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Lithograph-Moulded Poly-L-co-D,L Lactide Porous Membranes for Osteoblastic Culture

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Pore size, shape, wall morphology, porosity, and interconnectivity are important characteristics of the scaffolds. Lithography is a manufacturing technique that allows the production of tridimensional scaffolds with a controllable and reproducible inner architecture. The aim of this study was to use lithography to create a poly-L-co-D,L lactide (PLDLA) scaffold with symmetrical pore size and distribution, and to evaluate its biocompatibility with osteoblasts *in vitro*. Lithographic moulds were used to produce porous PLDLA membranes by a casting procedure. Osteoblasts were removed from calvarial bones and seeded onto porous and smooth PLDLA membranes after which cell viability and adhesion assays, cytochemical analysis and scanning electron microscopy were used to characterize the cells. Cell viability and adhesion assays, cytochemical analysis, and scanning electron microscopy were carried out. Cell viability was similar on porous and smooth PLDLA membranes but higher than on a polystyrene substrate (positive control). Although osteoblasts adhered to the surface of all the materials tested, cell adhesion to lithographed PLDLA was greater than to smooth PLDLA membranes. In conclusion, osteoblasts interacted well with PLDLA membranes, as shown by the viability and adhesion assays and by the enhanced collagen production.

Keywords: *bone tissue engineering, cell adhesion, lithography, osteoblastic cells, poly-L-co-D,L lactide, porosity, viability*

1. Introduction

In tissue engineering, cells cultured on biomaterial surfaces provide a simple method for screening the biocompatibility of materials prior to testing *in vivo* testing[1,2]. Pore size, shape, porosity and interconnectivity are important characteristics of scaffolds. These characteristics are important for cell attachment, growth and new tissue formation, diffusion of nutrients and metabolic waste products to and from the implant, and angiogenesis[3].

Bone tissue engineering is based on studies of bone cells cultivated on biomaterials *in vitro*[4]. Mesenchymal stem cells seeded on scaffolds can dramatically accelerate bone regeneration when compared to scaffolds without cells[5]. Osteoblasts synthesize collagen type I that is involved in bone mineralization and this collagen production is influenced by the biomaterial on which these cells are seeded. Consequently, the compatibility of biomaterials can be partially characterized by the amount of minerals produced as the cells grow on the polymer surface[6].

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The quality of the pores in scaffolds used for bone tissue engineering is important, with pores >100 μm in diameter being recommended for biomaterials used as bone substitutes. Soluble particles, including salts and carbohydrates, or hydrophobic systems are used to produce pores in a polymeric matrix but allow only partial control of porosity. In addition, porogen waste and solvent residues can be toxic when the scaffold is applied *in vitro* or implanted *in vivo*[7]. An alternative to porogen substances is the lithography, a controlled manufacturing technique that allows the production of tridimensional (3D) scaffolds with a controllable and reproducible inner architecture[8].

Patterned surfaces produced by lithographic techniques show improved adhesion of rat mesenchymal stem cells and greater proliferation on scaffolds containing hyaluronic acid biofunctionalized with peptides[9]; the proliferation of osteoblast-like MG-63 cells and rat mesenchymal stem cells on Bioglass®-based glass-ceramic scaffolds is also enhanced[10]. A micropatterned surface facilitates the alignment, elongation and colonization of human

osteoblastic-like cells[11] and rat mesenchymal stem cells[12] *in vitro*. The use of lithography to modify the surface morphology of titanium substrates enhances the osteointegration between implants and bone tissue[13].

Poly-L-lactide can be used as a scaffold for bone tissue engineering[14,15]. Poly-L-lactide (PLLA) is a biomaterial that shows increasing crystallinity during degradation[16]. The co-polymer poly-L-co-D,L lactide (PLDLA) has similar mechanical features to poly-L-lactide without the inconvenience of long degradation and high crystallinity. Moreover, PLDLA is a polyester that can be hydrolyzed into lactic acid monomers and eliminated through the tricarboxylic acid cycle[17]. Finally, PLDLA-based bioresorbable device are entirely replaced by cells and extracellular matrix[18].

