

## Surface Characterization and Osteoblast-like Cells Culture on Collagen Modified PLDLA Scaffolds

*Bruna Antunes Más<sup>a,b\*</sup>, Silvia Mara de Mello Cattani<sup>b</sup>, Rita de Cássia Cipriano Rangel<sup>c</sup>,*

*Gabrielle de Almeida Ribeiro<sup>d</sup>, Nilson Cristino Cruz<sup>e</sup>, Fabio de Lima Leite<sup>d</sup>,*

*Pedro Augusto de Paula Nascente<sup>e</sup>, Eliana Aparecida de Rezende Duek<sup>a,b</sup>*

<sup>a</sup>Department of Material Engineering, Faculty of Mechanical Engineering, Universidade Estadual de Campinas – UNICAMP, Rua Mendeleev, 200, CEP 13083-970, Campinas, SP, Brazil

<sup>b</sup>Laboratory of Biomaterials, Pontifícia Universidade Católica de São Paulo – PUC-SP, Rua Joubert Wey, 290, CEP 18030-070, Sorocaba, SP, Brazil

<sup>c</sup>Laboratory of Technological Plasmas, Universidade Estadual Paulista – UNESP, Av. 3 de Março, 511, CEP 18085-180, Sorocaba, SP, Brazil

<sup>d</sup>Department of Material Engineering, Universidade Federal de São Carlos – UFSCar, Rodovia João Leme dos Santos, km 110, 3031, CEP 18052-780, Sorocaba, SP, Brazil

<sup>e</sup>Department of Materials Engineering, Universidade Federal de São Carlos – UFSCar, Via Washington Luiz, km 235, CEP 13565-905, São Carlos, SP, Brazil

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Surface modification techniques based on the grafting of chemical functional groups and immobilization of bioactive molecules have been used to improve biocompatibility and clinical performance of bioabsorbable scaffolds in tissue engineering and medicine regenerative applications. This study aimed at developing and characterizing a biomimetic surface to stimulate bone regeneration by a simple and low-cost method of surface biofunctionalization of the poly (L-co-D,L lactic acid)-PLDLA scaffolds. The method was obtained by grafting reaction of carboxyl groups (-COOH) on their surface via acrylic acid (AAc) polymerization process, followed by immobilization of collagen type I (Col). Such approach resulted in a surface morphology markedly modified after treatment, with increase of pores and roughness on PLDLA-AAc surfaces and a network of fibrillar collagen deposition in non-specific areas of PLDLA-Col surfaces. The cytocompatibility of collagen-immobilized scaffolds was significantly improved in terms of cellular adhesion, proliferation, collagen synthesis and maintenance of osteoblast-like phenotype, indicating, therefore, the fundamental role of collagen protein over the biological interactions that occur by bio-recognition mimetic mechanisms at biomaterials interface. These results indicate that the surface modification method used here may be useful as a strategy to develop biofunctional scaffolds, which provide a more successful clinical application of biomaterials in the tissue engineering field.

**Keywords:** *bioabsorbable polymers, collagen immobilization, PLDLA scaffolds, surface modification, tissue engineering*

### 1. Introduction

The biggest challenges in tissue engineering and biomaterials science have been the production and development of biomaterials able to mimic the complexity, physicochemical specificity and dynamics of the extracellular matrix (ECM) of tissues in such a way as to stimulate biomaterial/host tissue interactions that will elicit specific biological responses and lead the functional repair of tissue<sup>1</sup>. To achieve this goal, the surface properties of biomaterials have been modified with mimetic functional molecules in order to enhance the biocompatibility of implantable devices by carrying specific cell signals or by preventing undesired biological events that occur at the interface of material surface and living tissues.

Poly ( $\alpha$ -hydroxyacids) polymers such as poly ( $\beta$ -caprolactone), poly (L-lactic acid), poly (glycolic acid) and co-polymers are well-known bioabsorbable polymers that have been widely investigated as tissue engineering scaffolds. The advantages of this class of materials include their biocompatibility, ease of processing and possibility of modulating their mechanical properties. However, the lack of natural cellular/tissue receptors that recognize these biomaterials and the hydrophobicity of a considerable number of polymers currently used in biomedical applications have severely limited the successful applications of biomaterials<sup>2,3</sup>.

Based on an understanding of the mechanisms of recognition between cells and biomaterials, surface

\*e-mail: [masbruna@fem.unicamp.br](mailto:masbruna@fem.unicamp.br)

modification techniques involving covalent immobilization of biomolecules have been developed to mimic ECM compounds and improve the bioreactivity of polymeric surfaces<sup>4,5</sup>. Some of the advantages associated with this approach include the enhancement of cell adhesion, migration, proliferation and maintenance of the cellular phenotype. Such modifications may also influence the success of polymeric materials in living tissue since they can increase the half-life of the biomolecule by preventing its metabolism and preserving the physical characteristics of the material<sup>6</sup>.

A commonly used approach for immobilizing natural macromolecules on polymeric substrates is through the formation of stable covalent bonds between nucleophilic terminal amino groups present in proteins and functional groups on the surface of activated material<sup>6</sup>. For this, a carboxyl functional group (-COOH) has often been introduced on the surface of poly ( $\alpha$ -hydroxy acids) through copolymerization of acrylic acid grafting; the latter is often activated by using a heterobifunctional chemical reagent such as carbodiimide hydrochloride<sup>4,6</sup>. The advantages of these functional group grafting and protein immobilization techniques include their low cost and mild reaction conditions that do not affect the bulk polymer or permanently alter the surface chemistry<sup>2,7</sup>.

Collagen, a natural protein polymer with a triple helix structure, is widely recognized by transmembrane receptors known as integrins that are present on the cell surface. The important role of collagen in cytoskeletal reorganization, focal complex formation and cell junctions, and in the signal transduction that regulates cell adhesion, proliferation, differentiation and maintenance of the cellular phenotype, are well known<sup>8,9</sup>.

Although various studies have described the advantages of using collagen in biomedical applications, the high cost of obtaining pure collagen, its high degradation rate and loss of mechanical properties, as well as its incompatibility with the regeneration of various tissues are still limiting factors to its widespread biomedical use<sup>10</sup>.

Based on the foregoing considerations, this study aimed at developing and characterizing a biomimetic surface on scaffolds of the poly (L-co-D,L-lactic acid) (PLDLA) obtained by grafting of carboxyl functional groups (-COOH) via acrylic acid polymerization process followed by immobilization of collagen type I. Simultaneously, cytocompatibility and potential application of modified surface scaffolds in bone tissue engineering were also evaluated *in vitro* by culturing osteoblast-like cells on materials surface.

