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Immobilization of silk fibroin on the surface of PCL nanofibrous scaffolds for tissue engineering applications

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ABSTRACT: Poly(ϵ -caprolactone) (PCL) is explored in tissue engineering (TE) applications due to its biocompatibility, processability, and appropriate mechanical properties. However, its hydrophobic nature and lack of functional groups in its structure are major drawbacks of PCL-based scaffolds limiting appropriate cell adhesion and proliferation. In this study, silk fibroin (SF) was immobilized on the surface of electrospun PCL nanofibers via covalent bonds in order to improve their hydrophilicity. To this end, the surface of PCL nanofibers was activated by ultraviolet (UV)–ozone irradiation followed by carboxylic functional groups immobilization on their surface by their immersion in acrylic acid under UV radiation and final immersion in SF solution. Furthermore, morphological, mechanical, contact angle, and Attenuated total reflection- Fourier transform infrared (ATR-FTIR) were measured to assess the properties of the surface-modified PCL nanofibers grafted with SF. ATR-FTIR results confirmed the presence of SF on the surface of PCL nanofibers. Moreover, contact angle measurements of the PCL nanofibers grafted with SF showed the contact angle of zero indicating high hydrophilicity of modified nanofibers. *In vitro* cell culture studies using NIH 3T3 mouse fibroblasts confirmed enhanced cytocompatibility, cell adhesion, and proliferation of the SF-treated PCL nanofibers. © 2018 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* **2018**, *135*, 46684.

KEYWORDS: biomedical applications; fibers; surfaces and interfaces

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INTRODUCTION

Tissue engineering (TE) has appeared as a promising approach in regenerative medicine. It involves the use of three-dimensional (3D) scaffolds as a mean of the structural base (matrix) for cell attachment and proliferation for effective tissue regeneration.¹

Thus far, a range of different techniques has been applied for fabrication of scaffolds in TE. Among them, electrospinning is an easy and cost-effective method for fabrication of fibrous scaffolds with the high surface area to volume ratio and interconnected pores from various natural and synthetic polymers.²

Poly(ϵ -caprolactone) (PCL) is a semicrystalline aliphatic polyester, has been approved by the U.S. Food and Drug Administration, and is widely studied for TE applications due to its biocompatibility, biodegradability, good mechanical properties, and low cost.^{3–9} However, poor tissue integration of scaffolds made from PCL due to its hydrophobic nature and lack of cell-binding motifs in the chemical structure of PCL are major challenges for application of PCL in TE.⁹ Surface modification using

different physical and chemical methods such as plasma treatment, alkali treatment, radio frequency, functionalization with different biomolecules, and immobilized proteins are the most common approaches to overcome the drawbacks of PCL scaffolds.^{2,3,7,10–12} Immobilization of biological molecules such as proteins, peptides, or polysaccharides on the surface of PCL scaffolds is a promising way to improve the biocompatibility of PCL scaffolds for TE applications.^{7,13} Due to the presence of specific protein sequences available in most of natural polymers structure, proteins existence on the scaffold's surface enhance cells attachment and improve their functions.²

Silk fibroin (SF) is a naturally occurring polymer, derived from *Bombyx mori* silkworm has been used in TE, due to its biocompatibility, low immunoreactivity, biodegradability, suitable oxygen and water vapor permeability, and good mechanical properties.^{14–20} Moreover, SF improves cell adhesion and proliferation for TE applications.^{14,16}

Combination of the two aforementioned polymers (PCL and SF) can be considered as a synthetic/natural polyblend to

improve cytocompatibility of the PCL. For instance, SF has been blended with PCL, and electrospun PCL/SF nanofibrous scaffolds have been fabricated in previous studies.^{21,22} Li *et al.* prepared PCL/SF core–sheath nanofibers via emulsion electrospinning and their results showed that the presence of SF in the structure of nanofibers significantly increases cells adhesion and proliferation.²³ Bonani *et al.* fabricated PCL–SF multilayered composite scaffolds for vascular TE applications.^{15,24} In spite of many publications regarding electrospinning of PCL/SF, a few kinds of research in the field of surface modification of PCL nanofibers with SF has been done.^{16,25} Bhattacharjee *et al.* compared two methods of immobilization of SF into PCL nanofibers and fabrication of PCL/SF nanofibers. They implanted the two scaffold types at bone defect sites in rabbit animal models and showed significantly better bone formation in SF-grafted matrices.²⁵

