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ORIGINAL ARTICLE

TERM

Grafting Collagen on Poly (Lactic Acid) by a Simple Route to Produce Electrospun Scaffolds, and Their Cell Adhesion Evaluation

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Increasing bioactivity and mechanical properties of polymers to produce more suitable scaffold for tissue engineering is a recurrent goal in the development of new biomedical materials. In this study, collagen-functionalized poly (lactic acid), PLA, was obtained by means of a simple grafting route, and electrospun scaffolds were produced to grow cells *in vitro*; their bioactivity was compared with scaffolds made of physical blends of PLA and collagen. Grafting was verified via nuclear magnetic resonance, attenuated total reflection-Fourier transform infrared and X-ray photoelectron spectroscopy. The cell adhesion performance of the scaffolds was studied using macrophages. Elastic modulus (74.7 megapascals) and tensile strength (3.0 megapascals) of the scaffold made from PLA grafted with collagen were substantially higher than the scaffolds made from physical blends of collagen and PLA: 32 and 2.16 megapascals, respectively, implying a more resistant material because of the chemical bond of the polypeptide to PLA. Besides, the fibers had more uniform diameter without defects. Scaffolds made from PLA grafted with collagen presented four-fold increase in cell adhesion than those of PLA blended with collagen. Furthermore, cell spreading within the scaffolds occurred only when collagen-functionalized poly (lactic acid) was used. These results open a new option for the easy tailoring of nanofiber-based scaffolds in three dimensions for tissue engineering.

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Key Words: Poly (lactic acid); Collagen; Grafting; Electrospun scaffold; Cell adhesion

INTRODUCTION

Most cells in the human body grow and live in a complex network of biomaterials usually known as the extracellular matrix, which is composed of various proteins and proteoglycans assembled into a highly ordered structure [1-3]. In regenerative medicine, when new tissue needs to be grown intra or extra corporally, there are fundamental aspects to be considered for the construction, development and functionality of living tissue: bioactive molecules together with a scaffold, used as a support for the cells, are needed to mimic the extracellular matrix and promote cell adhesion, proliferation, differentiation, and migration pathways [4,5].

Producing scaffolds for tissue engineering demands the development of new materials with bioactive molecules and an appropriated technique. Among the techniques to produce polymeric scaffolds, electrospinning is a simple and effective method to obtain nanofiber-based scaffolds resembling the functions of the extracellular matrix [6-10]. Regarding the materials, a number of natural and synthetic polymers have been used to produce electrospun scaffolds [11-15].

Poly (lactic acid) (PLA) is widely used for the generation of scaffolds for tissue engineering to allow precise control over their physicochemical and mechanical properties but exhibit limited cellular affinity. Natural biodegradable polymers, such as collagen, provide inherent binding sites for the promotion of cell adhesion and cellular growth. However, this natural material, obtained from animal or human tissues, is expensive and

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not readily available in large quantities [16].

Commonly, stability of the scaffolds made from pure collagen needs to be increased, and they are chemically cross-linked after the electrospinning process using glutaraldehyde. In one way, the chemical treatment reduces the solubility of collagen in the body fluids, and, in the other, it improves the biological and mechanical stability and ease of handling of the scaffolds. However, this chemical cross-linking may cause toxicity problems owing to the presence of catalysts or unreacted crosslinking agents in the final product [17,18].

In the search for better materials to produce efficient electrospun scaffolds, there are intensive investigations on the modification of PLA with different proteins that are part of the extracellular matrix such as collagen. Various chemical modifications and physical blends of PLA with collagen and other biopolymers have being conducted to combine the best properties of each one, to create unique materials with improved biocompatibility and enhanced mechanical stability [19-22]. Yang et al. [23] found that a physical blend of PLA and collagen is composed of a continuous phase of PLA and a discontinuous phase of collagen. Although the rate of biodegradation was increased by the addition of collagen, this blend exhibited poor mechanical properties.

Yang et al. [24] studied the effects of coupling collagen with PLA via gamma irradiation, using PLA as a coupling agent. They found that gamma irradiation induced degradation of the PLA backbone and a reduction of the mechanical properties of the material. Several authors have reported on the modification of the PLA's surface using a myriad of methods such as plasma treatment, hydrolysis and aminolysis [25-28] as well as with phosphorous pentachloride [29], with the final intent of obtaining a functional polymer that could be more attractive to bioactive molecules. However, the reactive groups introduced on the surface of PLA using these methods were transient, and the functionality was time-dependent. Wang et al. [30-32] introduced reactive groups onto the PLA chain by using a three-step approach. In the first step, maleic anhydride was grafted onto the backbone of PLA. The second step involved the reaction of these groups with diamines, and the third step included the coupling of the modified PLA with proteins. In all of the previously mentioned studies, chemical modifications of PLA were used and scaffolds were produced. These procedures required several reaction steps and because of their complexity, they were demanding in terms of cost and time preparation.

Previously, we reported [33] a preliminary study of coupling collagen onto PLA in only two steps with no reaction confirmation, and without cell adhesion tests. Therefore, the purpose of this study is to evaluate the use of maleic anhydride as unique intermediary for grafting collagen on PLA, and, at the same time, producing scaffolds by electrospinning technique from this polymer solution. Then, compare the macrophage adhesion and the mechanical strength of these scaffolds with scaffolds made from a physical blend of PLA and collagen. The aim of this paper is to provide a simple method for the modification of PLA, and to prepare scaffolds that promote good cell adhesion to make them useful for application in tissue engineering.

MATERIALS AND METHODS

Materials

The following materials were used as received: Poly (lactic acid) from NatureWorks, Minnetonka, MN, USA; lyophilized type I collagen (Col) from calf skin, (Mw=10^6 g/mol) from Elastin Products Co., Owensville, MO, USA. Maleic anhydride (MA), benzoyl peroxide, anhydrous dichloromethane, anhydrous di-ethyl ether, 2,2,2-trifluoroethanol, tetrahydrofurane, dimethylsulfoxide, and ninhydrin, macrophage cell line J774, Roswell Park Memorial Institute Medium (RPMI), fetal bovine serum, glutamine and β-mercaptoethanol from Sigma-Aldrich, St. Louis, MO, USA.

Functionalization of poly (lactic acid) with maleic anhydride

The MA was grafted onto PLA via a free radical reaction by using benzoyl peroxide as initiator. The PLA was dissolved in anhydrous dichloromethane and mixed with MA in a ratio of 100:10 (%w/w). Benzoyl peroxide was added in a proportion of 5% w/w with respect to the amount of MA. The mixture was stirred at room temperature until it became homogeneous. The solvent was removed, and the mixture was kept in vacuum for 12 h at 110°C under a nitrogen atmosphere. The reaction product was dissolved in anhydrous dichloromethane, and it was precipitated with anhydrous di-ethyl ether to remove the residues of maleic anhydride. Finally, the functionalized PLA (PLA-g-MA) was filtered and dried under vacuum at room temperature for 24 h [34]. The proportion of MA attached to the PLA was determined by the modified rhodamine-carboxyl interaction method [35].

