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Plasma treatment induces internal surface modifications of electrospun poly(L-lactic) acid scaffold to enhance protein coating

Hyok Jin Seo,[1,2] Mi Hee Lee,[1,2] Byeong-Ju Kwon,[1,2] Hye-Lee Kim,[1,2] Seung Jin Lee,[3] Bong-Jin Kim,[4] Kang-Kyun Wang,[4] Yong-Rok Kim,[4] and Jong-Chul Park[1,2,a)]

[1]*Cellbiocontrol Laboratory, Department of Medical Engineering, Yonsei University College of Medicine, Seoul 120-752, South Korea*

[2]*Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, South Korea*

[3]*Department of Industrial Pharmacy, College of Pharmacy, Ewha Womans University, Seoul 120-750, South Korea*

[4]*Department of Chemistry, Yonsei University, 50 Yonsei-ro, Seodaemun-Gu, Seoul 120-749, South Korea*

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Advanced biomaterials should also be bioactive with regard to desirable cellular responses, such as selective protein adsorption and cell attachment, proliferation, and differentiation. To enhance cell-material interactions, surface modifications have commonly been performed. Among the various surface modification approaches, atmospheric pressure glow discharge plasma has been used to change a hydrophobic polymer surface to a hydrophilic surface. Poly(L-lactic acid) (PLLA)-derived scaffolds lack cell recognition signals and the hydrophobic nature of PLLA hinders cell seeding. To make PLLA surfaces more conducive to cell attachment and spreading, surface modifications may be used to create cell-biomaterial interfaces that elicit controlled cell adhesion and maintain differentiated phenotypes. In this study, (He) gaseous atmospheric plasma glow discharge was used to change the characteristics of a 3D-type polymeric scaffold from hydrophobic to hydrophilic on both the outer and inner surfaces of the scaffold and the penetration efficiency with fibronectin was investigated. Field-emission scanning electron microscope images showed that some grooves were formed on the PLLA fibers after plasma treatment. X-ray photoelectron spectroscopy data also showed chemical changes in the PLLA structure. After plasma treatment, -CN (285.76 eV) was increased in C1s and $-NH_2$ (399.70 eV) was increased significantly and –N=CH (400.80 eV) and $–NH_3^+$ (402.05 eV) were newly appeared in N1s. These changes allowed fibronectin to penetrate into the PLLA scaffold; this could be observed by confocal microscopy. In conclusion, helium atmospheric pressure plasma treatment was effective in modifying the polymeric scaffold, making it hydrophilic, and this treatment can also be used in tissue engineering research as needed to make polymers hydrophilic. © *2013 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4818914]

I. INTRODUCTION

Biomaterials that are commonly used in tissue engineering should have good mechanical and biocompatible properties. Previous biomaterials were considered only to be inert or biocompatible, but present advanced materials must not only be inert or biocompatible, but also bioactive with regard to desirable cellular responses, such as selective protein adsorption, cell attachment, proliferation, and differentiation.

To enhance cell-material interactions, surface modifications are generally performed. Among the various approaches, atmospheric pressure glow discharge (APGD) plasma has been used to change hydrophobic polymer surfaces to hydrophilic surfaces.[1] Surface hydrophobicity is a critical parameter in polymer scaffolds.[2–5]

Poly(L-lactic acid) (PLLA) is used as a scaffold for regenerating cartilage, not only because it has similar mechanical properties to the target tissue, but also because of its good biological interactions with host cells when implanted.[6] However, PLLA-derived scaffolds lack cell recognition signals and its hydrophobic nature hinders cell seeding. To make PLLA surfaces more conducive to cell attachment and spreading, surface modifications may be used to create cell-biomaterial interfaces that elicit controlled cell adhesion and maintain differentiated phenotype.[7] Such modifications generally involve enriching the substrates with extracellular matrix (ECM) molecules and derivatives, such as collagen, chitosan, and gelatin, as surface modifiers, which have been found to improve the cytocompatibility of polyesters substantially.[8,9]