The aim of this study was to use lithography to create a PLDLA scaffold with a symmetrical pore size and distribution and evaluate its biocompatibility with osteoblasts *in vitro*.

2. Material and Methods

2.1. *Mould fabrication using lithography*

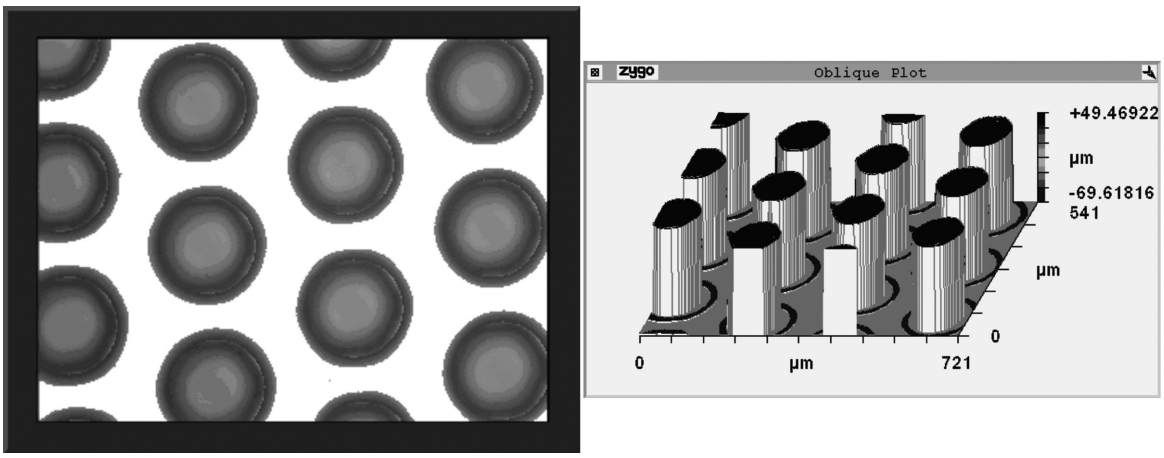
The mould was designed using the computer-aided design (CAD) software AutoCAD® (Autodesk Inc. San Rafael, CA, USA.) in drawing exchange format (DXF). The design was a bidimensional (2D) hexagonal array of closed polylines with 100 µm between two opposed vertices. The distance between two contiguous pillars was 200 µm. The design was converted to GDSII stream format (industry standard database binary file format) to generate a mask. The chrome dark field glass mask was produced using electron beam lithography equipment (Electron Beam Microfabricator EBMF 10.5 Leica Lithograph, Cambridge, UK) and a positive tone resist EBR9 (Toray Industries Inc., Tokyo, Japan) process with a resolution of±0.1 µm. A 1.52-mm-thick borosilicate glass plate was used as the mould substrate. The plate was immersed in sulfur acid for 7 min at 60 °C followed by spraying with deionized water (DI). The samples were immersed in a heated ultrasonic detergent bath for 7 min followed by a DI spray and immersion in an isopropyl alcohol dehydration bath for 7 min, after which they were dried in isopropyl alcohol vapor. The substrate was covered with SU-8 50 resist (Microchem, Newton, MA, USA) by spinning at 1,500 rpm for 40 s to yield a 100 µm thick film and then heated on a hot plate at 65 °C for 5 min and cured at 95 °C for 15 min. Subsequently, the substrate was exposed to ultraviolet light using a G-line mask copier (Tamarack model 155, Tamarack Scientific Co. Inc., Corona, CA, USA.) at an energy of 350 mJ/cm^2 after which the exposed film was immersed in SU-8 developer for 4 min. The resulting SU-8 structures were hardened by curing on a hot plate at 150 °C for 5 min. The lithographic process yielded an array of pillars 100 µm in height. The resulting mould was characterized by using an optical profilometer (Zygo NewView 5032, Zygo Corporation, Middlefield, CT, USA; Figure 1).