In the current study, surface treatment by ultraviolet (UV)–ozone was applied to activate the surface of PCL nanofibers for surface immobilization of carboxylic functional groups. Then SF was attached to the nanofibers surface via covalent bonds. In particular, when atmospheric oxygen is exposed to UV irradiation the oxygen absorbs UV to form O₃.²⁶ UV–ozone treatment is an easy and cost-effective surface treatment without the utilization of vacuum or chemicals^{26,27} by using atmospheric pressure and room temperature. Finally, *in vitro* cell culture studies with NIH 3T3, mouse fibroblasts were used to evaluate the effect of surface modification of PCL nanofibers with SF on cell attachment and proliferation.²⁸

EXPERIMENTAL

Materials

PCL ($M_w = 80,000$), methylene chloride (MC), dimethyl formamid (DMF), acrylic acid, and *N,N'*-dicyclohexylcarbodiimide were obtained from Sigma (St Louis, MO). Silk filaments were purchased from Lahijan Company (Iran). Calcium chloride and ethanol were obtained from Merck (Germany). CellTiter 96_ AQueous One solution reagent (MTS), used in the cell culture study, was purchased from Promega (Madison, WI). Phosphate buffered saline (PBS) were purchased from HiMedia (Mumbai 400086, India).

Fabrication of PCL Nanofibrous Scaffolds

Electrospun PCL nanofibers were fabricated through electrospinning of PCL solution dissolved in MC/DMF (80/20 vol/vol) with a concentration of 12% (wt/vol). Then 5 mL of the prepared solution was applied by a syringe with a needle diameter of 0.4 mm under a constant voltage of 12 kV and solution flow rate of 1 mL/h. The PCL nanofibers were collected on a flat aluminum plate placed at a distance of 12 cm from the needle tip.

Preparation of SF Solution

SF solution was prepared from silk filaments. First, silk filaments were degummed by boiling them in sodium carbonate solution (0.5% wt/vol) for 30 min. After washing with distilled water, silk filaments were dried and then dissolved in the solvent mixture of calcium chloride/ethanol/water (1:2:8 in molar ratio).²⁹ This solution was dialyzed using cellulose tubular

membrane in distilled water for 24 h to eliminate impurities from SF solution.

Covalent Attachment of SF on the Surface of PCL Nanofibers

The UV–ozone treatment of PCL nanofibers, as the first step of surface modification of nanofibers, was carried out in a commercial UV–ozone chamber (COG-2A/France) containing five parallel-UV-lamps (Philips-TUV-11-W mercury-vapor with 253.7 nm UV-radiations) placed horizontally at the top of the samples.

In this study, the nanofibers were placed at a distance of 10 mm from the lamp for 5, 10, 15, and 20 min. Ozone was generated at atmospheric pressure by the decomposition of oxygen upon exposure to UV.

After UV–ozone treatment, PCL nanofibers were immersed in the acrylic acid solution with a concentration of 1% wt/vol and irradiated under UV lamp at a distance of 10 mm for 5, 10, 15, and 20 min. For determining the amount of grafted acrylic acid on the surface of nanofibers, the samples were washed with distilled water three times after immersion in acrylic acid solution. After drying in a vacuum oven, the amount of grafted acrylic acid was calculated using eq. (1)

$$\text{Grafted acrylic acid\%} = (W_2 - W_1 / W_1) \times 100 \quad (1)$$

where W_2 and W_1 are the weight of dried samples after and before immersion in the acrylic acid solution, respectively.

Then the samples were washed with water thoroughly and immersed in *N,N'*-dicyclohexylcarbodiimide dissolved in ethanol (1% wt/vol) for 24 h at 4°C. After washing the samples with distilled water, the samples were immersed in SF solution for 2 h at room temperature.

Figure 1 shows a schematic illustration of covalent attachment of SF on the surface of PCL nanofibers as carried out in this study.

Characterization of Nanofibers

The morphology of nanofibers was observed using scanning electron microscopy (SEM, XL30, PHILIPS, the Netherlands) at an accelerating voltage of 20 kV after coating of nanofibers with gold using a sputter coater (SBC 12, Japan). The diameter of the fibers was measured using image analysis software (Image J, National Institutes of Health, MD).

The contact angle was measured by a video contact angle system (OCA 15 plus, Dataphysics) and water as the fluid. The droplet size was set at 4 μL for investigating the hydrophilicity of different nanofibers. At least three samples were tested for each type of nanofibers and the contact angle of three droplets was measured on each sample. The average value was reported with standard deviation ($\pm\text{SD}$).

Attenuated total reflection- Fourier transform infrared (ATR-FTIR) was used to study the changes in surface chemical properties of PCL nanofibers after modification with SF. ATR-FTIR spectroscopy of nanofibers was carried out over a range of 4000–400 cm^{-1} at a resolution of 2 cm^{-1} using a Nicolet spectrometer system.