Among the various surface modification approaches available for PLLA, plasma treatment seems the most promising. By choosing an appropriate plasma source, diverse functional groups can be introduced to the fiber surface to improve biocompatibility or allow subsequent covalent immobilization of various bioactive molecules.[10,11] However, a limitation of plasma surface treatments of porous polymer constructs is the depth of penetration of the modification, which depends on the treatment time and the power level.[12] Various ECM protein components, such as gelatin, collagen, laminin, and fibronectin, have been immobilized onto plasma-treated surfaces to

a)Author to whom correspondence should be addressed. Electronic mail: parkjc@yuhs.ac

enhance cellular adhesion and proliferation.[13,14] If three-dimensional (3D) structured scaffolds can have hydrophilic property not only at the surface but also on the inside, this could be effective for culturing cells on the scaffold and treating some kinds of ECM proteins, such as fibronectin, lamin, and vitronectin. In this study, (He) gaseous atmospheric plasma glow discharge was used to change the characteristics of a 3D-type polymeric scaffold from hydrophobic to hydrophilic on both the outer and inner surface of the scaffold. To overcome a limitation of plasma technique, a self-designed plasma system was used and various parameters of plasma system, such as dielectric barrier's gap distance, gas flow, power voltage, and frequency, were controlled. Penetration efficiency with fibronectin to scaffold was also investigated. The "inner surface" means inner area which is made up of many nano/micro fibers.

II. MATERIALS AND METHODS

A. Preparation of a 3D microfibrous scaffold

The process for fabricating the scaffold was described in detail previously.[15] Briefly, PLLA (intrinsic viscosity 0.63 dl/g, $MW = 2.5 \times 10^5$ g/mol) was purchased from Purac Biochem (Gorinchem, The Netherlands). A 20% (wt./vol.) PLLA solution was prepared in a mixed solvent of dichloromethane and acetone (Duksan Chemicals Co., Seoul, Korea) with volume ratios of 80:20. This PLLA solution was kept in a 10-ml syringe attached to a 25-gauge blunt-end needle and extruded from the syringe with a pump at a volume flow rate of 0.1 ml/min.

The electrospinning process was performed in a sterile environment at high voltage between 8 and 20 kV. The distance between the needle tip and the collector was 15 cm. Electrospun fibers were collected on a metal plate and formed non-woven microfibrous mats. Electrospun fibrous mats were expanded mechanically in all directions using a metal comb. After expansion, their volume enlarged up to approximately seven times. The completed electrospun scaffolds were dried for 3 days under vacuum at 70 °C to remove residual solvent.

B. Helium atmospheric pressure glow discharge plasma system

In this study, a typical planar type dielectric barrier discharge system (helium atmospheric pressure glow discharge, He-APGD) was constructed. A self-designed plasma head unit equipped with a high-voltage bipolar direct-current-pulse power source (HPI-500, FT-Lab, Korea) was used to generate atmospheric pressure plasma. The head unit consisted of two alumina plates as dielectric barriers, with an aluminum electrode attached on the outer side. To modify inner surface of scaffold, various parameters were controlled. The gap distance of the alumina plates was 3 mm and helium gas could be introduced through the gap between the alumina plates. The PLLA electrospun scaffold was placed between these alumina plates. After sealing the dielectric barriers, helium was introduced into the narrow space between them at a rate of 2 l/min. The parameters of the plasma system were 2 kV power voltage, 2 kHz frequency, and a 20% duty cycle. Plasma treatment time was set to 30 s, 5 min, and 10 min, and non-treated scaffold (0 s) were used as a control.

C. Surface morphology assessed by scanning electron microscopy

Morphological changes were observed with a field-emission scanning electron microscope (FE-SEM, S-800, Hitachi, Japan). The prepared samples were untreated scaffold (control) and plasma-treated scaffolds (30 s, 5 min, and 10 min). After a thin gold coating, the specimens were examined using a FE-SEM.