## 2. Material and Methods

### 2.1. Preparation of PLDLA membranes and scaffolds

PLDLA 70/30 (Mw= 180.000 g/mol) was synthesized by ring-opening copolymerization of L-lactide and D,L lactide monomers, using stannous octoate as the catalyst, essentially as described by Motta<sup>11,12</sup>. PLDLA scaffolds were obtained by the salt-leaching method. After complete PLDLA dissolution in dichloromethane (10% w/v), 70%

(w/v) sucrose (Synth) was added, with the pore size being controlled granulometrically to 250-500  $\mu$ m. The solution was poured into a silicon cylindrical mold 5 mm in diameter and kept in this recipient until the complete evaporation of solvent. Cylindrical samples were then punched out of the molds and placed in a 1% polyvinyl alcohol (PVA) solution to remove the sucrose particles. The physical and chemical characterization of the material surface after surface modification was done in PLDLA membranes prepared by casting. The PLDLA scaffolds were sterilized in 70% ethanol solution for 1 h and air-dried overnight at room temperature.

### 2.2. Surface modification

#### 2.2.1. Acrylic acid grafting copolymerization

Samples were immersed in a quartz tube containing 10 mL of hydrogen peroxide under UV light in a photochemical reactor manufactured according to Campos et al.<sup>13</sup>. The reactor was equipped with a 14-1000 W high-pressure Hg lamp and operated at a constant temperature of 40 °C for 40 min. After photo-oxidization, the samples were rinsed three times with deionized water to remove the excess hydrogen peroxide. To introduce carboxyl groups (-COOH) on the surface, photo-oxidized samples were immersed in 10 mL of 10% (v/v) acrylic acid (AAc) in a glass tube purged with nitrogen. Graft polymerization was done at 37 °C for 60 min, after which the samples were rinsed with deionized water to eliminate the non-grafted homopolymer.

#### 2.2.2. Collagen immobilization

PLDLA-AAc samples were immersed in 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDC, 10 mg/mL; Aldrich)/phosphate-buffered saline (PBS, pH 7.4) at 4 °C for 24 h. Subsequently, the samples were immersed in a solution of collagen I (calf skin, Sigma)-PBS (collagen concentration: 3 mg/mL) for 24 h at 4 °C. The PLDLA-Col membranes and scaffolds were rinsed with deionized water and dried under vacuum. The dried samples were stored at 4 °C until analysis.

### 2.3. Surface characterization

#### 2.3.1. Scanning electron microscopy (SEM)

The surface morphology of unmodified PLDLA, PLDLA-AAc and PLDLA-Col scaffolds was evaluated by scanning electron microscopy (SEM) using a JEOL JXA 860A microscope, operating at 10kV. All of the samples were dried and sputter-coated with gold before analysis.

#### 2.3.2. Atomic force microscopy (AFM)

The surface topography of PLDLA membranes before and after surface treatment was examined with a digital AFM (Veeco, diMultiMode model) run in tapping mode at room temperature. Images were scanned by using a cantilever with a tip radius of 12 nm (SNL-10 model), a nominal constant spring of 0.06 N/m and a scanning rate of 1 Hz. Three measurements were taken for each sample surface. Surface scans of AFM were analyzed using a freeware scanning probe microscopy software based on MS-Windows named WSXM.

### 2.3.3. Quantitative determination of the carboxyl groups

After AAC grafting polymerization, the amount of COOH grafted on PLDLA membranes was determined using the toluidine blue-O (TBO) method, in which the dye stains the deprotonated acid groups through ionic interaction. The experiment was performed into 96 well plate containing circular samples ( $n=5$ ) of PLDLA and PLDLA-AAc. In brief, 200  $\mu\text{L}$  of TBO solution (pH 10) were added on the samples and then shaken for 2h. After TBO binding, the supernatant was removed from well plate and the stained samples were washed under shaking conditions with NaOH solution (pH 10) for 1h. 200  $\mu\text{L}$  of 50% acetic acid was added to remove the TBO dye from carboxyl groups on PLDLA membrane during 30 min. Subsequently, 100  $\mu\text{L}$  aliquots of the solution in each well were transferred to a new well plate and the optical density then measured at 650 nm using a microplate reader. The concentration of the carboxyl groups was determined with a calibration curve containing several samples with different carboxyl group concentrations. The calculation is based on the assumption that 1 mol of TBO has complexed exactly with 1 mol carboxyl groups<sup>14</sup>.

### 2.3.4. Determination of contact angle

The contact angles and surface energies of PLDLA, PLDLA-AAc and PLDLA-Col were measured at room temperature and 60% relative humidity by using the sessile drop method and an automated goniometer (Ramé-Hart model 100-00). Deionized water and di-iodomethane were used as probe liquids. At least five measurements were obtained for each sample and the resulting values were averaged.

### 2.3.5. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-IR) analysis

ATR-FTIR spectra of PLDLA membranes were obtained with a Perkin Elmer 1800 FTIR spectrometer over the range of 1000 to 3500  $\text{cm}^{-1}$ . For this analysis, a solution of collagen I (3 mg/mL) was poured into a glass mold and lyophilized at  $-80^\circ\text{C}$  for 24 h. Samples of pure collagen were used to compare the presence of collagen absorption peaks on the PLDLA surface after treatment.

### 2.3.6. X-ray photoelectron spectroscopy (XPS) analysis

XPS was employed to analyze the chemical changes on PLDLA surface after acrylic acid grafting and collagen immobilization treatment. The measurements were performed in ultra-high vacuum using a Kratos XSAM HS spectrometer with Mg  $K\alpha$  X-ray source (1253.6 eV) and operated at 12 kV, 5 mA. The Shirley background, Gaussian (for C1s, O1s, N1s, and Si2p peaks), mixed Gaussian/Lorentzian (for doublet Cl 2p) functions, and a least-square routine were used for the fitting of the peaks<sup>15</sup>. The binding energies were referenced to the hydrocarbon component C1s (C-C; C-H) level set at 284.8 Ev. The accuracy for semi-quantitative analysis is  $\pm 15\%$ .

## 2.4. *In vitro* study

### 2.4.1. Cell culture

Osteoblasts were obtained from explants of calvariae from three 20-day-old New Zealand white rabbits. All procedures involving animals were approved by the Ethics Committee of the Universidade do Vale do Paraíba (UNIVAP), São José dos Campos, SP, Brazil (protocol no. A01/CEP/2009.). Briefly, the skin and soft connective tissues were removed. In a laminar flow, the calvariae were removed, cut into small pieces and distributed in T25 tissue culture dishes in which they were cultured in a 5%  $\text{CO}_2$  atmosphere at  $37^\circ\text{C}$  until confluence. Osteoblasts were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), gentamicin (50  $\mu\text{g}/\text{mL}$ ), amphotericin B (5 mg/mL), ascorbic acid (50 mg/mL) and 10 mM  $\beta$ -glycerophosphate. The cells were then harvested using trypsin/EDTA and seeded onto 96-well culture plates containing the polymeric scaffolds of interest. The culture medium was changed every 2 days.

