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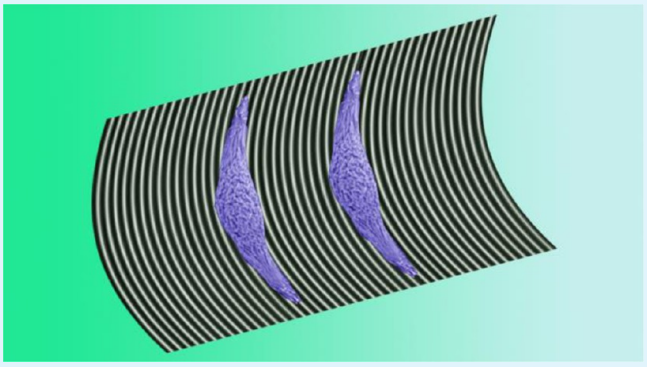
Electrospun Tubular Scaffold with Circumferentially Aligned Nanofibers for Regulating Smooth Muscle Cell Growth

Yanming Wang,[†] Haigang Shi,[†] Jing Qiao,[†] Ye Tian,[†] Man Wu,[†] Wei Zhang,[†] Yuan Lin,*[,‡] Zhongwei Niu,*[,†] and Yong Huang[†]

[†]National Research Center of Engineering Plastics, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Beijing 100190, China

[‡]State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China

ABSTRACT: Simulation for the smooth muscle layer of blood vessel plays a key role in tubular tissue engineering. However, fabrication of biocompatible tube with defined inner nano/micro-structure remains a challenge. Here, we show that a biocompatible polymer tube from poly(L-lactide) (PLLA) and polydimethylsiloxane (PDMS) can be prepared by using electrospinning technique, with assistance of rotating collector and parallel auxiliary electrode. The tube has circumferentially aligned PLLA fibers in the inner surface for cell growth regulation and has a PDMS coating for better compressive property. MTT assay showed the composite PLLA/PDMS tube was suitable for various cells growth. In vitro smooth muscle cells (SMCs) cultured in the tube showed that the aligned PLLA fibers could induce SMCs' orientation, and different expression of α-SMA and OPN genes were observed on the aligned and random PLLA fibers, respectively. The successful fabrication of composite PLLA/PDMS tubular scaffold for regulating smooth muscle cells outgrowth has important implications for tissue-engineered blood vessels.



KEYWORDS: *electrospinning, alignment, tubular scaffold, cell culture, smooth muscle cells, phenotype*

INTRODUCTION

Coronary artery disease (CAD) is one of the biggest killers in the world.[1] Suitable blood vessel substitutes, especially small-diameter (inner diameter < 6 mm) vessels, are in the great need for clinical CAD therapy. A healthy artery is typically composed of three concentric layers—intimae, media, and adventitia—in which the media is circumferentially oriented smooth muscle layer.[2−4] Among the three layers, the smooth muscle layer plays an important role in maintaining elasticity, mechanical strength, and vasoactive responsiveness of the blood vessels.[5,6] Therefore, generating a tubular scaffold that can mimic the function of a smooth muscle layer is a prerequisite for vascular tissue engineering. Biocompatibility (including non-thrombogenicity and high vasoactivity) and orientation are key requirements for this tubular scaffold.[7,8] In the body, except in the physiologic vascular environments, smooth muscle cells (SMCs) are spindle-shaped and aligned their long axis perpendicular to the blood vessel length. Thus, in order to obtain an artificial vascular tunica media, it is critical to create a tubular scaffold with circumferentially aligned surface morphology of the inner walls. Then, the SMCs can be guided along the oriented surface of the inner walls, just as the SMCs in the vascular tunica media. To fabricate tubular scaffold to culture SMCs, Jiang et al. exploited stress-induced rolling membrane technique,[9] and our former study provided a glass capillary tube with patterned tobacco mosaic virus inside.[10] To direct cell growth behaviors, macro- and/or micro-scale morphology,[11,12] as well as stress-induced SMC alignment, 2D pattern generation by mechanical stress and lithography is all widely used.[10,13,14]