Grafting collagen onto poly (lactic acid)

For the reaction of grafting collagen on PLA-g-MA, both components were dissolved in 2,2,2-trifluoroethanol and mixed at two different proportions. The mixtures were stirred during 24 h at room temperature to promote the chemical grafting of collagen on PLA-g-MA. The ratios of PLA-g-MA: Collagen in the reacting mixtures were 15:1 and 30:1 (w/w), respectively 6.26% and 3.22% of collagen content.

Preparation of poly (lactic acid)-graft-maleic anhydride/collagen and blends of poly (lactic acid)-collagen scaffolds by electrospinning

The polymeric solutions mentioned above (PLA-g-MA/Col) were used immediately after 24 hours for the fabrication of nanofibrous scaffolds through electrospinning technique. The electrospinning equipment and the procedure used were described elsewhere [36].

To compare the grafted polymer scaffolds with the scaffolds made of raw materials and physical blends, solutions of PLA (13% w/v), PLA-g-MA (13% w/v), collagen (5% w/v) and PLA-Col (30:1), were prepared also using 2,2,2-trifluoroethanol as a solvent. Each polymer solution was transferred to a 5 mL plastic syringe having a 0.7 mm diameter needle. Aluminum foil was used as fibers collector. The polymer solutions flow rate was kept at 0.4 mL h^{-1} and a potential difference of 10 kV (Spellman CZE 1000R) was applied between the needle and the collector. The distance between the needle tip and the collector was 10 cm.

Characterization

Grafting of MA onto PLA was analyzed by proton nuclear magnetic resonance (Bruker Advance III Micro Bay NMR spectrometer 400 MHz, Bruker, Billerica, MA, USA) using deuterated chloroform as a solvent. The average molecular weights (Mw) of PLA and PLA-g-MA were determined by high permeation liquid chromatography in a Series 200 HPLC (Perkin Elmer, Waltham, MA, USA) at room temperature. The polymers were dissolved in tetrahydrofurane, high permeation liquid chromatography grade, at a concentration of 2 mg/mL. Polystyrene standards were used for calibration.

The morphology of electrospun scaffolds was analyzed by scanning electron microscopy (SEM), using a Jeol JSM-7600F scanning electron microscope, Jeol, Tokyo, Japan. The samples were previously coated with a thin layer of gold to improve imaging resolution. The average diameter of the fibers and its distribution were determined by using the Image J software. From each SEM image, seventy fibers were measured at three different locations: at the middle and at the two end points.

Evidence of chemical coupling of collagen to PLA-g-MA in the electrospun scaffolds was obtained via attenuated total reflection-Fourier transform infrared spectroscopy from a Thermo Scientific Nicolet 6700 spectrometer, and via X-ray photoelectron spectroscopy using a VG Microtech Multilab ESCA 2000 spectrometer with an X-ray source of MgKa, operating at 15 kV (Thermo Scientific, Waltham, MA, USA).

The collagen content on PLA-g-MA/Col electrospun scaffolds was indirectly determined with the ninhydrin test by measuring the amine groups in the samples [37] from a calibration curve. Briefly, electrospun scaffolds (4 mg) were immersed for 45 s in a 0.05 M solution of ninhydrin in dimethylsulfoxide, then transferred to a vial and heated to 80°C for 10 min. The samples were solubilized with 2 mL of ninhydrin solution and 2 mL of dimethylsulfoxide, heated for 20 min more, and then cooled down at 25°C. The quantity of collagen grafted to PLA-g-MA was determined measuring the absorbance at 580 nm (UV300 UV-Visible Spectrophotometer, Thermo Scientific, Waltham, MA, USA).

The glass transition temperature (T_g) and the melting temperature (T_m) of the electrospun scaffolds were determined with a 2910 TA Instruments differential scanning calorimeter (TA Instruments, New Castle, DE, USA), at a heating rate of 10°C/min, under an inert nitrogen atmosphere. The mechanical properties of the scaffolds were determined under tensile stress in a universal mechanical testing machine (Instron model 5500 R, Instron, Norwood, MA, USA), at a crosshead speed of 10 mm/min, and 23°C. The samples were cut with a die according to the ASTM D-1708 norm [38], and all the tests were performed using five replicates.

Cell line cultured

Macrophage cell line J774 was cultured in RPMI supplemented with 10% (v/v) of fetal bovine serum, 2 mM glutamine and 0.05 mM β-mercaptoethanol. Cells were cultured at 37°C, in a CO_2 atmosphere humidified 5%. Viability and total cell concentrations were determined using a Hemacytometer (Fullerton, CA, USA).

Cell adhesion evaluation

Cell adhesion was evaluated on electrospun scaffolds of PLA-Col and PLA-g-MA/Col to analyze and compare the performance of the chemically grafted and physically blended polymers scaffolds to grow cells. The scaffolds were placed on the bottom of a 6-well cell culture cluster previously sterilized by using UV irradiation (254 nm). The macrophages were seeded at a dilution of 4×10^5 cells/well in 2 mL of RPMI, supplemented with 10% of fetal bovine serum and incubated at 37°C. As a control, cells were grown in a well without scaffold. After 48 hours of incubation, the scaffolds were removed and washed twice with cold phosphate-buffered saline. The cells adhered to the scaffold were fixed by immersing the scaffolds in a solution of 4% (v/v) formaldehyde in phosphate-buffered saline. After an immersion time of 20 min, the scaffolds were washed three times with cold fetal bovine serum. The electrospun scaffolds were then carefully placed on glass slides, air dried, and stained with a SlowFade® Gold antifade reagent containing DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride). The specimens were observed using a Zeiss Axio micro-

scope equipped with an ApoTome module (Carl Zeiss, Jena, Germany). Total cell count of DAPI-stained nuclei were obtained. A second set of scaffold specimens were gold sputtered for SEM imaging.

RESULTS

Proton nuclear magnetic resonance spectroscopy

Searching collagen was grafted onto PLA with maleic anhydride as a unique intermediary required using different spectroscopic techniques: attenuated total reflection-Fourier transform infrared spectroscopy, proton nuclear magnetic resonance and X-ray photoelectron spectroscopy. The results of proton nuclear magnetic resonance spectroscopy analysis in the electrospun scaffolds confirmed the grafting of maleic anhydride on the backbone of PLA (PLA-g-MA). The spectrum of PLA (Fig. 1A) clearly shows the two expected chemical shifts corresponding to this polymer: (a) δ_{CH}=5.15 ppm (q, J=7.1 Hz, 1H), (b) δ_{CH3}=1.57 ppm (d, J=7.2 Hz, 3H). The spectrum of PLA-g-MA is in Figure 1B. In this spectrum, additionally to the two signals from PLA, there are three new signals: (e) δ_{CH}=3.93 ppm (q, J=8.7 Hz), (d) δ_{CH2}=3.47 ppm (q, J=7.0 Hz), (c) δ_{CH3}=2.17 ppm (s). In the case of the signal (d), there are two protons in a geminal coupling, and a scalar coupling (e).