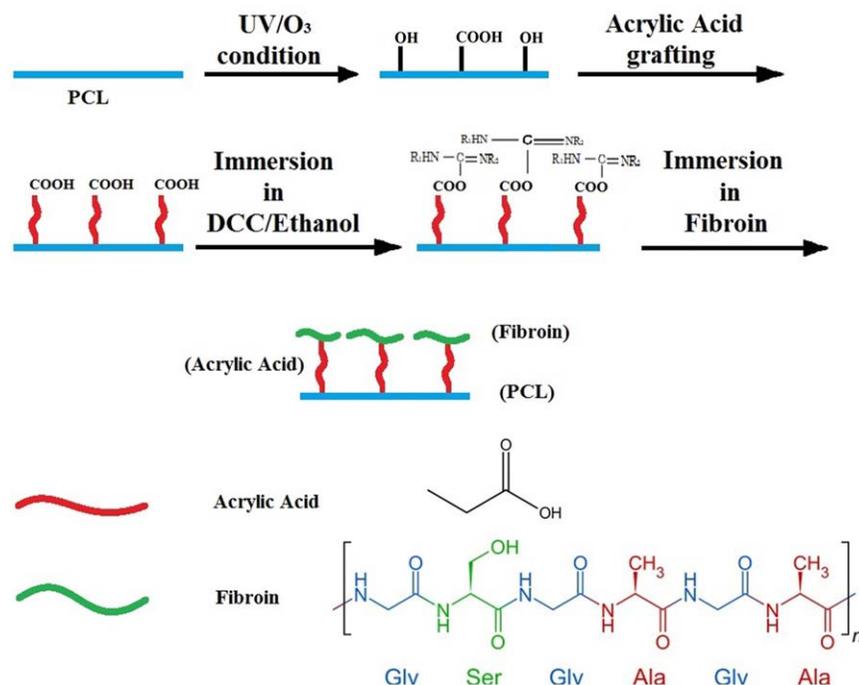


Figure 1. Schematic illustration of covalent attachment of SF on the surface of PCL nanofibrous scaffolds. [Color figure can be viewed at wileyonlinelibrary.com]

Tensile mechanical properties of the nanofibers (PCL, PCL-5, and PCL-10) were measured using the uniaxial tensile testing machine (Zwick 1446-60, Germany). Rectangular shaped samples ($50 \times 10 \text{ mm}^2$) were cut and placed between the grips of the machine; the distance between the grips was calibrated to be 3 cm, and 1 cm at each end of the test specimens was secured into the tensile grip. It is well known that thickness of nanofibrous mat influences the mechanical properties, for measuring mechanical properties, thickness has to be considered as an important parameter. The thicknesses of the nanofibrous mat were measured using a thickness tester (Bear 674, Swiss) at five points and the average was calculated and considered for measurement of mechanical properties. The thickness of the nanofibrous mat was about 0.27 mm. Tests were performed under a 20 N load cell at a crosshead speed of 10 mm/min until rupture occurred. The average of five measurements for each sample was reported.

In Vitro Cell Culture Studies

Cell Culture of 3T3 Fibroblasts. NIH 3T3 mouse fibroblasts (ATCC, UK) were cultured in dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum, 50 U/mL penicillin and 50 U/mL streptomycin, and incubated at 37°C with 5% CO₂. The medium was changed every 3 days. After reaching about 80% confluent, cells were detached by 0.05% trypsin/0.05% Ethylenediaminetetraacetic acid (EDTA) and seeded onto scaffolds at a density of 50,000 cells/cm².

Metabolic Activity and Proliferation of 3T3 Fibroblasts. Cell viability and metabolic activity of cells seeded on PCL nanofibers with and without SF and tissue culture polystyrene coverslip (TCP) as control were measured using MTS(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

tetrazolium) (MTS) cytotoxicity assay after 2 and 7 days of cell seeding. Cells washed with PBS and 500 μL of culture medium was added to each well and incubated for 4 h in a humidified incubator at 37°C and 5% CO₂. The absorbance was read at 490 nm in a spectrophotometer Microplate Reader (Wallac VICTOR3 1420 multi-label counter, PerkinElmer, Waltham, MA, USA).

Cell Morphology. The morphology of cells on different substrates was studied using SEM after 1 and 7 days of cell seeding. Specimens were fixed in 3% glutaraldehyde for 2 h and subsequently, samples were dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 90%, and 100%) for 10 min each. Finally, the constructs were treated with hexamethyldisilazane (HMDS) for further water extraction. After sputter-coating with platinum, SEM was used to observe the morphology of cells on scaffolds.