### 2.4.2. Cell adhesion

Osteoblast-like cells adhesion on scaffolds was assessed by the MTT colorimetric assay in which MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was reduced to a purple formazan product by the mitochondria of living cells.  $1 \times 10^5$  cells/mL in 200  $\mu\text{L}$  of DMEM supplemented with 10% FBS were seeded in wells containing the scaffolds and incubated for 2 h and 24 h. The culture medium was removed and the wells then washed twice with 0.1 M PBS, pH 7.4, at  $37^\circ\text{C}$  followed by the addition of 180  $\mu\text{L}$  of DMEM and MTT assay mixture (20  $\mu\text{L}/\text{well}$ , containing 5 mg of MTT reagent/mL). After incubation for 4 h at  $37^\circ\text{C}$ , the cells were lysed by adding 200  $\mu\text{L}$  of dimethylsulphoxide (DMSO). Subsequently, 100  $\mu\text{L}$  aliquots of the solution in each well were transferred to a new plate and the absorbance then measured at 570 nm using a microplate reader.

### 2.4.3. Cell proliferation

Cell proliferation was examined using the Quant-iT™ PicoGreen assay kit (Molecular Probes), according to the manufacturer's instructions. The normal procedure described in the kit protocol and the accompanying DNA standards (up to 1,000 ng/mL) were used. Cells ( $1 \times 10^5/\text{mL}$  in 200  $\mu\text{L}$  of DMEM supplemented with 10% FBS) were seeded in wells containing the scaffolds and incubated for 1, 7, 14 and 21 days. At the end of each period, the medium was aspirated from the wells and the scaffolds were washed five times with 200  $\mu\text{L}$  of PBS. Subsequently, 200  $\mu\text{L}$  of Tris-EDTA (TE) buffer was added to each sample, frozen at  $-80^\circ\text{C}$  overnight and then thawed at  $37^\circ\text{C}$ . The seeded scaffolds were sonicated for 5 min each and samples then aliquoted in duplicate (100  $\mu\text{L}$ ) into a 96-well flat bottomed plate. PicoGreen reagent (100  $\mu\text{L}$ ) was added to the wells containing standard or sample and the plate then read at excitation/emission wavelengths of 485/538 nm in a Perkin Elmer fluorometer. The resulting fluorescence was compared with a standard curve of DNA and the cell number was calculated by assuming 7.7 pg of DNA/cell<sup>16,17</sup>.

#### 2.4.4. Collagen quantification

Collagen secretion by cultured osteoblasts was determined by using the Sirius Red colorimetric assay that is based on the selective binding of Sirius red, an anionic dye, to collagen proteins; this method has been widely used to estimate collagen production by cultured cells. The cells were cultured on scaffolds for 2 h, 24 h, 5 days and 10 days after which the scaffolds were fixed in Bouin solution for 1 h and stained with Sirius red. After 1 h, the scaffolds were washed with 0.01 M HCl for 30 s and the excess dye then removed from the cell layers by washing with 0.1 M NaOH for 30 min. Aliquots (100  $\mu$ L) of the solution in the wells were transferred to a new plate and the absorbance of the samples (as an indicator of collagen content) was measured at 570 nm using a microplate reader.

#### 2.4.5. Cell morphology

Cell morphology was assessed by scanning electron microscopy (SEM) after 3, 5 and 12 days of culture. The samples were rinsed with PBS and fixed for 1 h at room temperature in a solution prepared by dissolving 2.5% paraformaldehyde, 2.5% glutaraldehyde, 0.03% picric acid and 1% tannic acid in PBS, and was followed by two rinses in PBS. After immersion in 1% (v/v) osmium tetroxide ( $\text{OsO}_4$ ) solution for 1 h, the samples were rinsed twice in deionized water and dehydrated in a graded ethanol series (70%, 80%, 95% and 100%) for 15 min each. The fixed samples were freeze-dried, sputter-coated with gold and examined with a JEOL JXA-840A microscope at 10kV.

#### 2.4.6 Statistical analysis

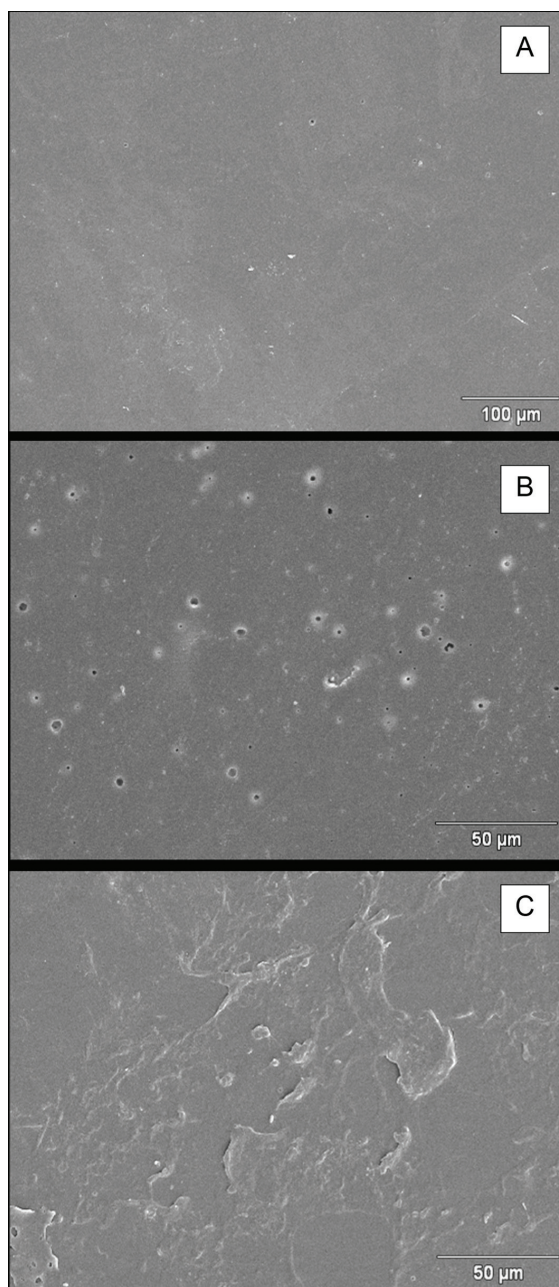
The data for cell viability, cell proliferation and collagen quantification were expressed as the mean  $\pm$  standard deviation (SD) for  $n=5$  experiments or determinations. Statistical comparisons were done using analysis of variance (ANOVA) followed by the Tukey test with ( $p < 0.05$ ) indicating significance. All data analyses were done using BioEstat<sup>®</sup> 5.0 statistical software.

### 3. RESULTS

#### 3.1. Scanning electron microscopy (SEM)

Figure 1 shows SEM images of the surfaces of PLDLA membranes obtained by solvent casting before and after acrylic acid grafting and collagen immobilization. The surface of unmodified PLDLA membrane was smooth (Figure 1A). After acrylic acid grafting, micropores of irregular sizes were distributed homogeneously on the membrane surface (Figure 1B). Images of collagen immobilized PLDLA membranes showed that collagen was deposited as a heterogeneous bidimensional layer on membrane surface (Figure 1C).