Attenuated total reflection-Fourier transform infrared spectroscopy

The attenuated total reflection-Fourier transform infrared

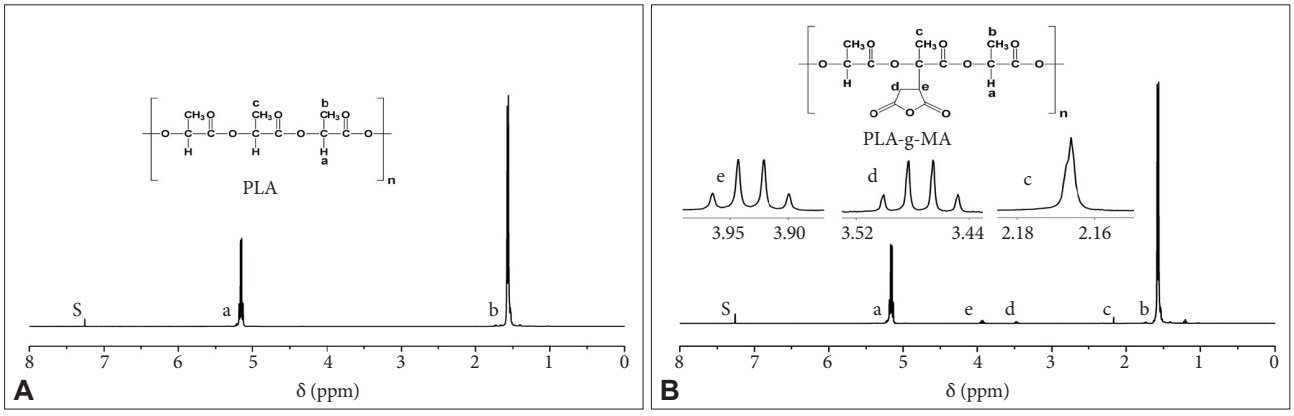

Figure 1. ^{1}H-NMR spectra of (A) PLA and (B) PLA-g-MA in $CDCl_3$. PLA: poly (lactic acid), PLA-g-MA: poly (lactic acid) grafted with maleic anhydride.

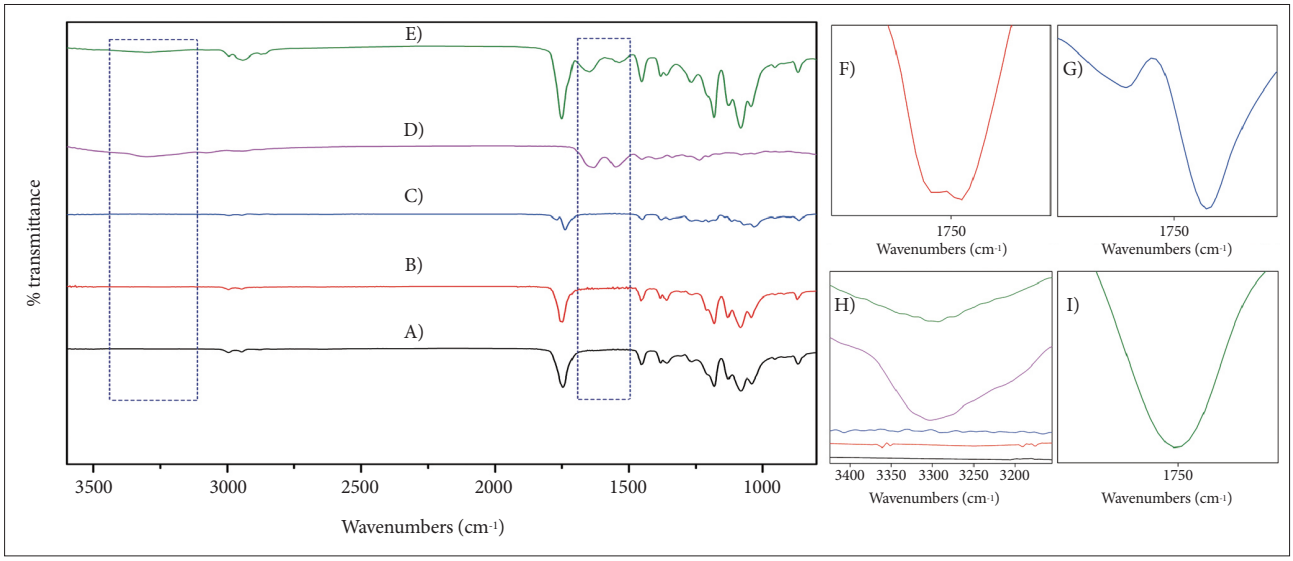

Figure 2. ATR-FTIR spectra of (A) PLA, (B) PLA-g-MA, (C) differential spectra between PLA and PLA-g-MA, (D) Collagen, (E) PLA-g-MA/Col nanofibers, (F) magnification of carbonyl group in PLA, (G) magnification of differential spectra, (H) Amino groups (ν_{N-H} in NH_2) of collagen and PLA-g-MA/Col and (I) disappearance of the double signal from the carbonyl group in the PLA-g-MA. ATR-FTIR: attenuated total reflection-fourier transform infrared spectroscopy, PLA: poly (lactic acid), PLA-g-MA: poly (lactic acid) grafted with maleic anhydride.

spectroscopy spectra of from ATR-FTIR for scaffolds of PLA, PLA-g-MA, differential spectra between PLA and PLA-g-MA, collagen Type I and PLA-g-MA/Col scaffolds are presented in Figure 2. The spectrum of scaffolds of PLA (Fig. 2, A) have one absorption band observed at 1747 cm^{-1} corresponding to its ester group, while the spectrum of PLA-g-MA (Fig. 2, B) reveals a doublet containing two bands: at 1755 and 1748 cm^{-1}. This doublet in the PLA-g-MA sample is a result of the overlapping of two absorption bands: the band located at 1748 cm^{-1} is associated with the stretching of the carbonyl group in the ester group in PLA, and the band at 1755 cm^{-1} assigned to the grafted anhydride. This signal is because of the presence of a five-membered cyclic anhydride who exhibit an intense absorption band between 1750 and 1794 cm^{-1} owing to the symmetrical stretching of C=O [39]. The magnification of this peak is in F (Fig. 2). Besides, as shown in G (Fig. 2), the differential spectra of PLA and PLA-g-MA also displayed double bands at 1738 cm^{-1} and 1768 cm^{-1} ($\nu_{C=O}$); these results are complementary evidence indicating that MA was successfully grafted onto PLA as per the results obtained by Muenprasat et al. [40]. The spectrum of nanofibers made from PLA-g-MA/Col solutions E (Fig. 2) shows the appearance of three new signals that are not observed in the spectra of PLA-g-MA. The first signal at 3300 cm^{-1} corresponds to the amino groups (ν_{N-H} in NH_2) H (Fig. 2). The second and the third signals are located at 1648 and 1537 cm^{-1} (δ_{N-H} in -$CONH^-$), corresponding to amide groups I and II, and are in agreement with the results found by Li et al. [41]. As it can be observed in D (Fig. 2), these signals are characteristic of the collagen.