Statistical Analysis

Statistical analysis was performed using single factor analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Effect of UV–Ozone Radiation on Properties of PCL Nanofibers

Uniform and bead-free PCL nanofibers with an average diameter of $760 \pm 30 \text{ nm}$ were fabricated and then exposed to UV–ozone radiation. The radiation treatment results in significant changes ($P < 0.05$) in the hydrophilicity of the nanofiber surface as denoted by the decrease in the water contact angle as a result of increasing the irradiation time. The water contact angle was found to change from $115^\circ \pm 3^\circ$ for untreated PCL nanofibers to $94^\circ \pm 5^\circ$ and $71^\circ \pm 4^\circ$ for PCL nanofibers irradiated for 5

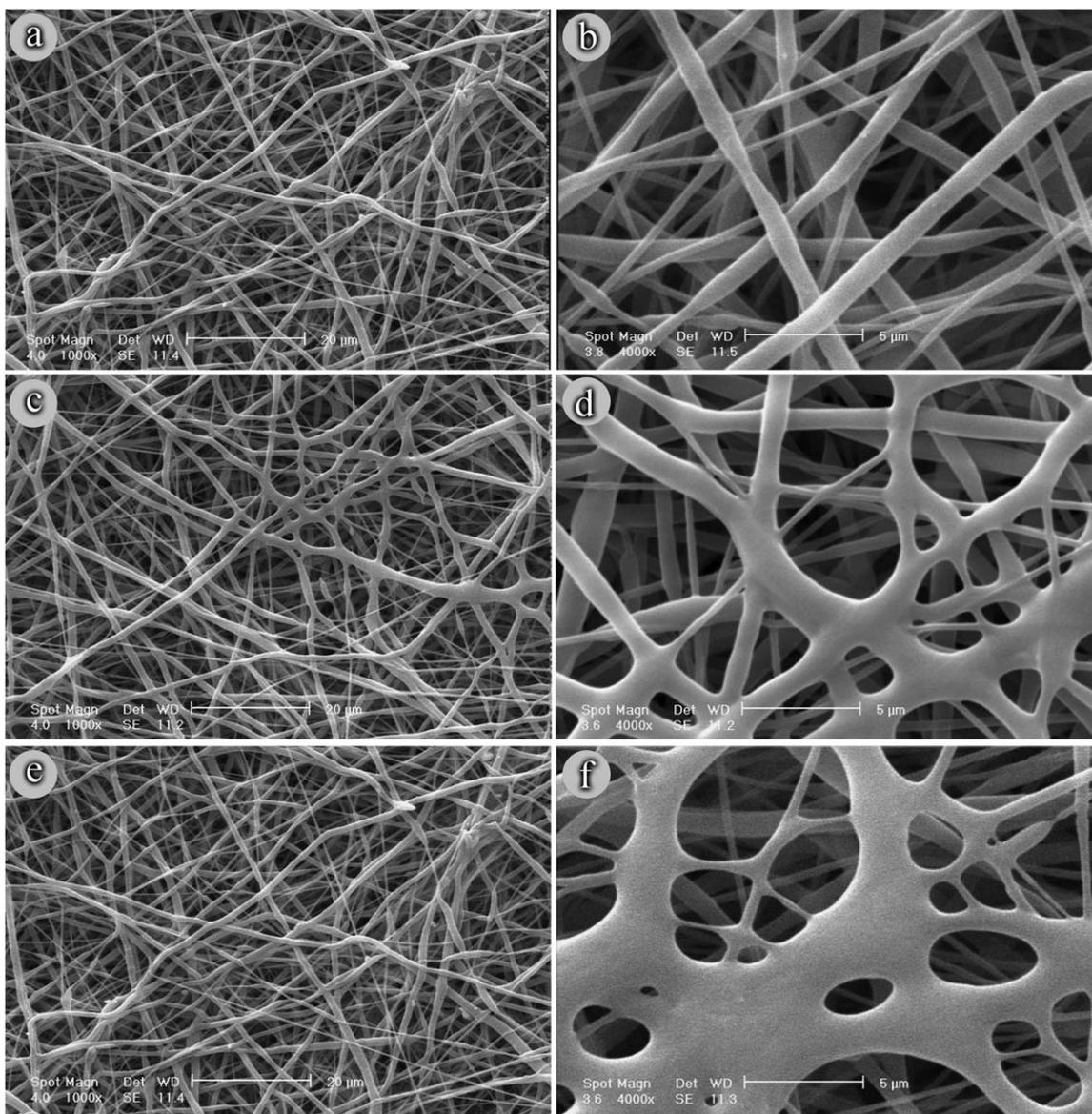


Figure 2. SEM images of (a,b) untreated PCL, (c,d) PCL nanofibers exposed to UV-O₃ for 5 min, (e,f) PCL nanofibers exposed to UV-O₃ for 10 min.

and 10 min, respectively. This can be due to the high oxidation power of the UV-ozone treatment with the introduction of OH and COOH functional groups on the nanofibers surface which is in accordance with previous studies.

