradation time *in vitro*.[11] The biocompatibility of PLGA has also been demonstrated in several biological tissues.[12] Notwithstanding the good results obtained with PLGA copolymer pins in cell/tissue interactions, these materials do not possess the same mechanical properties as bone and should not be implanted for long periods.[3]

Another polymer that has been thoroughly used in the last 30 years to produce bioresorbable sutures, drug delivery systems, and is now being explored to substitute bone graft, is poly(ε-caprolactone) (PCL).[13] This biodegradable polymer was noticed due to its high biodegradability, biocompatibility, and good mechanical properties.[14]

The polymeric blends are obtained by physically mixing two or more types of polymers, which have been previously investigated, and their physical and mechanical properties can be improved when compared with pure polymer.[15] Some blends, which include the mixture of a synthetic polymer and a biological polymer (for instance, collagen), have been prepared with the purpose of improving the biocompatibility of the synthetic polymer.[16] In general, the preparation of blends has been carried out with the main objective of controlling the degradation speed of the biodegradable polymer. The degradation time of material constituted of biodegradable polymer for biomedical applications can vary from months to years, depending on their hydrophilic/hydrophobic amorphous/crystalline balance. This balance can be controlled by the amount and type of polymer used in the preparation of blends.[15]

In this study, we have prepared PCL and PLGA blends with the proportions of 70/30, 50/50, and 30/70, respectively, to obtain a support that is compatible with the bone tissue and an acceptable degradation time. This work aimed at evaluating the interaction between osteoblastic cells and membranes by carrying out cytotoxicity and cell adhesion assays and cell–cell and cell–membrane interaction studies by scanning electronic microscopy (SEM).

METHODS

Preparation of blends

Membranes were prepared using poly(D,L-lactic acid-*co*-glycolic acid) (50/50) (PLGA50) (M_w 65,000 g/mol) supplied by Purac (Groningen, The Netherlands) and PCL (M_w 100,000 g/mol) supplied by Sigma (St. Louis, EUA) at PCL/PLGA50 concentrations of 70/30, 50/50, and 30/70 (w/w). Initially, a 10% (w/v) solution of polymer dissolved in methylene chloride (Merck KgaA, Darmstadt, Germany) was used to prepare the samples at room temperature. Then a polymer methylene chloride solution containing 50% (w/v) trisodium citrate ($C_6H_5Na_3O_7{\cdot}5H_2O$) was prepared (Merck, Darmstadt, Germany). Prior to use, the citrate salt was sieved to give particles of 180–250 μm in diameter. The blends were cast on glass plates and dried for ~ 24 h in a closed chamber with a constant flow of dry filtered air. After casting, the salt was removed by immersion in distilled water for 24 h followed by rinsing in ethanol for 2 h. The samples were vacuum-dried and stored in a dessicator for 5 days to ensure the total removal of solvent.

Cell isolation, seeding, and culture

Osteoblastic cells were obtained from 10 calvaria of young male adult (20 days old, 150–170 g) Wistar rats (*Rattus norvegicus*), from CEMIB (Centro Multidisciplinar para Investigação Biológica, Campinas, SP, Brazil). Following euthanasia by cervical displacement, the calvaria were aseptically excised, cleaned with a soft tissue, and washed in Dulbecco's modified eagle's medium (DMEM) (Nutricell-Nutrientes Celulares, Campinas, SP, Brazil) containing 0.2*M* L-glutamine (Sigma Chemical, St. Louis, MO) and 150 μg/mL gentamicin sulfate (Sigma). This concentration of antibiotics was three times the normal amount used in cell culture and was used as a precautionary measure to avoid contamination during harvest. In sterile atmosphere, the calvaria were fragmented and submitted to enzymatic digestion for cellular isolation in DMEM medium and 1 mg/mL of collagenase type 1A (Sigma Chemical) during 2 h at 37°C. After this procedure, it was submitted to a three-stage centrifugation for 10 min at 240 g force, and subsequently resuspended in DMEM medium containing 10% of fetal bovine serum (FBS) and antibiotic. After centrifugation, the pellet was resuspended in DMEM medium and cellular viability was quantified by staining with Tripan Blue vital stain (Sigma) in a Neubauer camera. Osteoblasts were seeded at a density of ~ 10^5 cells/mL in culture flasks (TPP-Techno Plastic Products, Trasadigen, Switzerland) containing DMEM medium, supplemented with 10% SFB and 25 μg/mL