Broad scanning X-ray photoelectron spectroscopy

The broad scanning X-ray photoelectron spectroscopy spectra for the C 1 s orbital in the electrospun scaffolds made from PLA-g-MA, PLA-Col, and PLA-g-MA/Col are shown in (Fig. 3A, B, and C). For the PLA-g-MA sample (Fig. 3A), the spectrum consists of three peaks with a binding energy at 285.00 eV for C-H bonds, at 286.97 eV for C-O, and at 288.94 eV O-C=O

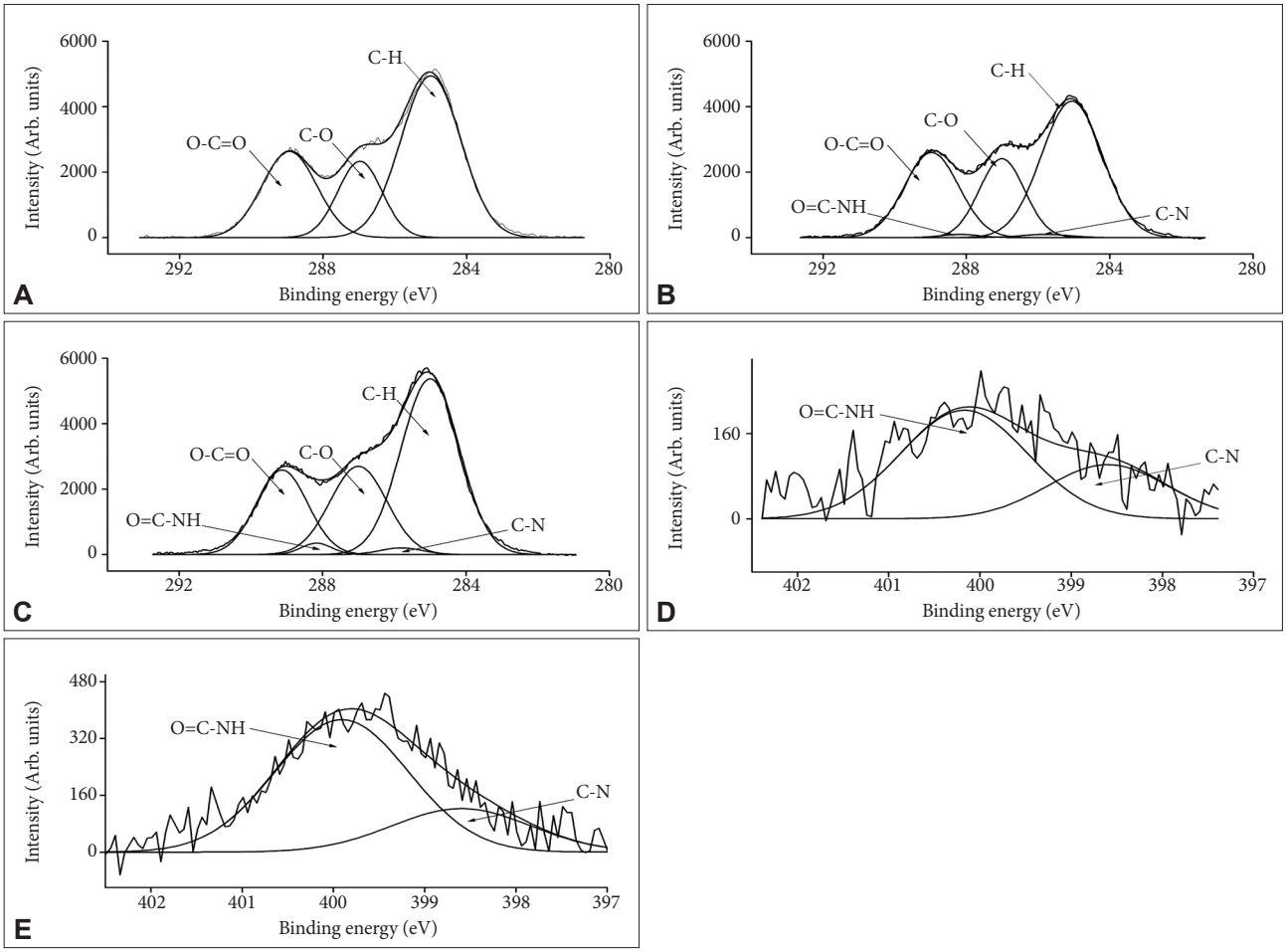

Figure 3. XPS C 1 s orbital spectra of (A) PLA-g-MA, (B) PLA-Col (30:1), (C) PLA-g-MA/Col (30:1) nanofibers and N 1 s orbital spectra of (D) PLA-Col (30:1) and (E) PLA-g-MA/Col (30:1) nanofibers. XPS: X-ray photoelectron spectroscopy, PLA-g-MA: poly (lactic acid) grafted with maleic anhydride, PLA-Col: blend of poly (lactic acid) with collagen.

bonds. In the spectrum of PLA-Col (Fig. 3B), two new peaks appeared associated with the C-N and O=C-NH bonds at 285.80 and 288.17 eV. In the spectrum of the PLA-g-MA/Col sample (Fig. 3C) these two peaks also appear with the difference that the intensity and the area of each peak are larger. Finally, the spectra of N 1s for PLA-Col and PLA-g-MA/Col are shown in Figure 3D and E, which presents two peaks; at a binding energy of 398.60 eV for the C-N bonds and at 399.91 eV for O=C-NH bonds. In this case, the difference is also the intensity of the peak corresponding to O=C-NH bond which is greater in the PLA-g-MA/Col.

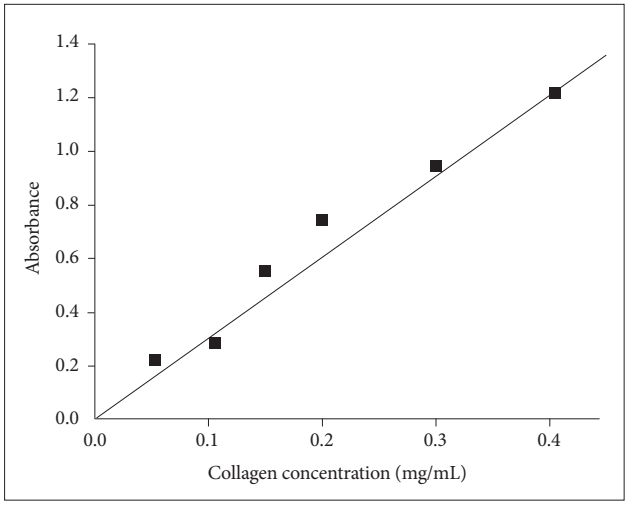

Figure 4. Calibration curve of absorbance with collagen concentration.

Determination of collagen content

For the determination of the collagen content in the electrospun scaffolds was conducted from a calibration curve. The calibration curve was built with absorbance (Abs) in the y axis versus concentration of collagen in ninhydrin [C] in the x axis, and it is shown in Figure 4. The obtained linear equation was Abs=0.0454+3.0115C; with a correlation (R^2) of 0.98. The amount of collagen was determined in the electrospun scaffold samples with a content of 15:1 and 30:1 of PLA-g-MA:Col, and the results were 5.75% and 3.14%, respectively.