2.2. *Membrane preparation*

Poly-L-co-D,L lactide (PLDLA; Mw=205,000 Da) was prepared by ring-opening polymerization, as previously described by Motta and Duek[19] using of L-lactide and D,L-lactide monomers (70:30, w/w) (Purac Biomaterials, Schiedam, The Netherlands). The membranes were obtained by casting. The co-polymer was dissolved in 5 % (w/v) chloroform (Sigma-Aldrich) for 2 h with mixing and poured into the mould created with the lithograph. After the solvent evaporation, the membrane was removed manually from the plate under sterile conditions. Smooth membranes were used as a control to determine whether the scaffold morphology influenced the cellular responses. The membranes were sterilized in a sterile laminar flow biohazard cabinet (Pachane, Piracicaba, SP, Brazil) with ultraviolet irradiation for 30 min and then placed in 96-well plates for cell culture.

2.3. *Osteoblast isolation and culture*

Osteoblasts cells were removed from calvarial bones of 20-day old Wistar rats, as described by Yamamoto et al.[20]. The rats were euthanized by cervical displacement followed by decapitation. The calvaria were removed, immersed in Dulbecco's Modified Eagle's Medium (DMEM) containing



(a) (b)

Figure 1. Microscopic view (a) and oblique profilometry plot (b).

gentamicin (150 µg.mL^{-1}) and amphotericin B (15 µg.mL^{-1}) and subsequently fragmented (about 1 cm^2) and subjected to enzymatic digestion with type I A collagenase (1 mg.mL^{-1}; Sigma-Aldrich) in DMEM for 2, 4 and 6 h at 37 °C. The cell suspensions were centrifuged three times at 250×*g* for 10 min after each interval. Viable cells were quantified by Trypan blue dye (Sigma-Aldrich) exclusion in a hemocytometer. The cells (10^5 cells.mL^{-1}) were plated in polystyrene tissue culture flasks (Techno Plastic Products) and grown at 37 °C in a 5% CO^2 atmosphere for 4-5 passages prior to testing. Samples of PLDLA membranes measuring 6 mm in diameter were placed in each well of a 96-well plate (Techno Plastic Products) and DMEM was added. The plates were incubated for 24 h at 37 °C in a 5% CO^2 atmosphere before cell seeding. The osteogenic medium used in the cell experiments consisted of DMEM containing 10% (v/v) fetal bovine serum (FBS; Nutricell – Nutrientes Celulares), L-ascorbic acid (50 µg.ml^{-1}), 100 nM dexamethasone, 10 mM β-glycerophosphate, 0.7 mM calcium chloride, gentamicin (50 µg.mL^{-1}) and amphotericin B (5 µg.mL^{-1}) (all from Sigma-Aldrich)[21,22].

2.4. *Cell viability and adhesion assays*

The cell viability and adhesion assays were based on reports by Mosmann[23], Lucchesi et al.[24] and Uzumaki[25]. Polystyrene (the culture plate itself) was used as a positive control in both assays. Phenol (1% solution) was used as a negative control for the cell viability assay while polytetrafluoroethylene (PTFE) dishes were used as a negative control for adhesion[26]. Six samples of each membrane type and the controls were tested (n=6). A 100 µL aliquot containing 2×10^5 cells.mL^{-1} was seeded onto membranes and controls in DMEM containing 10% FBS. Osteoblast viability and adhesion were assessed after 24 h and 2 h, respectively. Subsequently, the medium was removed and the samples were washed 3-4 times with phosphate buffered saline (PBS) after which 100 µL of a solution containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 0.5 mg.mL^{-1}; Sigma-Aldrich) in DMEM was added to each well followed by 4 h of incubation (Shel Lab 5212, Cornelius, NC, USA). The MTT solution was subsequently replaced with 200 µL of dimethyl sulfoxide (DMSO; Merck) and 25 µL of glycine/Sorensen buffer (Nutricell – Nutrientes Celulares). The resulting absorbance was determined at 570 nm using a microplate reader (Elx-800-UV, Bio-Tek Instruments, Winooski, VT, USA).