Darain *et al.* also investigated the surface modification of PCL membrane using UV-ozone treatment and suggested that the formation of OH and COOH groups on the surface of PCL nanofibers is due to chain scission of PCL backbone and an increase of the end groups on the surface of PCL membrane.³⁰

Moreover, by increasing the radiation exposure time, PCL nanofibers morphological changes were observed. Figure 2 compares the morphology of nanofibers after 5 and 10 min exposure to UV-ozone irradiation with the non-treated PCL nanofibers. No significant changes were observed for PCL nanofibers exposed to UV-ozone for 5 min. However, morphological changes were observed for nanofibers exposed to UV-ozone for 10 min as the

nanofibers melted and fused together. After 15 and 20 min of UV-ozone exposure, deterioration and shrinkage of the fiber morphology were observed (Figure 3) due to the increase of the chamber temperature by increasing of UV-ozone irradiation. It is worth mentioning that while UV-ozone treatment is a surface modification technique, due to the high surface area of the fiber especially nanofibers at longer exposure time (more than 5 min), the bulk properties will also be affected by the high oxidation power and irradiation conditions. In this case, the fiber nature and temperature of the cabinet after 5 min irradiation resulted in enough high temperature to melt some part of the fibers with fused configuration.

Figure 4 shows the FTIR spectra of non-treated PCL nanofibers and PCL nanofibers irradiated for 5 and 10 min. The addition of carboxyl functional group on the surface of PCL nanofibers after UV-ozone treatment is visible.



Figure 3. Images of PCL nanofibers exposed to UV-ozone irradiation for (a) 15 min, (b) 20 min. [Color figure can be viewed at wileyonlinelibrary.com]

The intensity of the peak at the wavelength of 1725 cm^{-1} (regarding C=O stretch of ester group) decreased by increasing the irradiation time which is due to breakage of the ester group. The intensity of the peak at the wavelength of 3400 cm^{-1} (the stretch of OH group) was found to decrease by increasing the irradiation time, which is likely due to the interaction of OH groups and radicals of oxygen.

Furthermore, the tensile strength of the nanofibers was found to decrease significantly ($P < 0.05$) by increasing the duration of UV-ozone irradiation (Figure 5). Long radiation times (beyond 5 min) increased the polymer chain scission leading to significant change in the mechanical properties of the PCL nanofibers whereas untreated PCL nanofibers demonstrated highest strength and elongation at break ($2.57 \pm 0.65\text{ MPa}$ and $101.87\% \pm 4.57\%$, respectively) compared with PCL-5 ($1.20 \pm 0.02\text{ MPa}$ and $70.6\% \pm 7.60\%$) and PCL-10 ($0.81 \pm 0.15\text{ MPa}$ and $26.47\% \pm 5.57\%$). Due to the high surface area of the nanofibers, most probably the irradiation affects both the surface and the bulk properties of the fibers.

Based on the mechanical studies, PCL nanofibers radiated for 5 min were selected as optimal samples for SF immobilization. It is worth noting that the mechanical properties of PCL-5 are still

in the appropriate range for some of the soft tissue regeneration applications.^{31–33}

Graft Polymerization of Acrylic Acid on the Surface of PCL Nanofibers

The carboxyl group is one of the most functional groups for chemical reactions between amino groups from proteins and carboxyl functional groups of the substrate.³⁰ Graft polymerization of acrylic acid on the polymeric surfaces has been reported in previous studies to introduce carboxylic groups on the surface of scaffolds where acrylic acid acts as a spacer between proteins and the surface.³⁴ In the present study, graft polymerization of acrylic acid on the surface of nanofibers was performed under UV. In particular, PCL nanofibers irradiated under UV-ozone for 5 min, then immersed in the acrylic acid solution and exposed to UV radiation for 5, 10, 15, and 20 min. Table I summarizes the weight of acrylic acid grafted on the surface of PCL nanofibers at different exposure times to UV. The maximum amount of acrylic acid grafted on the surface of nanofibers (without any destruction of the nanofibers surface) was obtained by immersion of PCL nanofibers in acrylic acid solution under UV irradiation for 15 min and this condition was chosen as optimum for graft polymerization of acrylic acid on the surface of PCL nanofibers for subsequent studies.