Electrospinning technology is a facile and versatile method, which can control the composition, structure, and mechanical properties of fibrous scaffolds ranging from nano- to micro-scale.[15−18] The fibrous scaffolds have high porosity,[19,20] which can mimic the structure and function of native extracellular matrix (ECM).[21,22] Moreover, by electrospinning, aligned fibrous scaffold can be simply obtained through many techniques, such as a high speed rotating drum and parallel Si array as collectors.[23,24] However, it still remains a challenge to fabricate tubular scaffold with oriented inner structure for in vitro SMCs study. In this work, we used the electrospinning technique to fabricate the tubular biocompatiable poly(L-lactide) (PLLA) scaffolds (inner diameter 4 mm) with aligned inner surface, under assistance of rotating collector and parallel electrodes. The strength of tubular scaffolds was enhanced by coating a layer of polydimethylsiloxane (PDMS) on the outside of the PLLA surface. Such a composite tubular scaffold of

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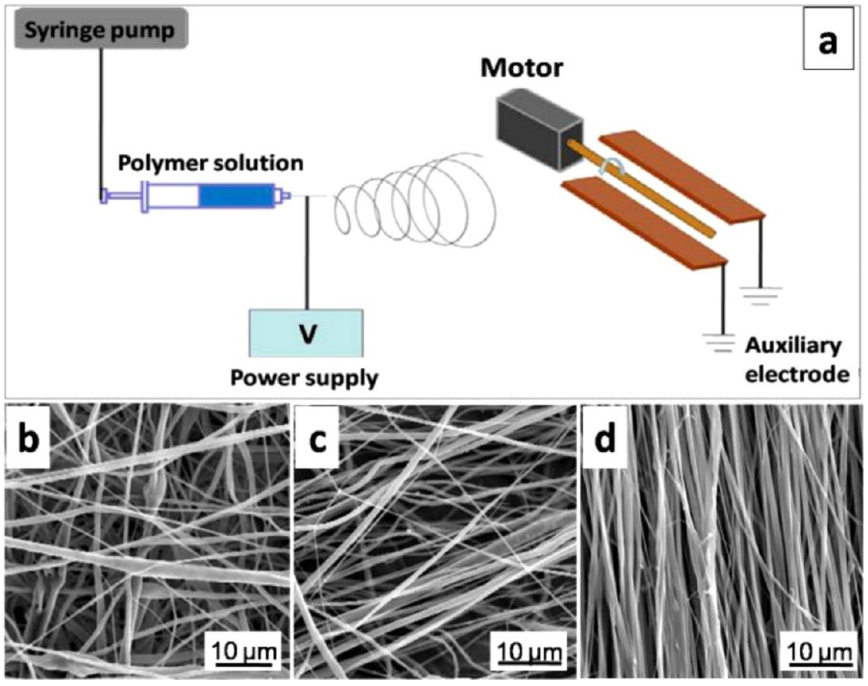

Figure 1. (a) Schematic illustration of the electrospinning setup. (b~d) The SEM images of electrospun PLLA fibers with different rotation speeds of the collector: (b) 200 rpm, (c) 500 rpm, and (d) 1000 rpm. Other electrospun parameters were kept the same (concentration of PLLA solution 7 w/v%, applied voltage 10 kV, flow rate 1.0 mL h^{-1} and work distance 15 cm).

PLLA/PDMS provides an excellent morphological mimic of the tunica media of the vessel and can be applied for the in vitro culturing of the SMCs. Morphological characterization and MTT assay were performed to test fiber orientation and biocompatibility of the composite scaffolds. Because of the contact guidance effect of the aligned nanofibers, the growth orientation of the SMCs can be aligned along the circumferential direction of the tubular walls.[25,26] Furthermore, this work highlighted and researched the typical gene expressions of vessel SMCs in the composite tube scaffolds by real-time polymerase chain reaction (RT-PCR) detection.

EXPERIMENTAL SECTION

Materials. Poly(L-lactide) (PLLA, Mn ~100 000 g mol^{-1}) was obtained from Jinan Daigang Biomaterial Co., Ltd. Dichloromethane (DCM) and dimethyl formamide (DMF) were purchased from Beijing Chemical Works and dehydrated by molecular sieve drying before used. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent was purchased from Beijing Solarbio Biomaterial Company. Electrospinning stainless steel needle (length 8 cm, diameter 4 mm) and copper boards (length 10 cm, width 1.5 cm and height 0.2 cm) were homemade. Fibroblast-like L929 cells (mouse fibroblast-like cells), MC3T3-E1 cells (mouse osteoblast cells), MCF-7 cells (human breast cancer cells) and rabbit vessel SMCs were all obtained from Cell Resource Center (IBMS, CAMS/PUMC).