2.5. *Cytochemical analysis*

A 100 µL aliquot of osteoblasts (2×10^4 cells.mL^{-1}) was seeded on porous and smooth PLDLA membranes and cultured with osteogenic DMEM. The plates were incubated in a 5% CO^2 atmosphere at 37 °C and the culture medium was replaced every two days. After 6 h and 48 h, and 7, 14 and 21 days in culture the samples were fixed with formaldehyde, dehydrated in ethanol and stained with toluidine blue (TB), a dye that bind to basic anionic groups, xylidine ponceau (XP), a dye that binds to cationic groups, von Kossa (VK), which stains mineralization nodules, and picrosirius (PS) which stains collagen type I/III. The samples were observed with a light microscope (Eclipse E800, Nikon Americas Inc., Melville, NY, USA) and images were captured with an FDX-35 camera (Nikon) attached to the microscope.

2.6. *Scanning electron microscopy (SEM)*

The culture conditions (cell number, culture medium and length of culture) were the same as used for the cytochemical analysis. At the end of the culture period, the samples were fixed for 30 min at room temperature in in fixative containing 2.5% paraformaldehyde, 2.5% glutaraldehyde, 1% picric acid and 1% tannic acid dissolved in 0.1 M PBS, pH 7.4. Subsequently, the samples were post-fixed in 1 % osmium tetroxide for 15 min in the dark, washed in water, dehydrated with ethanol (all reagents from Sigma-Aldrich, St. Louis, MO, USA), critical point dried (Balzers CDT 030, Balzers Inc., Elgin, IL, USA) and coated with gold in a sputter coater (Balzers CDT 050, Balzers Inc., Elgin, IL, USA). The coated specimens were examined with a JEOL JXA-840A scanning electron microscope (JEOL Ltd., Peabody, MA, USA).

2.7. *Statistical analysis*

Numerical results are reported as the mean ± standard deviation. Cell viability and adhesion were analyzed statistically with one-way analysis of variance (ANOVA) followed by the Tukey test for *post hoc* analyses. A value of p<0.05 indicated significance. All data analyses were done using BioEstat version 5.0.

3. Results

3.1. *Cell viability and adhesion*

Osteoblast viability was similar in porous and smooth PLDLA membranes and not significantly different from that seen with polystyrene (Figure 2). In contrast, cell adhesion to lithographed PLDLA membranes was greater than for smooth PLDLA membranes (Figure 3).

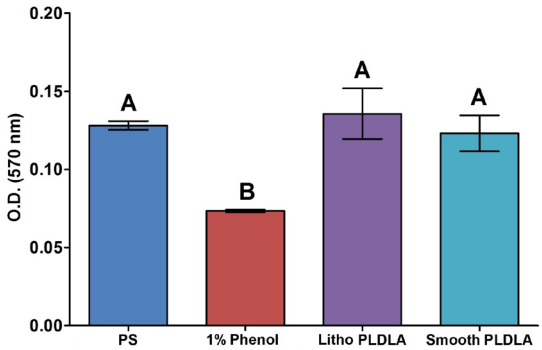


Figure 2. Cell viability assessed by the MTT assay in 24-h osteoblast cultures. The columns represent the mean ± standard deviation (n=6). Columns with the same letter did not differ significantly. There were no significant differences in viability between the positive control (polystyrene, PS) and lithographed (Litho PLDLA) and smooth (Smooth PLDLA) membranes. Cell viability in these three groups was significantly greater (p<0.01) than in the the negative control (1% phenol).