Morphology of the electrospun scaffolds

The morphology of the electrospun scaffolds is one of the several factors that influence adhesion and cell proliferation in tissue engineering. Other factors include surface properties, porosity and chemical composition, which should be also considered during cell culture studies [42]. For every electrospun scaffold, a SEM micrograph (Fig. 5A, B, and C) and a frequency histogram (Fig. 5D, E, and F) are shown, corresponding to the fiber's morphology and fiber diameters distribution. The SEM image for PLA scaffold illustrates a network of highly uniform and randomly oriented fibers, without the presence of beads defects (Fig. 5A). The average diameter of the PLA nanofibers

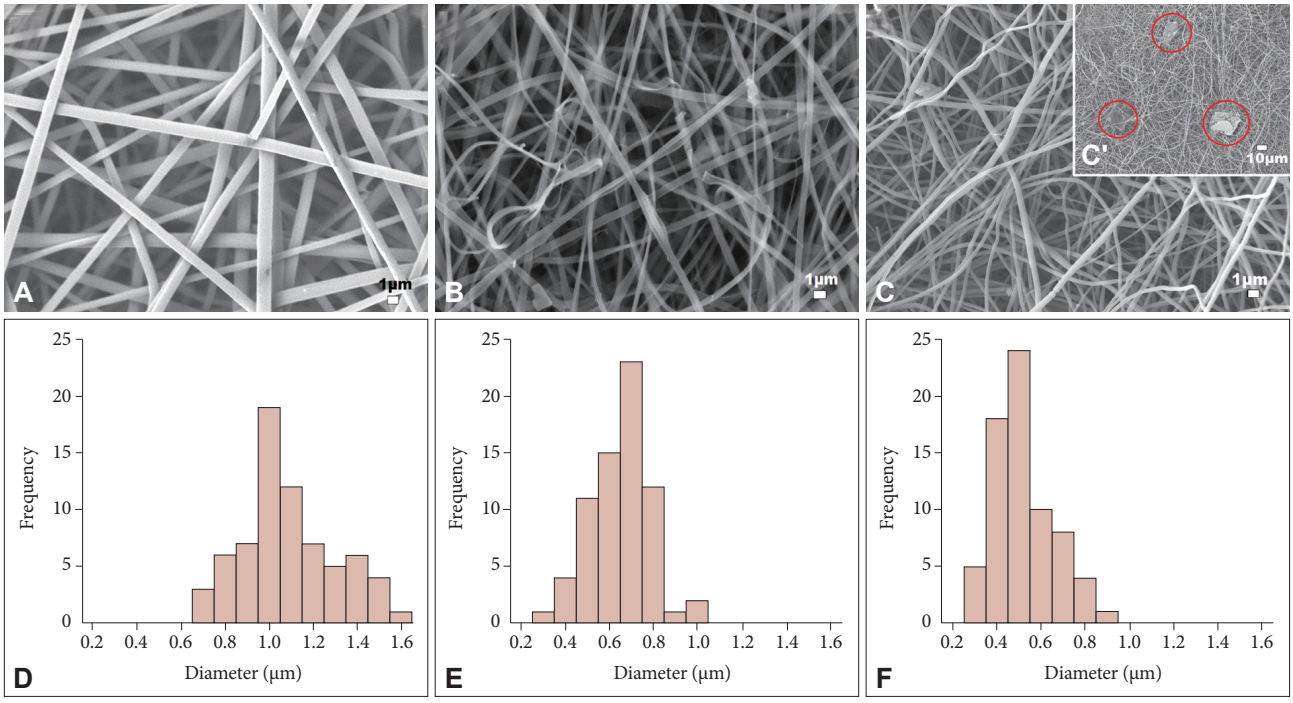

Figure 5. SEM micrographs of nanofiber's scaffolds of (A) PLA, (B) Col and, (C) PLA-Col blend, (C') Collagen clusters. Diameters histograms: (D) PLA, (E) Col, and (F) PLA-Col blend. SEM: scanning electron microscopy, PLA: poly (lactic acid), Col: collagen.

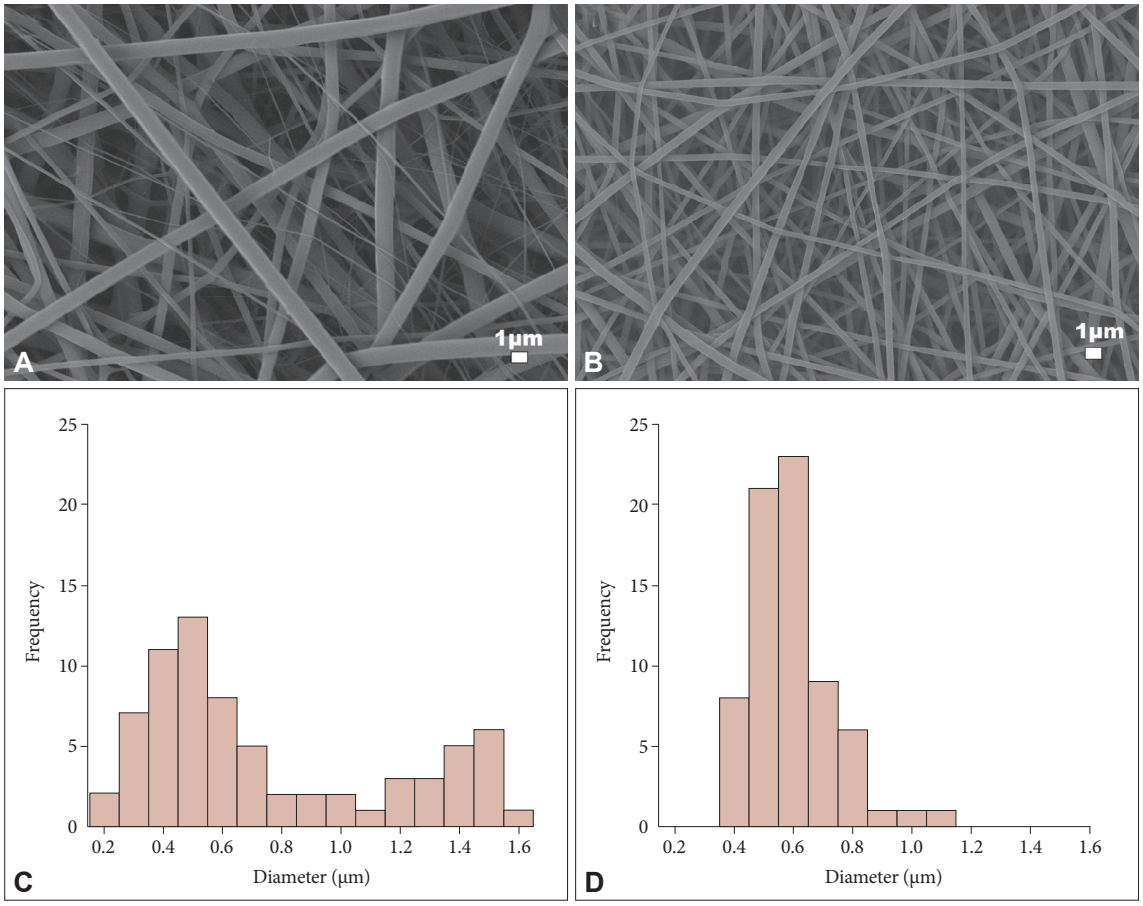

Figure 6. SEM micrographs of nanofiber's scaffolds of PLA-g-MA/Col nanofibers at different concentrations of PLA-g-MA:Col (A) 30:1, and (B) 15:1. Diameters histograms: (C) 30:1, and (D) 15:1. SEM: scanning electron microscopy, PLA-g-MA: poly (lactic acid) grafted with maleic anhydride, PLA-Col: blend of poly (lactic acid) with collagen.