gentamicin, 10 m*M* β-glycerol phosphate (Sigma), and 50 mg/mL L-ascorbic acid (Sigma) containing 10 n*M* dexamethasone (Sigma) to promote cells with osteoblastic phenotype, which were used in experiments after the third subculture. The flasks were incubated in a humidified incubator at 37°C (5% CO_2/balanced air). When cells reached 80% of confluence, cell cultures were considered to have reached full growth and the cells were enzymatically lifted from the flask by using a 625 mg/mL trypsin solution and monitored daily with an inverted microscope, Eclipses TS 100 (Nikon, Tokyo, Japan). Statistical differences ($P < 0.05$) were determined using variance analysis (ANOVA) followed by Tukey's test for multiple comparisons.

Cell adhesion and cytotoxicity assays

Identification of cell adhesion and late cytotoxicity on blends were carried out by performing the MTT assay, a modification of Mosmmann[17] method, which was used for both cell adhesion and direct cell cytotoxicity assays.[18] Previously, the sterilized blends ($n = 6$) were placed in a 96-well plate (Corning, USA) with 100 μL of culture medium and incubated at 37°C for 24 h. After incubation, 2×10^5 cells/mL in 100 μL DMEM medium supplemented with 10% FBS were added to the wells containing the membranes. The cells were cultured for 2 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 DMEM medium, a MTT assay mixture [10 μL per well, containing 5mg/mL of 3-(4,5-dimethylthia zol-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 sulfoxide (DMSO, Sigma) and 25 μL of glycine/Sorensen buffered solution replaced the assay mixture in each well to dissolve the formazan crystals, according to Santos et al.[18] Absorbance was quantified by a spectrophotometer at 540 nm, using a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA). MTT is a colorless tetrazolium salt that forms a dark compound when oxidized by mitochondria, which is detected by spectrophotometer. For the cytotoxicity assay, latex membranes were used as positive control, and the culture plate (polystyrene) was used as negative control. For cell adhesion evaluation, Teflon dishes were used as negative control, and the culture plate itself was used as positive control. The membranes were sterilized by UV irradiation for 30 min. Absorbance of all experimental conditions 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

The blends were sterilized by UV irradiation for 30 min and placed in 96-well plates. Approximately 2×10^5 cells/mL osteoblastic cells in DMEM medium supplemented with 10% FBS (Sigma, St. Louis, MO) were seeded in each well containing the membranes and cultured at 37° C. After 6, 24, and 168 h, the samples were fixed in a fixative solution prepared by dissolving 2.5% paraformaldehyde, 2.5% glutaraldehyde, 0.06% picric acid, 1% tannic acid in 0.1*M* cacodylate buffer, and the same volume of DMEM medium for 1 h at room temperature (RT), washed in 0.1*M* cacodylate buffer, postfixed 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 SEM.

RESULTS AND DISCUSSION

After enzymatic digestion, the adherence of osteoblastic cells to the culture flask TPP (Techno Plastic Products, Trasadigen, Switzerland) was delayed in ~ 24 h. Initially, they presented round morphology, and after 48 h, the cultivated cells already presented dispersed proliferation and cytoplasmatic projections. When in confluence, the cells acquired a cuboid morphology, which is typical of osteoblastic cells in culture.[6]

Cytotoxicity analyses of blends

The results expressed in absorbance obtained after the samples were read in the microplate reader (540 nm) are presented in Figure 1. The variance analysis (ANOVA) showed significant differences between the positive, negative controls, and the samples studied ($P < 0.05$). Through Tukey's test, it was shown that the blend composition 70/30 presented a better result when compared with the blend 50/50 ($P < 0.05$). All blends assayed showed absorbance indexes higher than the negative control ($P < 0.05$), demonstrating that polymeric materials or their 70/30, 50/50, and 30/70 compositions do not present cytotoxicity indexes. All the tests developed are in agreement with standardization norms of ISO and evaluation of biomedical devices.[19,20]