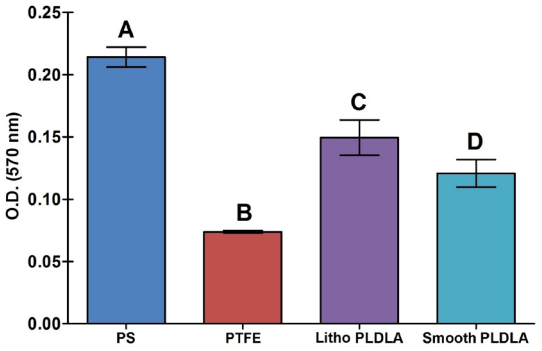


Figure 3. Osteoblast adhesion to PLDLA scaffolds. The columns represent the mean ± standard deviation (n=6). Columns with different letters were significantly different from each other. Polystyrene (PS; positive control) showed the highest adhesion and polytetrafluoroethylene (PTFE; negative control) the lowest (p<0.05 compared to smooth PLDLA and p <0.01 compared to lithographed (Litho) PLDLA). Porosity significantly increased cell adhesion (Litho vs. Smooth PLDLA; p<0.05).

3.2. *Cell morphology*

Osteoblasts adhered to the surface of all materials tested. The morphology of these cells on the surfaces of porous and smooth polymeric membranes was examined. On the 14th day and 21st day after seeding, some cells formed multiple layers suggestive of cellular proliferation and synthetic activity. The cell nuclei and cell borders were difficult to visualize in these layers. Six hours after seeding, the cells had not yet completed the spreading process because their morphology was spherical and sometimes slightly flat (Figure 4a and b; Figure 5a). After 48 h the cells had adhered and showed long, thin protrusions but were still separated from each other (Figure 4c and d). After 7 days, the cells were flat, long and spindle-shaped and were connected to each other by cellular protrusions such as filopodia and lamellipodia (Figure 5b and c); there was also marked intercellular contact indicative of proliferation (Figure 4e and f). After 14 days, several pores colonized by a cellular monolayer were observed on the lithographed membrane. Cells outside these pores maintained contact with cells inside the pores (Figure 4g and h; Figure 5d). The cell number after 14 days was greater than at previous times. By 21 days post-seeding, the cells formed very tight, multilayered structures that made it difficult to visualize nuclei and the areas inside and outside the pores (Figure 4i and j; Figure 5g and h). The presence of collagen nets showed that the cells were able to synthesize extracellular matrix (Figure 4j) and the occurrence of mineralized nodules indicated that the cells were depositing calcium phosphate crystals (Figure 4i). Some cells stretched from the bottom of the pores to their edge (Figure 5e, f).

4. Discussion

In this study, the interaction between osteoblasts and porous and smooth PLDLA membranes was evaluated. A computer designed metallic mould produced by lithography was used to obtain porous membranes. The MTT assay was used to assess PLDLA cytotoxicity (based on mitochondrial activity) and to measure cell adhesion. Morphological alterations were assessed by light microscopy after selective staining and SEM was used to examine ultrastructural features.

The measurement of mitochondrial activity is a suitable criterion for assessing cell viability since toxic substances affect not only the molecular structure but also several cellular functions[24]. PLDLA (70:30) has already been tested with regards to cytotoxicity, and the MTT, agar and filter diffusion assays have shown good cytocompatibility for this copolymer[27]. In addition, the mortality of human osteoblasts in extracts containing PLLA degradation products is ~30%, indicating low cytotoxicity[28]. These results support the low cytotoxicity reported here.

A number of studies that have investigated the use of PLLA in tissue engineering *in vivo* have reported satisfactory results for bone regeneration[29,30], guided tissue regeneration[31], nerve peripheral regeneration[32], and cartilaginous tissue[33]. PLDLA copolymer is compatible with[34] and suitable for use as a bone graft substitute[35-38], meniscus replacement[39,40], suture cords/threads[41] and axon regeneration[42].