D. Surface chemistry survey

Chemical changes on the surface caused by He-APGD treatment were analyzed using X-ray photoelectron spectroscopy (XPS, K-alpha, Thermo UK). To observe the surface chemistry, ion-beam etching was not performed.

E. Changes in hydrophilicity

Changes in hydrophilicity after He-APGD plasma treatment were assessed using a digital camera (Nikon PowerShot SX20 IS, Japan). Trypan blue solution (10 μl) was dropped on the PLLA scaffold from the tip of a needle and photographic views were captured with the digital camera.

F. Effects of plasma treatment in enhancing inner surface coating with fibronectin

The effects of He-APGD plasma treatment on fibronectin adsorption into the inner surfaces of a PLLA scaffold were observed using confocal laser scanning microscopy (LSM 700, Carl Zeiss, Jena, Germany). Fibronectin (HiLyte Fluor 488-labeled; Cytoskeleton, Inc.) was diluted 1:1 with phosphate-buffered saline. Diluted fibronectin solution (10 μl) was dropped on the PLLA scaffolds from the tip of a needle. The scaffolds were placed on a slide glass and viewed at 5× magnification. Z-stack analysis was then performed for a 3D analysis, to assess whether the fibronectin solution had penetrated to the inner surfaces of the scaffold.

III. RESULTS

A. Changes in hydrophilicity of the PLLA electrospun scaffold

He-APGD plasma was treated to PLLA electrospun scaffolds for 10 min and compared them with untreated scaffold. After plasma treatment, trypan blue solution (~10 μl, using a micro pipette) was dropped and pictures were taken with a digital camera (Fig. 1). Trypan blue solution spread on the surface of the plasma-treated scaffold, but it did not spread on the surface of the untreated scaffold. The untreated scaffold was too hydrophobic to allow permeation by a polar liquid. Based on this result, He-APGD plasma treatment was effective in modifying the surface of the scaffold.

B. Surface morphology by FE-SEM

Figure 2 shows the surface morphology of PLLA scaffolds by FE-SEM. Plasma treatment did not result in any

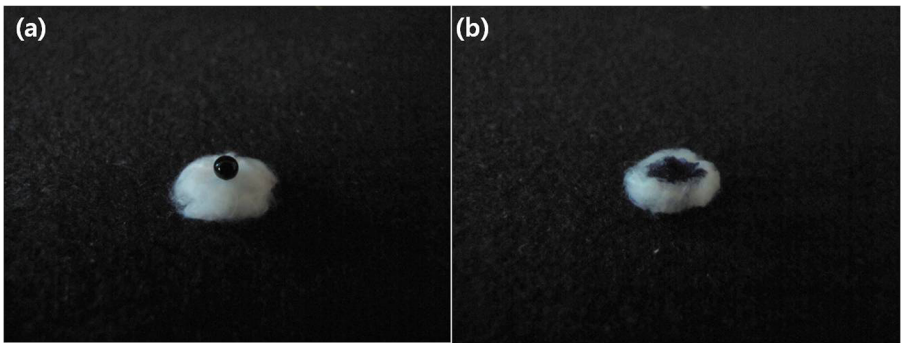

FIG. 1. To confirm the effects of plasma treatment, trypan blue solution was dropped onto the PLLA scaffold: (a) untreated PLLA scaffold, (b) 10 min plasma-treated PLLA scaffold.

irreversible change, except for some grooves. Some grooves were formed on plasma-treated scaffolds (Figs. 2(f)–2(h)). On the other hand, smooth surfaces were observed on untreated scaffolds (Fig. 2(e)). These grooves, formed on the plasma-treated scaffolds, might affect both outer and inner surfaces of the PLLA scaffold, and made the surfaces hydrophilic. Because these morphological changes alone would not seem to account for the change in hydrophilicity, X-ray photoelectron spectroscopy was performed.