The yellow stain of the reagent MTT is converted inside the mitochondria into a dark blue stain, formazan (translated by the largest absorption of MTT), which demonstrates that cellular mitochondrial activity is present. Although toxic substances do not

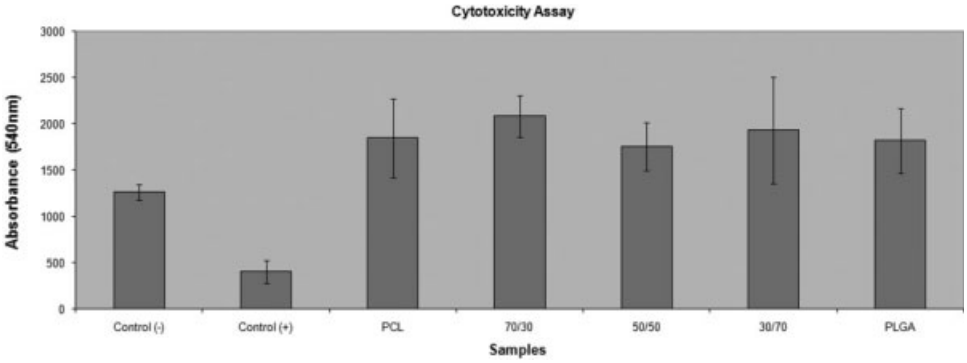

Figure 1 Cytotoxicity assay results obtained after 24 h of cell culture (negative control = culture plate; positive control = latex membranes embedded in phenol; $n = 6$).

act in a specific cellular level, they can affect their functions,[21] thus early determination of mitochondrial activity in terms of cellular sensitivity, in our opinion, was enough to evaluate cellular viability with respect to subsequent proliferation. Absence of cytotoxicity does not check on the material biocompatibility; yet the determination of cytotoxic potential is an important issue in standard tests such as ISO.[19]

Our results are in agreement with the literature. Rezende et al.[12] used PLGA membranes as skin curative in Wistar rats and did not observe cytotoxic effects on tissues. However, the rate of PLGA degradation should be evaluated on the short and long terms, once this copolymer possesses as characteristic a high degradation rate depending on its composition.[22] The degradation products of α-hydroxy acids alter the pH and could cause cytotoxic effects, in which degradation is greater than "rebalance" of local pH.[23] According to Seal et al.,[3] this degradable material serves as support for cell proliferation and secretion of the extracellular matrix. Currently, other products have been studied for specific applications in skin reconstruction, including fibroblasts and keratinocytes culture in PLGA microspheres and other materials. Kweon et al.[24] reported that PCL also allowed the growth of osteoblastic cells on samples. In this report, they have shown that the cells were capable of proliferating on different PCL scaffolds. Other reports show that PCL membranes and blends of PCL-Nafion do not produce lethal effects to osteoblastic human cells.[25] The compositions assessed in our study are physical mixtures of PLGA and PCL, and it was already anticipated that the blends would not result in toxicity. The cytotoxicity assays of these polymers indicate good tolerance of osteoblastic cells and absence of toxicity. However, confirming that a material is not toxic to cells does not mean that it will be a good substratum. The interaction of the biomaterial with the cells through cellular adhesion is also necessary.

The first step for the selection of a material to be used as implant in humans is to evaluate the toxicity and damages that it might cause to tissues. The great majority of biomaterial compositions developed is rejected in this stage because of their toxic effects that can lead to cell death or promote strong tissue reaction, such as inflammatory processes or even tissue necrosis. Thus, toxicity evaluation of materials is a problem to be solved.

Cell adhesion analysis of blends

The results obtained through cell adhesion assays are shown in Figure 2. The statistical analysis of the materials showed significantly superior indexes to the negative control (Teflon disks) ($P < 0.05$). The blend composition 30/70 presented indexes similar to the positive control (polystyrene plate) ($P < 0.05$). There were differences between the 70/30 and 30/70 compositions, and the blend 30/70 presented a better absorbance index than 70/30 composition ($P < 0.05$).