Table 1. Thermal properties of electrospun scaffolds of pure PLA, PLA physically blended with collagen, PLA functionalized with MA, and PLA grafted with collagen (30:1 and 15:1)

Sample	Tg (°C)	Tm (°C)
PLA	60.36	151.75
PLA-Col	60.44	151.75
PLA-g-MA	52.11	148.71
PLA-g-MA/Col (30:1)	61.82	152.3
PLA-g-MA/Col (15:1)	61.73	152.51

PLA: poly (lactic acid), MA: maleic anhydride

was determined to be 1093±210 nm. The corresponding diameters histogram (Fig. 5D) shows that the most frequent diameters of fibers were from 900 nm to 1100 nm, and the range of the diameters, between the thinner and the thicker, was approximately 900 nm. Collagen nanofibers exhibited an average diameter of 651±135 nm. By analyzing Figure 5B, the main difference found between the fibers of collagen, PLA and PLA-Col blends is that the collagen fibers look transparent. The most frequent diameter of collagen fibers was around 700 nm with a distribution width of 600 nm (Fig. 5E). From the SEM micrographs, the average diameter for PLA-Col blends (30:1) nanofibers was determined to be 520±125 nm (Fig. 5C). The nanofibers of PLA-Col blends (30:1) also showed the presence of large clusters, circles in Figure 5C', indicating that collagen was not completely miscible with PLA. The corresponding histogram illustrates that the most common diameters were between 400 to 500 nm, with a distribution width of 600 nm (Fig. 5F). A compilation of SEM micrographs of PLA-g-MA/Col nanofibers obtained at concentrations of: 30:1 (3.22% of Col) and 15:1 (6.25% of Col), respectively, are presented in Figure 6A and B. The average diameter of the fibers of the concentration 30:1 was determined to be 746±424 nm, and for the concentration 15:1 was 595±139 nm. The high deviation for the concentration 30:1 in Figure 6A was because this sample presented a bimodal distribution of diameter with two different means (Fig. 6C); the first peak corresponds to an average diameter of 500 nm with an amplitude of 800 nm, and the second one has a maximum at 1500 nm with a distribution of sizes in the range of 500 nm. In high contrast, the histogram of

the PLA-g-MA/Col 15:1 (Fig. 6D) presents smaller amplitude; the most frequent diameters were between 500 and 600 nm with a sharp distribution between 400 and 800 nm, and then a plateau with some larger fibers of 900, 1000, and 1100 nm.

Thermal properties of the electrospun scaffolds

The values of glass transition temperature (Tg) and melting temperature (Tm) of the electrospun scaffolds made from pure PLA, PLA-Col, PLA-g-MA, and PLA-g-MA/Col are displayed in Table 1. As expected, Tg and Tm of PLA (Mw=192, 620 g/mol) were higher than Tg and Tm of PLA-g-MA (Mw=160, 889 g/mol), indicating their differences in molecular weight, and providing indirect confirmation of the grafting effectiveness, according to the results obtained by Hwang et al. [43].

Mechanical properties of the electrospun scaffolds

As the electrospun scaffolds must withstand the growth and cell proliferation for tissue regeneration during significant periods of time, determining their mechanical properties is of utmost importance [36]. Mechanical properties measured in the electrospun scaffolds produced from solutions of PLA, collagen, PLA-Col 30:1 and PLA-g-MA/Col 30:1 are displayed in Figure 7. Stress-strain curve of pure PLA in Figure 7A showed a maximum stress after the linear part of the curve that is not present in the rest of the samples. Particularly, pure PLA had the maximum values in elastic modulus (Fig. 7B) tensile strength (Fig. 7C) and percent of strain (Fig. 7D), while collagen had the poorer mechanical properties. It is hypothesized that the poor mechanical properties of collagen nanofibers are related to the transparency of the fibers presented in Figure 5B; these fibers may be too weak to support cells growing and adhesion. When comparing the mechanical properties of PLA-Col and PLA-g-MA/Col in Figure 7, PLA-Col blend had poorer mechanical properties due to the low miscibility of the phases, leading to weak adhesion. The increase in mechanical properties of the specimen containing the grafted MA and collagen highlights that PLA-g-MA/Col nanofibers exhibited greater strength and strain than the scaffolds of just blends of PLA and collagen. These mechanical measurements are in agreement with the attenuated total reflection-Fourier transform infrared spectros-

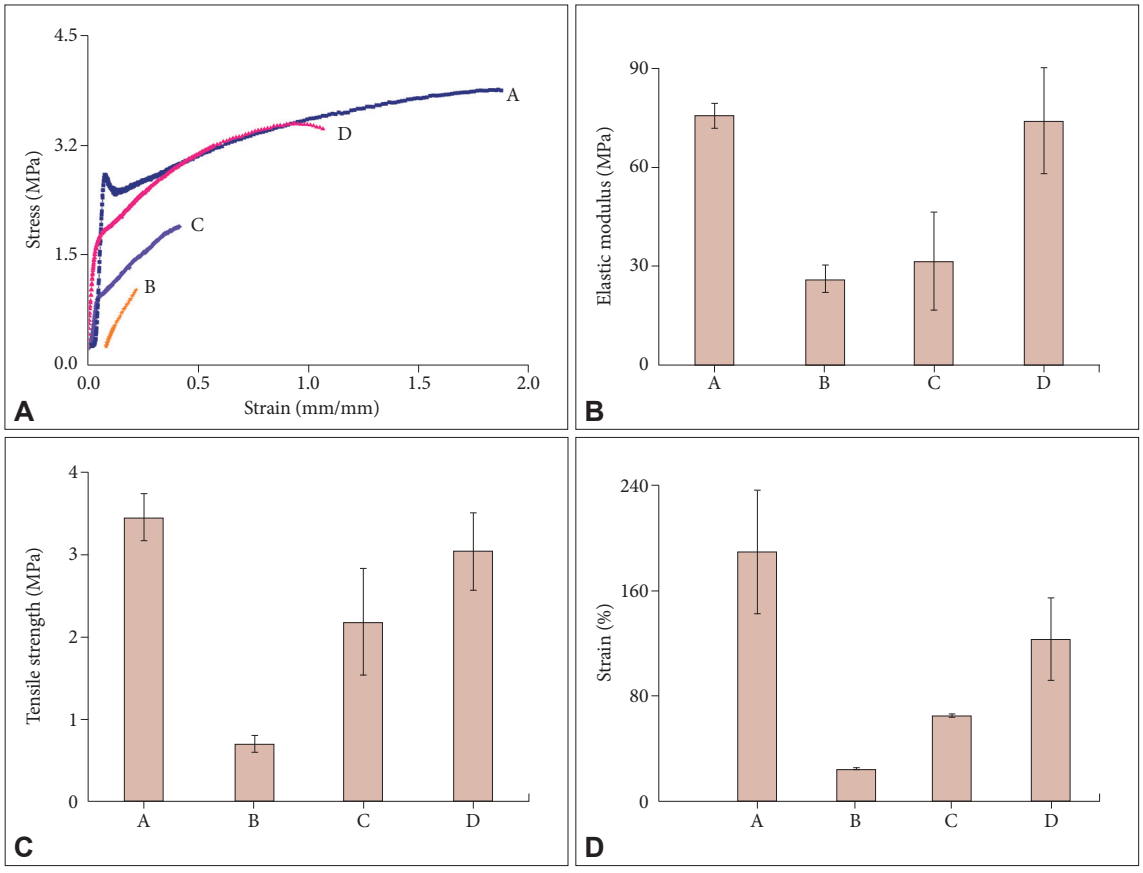

Figure 7. Mechanical properties of nanofiber scaffolds of PLA=A, collagen=B, PLA-Col=C, and PLA-g-MA/Col=D. (A) Stress-strain curve, (B) elastic modulus, (C) tensile strength, and (D) percent of strain. PLA: poly (lactic acid), PLA-Col: blend of poly (lactic acid) with collagen, PLA-g-MA/Col: poly (lactic acid)-graft-maleic anhydride/collagen.