C. Surface characterization by XPS

XPS spectra give a detailed elemental composition of the surface. Figure 3 shows change in elemental composition by plasma treatment. The C1s (Fig. 3(a)) consist of –CH (284.59 eV), -CN (285.76 eV), -C-O-C (286.49 eV), -C=O (288.39 eV) and –O=C-O (288.86 eV). After plasma treatment (Fig. 3(b)), -CN peak was increased in C1s. This alteration could be found more detail in N1s. In Figure 3(b), before plasma treatment, $-NH_2$ was assigned at 399.35 eV but after plasma treatment, the graph was shifted and $-NH_2$ was assigned at 399.70 eV. Furthermore, –N=CH (400.80 eV) and $-NH_3^+$ (402.05 eV) were newly appeared after plasma treated.[16–20]

D. Effect of plasma treatment to modify inner side and surface of scaffold

The modifying effect on the scaffold was examined by confocal microscopy. Fibronectin labeled with green fluorescent protein can be identified by its green color; fibronectin penetration into the scaffold was increased by plasma treatment, time-dependently (Fig. 4). As plasma treatment time increased, fibronectin penetration increased. After 10 min of plasma treatment, the whole body of the scaffold was dyed green, whereas the untreated scaffold was not. Green-colored fibers were barely seen in non-treated scaffold because the untreated scaffold is very hydrophobic and does not absorb the fibronectin solution.

IV. DISCUSSION

Atmospheric gaseous plasma treatment is a well-known method for modifying the surface of polymeric materials. Most polymeric materials are hydrophobic and many attempts have been made to change the hydrophilicity, such as pre-wetting the material with ethanol before cell seeding,[4,5] hydrolysis with NaOH,[4,21] protein coating,[22] and plasma treatment[23] before experiments. In this study, He gaseous atmospheric plasma glow discharge was used to change the characteristics of a 3D-type polymeric scaffold from hydrophobic to hydrophilic not only on the outer surface but also on inner surfaces of the scaffold.

Gaseous plasma consists of various excited species, such as radicals, ions, electrons, and photons, and these excited species are potent in breaking molecular bonds.[1,24] Such broken bonds then react with other atoms to form new bonds, such as in the processes of grafting or polymerization. Thus, the change in hydrophilicity can be explained as a

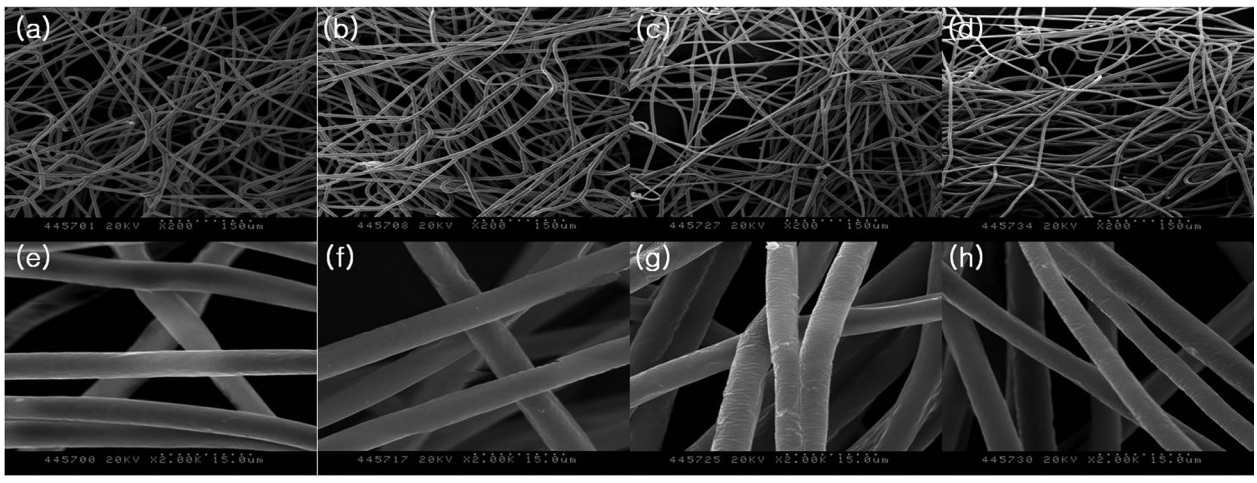

FIG. 2. SEM images of PLLA electrospun scaffold. (a), (e) untreated scaffold; (b), (f) 30 s plasma-treated scaffold; (c), (g) 5 min plasma-treated scaffold; and (d), (h) 10 min plasma-treated scaffold.