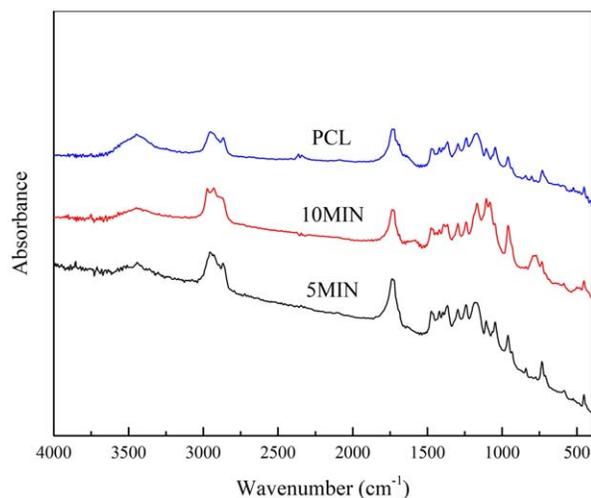


Figure 4. FTIR spectra of untreated PCL and PCL nanofibers irradiated for 5 and 10 min. [Color figure can be viewed at wileyonlinelibrary.com]

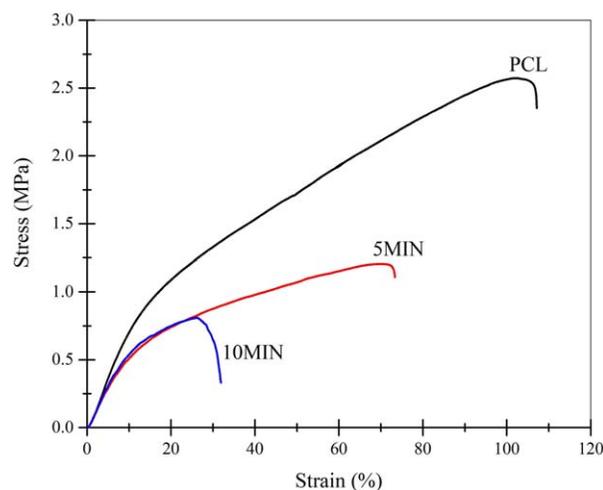


Figure 5. Typical stress-strain curves of untreated PCL nanofibers and PCL nanofibers after being exposed to UV-ozone for 5 and 10 min. [Color figure can be viewed at wileyonlinelibrary.com]

Table I. Weight Gain of PCL Nanofibers (in %) After Grafting of Acrylic Acid on the Surface of PCL Nanofibers at Different Exposure Times to UV

Samples immersed in acrylic acid solution and exposed to UV for different times	Weight of acrylic acid grafted on the surface of PCL nanofibers
5 min	0
10 min	1%
15 min	9.6%
20 min	Shrinkage of samples

Figure 6 compares the ATR-FTIR spectra of untreated PCL nanofibers and PCL nanofibers grafted with acrylic acid for 15 min.

Covalent Attachment of SF on the Surface of PCL Nanofibers

SF is a highly biocompatible natural polymer with suitable functional properties for TE applications.³⁵ Simple physical coating of proteins on the surface of the scaffolds can enhance cell adhesion and proliferation. However, poor stability of the protein layer on the surface is the major drawback of this method. Nevertheless, covalent attachment of proteins on the surface of scaffolds is a favorable method for obtaining a stable protein layer on the surface. As mentioned earlier, there are a few studies on immobilization of SF on the PCL nanofibers surface. In the study by Bhattacharjee *et al.* they grafted SF on the aminolyzed electrospun PCL nanofibers where aminolysis was used to introduce functional NH_2 groups onto PCL nanofibers.²⁵ In the present study, a new approach for grafting SF on the surface of nanofibers has been used. The PCL nanofibers were exposed to UV–ozone radiation for 5 min, then treated with the acrylic acid solution under UV radiation for additional 15 min, and subsequently were immersed in SF solution for 2 h. The advantage of this method, in comparison with the aminolysis method used by Bhattacharjee *et al.* is the utilization of a reduced amount of chemicals and the formation of more biocompatible PCL nanofibers.²⁵

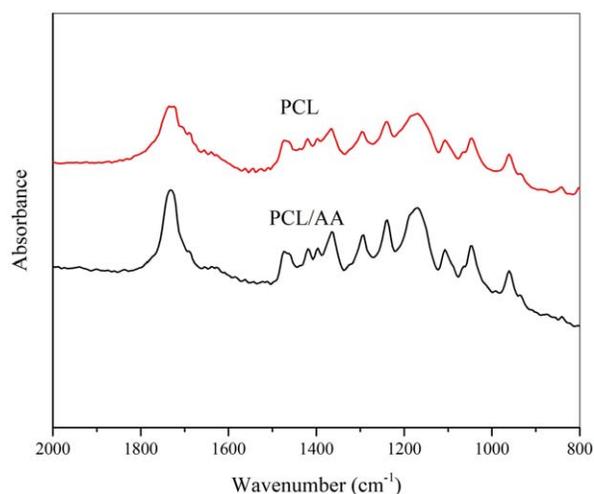


Figure 6. ATR-FTIR spectra of untreated PCL nanofibers and PCL nanofibers grafted with acrylic acid for 15 min. [Color figure can be viewed at wileyonlinelibrary.com]