Cellular adhesion to biomaterials is extremely important in material sciences. As shown here, more osteoblasts adhered to the surface of porous PLDLA than to smooth PLDLA. Once adhered to the substrate, the cells migrate and proliferate or show specific physiological activities, such as the production of extracellular matrix[43,44]. Wu et al.[45] reported enhanced osteoblast adhesion to scaffolds with larger pores (300-500 μm) compared to those with smaller pores (150-180 μm), and adhesion to the latter was greater than to smooth surfaces. However, other cellular responses such as proliferation and osteogenic function are not significantly influenced by pore size. Micro- and nano-scale structures on PLLA and polystyrene improve the efficiency of adhesion when compared to smooth substrates[11]. Porous PLDLA membranes (50-70 μm) enhanced mesenchymal cell proliferation, differentiation and activity when compared to smooth membranes[46]. Nevertheless, the influence of porosity and pore size is a controversial. Whiston et al.[22] showed that the surface relief (micro- and nano-scale structures) of PLLA did not enhance the metabolic activities of osteoblasts after two days, as assessed by the MTT test[11]. Bet et al.[47], reported that fibroblast adhesion to PLLA scaffolds was low and there was no difference in adhesion among membranes containing pores of different sizes. Pore quality and quantity do not influence the proliferation of osteogenic cells from rat calvaria cultivated on PLGA (75:25) for different periods of time[48]. Thus, as other studies have demonstrated, the interaction between osteoblasts and materials with variable porosities and pore sizes does not affect the cellular response[49,50]. Consequently, different cell types respond differently to the substrate surface topography[11].

The light microscopy and SEM findings described here indicate that osteoblasts adhered to and spread over smooth and porous membranes, in addition to showing the spindle-shaped and polyhedral cells characteristic of osteoblasts. None of the images showed cells bridging or