Figure 2 shows SEM images of the surface of PLDLA scaffolds prepared by particulate leaching followed by surface modification. The presence of sucrose particles resulted in homogeneously distributed irregular pores (250-500  $\mu$ m in diameter) that are characteristic of this method (Figure 2A). PLDLA scaffolds treated with acrylic acid had a pore structure similar to unmodified scaffolds, although there was an increase in surface roughness and the

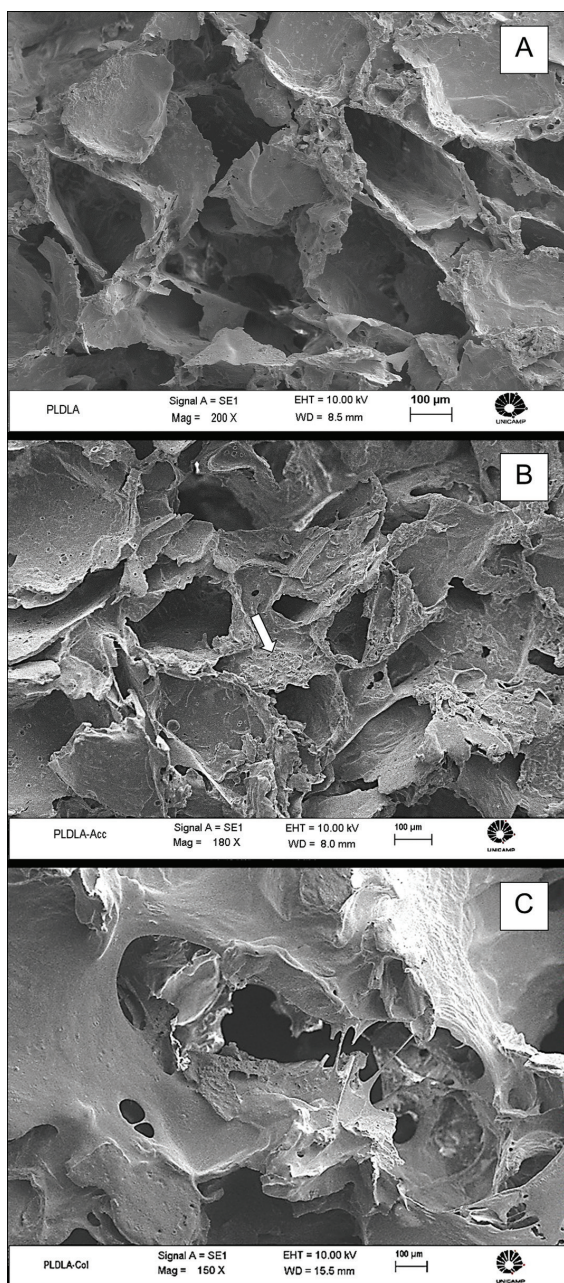


**Figure 1.** SEM images of (A) unmodified PLDLA membranes, (B) PLDLA-AAc membrane showing distributed micropores on the membrane surface and (C) PLDLA membranes with immobilized collagen covering the membrane surface.

pore walls showed an irregular, microporous morphology (Figure 2B). The surface of PLDLA-Col scaffolds was unevenly covered with collagen that formed a discontinuous layer which partially or entirely clogged some pores on the surface (Figure 2C).

#### 3.1.2. Atomic force microscopy (AFM)

Surface topography of PLDLA membrane after acrylic acid grafting copolymerization and collagen immobilization was examined by AFM. Figure 3 shows AFM 2D and



**Figure 2.** SEM images of the surface of (A) pure PLDLA scaffold, (B) PLDLA-AAc scaffold, showing the surface roughness and the appearance of smaller pores with an irregular morphology (white arrow), and (C) PLDLA-Col scaffold in which collagen formed a discontinuous and heterogeneous network on the surface of PLDLA scaffold.

3D images, respectively, of unmodified PLDLA surface (Figure 3A), PLDLA-AAc (Figure 3B) and PLDLA-Col (Figure 3C). Notable changes could be observed after (AAc) grafting process onto smooth PLDLA membrane, which generated many deep pores ranging from several nanometers to few micrometers, uniformly, distributed over entire material surface. Collagen deposition with net-like fibrillar structure and higher deposition in non-specific sites of

PLDLA surface promoted an increase in average roughness (Ra) from 6.3 nm (PLDLA pure) to 30.7nm (PLDLA-Col). These results confirm that the collagen immobilization method employed not only allows covalent collagen binding onto polymer surface but also improves surface roughness.

### 3.1.3. Quantitative determination of the carboxyl groups

As an evidence of AAc grafting polymerization, the amount of  $-COOH$  groups in the PLDLA and PLDLA-AAc membranes (Figure 4) was measured by toluidine blue method as 0.041 and 0.064 mmol/cm<sup>2</sup>, respectively. Pure PLDLA contains ester bonds at its ends and chains that can be broken under the alkaline conditions of the dye solution, producing carboxylic groups and hydroxyl groups on the material surface which may react with TBO dye during the experiment. Thus, unmodified PLDLA membranes were used as control.

### 3.1.4. Contact angle measurements

Table 1 shows the contact angle measurements of PLDLA membranes before and after acrylic acid grafting and collagen immobilization. The mean contact angle for PLDLA was  $82 \pm 1$ . This decreased to  $74 \pm 3$  after treatment with AAc, indicating that a hydrophilic carboxylic functional group had been introduced onto the PLDLA surface. Subsequent collagen immobilization increased the contact angle to  $77 \pm 5$ .

### 3.1.5. ATR-IR spectra

Figure 5 shows the ATR-FTIR spectra of PLDLA membranes. All of the spectra showed the characteristic polylactide bands at 1079, 1081 and 1266 cm<sup>-1</sup> ( $=C-O$  stretching), 1361 and 1380 cm<sup>-1</sup> ( $CH_2$  wagging), 1450 cm<sup>-1</sup> ( $CH_3$  bending), 1748 cm<sup>-1</sup> ( $C=O$  stretching in the ester group), 2940 cm<sup>-1</sup> ( $CH$  stretching) and 2994 cm<sup>-1</sup> ( $CH_3$  stretching). However, for the PLDLA-Col membranes spectrum new absorption peaks were observed at 1639 cm<sup>-1</sup> ( $N-H$ ) and 1544 cm<sup>-1</sup> ( $C-N$  stretching) that corresponded to amides I and II. The absorption band at 3308 cm<sup>-1</sup> was ascribed to the stretching vibration of ( $O-H$ ). The same bands were found in the spectrum of pure collagen.

### 3.1.6. XPS analysis

The surface chemical composition of PLDLA, PLDLA-AAc and PLDLA-Col membranes was assessed by XPS analysis and compared to the pure collagen after surface treatment. Table 2 summarizes the binding energies of the main peaks and the atomic concentration of carbon, oxygen and nitrogen in each sample. XPS spectra (not shown) revealed that the C1s and O1s peaks of PLDLA and PLDLA-AAc are very similar to each other. The C1s spectra were decomposed into three components, at respectively 284.8 eV (C-C or C-H), 287.1 eV (C-O), and 289.3 eV ( $-CO-O$ ) which correspond to the well known components, corresponding to the main carbon atoms expected in saturated hydrocarbons<sup>18,19</sup>.