Electrospinning of PLLA Tubes. PLLA was dissolved in DCM/DMF solution (2:1 in volume ratio) with concentration 7 w/v% for 3 h at room temperature by stirring. At a relative humidity of approximately 50%, PLLA solution was pumped to the needle by a syringe infusion pump at the flow rate of 1.0 mL h^{-1}. The eletrospun fibers were captured by the rotating stainless stain collector between two parallel copper boards during electrospinning at ambient temperature. Different rotation speed and positive voltages of 10 kV were applied to the needle during electrospinning. The whole experimental setup is illustrated in Figure 1a. The obtained PLLA tubes were dried in a vacuum oven, then sputter-coated with gold–palladium for 60 s. The morphology of fibers of the tubes was observed using a JEOL S-4300 field emission scanning electron microscope (FE-SEM) operated at an accelerating voltage of 10 kV.

Fabrication of Composite PLLA/PDMS Tube. The strength of tubular scaffolds was further enhanced by coating a layer of polydimethylsiloxane (PDMS) on the outside of the tubes. The ratio of pre-polymer and curing agent of PDMS (Sylgard 184, Dow Corning, MI, U.S.) was 10:1. In a typical experiment, in order to avoid the PDMS entering into the inside of the electrospun tube, after electrospinning, the electrospun scaffold together with the electrode was directly rolling on the PDMS solution surface. After that, the PLLA tubes containing fibers with a coated layer of PDMS were heated at 60 °C for 20 min, then the composite rod was heated at 60 °C for another 5 h to cure the PDMS completely. Afterwards, the composite PLLA/PDMS tubes were peeled off from the collector, in which process the morphology of inner electrospun PLLA fibers can be sustained.

MTT Assay. The proliferation of the cells was investigated by MTT assay.[27] Tissue culture polystyrene (TCP) culture plate was served as control. The composite PLLA/PDMS tubes were cut into 5 mm × 5 mm pieces and digested by complete medium (high Glucose DMEM (Hyclone) supplemented with 10% calf serum (Gibco)) (0.2 g mL^{-1}) and 1% penicillin and streptomycin sulphate at 37 °C for 24 h, followed by filter sterilization. At the end of the culturing, the seeded L929 cells were washed three times with PBS and then 20 μL MTT (5 mg mL^{-1} in PBS) was added. After incubation at 37 °C for 4 h, the reaction solution was carefully removed from each well and 160 μL DMSO was appended. Then, the plate was lightly agitated until the formed precipitate was dissolved. The optical density (OD) values were measured with iMark Microelisa Reader (Bio-Rad, 13096) at 490 nm.

Cell Culture and Characterization. All tubular scaffolds with aligned/random PLLA fibers were cut into 1 cm length and soaked in 75 % ethanol for 30 min, and then, the samples were autoclaved for 20 min at 121 °C under 0.1 MPa. After that, the scaffolds were placed on the bottom of a 24-well plate, followed by washing twice with 0.1 M sterilized phosphate buffered saline (PBS, pH 7.2); then, the samples were soaked in complete medium (high Glucose DMEM (Hyclone) supplemented with 10% calf serum (Gibco) and 1% of penicillin and streptomycin sulphate (Solarbio)) overnight. 1 × 10^4 SMCs in 2 mL

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Table 1. Primer Sequences Used in the Experiment[a]

genes	forward primer sequence (5′-3′)	reverse primer sequence (5′-3′)
α-SMA	TGTACCCTGGCATTGCTGACCG	TGTGGGCTAGAAACAGAGCAGGG
OPN	CCACACGCCGACCAAGGAACAA	AGCTGCCCGAATCAGCGTGT
GAPDH	CTTCAACAGTGCCACCCACTCCTCT	TGAGGTCCACCACCCTGTTGCTGT

[a]All primers are the same for rabbit vessel SMC cell lines.

complete medium was added to each scaffold to replace the previous medium and incubated in 5% CO_2 at 37 °C. The culture medium was changed every two days. After different culture times, the samples were rinsed with PBS twice, fixed in 4.0 % paraformaldehyde overnight, and washed again with PBS twice. After that, samples were dehydrated in the increasing concentrations of ethanol and tert-butanol frozen for later use. Samples for SEM analysis were dried under freeze drying for 24 h and then sputter-coated with gold−palladium for 100 s. The morphology of samples was analyzed using a JEOL S-4300 field emission scanning electron microscope (FE-SEM) operated at an accelerating voltage of 10 kV.