copy and X-ray photoelectron spectroscopy experiments presented earlier, because of the functionalization with MA and posterior grafting of collagen onto PLA improves compatibility between PLA and collagen. This increase in compatibility has a substantial effect on the elastic modulus (Fig. 7B) of the scaffolds: it is 130% higher than the elastic modulus of the physically blended samples. The tensile strength (Fig. 7C) and the strain percentage (Fig. 7D) of the PLA-g-MA/Col scaffolds also increased in a 41% and an 88%, with respect to the scaffold made of physical blends of PLA-Col.

Cell adhesion on the electrospun scaffolds

Macrophages play an important role in the cellular matrix: when they are present in the restoration area of a living tissue promote and protect the cell growth of specialized tissue. Macrophages are dominant infiltration cells that respond rapidly to

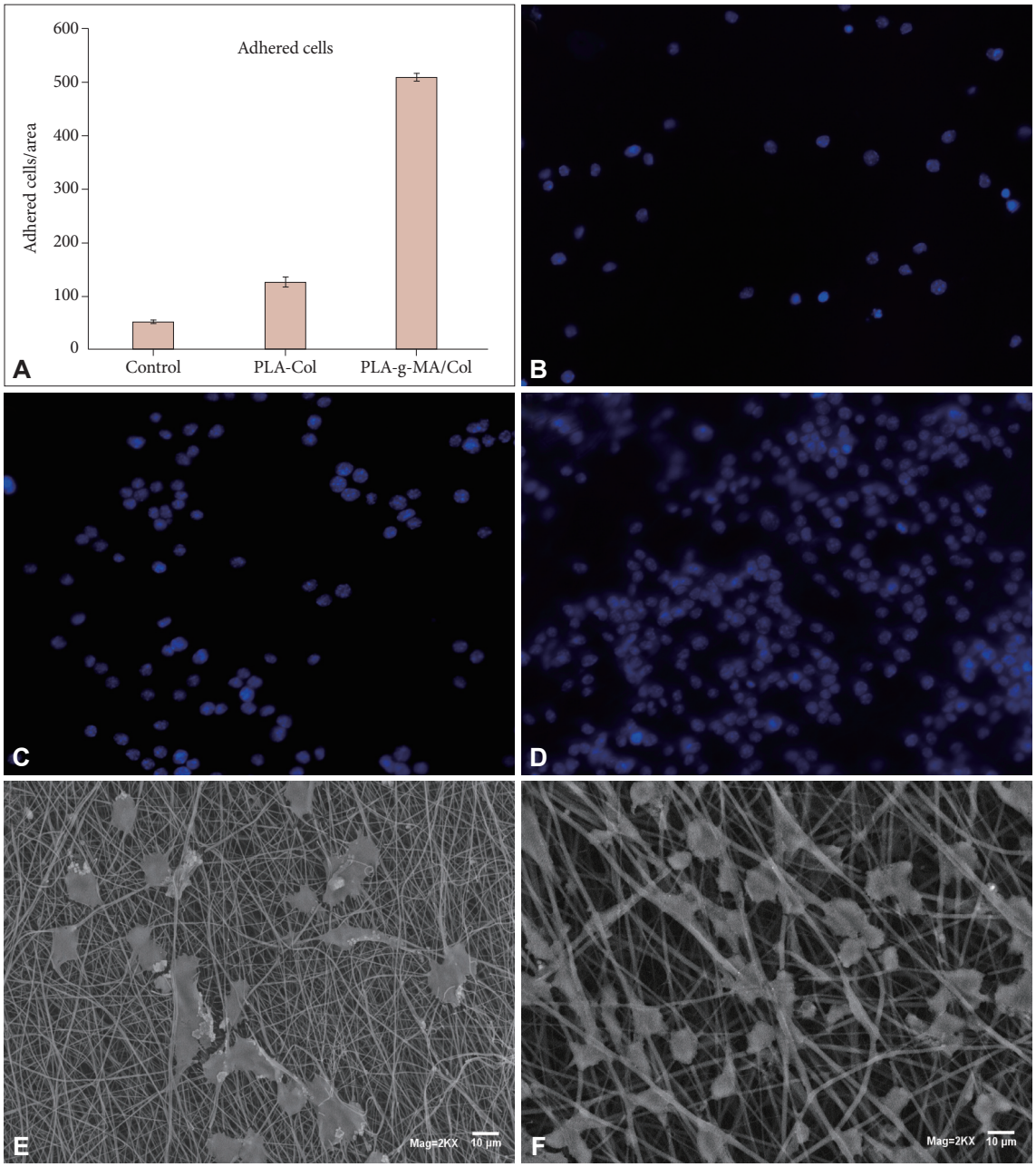

Figure 8. Cell culture of J774 Macrophages: (A) Cells count. DAPI-staining micrographs of: (B) Control, (C) PLA-Col (30:1), (D) PLA-g-MA/Col (30:1). SEM micrographs (E) PLA-Col (30:1), (F) PLA-g-MA/Col (30:1) electrospun scaffold. DAPI: 4', 6-diamidino-2-phenylindole, PLA-Col: blend of poly (lactic acid) with collagen, PLA-g-MA/Col: poly (lactic acid)-graft-maleic anhydride/collagen, SEM: scanning electron microscopy.

biomaterial implantation, and play a crucial role in regulating the inflammatory response and tissue remodeling, by secreting large amounts of bioactive mediators that can initiate inflammation, cell adhesion and differentiation, tissue remodeling and blood vessel formation [44,45]. In this work, the J774 macrophages were cultured for 48 hours on the electrospun scaffolds of PLA-Col (30:1) and PLA-g-MA/Col (30:1) to evaluate the effect of grafting collagen onto PLA. The number of cells adhered to the scaffolds are in Figure 8A. The count of cells was conducted from fluorescence microscopy images showed in Figure 8B, C and D, of the control and the electrospun scaffolds, respectively. For this analyzing technique, the DAPI staining method allowed the observation of the adhesion of cells by means of blue coloration of the nuclei of the cells. Stained cells were found in the two-electrospun scaffolds, and in a minor scale in the control, demonstrating positive cell adhesion. However, PLA-g-MA/Col scaffolds showed a better response, as the number of cells adhered to this scaffold was over 509, and the number of cells adhered to the PLA-Col scaffold was 127 (Fig. 8A). This four-fold increase in the number of cells is evidence that grafting collagen to PLA-MA promotes a greater degree of cell growth. This increase in cell growth can also be attributed to the increased bio-stability of the PLA-g-MA/Col scaffold as its mechanical properties are higher, providing a more stable substrate. SEM micrographs of scaffolds with cultured cells are shown in Figure 8E and F. These micrographs allowed us to see the cultured cells in three dimensions, and it can be noticed that most of the cells in the PLA-Col scaffold are adhered to the surface only, while the cells in the PLA-g-MA/Col scaffold are adhered not only to the surface but also inside the scaffold. The growth of cells within the scaffold is a desired feature since it may allow cell growth in three dimensions. In both cases, cell morphology is elongated and they grew in the direction of the fibers, which is in agreement with previous reports [46]. Another factor that promoted more macrophage adhesion in this scaffold is that, from SEM images, the PLA-g-MA/Col fibers (Fig. 6A and B) have larger diameters than the fibers of the blends of PLA-Col (Fig. 5C).