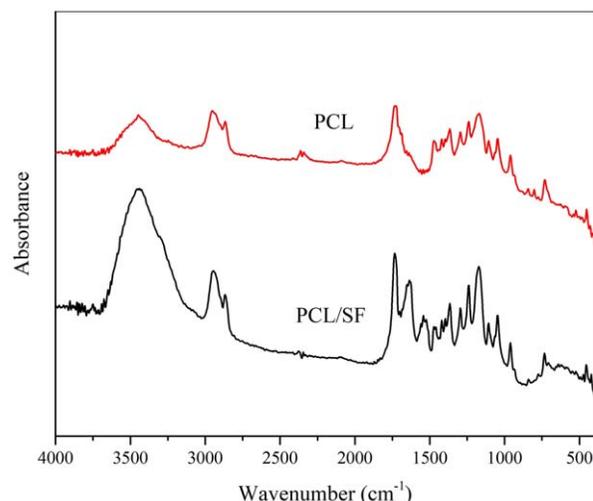


Figure 7. ATR-FTIR spectra on PCL nanofibers with and without SF. [Color figure can be viewed at wileyonlinelibrary.com]

The presence of SF on the surface of PCL nanofibers was confirmed using ATR-FTIR (Figure 7). The characteristic absorption bands of PCL including 2950 (asymmetric CH_2 stretching), 2870 (symmetric CH_2 stretching), 1725 (carbonyl stretching), 1295 (C—O and C—C stretching), 1240 (asymmetric COC stretching) cm^{-1} were observed in the ATR-FTIR spectra of PCL nanofibers with and without SF. The presence of absorption bands at 1635 and 1542 cm^{-1} in the ATR-FTIR spectra of PCL grafted with SF clearly indicates the presence of amide I and amide II which are available in the structure of SF. Moreover, the contact angle of PCL nanofibers grafted with SF was found to be zero indicating the high hydrophilicity of modified nanofibers due to the presence of SF on the surface.

In Vitro Cell Culture Study

In vitro cell culture studies were performed to investigate the effect of surface modification on cell-scaffold interactions. Figure 8 compares metabolic activities of fibroblasts seeded on TCP, PCL, and modified PCL (M-PCL) nanofibers. The

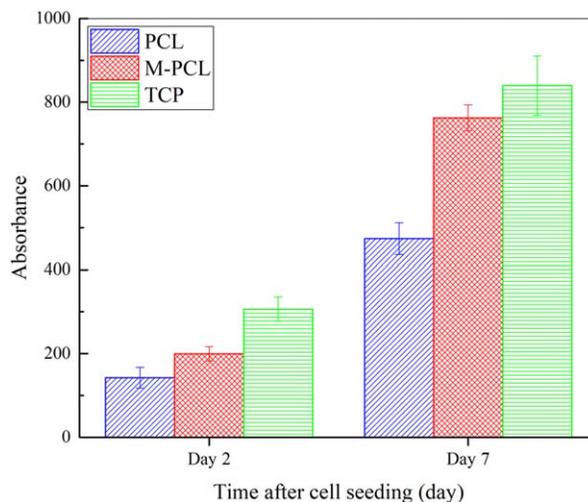


Figure 8. MTS results of cells on PCL, PCL–SF, and TCP after 2 and 7 days of cell seeding. [Color figure can be viewed at wileyonlinelibrary.com]