Real-Time Polymerase Chain Reaction (PCR). The expression levels of mRNA in α-SMA and OPN of SMCs were tested to show different phenotypes of SMCs. The SMCs were seeded on composite PLLA/PDMS tube with aligned and random PLLA fibers and cultured for 7 and 14 days, respectively. The total RNA was extracted with Trizol (Invitrogen, U.S.A.), and then, the total RNA was converted to cDNA using the PrimeScript RT reagent kit (TaKaRa, Japan). The Real-time PCR reactions were performed using SYBR Green assay (TaKaRa, Japan) on the Fast 7500 Real-time PCR System (ABI, U.S.A.), in order to evaluate the gene expressions of the α-SMA and OPN. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a housekeeping gene, was used as endogenous normalization controls for protein-encoding genes. The primers are listed in Table 1.

RESULTS AND DISCUSSION

It is well known that the concentration of the solution and the applied voltage determine the uniformity of the electrospun fibers.[28] During our experiment, the smooth surface of PLLA fiber was obtained at concentration of 7 w/v% and applied voltage of 10 kV. Other electrospun parameters were flow rate 1.0 mL h^{-1} and work distance 15 cm. To get a tubular scaffold with circumferentially aligned inner surface, we improved the collector as shown in Figure 1a. A rotating stainless steel needle plays as the fiber collector, which can draw the fibers into tubular configuration as a support and furthermore control the alignment of fibers of PLLA tube. The parallel copper boards provide a uniform electric field, which can assist the alignment of the PLLA nanofibers from rotating mechanical force.[29,30] The rotating speed influences the alignment seriously. When the rotation speed is 200 rpm, only random fibers were collected on the stainless steel rod (Figure 1b). As the rotation speed increased to 500 rpm, partial alignment of PLLA was visualized (Figure 1c). If the rotation speed is increased to 1000 rpm, well aligned PLLA fibers could be obtained (Figure 1d). By applying this setup, we can prepare PLLA fibrous tubes with length around 8 cm, inner diameter about 4 mm (Figure 2a and c), and wall thickness varied from 10 μm to 50 μm depending on the spinning time. Artificial vessels with small diameter are very useful because many smaller regions of the vascular system, as the coronary or peripheral arteries have inner diameters smaller than 6 mm.[31] However, both random and aligned fibers of PLLA tubes showed poor compressive property, which make the tubes being easily deformed after being peeled off from the stainless steel collector. In order to enhance the compressive strength of the PLLA fibrous tubes for further applications in cell culture, PDMS was chosen as a coating layer on the outside surface of PLLA tubes before peeling off from the collector. Parts b and d of Figure 2 are the free standing composite PLLA/PDMS tubes. The thickness of PDMS can be controlled. Compared to the uncoated PLLA fibrous tubes, the outside surface of composite PLLA/PDMS tubes becomes very smooth. Parts e and f of Figure 2 show the cross section of the composite tubes. The thickness of the PDMS layer is around 80 μm, and the PDMS only wraps the outside surface of the PLLA tubes, not penetrating through the underlayer. Moreover, the inner fibers still sustained well aligned morphology along circumferential direction of the tubes, which is beneficial for the aligned regulation of SMCs growth.