In the pure collagen samples, the C1s is characterized by three main components, due to carbon single bond to carbon or hydrogen (C-C or C-H) at 284.8 eV, carbon single

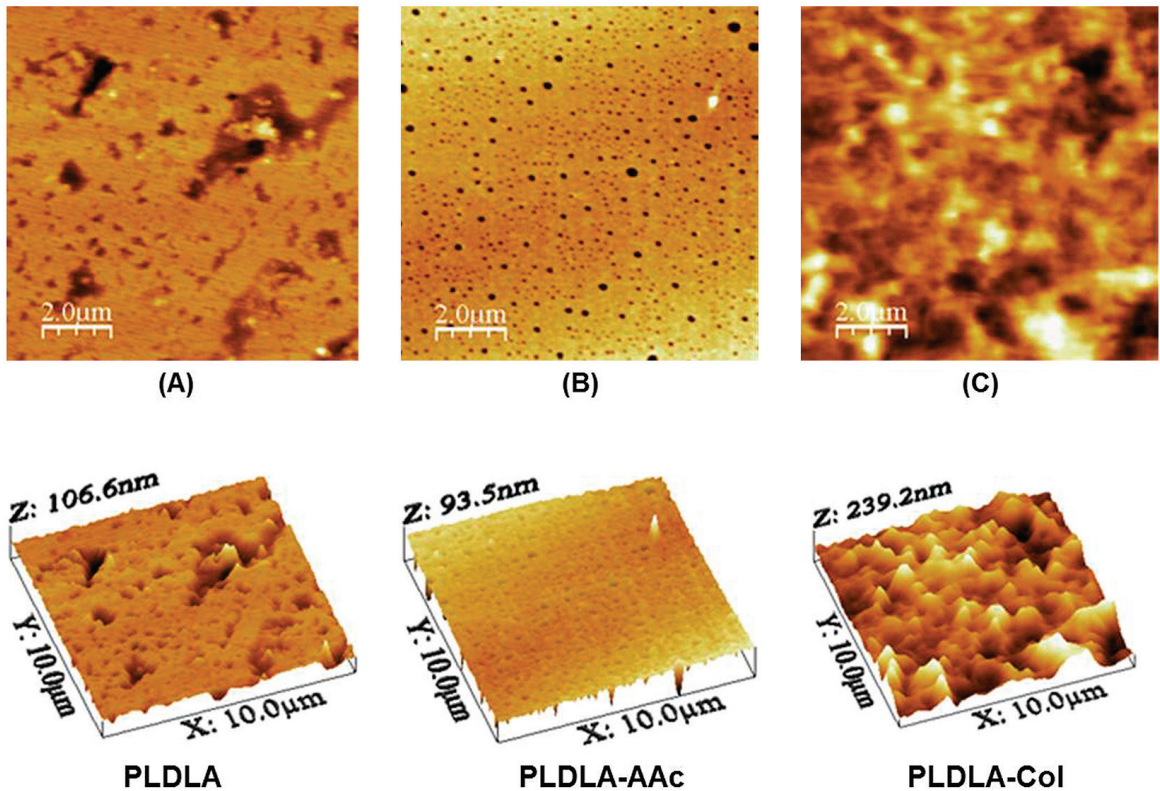


Figure 3. 2D (upper row) and 3D (lower row) AFM images of PLDLA (A), PLDLA-AAc (B) and PLDLA-Col (C) membranes.

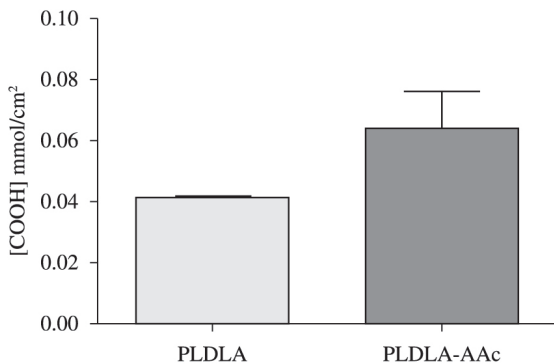


Figure 4. Density of (-COOH) groups on membrane surface obtained by toluidine blue staining.

bond to oxygen or nitrogen (C-N or C-O) at about 286.2 eV, and due to carbon involved in the peptide binding (C=O or O=C-N) at about 288.1 eV<sup>20,21</sup>. O1s peak was decomposed in two components, being the main, at 531.8 eV associated to (O=C-N) and the second, at 533.9 eV that may be related to water adsorption on material surface. N1s peak was adjusted using one component at 400.0 which corresponds to N-C.

The comparison between pure Collagen and PLDLA-Col reveals that the chemical elements, with the same or similar binding energies values for C1s, O1s and N1s are present in both samples. Furthermore, a second component for N1s peak is present on the membranes surface at 401.8 eV, which can be attributed to nitrogen bonded with oxygen. Besides, it

Table 1. Static contact angle of PLDLA membranes before and after acrylic acid (AAc) grafting and collagen (Col) immobilization.

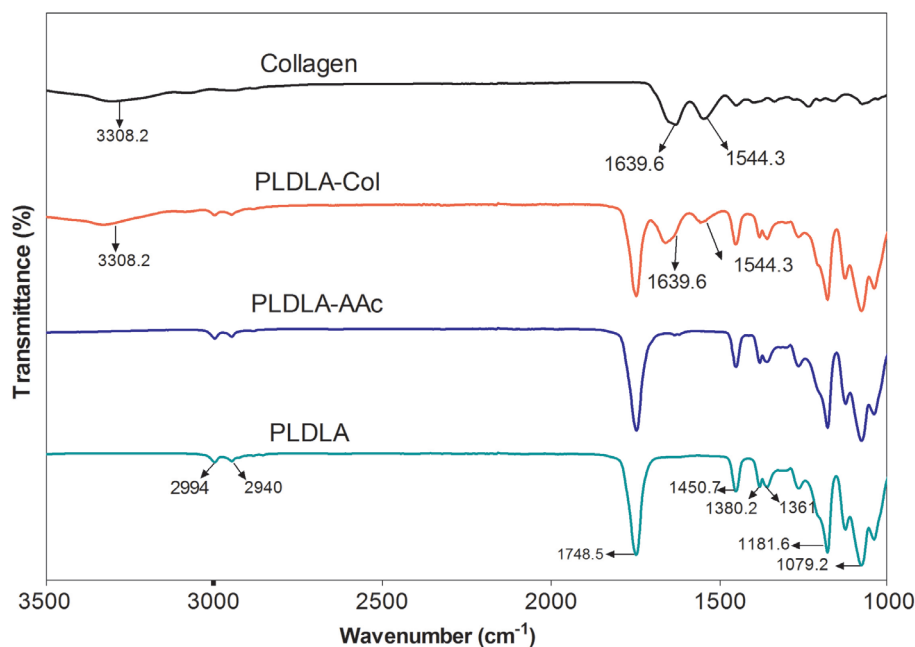
Sample	Water contact angle (°)
PLDLA	82±1
PLDLA-AAc	74±3
PLDLA-Col	77±5

can be seen that after collagen immobilization, the surfaces were enriched with nitrogen atoms from collagen amine groups and amide bonds on PLDLA-Col, while there is no detectable nitrogen on PLDLA and PLDLA-AAc samples, confirming, therefore, the presence and immobilization of collagen on PLDLA surface.