All the evaluated membranes, independent of their composition, presented adhesion capacity; however, the ultrastructural differences must be emphasized. According to Vogler and Bussian,[26] the rate of initial adhesion in plastic substratum is a good parameter to characterize the compatibility of materials. Despite the slow cell adhesion in synthetic materials described in the literature,[27] in our studies, the polymers showed satisfactory results in the analysis of cell adhesion independently of the composition used. It is also described in the literature that the surface of polyesters is hydrophobic[23] and that most of the cells adhere more easily to hydrophilic surfaces.[28] Nevertheless, van Kooten et al.[29] mentioned that only wettability is not a decisive factor to improve adhesion and cellular spread. In our studies, cell adhesion was not apparently affected by surface hydrophobicity, but other factors might have interfered in this process, once the adhesion between

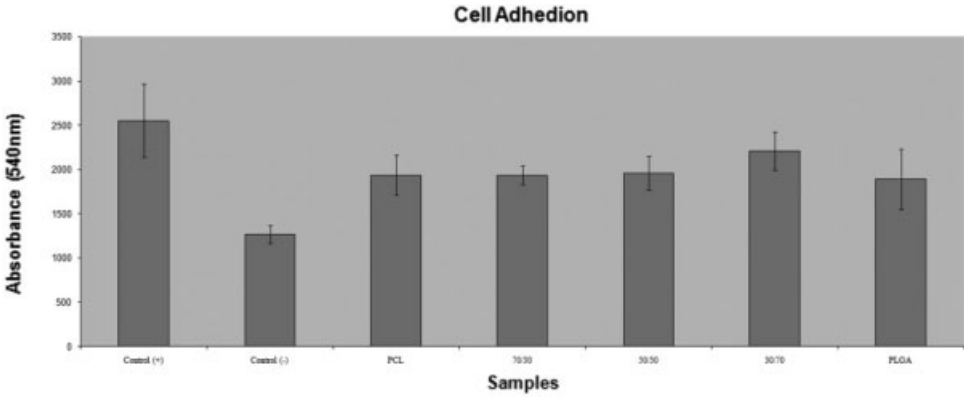

Figure 2 Cell adhesion assay results obtained after 2 h of cell culture (positive controls = culture plate; negative control = teflon dishes; $n = 6$).

osteoblasts and the surface of synthetic materials depends on the adhesion of molecules, specific membrane receivers, besides factors such as interaction of electric charges, hydrogen bonds, electrostatic and characteristic topographical forces of the polymer,[30] and the properties of the surface of the material that have a crucial role in cell adhesion behavior. Similar results were found by other researchers who have cultivated osteoblastic human cells in PLLA and PLGA membranes and reported that osteoblasts have a quite slow pattern of adhesion on PLLA substrate, but PLGA was shown to be a more adhesive substratum to this cellular type.[8] PCL was also demonstrated to be a polymer that is capable of allowing not only the cell adhesion but also its proliferation.[25]

Tang and Hunt[31] carried out studies with PCL and PLGA in the compositions 90/10, 80/20, and 70/30. The blends showed a rough and porous morphology after degradation in culture medium mainly due to PLGA degradation, and the 70/30 blend presented the most porous structures. This study is in agreement with our results that reported that the 30/70 blend containing more PLGA is more porous than the other blends, which is probably the reason it provides better structures to the adherence of osteoblasts.

Ultrastructural analysis of cell adhesion

Through SEM, it was observed that cells adhered to all the samples studied, allowing osteoblasts to establish on surfaces and start cell polarization for the extension of cytoplasmatic projections; however, morphologic alterations were observed depending on the composition of blends [Fig. 3(A,B)]. These results are in agreement with the literature that describes that once the adhesion on substratum is established, the cells start to respond to it. Usually, in response to the "signs" originated by the substratum, cells alter their morphology, growth and proliferation, differentiation pattern, and behavior or even several of the parameters mentioned herein, simultaneously.[27]

SEM analysis allowed visual confirmation of the good polymer/osteoblastic cell interaction. After 6 h of culture, the osteoblastic cells adhered to the substrates presented prolonged morphology with ramifications of cytoplasmatic projections in most of the samples, and the presence of cells with round morphology on 100/0 and compositions with higher amounts of PCL (70/30) was observed. It was not possible to observe significant differences in the number of cells on the different membranes; however, the blend 0/100 showed the greatest amount of particulate material on cellular surfaces.