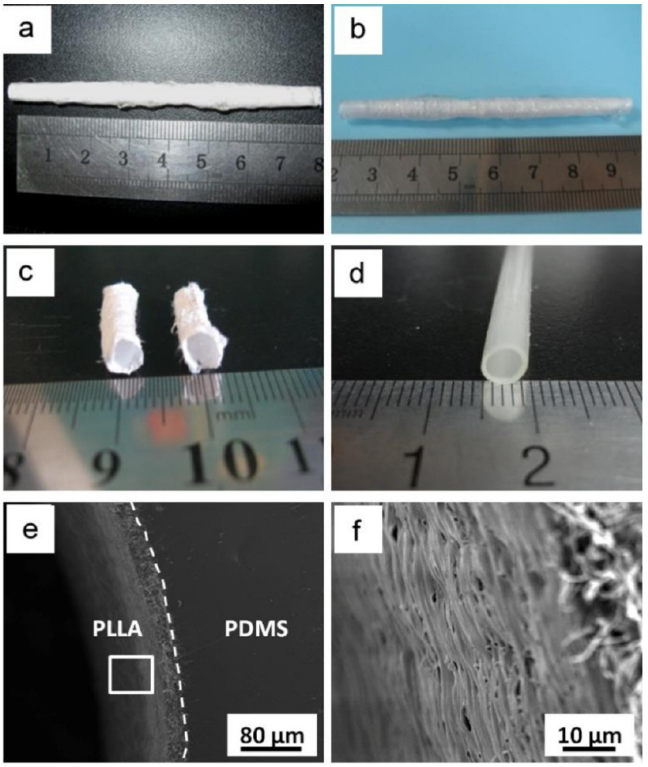

Figure 2. Photos of the electrospun PLLA tube (a, c) and the composite PLLA/PDMS tubes (b, d). The SEM image (e) is the cross-section images of composite PLLA/PDMS tube. The white dashed line indicates the boundary of PLLA and PDMS, and the square region was enlarged as SEM image (f).

Biocompatibility of the scaffold is the first important parameter for vascular tissue engineering. PLLA and PDMS are both approved by Food and Drug Administration (FDA). From OD values of MTT assay data, we can indirectly get the cell viability at different times.[27] Based on their biocompatibility, cells showed high proliferation (more than 100%) inside the composite PLLA/PDMS tubes from 24 h to 120 h (Figure 3). Thus, the composite PLLA/PDMS tube is suitable for the regulation of cell growth.

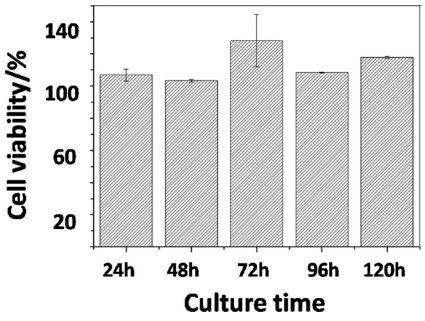

Figure 3. Cell proliferation histogram with different culture times.

In order to investigate the universality of this method, MC3T3-E1 cells and MCF-7 cells were cultured in the aligned and random composite PLLA/PDMS tubes. As shown in Figure 4, MC3T3-E1 and MCF-7 cells both grow along the

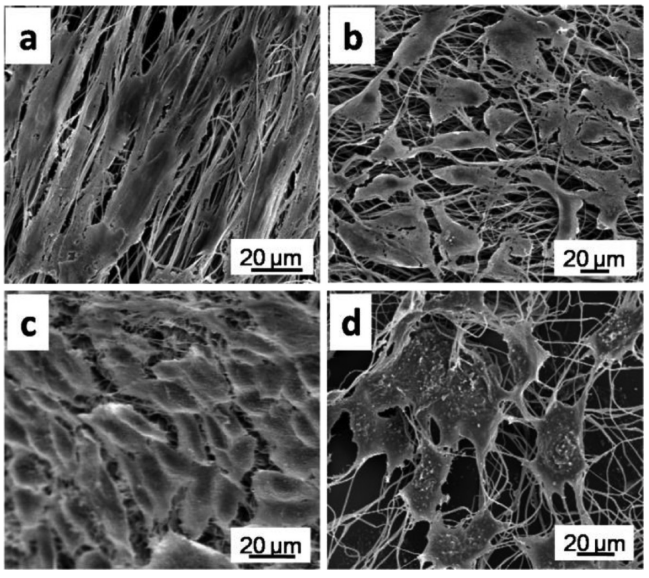

Figure 4. SEM images of MC3T3-E1 cells (a, b) and MCF-7 cells (c, d) cultured in aligned (a, c) and random (b, d) composite PLLA/PDMS tubes for 7 days.

orientated direction of the PLLA fibers on aligned scaffold, while present polygon morphologies on the random scaffold. Therefore, by this electrospinning method with rotating collector and parallel auxiliary electrode, we can simply prepare aligned fibrous tubular scaffold for guidance of various cells aligned growth.