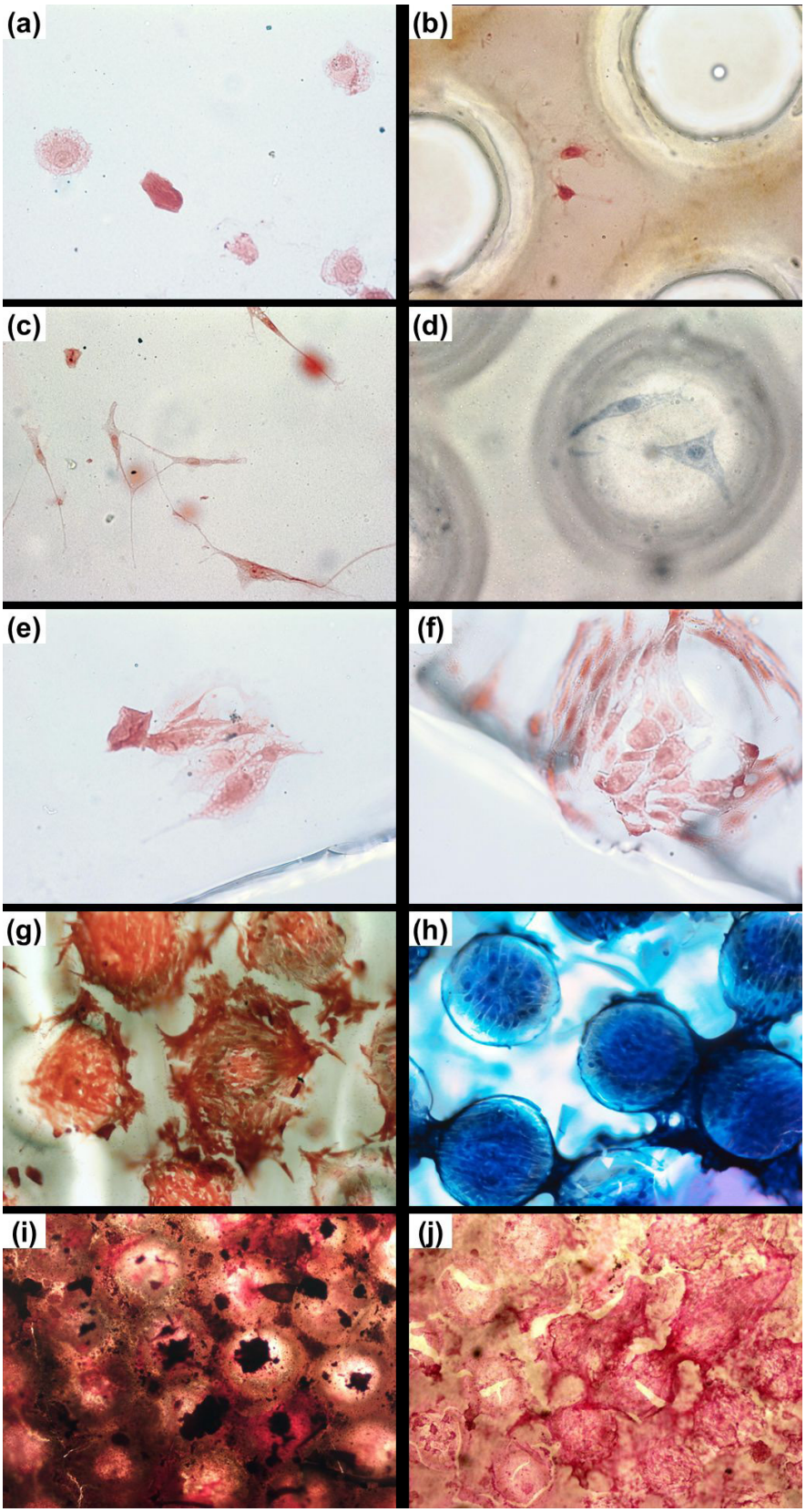


Figure 4. Osteoblasts cultured on PLDLA for 6 h (a and b) and 48 h (c and d), and 7 (e and f), 14 (g and h) and 21 (i and j) days using porous (b, d, f, g-j) and smooth (a, c and e) membranes. Staining with xylidine ponceau (a-c, e-g), toluidine blue (d and h), picrosirius (j) and von Kossa (i) dyes. Magnifications: 400× (a, b, d-f), 200× (c and g) and 100× (i and j).