### 3.2. In vitro study

#### 3.2.1. Cell adhesion

To assess the influence of surface treatment on cell adhesion, osteoblast-like cells were cultured on PLDLA, PLDLA-AAc and PLDLA-Col scaffolds for 2 h and 24 h. Cell culture on tissue culture polystyrene plate (TCPS) was used as positive control while the cell culture performed on TCPS containing a toxic substance, phenol 1%, was used as negative control. Cell adhesion and viability was determined by MTT assay and is shown in Figure 6. By analyzing the results obtained, one can observe that the cells adhered to the surface of all scaffolds at first 2h and the cellular viability levels were kept after 24 h of culture, thus, indicating, that the surface treatment did not exert



**Figure 5.** ATR-FTIR spectra of PLDLA, PLDLA-AAc, PLDLA-Col and pure collagen. Spectrum of PLDLA-Col showing bands at  $1639\text{ cm}^{-1}$  (amide I),  $1544\text{ cm}^{-1}$  (amide II), also seen in pure collagen.

**Table 2.** Binding energy (eV) of the main XPS peaks and atomic concentrations found on PLDLA membranes before and after surface treatment. The percentages in parentheses refer to the relative amounts of each component of a particular absorption peak.

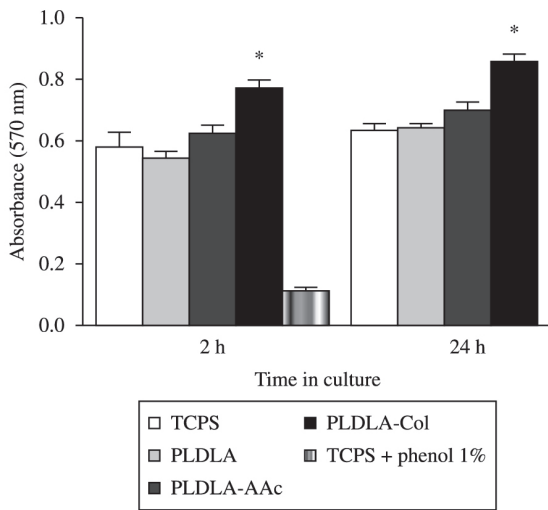
Sample	Binding Energy (eV)					Atomic concentration (%)				
	C 1s	O 1s	N 1s	Other elements		C	O	N	Other elements	
				Si 2p	Cl 2p3/2				Si 2p	Cl 2p3/2
PLDLA	284.8 (51%)	532.1 (72%)		102.2		63.05	28.73		8.17	
	287.1 (16%)	533.8 (28%)	----		----					----
	289.3 (15%)									
PLDLA-AAc	284.8 (64%)	532.2 (70%)		102.3		64.46	29.13		6.42	
	286.0 (9%)	533.8 (30%)	----		----					----
	287.2 (13%)									
	289.3 (15%)									
PLDLA-Col	284.8 (52%)	531.9 (78%)	399.8 (84%)	102.3	197.7 (80%)	62.25	23.59	8.9	3.47	1.79
	286.2 (28%)	533.6 (22%)	401.8 (16%)		200.1 (20%)					
	287.8 (12%)									
	289.1 (8%)									
Collagen	284.8 (51%)	531.8 (95%)	400	102.3	197.6 (70%)	60.69	21.73	11.97	4.51	1.12
	286.2 (30%)	533.9 (5%)			199.6 (30%)					
	288.1 (19%)									

any cytotoxic effect on osteoblast-like cells adhesion. No significant differences in the number of viable cells were found among the positive control, PLDLA and PLDLA-AAc scaffolds ( $p > 0.01$ ); however, cell adhesion was significantly improved by PLDLA-Col scaffolds compared with other samples ( $p < 0.01$ ), confirming that the collagen immobilized on PLDLA scaffolds enhance the surface properties of the material for cell adhesion, as expected.

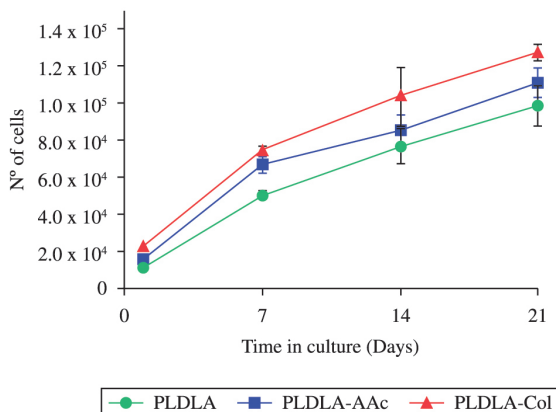
### 3.2.2. Cell proliferation

Cell proliferation was evaluated by the total DNA quantification assay, using Picogreen reagent. The cell growth curve was calculated assuming the concentration of DNA/ cells equal to  $7.7\text{pg}^{16}$ . Figure 7 shows that the number of cells increased progressively in all the three treatments; however, the cell growth rate was varied in

function of surface characteristics of scaffolds. Consistent with cell adhesion results obtained by MTT assay, the number of cells found in the PLDLA-Col scaffolds after 1 day of culture was significantly greater ( $p < 0.05$ ) than those found in the PLDLA and PLDLA-AAc scaffolds. Nevertheless, after 7 days of culture both PLDLA-AAc and PLDLA-Col scaffolds presented a similar number of cells ( $p > 0.05$ ), demonstrating that although cell adhesion has not been improved in the PLDLA-AAc scaffolds after 1 day of culture, the carboxyl groups introduced on PLDLA surface were also able to stimulate significantly the osteoblast proliferation during the first 7 days. Only after 14 days of culture, the average number of cells found in the unmodified PLDLA samples reached the values found



**Figure 6.** Adhesion and viability of osteoblasts-like cells cultured on PLDLA scaffolds before and after surface modification for 2 h and 24 h. Osteoblast cells culture on TCPS were used as positive control and cell culture performed on TCPS containing the toxic substance phenol 1% were designed as negative control. The columns represent the mean  $\pm$  SD of five independent experiments. \* Data significant statistically compared to other groups ( $p < 0.01$ ).



**Figure 7.** Osteoblast proliferation on PLDLA, PLDLA-AAc and PLDLA-Col scaffolds. The points represent the mean  $\pm$  SD of five independent experiments.

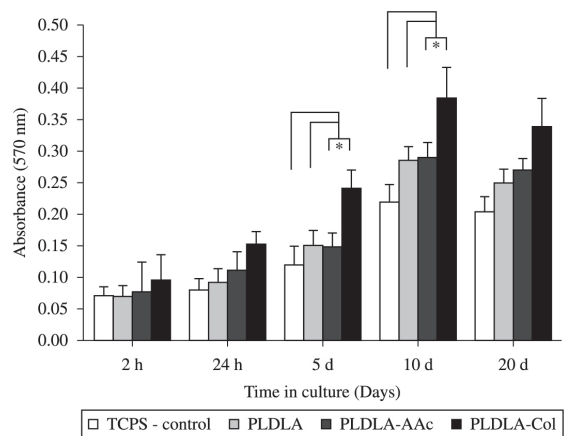
for PLDLA-AAc and PLDLA-Col scaffolds, which did not present a significant cell growth for the period of culture between 7 and 14 days. After 21 days of culture, the cell growth was resumed in the PLDLA-AAc and PLDLA-Col samples, exhibiting a greater number of cells compared with PLDLA scaffolds ( $p < 0.01$ ).