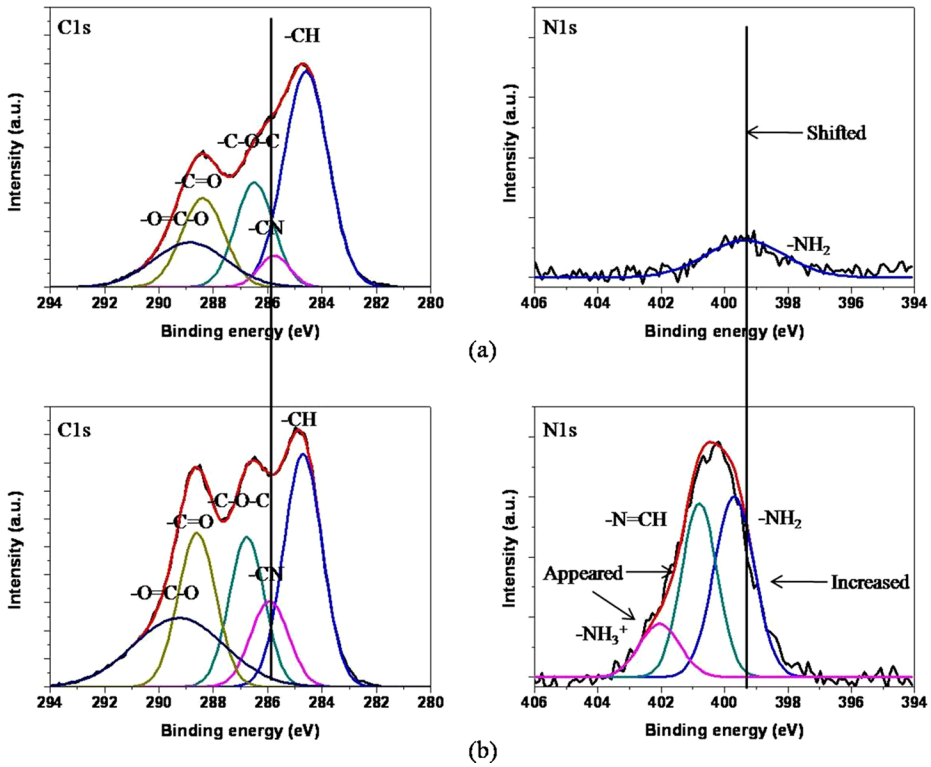

FIG. 3. XPS spectra of (a) untreated PLLA scaffold and (b) 10 min He-APGD plasma treated PLLA scaffold.

result of bond breaking and forming mechanisms, as well as by this alteration being caused by grafting of polar functional groups, which originate from the gaseous plasma.

XPS analysis can show the results of the gaseous plasma treatment. An advantage of He-APGD treatment is that the high population of excited species in the higher pressure discharge, can modify the surface more effectively.[25,26] In this experiment, -CN was increased in C1s peak after plasma treatment. In N1s peak, location of -NH_2 was changed because the graph was shifted after plasma treatment. –N=CH and –NH_3^+ were newly appeared in N1s. Nitrogen gas was from air because the plasma system which was used for this study is not operated in a vacuum. Nitrogen is constituted 78.09% by volume of Earth's atmosphere. As a result, primary amine (-NH_2), imine (-N=CH), and –NH_3^+ (these reactions could be existed differently by environmental condition) were assigned in N1s and these bonds affect the hydrophilicity of the polymer. This presumed reaction is shown figuratively in Fig. 5. On the other hand, mechanical strength of the PLLA scaffold could be reduced by plasma but the