After 24 h of culture, it was observed that osteoblastic cells cultured on PLGA blends and its compositions, 30/70 and 50/50 [Fig. 3(C–E)], presented more prolonged morphology, whereas on PCL blends and composition 70/30 [Fig. 3(A,B)], osteoblastic cells were shown to be more dispersed with greater amounts of fine fillopodes, besides large lamellipodes occupying a larger area of the samples in relation to the other compositions. In all blends, the presence of structures similar to microvillosities was noticed. The presence of microvillosities in cellular surfaces evidences that the cell maintained its biosynthetic capacity. Moreover, cell adhesion to the substratum not only stimulates proliferation but also its biosynthetic activity.[32] A greater amount of cells was observed in 70/30 blends and pure PCL. These cells presented cytoplasmatic projections extended from other osteoblastic cells showing a sensitive change in its form, the cells assumed a flat morphology, some times prolongated other times round on the substratum. In fact, if cells are very flat, it is an indication that a great cellular interaction with the substratum has occurred.

It was possible to observe that the osteoblastic cells present in all samples showed metabolic

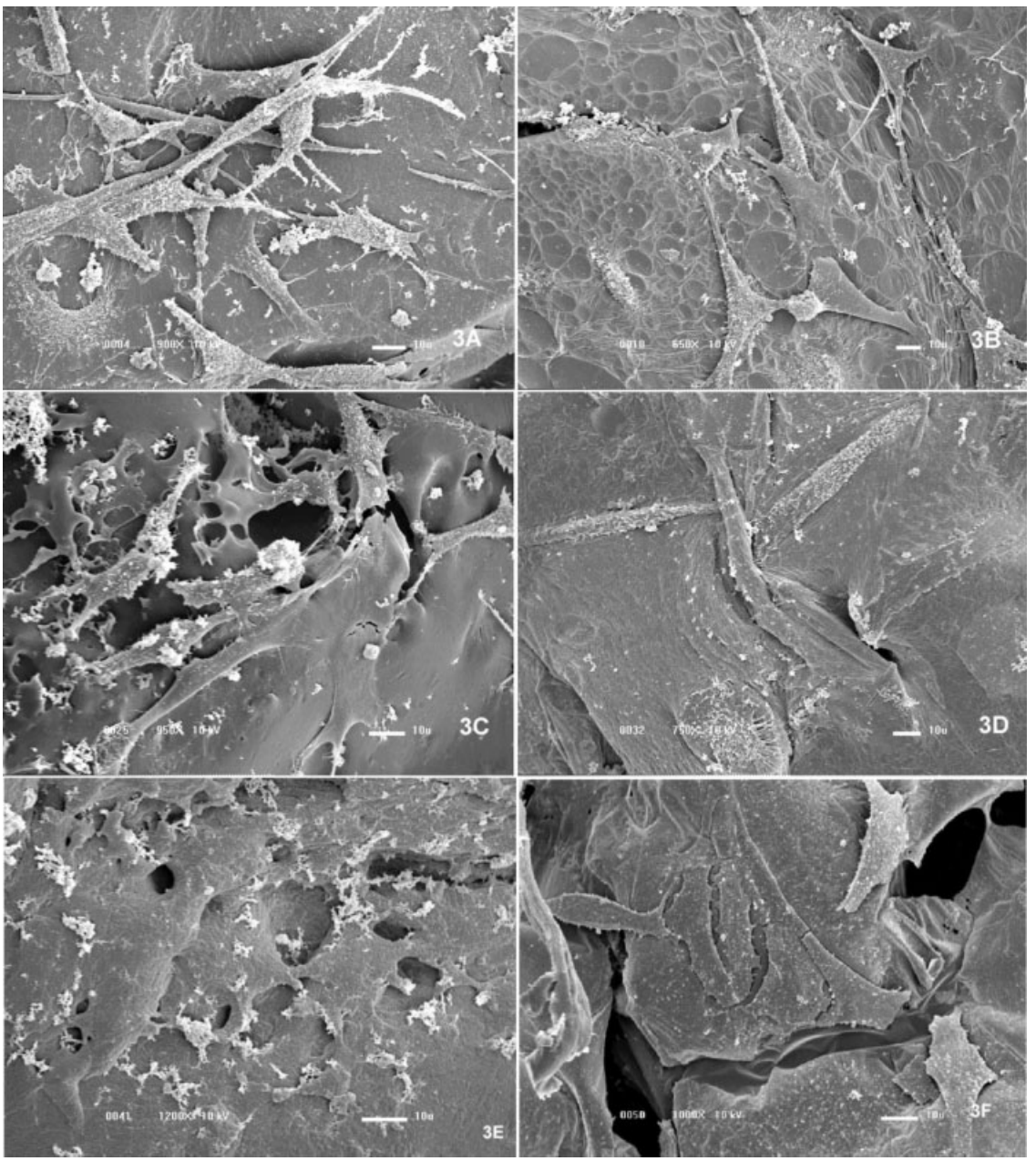

Figure 3 Scanning electronic microscopy of osteoblastic cells cultivated on the different substrates. (A) After 24 h of culture: (A) 100/0 blend; (B) 70/30 blend; (C) 50/50 blend; (D) 30/70 blend; (E) 0/100 blend; (F) Note: The organic material on cell surfaces and confluence was observed in all compositions after 168 h in culture.

activity because of the emergence of vesicles, which suggests deposit of organic material on the polymeric substrates. The interaction of cells with porous PCL and PLGA and their compositions is high, given that they are capable of growing and proliferating on those blends. Ciapetti et al.[33] studied osteoblastic cells growing on PCL macro and microporous supports with the addition of hydroxyapatite and noticed that in both supports the cells were interlinked by cytoplasmatic prolongations. In addition, they observed signs of bone differentiation such as the production of alkaline phosphatase and deposition of calcium salts on the substrate after 4 weeks of culture.