### 3.2.3. Collagen quantification

Figure 8 shows the collagen deposition by osteoblasts cultured on PLDLA, PLDLA-AAc and PLDLA-Col scaffolds for 2 h, 24h, 5, 10 and 20 days, as assessed by staining with Sirius red dye. There was a progressive increase in collagen deposition during the first 10 days in all groups, after which the collagen level stabilized or decreased slightly. There were no significant inter-group differences in collagen deposition after 2 h and 24 h. However, from day 5 onwards cells grown on PLDLA-Col deposited significantly greater ( $p < 0.01$ ) amounts of collagen compared to PLDLA and PLDLA-AAc; the collagen deposition in the latter two groups was similar.

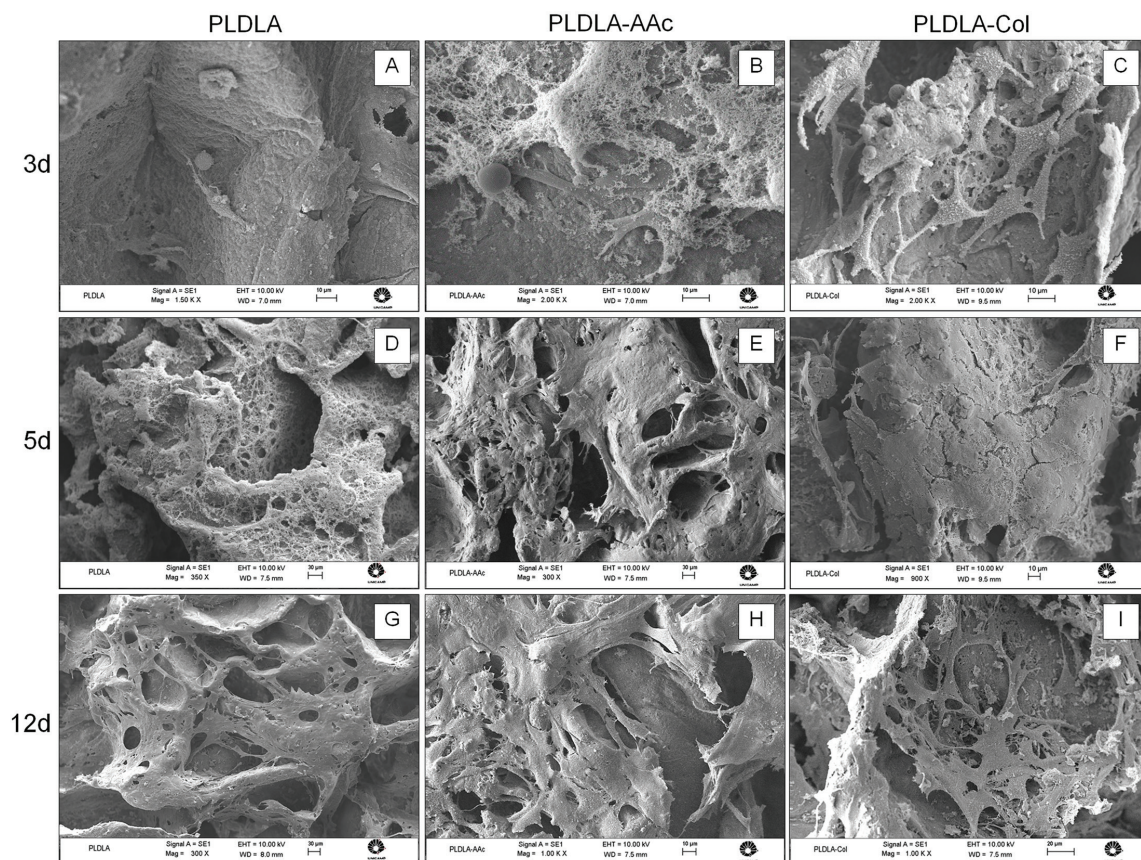
### 3.2.4. Cell morphology

Figure 9 shows SEM images of osteoblasts grown on scaffolds for 3, 5 and 12 days. The cells attached, spread and proliferated on all three scaffolds; however, the morphology and cell behavior varied according to the surface treatment. On unmodified PLDLA (Figure 9A), the cells tended to grown in isolation and did not spread well after 3 days in culture, whereas on PLDLA-AAc and PLDLA-Col, the cells tended to be grouped and well-spread, with cytoplasmic projections extending away from the central body to adjacent cells. Cells grown on PLDLA-acrylic acid deposited an abundant fibrillar extracellular matrix (Figure 9B) while those grown on PLDLA-Col showed good attachment to collagen (Figure 9C). From day 5 onwards, the osteoblasts started to form monolayers at non-specific areas on the scaffold surface. On PLDLA, most of the surface was covered with a fibrillar network



**Figure 8.** Collagen secretion by osteoblasts-like cells cultured on PLDLA substrates for up to 20 days. Collagen was quantified using a Sirius red-based colorimetric assay. The columns are the mean  $\pm$  SD of five independent experiments. \* Data significant statistically compared to other groups ( $p < 0.01$ ).





**Figure 9.** Morphology of osteoblasts grown on PLDLA scaffolds before and after surface treatment for up to 12 days.

(Figure 9D), whereas on PLDLA-AAc and PLDLA-Col the cells formed a progressively compact layer that filled some of the pores and covered the scaffold surface (Figure 9E and F). On day 12, the osteoblast morphology predominated on PLDLA-AAc (Figure 9H) and PLDLA-Col (Figure 9I) scaffolds, with cells having a polygonal or spindle shape and homogeneous growth.

#### 4. Discussion

Surface modification techniques based on the addition of functional chemical groups and immobilization of biomimetic molecules have been widely used in the biofunctionalization of polymeric material surfaces in order to improve interactions between cells/tissues and biomaterials during tissue regeneration. In this study, the surface of PLDLA scaffolds was modified by photo-oxidation followed by acrylic acid grafting polymerization and the immobilization of type I collagen. Collagen was chosen as the biomimetic molecule because it is the main structural protein found in bone. We examined the cytocompatibility and influence of scaffold surface on osteoblast affinity for the substrate, attachment and proliferation before and after surface treatment.

Various studies have shown that the surface properties, e.g., topography, chemistry and wettability of polymeric scaffolds play a fundamental role in cell-material interactions<sup>22,23</sup>. In the current study, the characterization

of PLDLA membrane and scaffold surfaces shows that the surface modification method employed is able to promote both collagen coating and immobilization on the material surface.