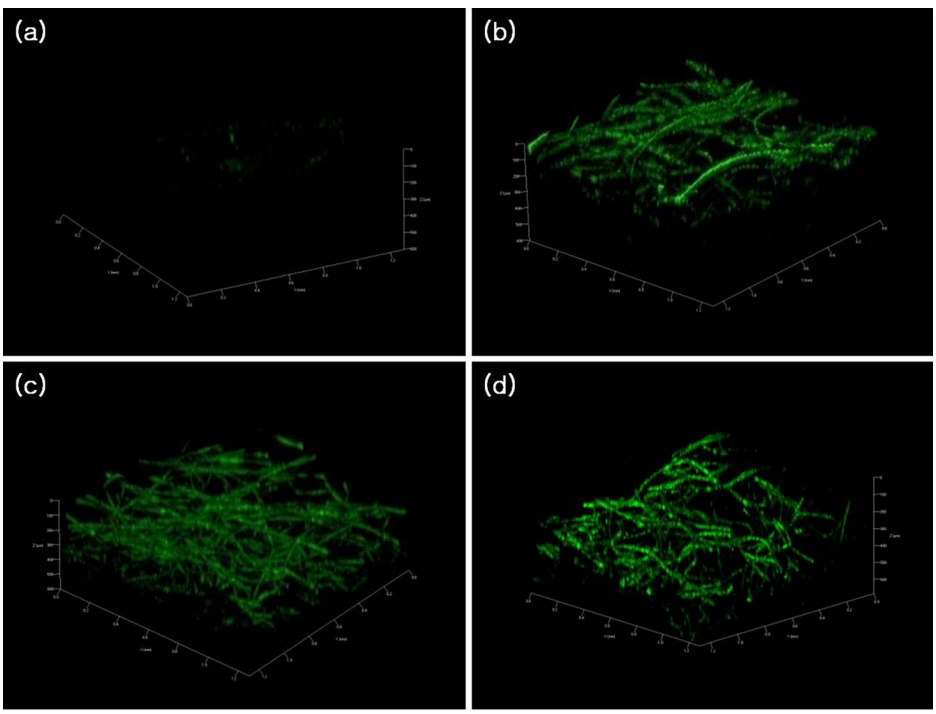

FIG. 4. Confocal microscopy images of PLLA electrospun scaffold. (a) Untreated scaffold, (b) 30 s plasma-treated scaffold, (c) 5 min plasma-treated scaffold, (d) 10 min plasma-treated scaffold (*x*-axis: 1.2 mm, *y*-axis: 1.2 mm, *z*-axis: 600 μm).