DISCUSSION

In this study, collagen was successfully grafted to the backbone of PLA with maleic anhydride as a unique intermediary to improve the biocompatibility of the synthetic polymer. Once the PLA was grafted, we focused on producing scaffolds by electrospinning to grow cells. The grafting reaction, in the first stage with maleic anhydride and then with collagen, was confirmed from attenuated total reflection-Fourier transform infrared spectroscopy, proton nuclear magnetic resonance and X-ray photoelectron spectroscopy results.

The proton nuclear magnetic resonance spectrum of PLA-g-MA in Figure 1B showed the two signals from PLA, additionally, there are three new signals. One of them, signal (d) (δ_{CH2}= 3.47 ppm) is close to the chemical shift of the succinic anhydride ($\delta_{CH2}\approx$3 ppm) [47]. These results are in agreement with those calculated by using the method of Pretsch et al. [48]: (e, δ_{CH})=4.22 ppm, for (d, δ_{CH})=3.10−2.85 ppm and (c, δ_{CH3})=1.60 ppm, but disagree with reports of previous authors [31,49]. The percentage of grafting of MA onto PLA determined with the rhodamine method was 0.32%. The presence of these chemical shifts found in the proton nuclear magnetic resonance spectrum of PLA-g-MA, Figure 1B, is a positive evidence of the effective grafting of MA onto PLA.

Attenuated total reflection-Fourier transform infrared spectroscopy permitted to confirm both maleic anhydride and collagen grafting. The differential spectra between PLA and PLA-g-MA in C and G (Fig. 2) showed the evidence of the anhydride grafting. Moreover, the disappearance of the double signal from the carbonyl group and the appearance of the signal at 3300 cm^{-1} corresponding to the amino groups in the PLA-g-MA/Col spectrum (Fig. 2, E and I) confirm the reaction of the anhydride groups with amino groups of the collagen [50] showing that, effectively, collagen has been grafted onto the PLA-g-AM in the scaffold.

The results from the broad scanning X-ray photoelectron spectroscopy spectra for the C 1 s and N 1 s orbitals in the Figure 3B-E for the samples PLA-Col and PLA-g-MA/Col are complementary evidence of the grafting of collagen onto the PLA-g-MA in the scaffold [51] hence confirming the results from the attenuated total reflection-Fourier transform infrared spectroscopy analysis.

The collagen content was determined in the electrospun scaffolds throughout a calibration curve of absorbance (Abs) versus concentration of collagen in ninhydrin (C) in a UV spectrophotometer. The samples prepared with the initial proportions of 15:1 and 30:1 of PLA-g-AM:Col were analyzed, and the grafting results were 5.75% and 3.14%, respectively. However, this amount of grafting is 15% higher than the obtained in a recent report [29]. This new graft polymer, collagen being a more bioactive molecule than PLA, yielded electrospun scaffolds with fewer morphological defects than the scaffold produced with the physical blends of PLA and collagen despite the small concentration of collagen in the graft polymer. A limitation of this work is the small amount of grafted collagen onto PLA. Further work suggests new attempts to increase the efficiency of the reaction to achieve a higher proportion of grafted collagen.

The extracellular matrix is the base for the attachment growing and proliferation of cells in a living tissue. Scanning elec-

tron microscopy is the technique to compare the original fibrous structure with the synthetic scaffolds. The scaffolds for tissue engineering are fibrous structures fabricated with polymers and natural molecules to substitute the function of the extracellular matrix, and they are an option to grow tissue *in vitro*. Some of the features of the scaffolds to succeed is that the fibers have the diameter and the porosity that mimic the natural one. In the electrospun fibers obtained in this work, it was observed that the diameter decreased with increasing concentration of collagen, with no variation nor defects along the fibers. A possible explanation for this trend is that as the amount of collagen increased, the viscosity of the solution decreased. Then, the higher concentration of amino acids improves the stretching ability of the polymer solution during the electrospinning process hence resulting in fibers of small diameters [52]. Furthermore, the samples made from PLA-g-MA/Col solutions do not showed precipitates or agglomerates after they were electrospun, as it was observed in SEM images of PLA-Col blends in Figure 5C', indicating that collagen was more compatible with PLA-g-MA than with pure PLA and this polymer solution was more amenable to be electrospun.

The thermal behavior of the scaffolds produced from PLA-Col appears to be very similar to that of the scaffolds produced from pure PLA; the thermal properties of the minority component, collagen, were not explicit in the thermogram. The samples of scaffolds produced from the PLA-g-MA/Col precursor solutions exhibited higher glass transition and melting temperature values than those produced from PLA-g-MA solutions. It is thought that collagen modifies slightly the thermal properties of PLA-g-MA because of the molecular interactions between collagen and the modified PLA backbone.

For the scaffolds for tissue regeneration it is required to have enough mechanical properties to support the cell growing and the fluid contact during a period of time to regenerate the live tissue. The scaffolds with the higher mechanical properties were made of PLA-g-MA/Col, and they had more cell growing than the scaffolds of PLA only or the scaffolds made of PLA-Col. The higher compatibility of the PLA-g-MA/Col scaffolds also leaded to better mechanical properties, and these results are in agreement with the attenuated total reflection-Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy experiments presented earlier, because of the functionalization with MA and posterior grafting of collagen onto PLA improves compatibility between PLA and collagen. Thus, the chemical grafting of collagen not only immobilized the collagen increasing the bioactivity of PLA, but also augmented its mechanical properties, compared to scaffolds made of physical blends of PLA-Col.

Developing new materials for tissue engineering ends with cells growing as the most important test before the *in vivo* experiments. The count of cell from fluorescence microscopy allowed us to find more cell adhesion in the scaffolds with grafted collagen than the pure PLA. The scaffold made from grafted collagen on PLA presented four-fold increase in cell adhesion than the scaffolds of PLA blended with collagen, with reduced diameter in the resulting electrospun nanofibers. The result is in agreement with a previous work where the influence of the polymer fiber diameter on macrophage adhesion and growing was analyzed using RAW 264.7 cells. It was found that 500 nm to 2 μm fiber diameters provided the best adhesion and spreading of macrophages compared to smaller fiber diameters or flat surfaces [53]. The growth of cells in the interior of the PLA-g-MA/Col scaffolds is indicative of the potential of this new material to create scaffolds that could sustain cell growth in three-dimensions. Finally, from the experiments and characterization protocols presented here, it can be inferred that the presence of anhydride groups in the PLA favors collagen grafting, better mechanical properties as well as more cell adhesion than PLA-Col physical blend. The presented procedure can open a new avenue for growing cells in three dimensions with the production of stable scaffolds with more bioactive molecules that promote tissue regeneration.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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