SEM and AFM analysis of the membranes and scaffolds demonstrated that the surface morphology and topography were markedly modified after the incorporation of carboxyl groups on the polymer surface by acrylic acid grafting and collagen immobilization. Acrylic acid grafting generated nano- and micropores on the membrane and scaffold surfaces. However, the micropores in the scaffolds were originated mainly in the pore walls, where the polymeric matrix was weaker and more susceptible to chain scission by acid hydrolysis during acrylic acid grafting<sup>24,25</sup>.

Coating the surface with collagen resulted in the obstruction of some pores. Although porosity and pore size distribution are important parameters for successful cell-biomaterial interactions, the results of the present study suggest that the closing of some pores caused by collagen coating did not adversely affect the cytocompatibility and performance of modified scaffolds. This finding is in accordance with other reports that mention the difficulty in controlling the extent of acrylic acid grafting and covalent collagen immobilization used to modify surface properties<sup>24,26</sup>.

In the present study, the time of 60 minutes for AAc grafting polymerization on material surface was determined

according to literature reports<sup>27,28</sup>. The authors have demonstrated that highest densities of -COOH groups grafted on polyester surfaces can be achieved within 30-60 min of reaction.

From the results obtained by toluidine blue method, we assume that the reaction time chosen for AAc grafting polymerization on material surface was proved to be effective, since the density of -COOH groups present on PLDLA-AAc (0.064 mmol/cm<sup>2</sup>) was higher than that found on unmodified PLDLA surfaces (0.041 mmol/cm<sup>2</sup>).

Characteristics adsorption peaks of acrylic acid grafted on PLDLA surface were not clearly detected by ATR-IR spectra. This can be explained due to PLDLA and AAc have the same stretching bonds in chemical structure. Besides, the adsorption peaks of carboxyl functional group at around 1730 cm<sup>-1</sup> from polymerized AAc, can be overlapped by the inherent carboxyl functional groups present in the PLDLA<sup>27,29,30</sup>.

After collagen immobilization, ATR-IR spectrum of the PLDLA-Col membrane confirmed the formation of amide bonds on the material surface by the appearance of amide I and amide II absorption bands, characteristics of collagen molecule. Furthermore, XPS analysis revealed that the surface of collagen-immobilized PLDLA membranes was enriched with nitrogen atoms, presenting similar binding energies to pure collagen, whereas no detectable nitrogen peaks were found for unmodified PLDLA and PLDLA-AAc. These results are indicative that collagen has been successfully immobilized on PLDLA surface.

It is known that the hydrophilicity/hydrophobicity balance has a significant influence on adsorption of adhesive proteins onto substrates, which are able to stimulate cell adhesion and proliferation<sup>31,32</sup>. As shown here, the hydrophilicity of PLDLA was not significantly improved by acrylic acid grafting, whereas collagen immobilization resulted in a slight decrease in hydrophilicity. These findings may reflect the high percentage of hydrophobic amino acid moieties present in the collagen molecule. In this regard, the rearrangement and reorganization of chemical groups on polymeric surfaces can lead to a heterogeneous distribution of polar and apolar domains on the surface that can influence the contact angle measurements<sup>33,34</sup>.

In addition, during collagen immobilization, some of the carboxyl functional groups grafted onto the polymer surface may be blocked by interacting with collagen hydrophilic groups, thereby exposing the hydrophobic amino acids of the protein molecule on the material surface<sup>27</sup>. Several studies have shown that a lack of improvement in hydrophilicity does not adversely affect cell interactions with the surfaces<sup>33,35</sup>. A similar observation was made in this study, as shown by the cell viability assay and SEM analysis of osteoblasts cultured on PLDLA-Col scaffolds.

Cellular attachment to ECM molecules is mediated by interaction between integrins (cell surface transmembrane receptors) and specific amino acid sequences in ECM macromolecules such as fibronectin, vitronectin, laminin and collagen<sup>36</sup>. Amongst these sequences, the RGD peptide (arginine-glycine-aspartic acid) has an important role in cell adhesion, proliferation and maintenance of the cell phenotype<sup>8,37,38</sup>.

As shown here, collagen had a significant positive influence on the attachment, proliferation and collagen synthesis of osteoblasts compared to unmodified PLDLA scaffolds. The affinity of osteoblasts for collagen was confirmed by SEM that revealed groups of cells with a well-defined osteoblast-like morphology attached mainly to a collagen fibrillar network on the scaffold surface.

Interesting results were obtained from the comparison of cell growth on PLDLA-AAc and PLDLA-Col scaffolds. Although the adhesion of osteoblasts on PLDLA-AAc was significantly lower than on PLDLA-Col scaffolds, the cell proliferation and morphology were similar on scaffolds polymerized with acrylic acid. This finding probably reflects the fact that the grafting of a hydrophilic (-COOH) group onto a polymeric surface promotes the adsorption of proteins from the culture medium and that this, in turn, stimulates several biological signaling pathways<sup>39,40</sup>.

Another point to be considered for the suitable cell proliferation and morphology found on PLDLA-AAc and PLDLA-Col scaffolds is the topographic features obtained on the materials surface after treatment. As commented above, porosity and surface roughness have a fundamental influence on biocompatibility of scaffolds, since it allows cell migration into the scaffolds and improves osseointegration by increasing cells-surface material contact, respectively<sup>41,42</sup>.

If, on the one hand, cell proliferation and morphology seem to have been stimulated in PLDLA-AAc scaffolds by obtained micropores formation and increase of surface roughness resulting from acrylic acid polymerization process, on the other hand, the biomimetic properties of collagen protein, combined with its heterogeneous distribution which also improved surface roughness (shown by SEM and AFM images), it seems to have accelerated osteoblast cells interactions with the material surface, promoting first, cell adhesion.

Previous reports have described the significant influence that the chemical grafting of carboxyl groups onto polymeric surfaces has on osteoblast cell adhesion<sup>39,43</sup>. However, such an effect was not observed here. We hypothesize that residual monomers from the acrylic acid copolymerization reaction may have affected the affinity of the cells for the substrate since faster acidification of the culture medium was observed, even when the scaffolds were washed several times before being used for cell culture<sup>44</sup>.

We consider that this result may not have any negative impact on the method application. Firstly, because during the cell culture acid monomer residues are naturally removed and neutralized through the periodic change of culture medium (pH = 7.4). Secondly, along the 20 days of cell culture, osteoblasts regained cell growth, showing osteoblast-like cell morphology.

The secretion of extracellular proteins such as collagen is an important marker of osteoblast maturation<sup>45</sup>. The collagen content measured during 20 days in culture showed that all types of PLDLA treatment stimulated collagen secretion by osteoblasts. The greater collagen content observed with PLDLA-Col compared to unmodified PLDLA and PLDLA-AAc scaffolds indicated that collagen coating stimulated cell differentiation and maintenance of the osteoblast cell phenotype.

## 5. Conclusion

The results of this study indicate that the surface-modification method used here allowed both collagen type I coating and immobilization on the surface of PLDLA membranes and scaffolds. The presence of collagen on the surface of the scaffolds played an important role in stimulating cell adhesion, proliferation and collagen biosynthesis. These findings suggest that the immobilization of natural macromolecules on polymer scaffolds is a viable strategy, with potential applications in the development

of biomaterials for tissue engineering and regenerative medicine.

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