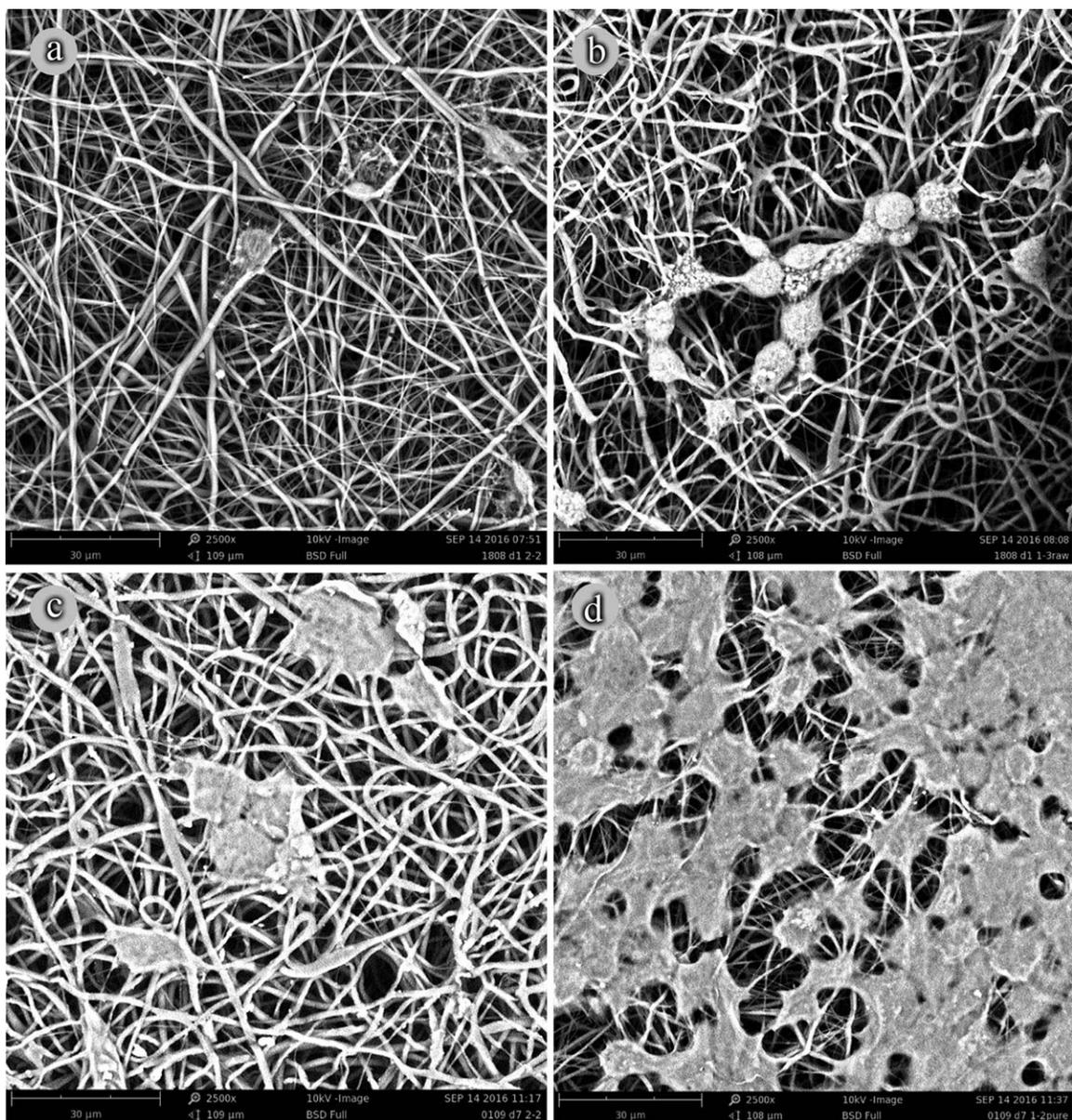


Figure 9. Morphology of fibroblasts on PCL and modified PCL nanofibers at different time points: (a) PCL nanofibers after 2 days of cell seeding, (b) modified PCL nanofibers after 2 days of cell seeding, (c) PCL nanofibers after 7 days of cell seeding, and (d) modified PCL nanofibers after 7 days of cell seeding.

proliferation of fibroblasts on the surface of SF-modified PCL nanofibers was significantly higher than that on pure PCL nanofibers after 7 days of cells seeding (Figure 8). After 2 days of culture, the metabolic activity of cells seeded on TCP was significantly ($P < 0.05$) higher compared to that for PCL and M-PCL nanofibers. However, there was a significant increase in metabolic activity (proliferation) of fibroblasts seeded onto M-PCL nanofibers, so that no significant difference ($P \geq 0.05$) was observed between the proliferation of cells on M-PCL and TCP after 7 days of cell seeding. The higher cell proliferation on modified PCL nanofibers compared to that on pure PCL nanofibers reveals the positive effect of SF grafting on the surface of nanofibers on cell proliferation.

Figure 9 indicates the attachment and morphology of cells on PCL and modified PCL nanofibers after 2 and 7 days of cell seeding. Enhanced cell attachment and proliferation was observed on the surface of modified PCL nanofibers, both after 2 and 7 days which is in accordance with the MTS results. Obviously, the presence of grafted SF on the surface of nanofibers enhanced the hydrophilicity of the PCL nanofibers and improved cell attachment and proliferation.

CONCLUSIONS

In the present study, a new technique for immobilization of SF onto PCL nanofibers was introduced. In particular, the PCL nanofibers were treated with UV–ozone irradiation followed by

their immersion in acrylic acid under UV radiation. This procedure introduces carboxylic functional groups on the surface of nanofibers for immobilization of SF via covalent bonds. The surface treatment procedure improved the hydrophilicity of PCL nanofibers and introduced functional groups on the surface of PCL nanofibers. Moreover, *in vitro* cell culture studies revealed enhanced cell attachment and proliferation on the SF-modified PCL nanofibers compared to that for untreated PCL nanofibers.

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