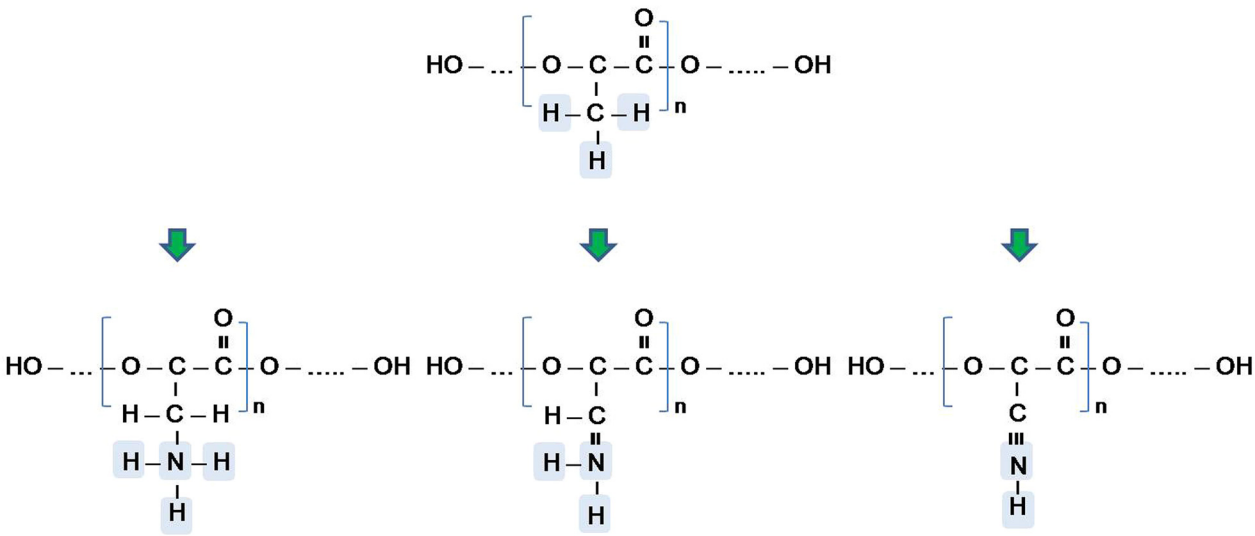

FIG. 5. Potential nitrogen grafting reactions occurring in the PLLA scaffold with helium gaseous atmospheric pressure plasma treatment.

scaffold is not made to bear mechanical load. In constructing scaffolds for tissue engineering, it is ideal to provide cells with an environment which closely resembles their native ECM. The spinning of nanofibers allows for a connected and porous scaffold which can mimic the ECM of many tissue types structurally, chemically, and mechanically.

FE-SEM analysis was also performed to confirm the surface morphology of the plasma-treated PLLA electrospun scaffold. Fig. 2 shows the alteration of the fibers by plasma treatment. Despite the plasma treatment, overall, the fibers, which consist mainly of PLLA in the electrospun scaffold, were not deformed or damaged. Instead, some grooves were formed on the fiber surface. These grooves provide a roughened surface and may be involved in the scaffold becoming more hydrophilic. Surface roughness is a major component in making polymers hydrophilic. In this research, the values of roughness were not determined, but it can reasonably be predicted that these grooves, formed on the fibers, may provide a rougher surface.

As described above, chemical and physical changes were detected on the PLLA electrospun scaffold after plasma treatment. To confirm the effects of the modification on the outer and inner surfaces of the scaffold, the water drop method was performed with trypan blue solution and fibronectin solution. The outcomes were captured with a digital camera and confocal microscopy. The trypan blue solution dropped on the PLLA scaffold spread out and went inside the scaffold, as expected. Trypan blue solution is not related to protein. It was used just confirming hydrophilicity because this solution has blue color and it can be detect easily compare with de-ionized water. Fibronectin is a well-known protein that plays a key role in cell adhesion, growth, migration, and differentiation. Fibronectin-coated polymer samples allow cells to attach more readily than uncoated samples, and this can also be assessed with a fluorescence microscope after green fluorescent protein is conjugated to fibronectin. This is the reason why green fluorescent protein-labeled fibronectin was used for the test. After fibronectin solution was dropped on the scaffold, confocal microscopy was performed to observe the inner surfaces of the scaffold, and images were displayed in three dimensions, with a z-stack program, which can show the depth penetration of fibronectin into the scaffold. This analysis indicated that the inner surfaces of the scaffold were coated with fibronectin and this might affect cellular adhesion to the outer and inner surfaces of the scaffold. Thus, although the plasma treatment conditions should be controlled properly, this may be a suitable method for making inner surfaces of 3D scaffolds hydrophilic.

V. CONCLUSIONS

In this study, He gaseous atmospheric pressure plasma treatment was used to modify internal surface properties of an electrospun PLLA scaffold to enhance protein coating. The plasma-treated scaffolds have hydrophilic outer and inner surfaces. This change enabled fibronectin to penetrate into the PLLA electrospun scaffold. Thus, He atmospheric pressure plasma treatment is an effective method for modifying polymeric scaffold hydrophilicity and could be useful in applied tissue engineering research.

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