After 168 h of culture, a greater density of cells on the blends was noticed [Fig. 3(F)], i.e., the cells covered the samples in monolayer and presented cuboid morphology which is characteristic of osteoblastic cells when *in vitro* confluence.[6] These cells were also observed to grow very close to each other suggesting an intimate connection among them [Fig. 3(A–D)]. In this condition, in some areas, the delimitation of the cells and their respective cellular limits is difficult to be noticed [Fig. 3(B,C)]. The presence

of organic material on cell surfaces was shown to be evident in membranes, except on PCL blends and 70/30 composition, whereas the amount of organic material seemed to be greater on PLGA blends [Fig. 3(E)]. Briefly, PCL and 70/30 blend with a higher proportion of PCL presented initially higher amounts of organic material, which was inverted after 168 h of culture, whereas larger amount of organic material was observed on PLGA blends and on 30/70 composition in the same period of culture. This is probably due to the fact that cells are in different maturation stages depending on the polymeric composition studied.

The cells also seemed to have preference for macro and micropores formed when they were formed with sodium citrate lixiviation. They adhered to the innerside of macropores and also cast out cytoplasmatic projections over them. Whenever cells adhered inside micropores, a similar behavior was noticed; however, there were instances they were avoided. According to Berry et al.,[34] the cells are sensitive to topography alterations, which influences cellular mobility and possibly cell proliferation. In studies in which the PCL surface was modified by the alteration of its nanotopography, an increase in the adhesion rate of osteoblastic cells was shown.[32] However, according to Anselme,[6] through the melting method employed to obtain pores with sodium citrate salt, the interconnection that is necessary for cell–cell contact among the pores cannot be preserved.

Our results are in agreement with similar studies with PCL and PLGA blends developed by Tang et al.,[35] in which they reported that osteoblasts adhered and spread throughout all blends that presented polygonal shaped cells.

SEM results indicated that PCL, PLGA polymers, or their blends are promising for the development of devices for bone tissue engineering, as they bring together other features, for instance, mechanical properties.

CONCLUSION

The results obtained demonstrated that all blends presented characteristics that are desirable in devices for bone tissue applications, such as adhesion, growth and cellular proliferation, and absence of cytotoxic effects.

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