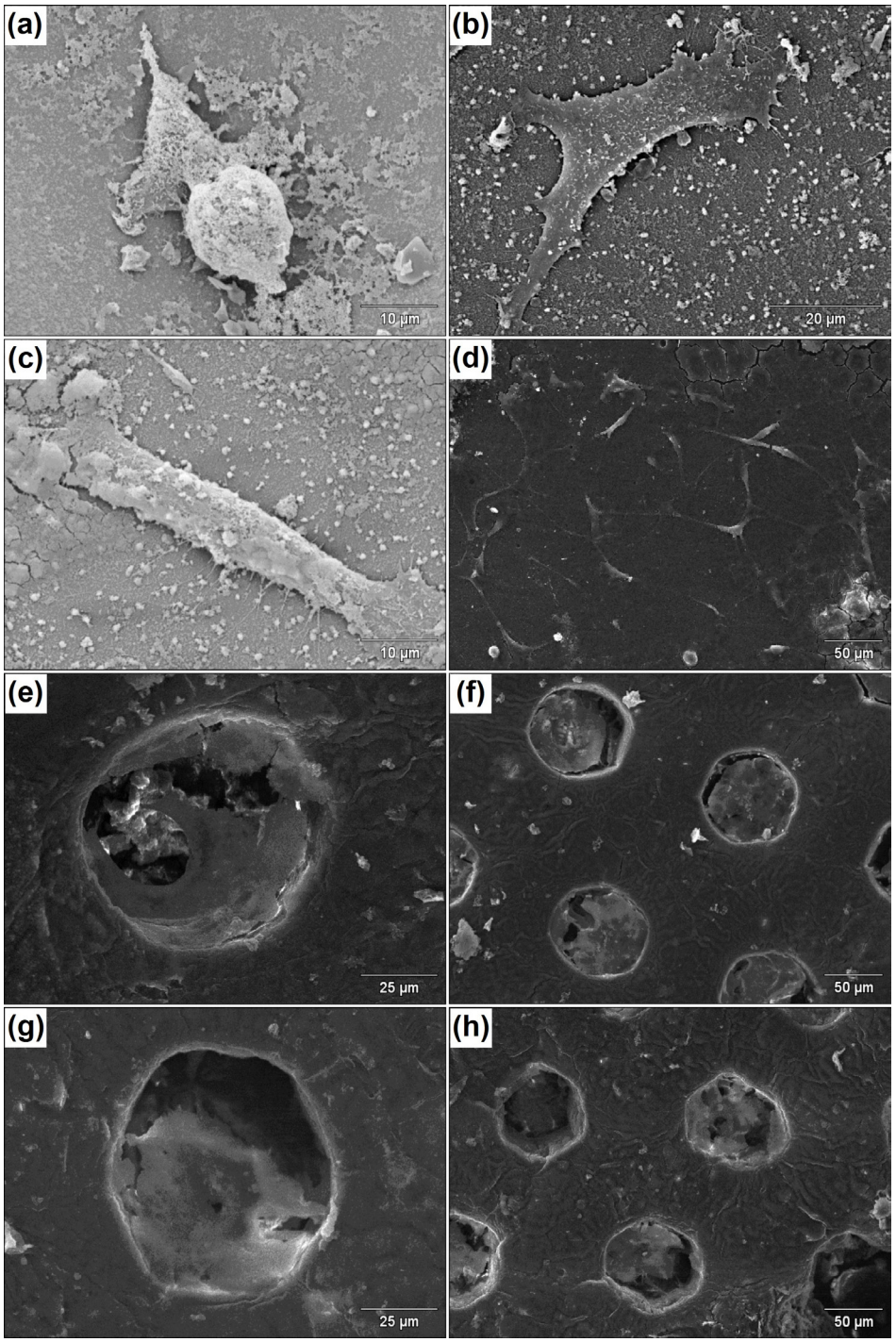


Figure 5. Scanning electron micrographs of osteoblasts on PLDLA membranes. (a) Cell showing incomplete adhesion after 6 h. (b and c) Presence of filopodia and lamellipodia after 2 days. (d) Cells in contact with each other after 21 days. Osteoblasts colonized the pores after 14 (e and f) and 21 (g and h) days. Scale bars: (a and c) 10 µm, (b) 20 µm, (e and g) 25 µm and (d, f and h) 50 µm.

covering the pores; rather, they were frequently observed within the pores (lining the bottom or wall). Pores generally increase the surface areas of porous membranes compared to smooth membranes and it was therefore expected that cell adhesion would be greater on porous membranes. Several studies have used SEM to demonstrate the extensive colonization of biomaterials[51-53]. Although these studies have used different cell types, culture conditions and polymeric substrates, in all cases confluent monolayers with poorly defined cellular limits form a continuous cellular mat covering the scaffold surface[51-53]. In contrast to these findings, as shown here, instead of a confluent layer of cells,

large groups of cells growing separately in various areas of the membranes were observed, especially 14 and 21 days after seeding. Light and scanning electron microscopy revealed a non-homogeneous cell distribution on the membrane surfaces, with osteoblasts always concentrated in small, condensed groups; this finding agrees with the observation that cellular aggregation is an important step in ossification[54]. Indeed, membrane pores may favor cellular aggregation. Substrates with low capability to stimulate adhesion may be able to sustain cellular adhesion[55]. In addition, the differentiation and synthesis of extracellular matrix can be stimulated by materials with low adhesion and proliferation rates[8,12,13,45]. As shown here, cells that adhered to the membranes were capable of producing collagen.

The micropatterned silica films were capable of inducing guided osteoblastic cell adhesion, spreading and propagation[56]. Isotropic and anisotropic surfaces changes cell-material and cell-cell interactions[57]. Therefore, the surface topography can modulate the way the cells adhere to[57] and proliferate on[58] the material.

Pelaez-Vargas et al.[11], reported that the surface microtexture modified cell morphology and spreading, which could influence important factors such as cell alignment, migration, implant surface colonization, and function. This suggests that the surface topography and pore uniformity may control cells responses in a different manner than the increased porosity offered by alternative fabrication techniques such as solvent casting[59].

5. Conclusion

Based on the results described here, we conclude that osteoblasts interact well with PLDLA membranes. These membranes can sustain adhesion and maintain viable cells, as shown by the ability of cells to produce collagen. PLDLA membranes represent a suitable biomaterial for cultivating osteoblasts and their potential usefulness in vivo deserves further investigation. An increase in the porosity of PLDLA can enhance cellular adhesion.

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