To mimic the blood vessel structure, SMCs were cultured inside the composite tubes. Herein, we prepared the random composite tubes at rotating speed of 200 rpm (Figure 1b) and aligned composite tubes at 1000 rpm (Figure 1d). In contrast to the SMCs cultured in the random composite tubes (Figure 5b and d), SMCs grew along the orientation of PLLA fibers inside the aligned composite tubes (Figure 5a and c). In random composite tubes, the morphology of SMCs was epithelioid or rhomboid, which is typical of the pathogenic synthetic phenotype. However, the SMCs cultured on the aligned composite tubes tended to be spindle-shaped, which is typical contractile phenotype. These two phenotypes are both the possible phenotypic states of the vascular SMCs. Synthetic phenotype means SMCs proliferate and migrate at an extremely high rate and produce extracellular matrix (ECM) components

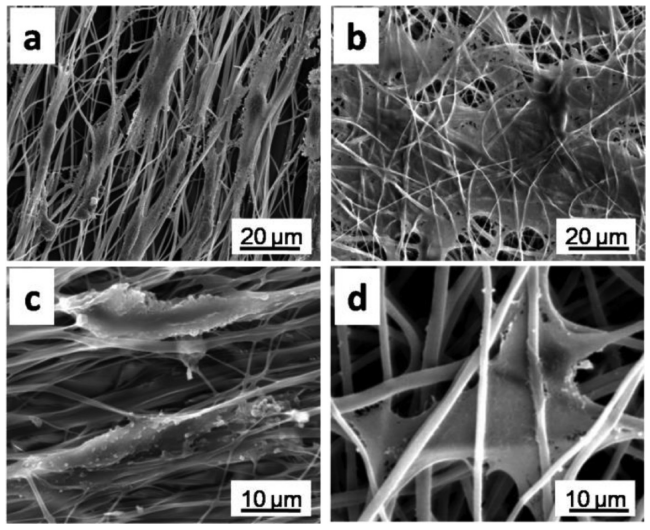

Figure 5. SEM images of SMCs cultured in composite tubes after 7 days (a) on oriented fibers obtained under 1000 rpm and (b) on random fibers obtained under 200 rpm. Magnified view of the aligned cells (c) and random cells (d).

such as collagen, elastin, etc.,[6] while contractile phenotype means SMCs mature and have abundant myofilaments and hardly produce ECM.[6] In a healthy adult vessel, SMCs are contractile phenotype and align their long axis perpendicular to the blood vessel length. This orientation is crucial for proper blood vessel function.[6] Thus, the SMCs phenotype on aligned composite tubes agreed with the healthy tunica media of the blood vessels.

It is commonly known that the two blood vessels SMCs phenotypes (pathogenic synthetic phenotype and contractile phenotype) have different gene expressions. The α-smooth muscle actin (α-SMA) is the main representative gene in contractile phenotype, while the osteopontin (OPN) is the representative gene of synthetic phenotype. Normally, α-SMA gene of SMC expresses higher in the healthy tunica media of vessel while OPN expression low. In another words, higher OPN expression shows high proliferation of the SMCs and presents the unhealthy tunica media of the vessel tissues. Therefore, mRNA levels of α-SMA and OPN of SMCs have important implication for SMCs culture in vitro.[2] We monitored the mRNA levels of SMCs on aligned and random tubes, which were cultured for 7 and 14 days, respectively. As shown in Figure 6, the relative quantity (RQ) of α-SMA mRNA levels on both of the aligned and random substrates shows higher expression than that of OPN after 7 days culturing, which means SMCs remain spreading themselves in the electrospun scaffolds. After 14 days, the mRNA level of α-SMA on the aligned scaffold still presents higher expression than that of OPN; however, the OPN level shows higher expression than α-SMA on the random scaffold. The OPN level of SMCs on the random substrate is higher, which means the SMCs are still proliferating in a vigorous way on the random PLLA fibers. SMCs cultured on tissue culture plates had almost same α-SMA and OPN expression at either day 7 or day 14 (data not shown). The phenomenon of the higher SMCs proliferation is very similar to the unhealthy tunica media of the vessel tissues. Hence, the random fibrous substrate induced SMC had a more synthetic phenotype. Therefore, the circumferentially aligned PLLA in the composite PLLA/PDMS tube can facilitate the SMCs to differentiate to health

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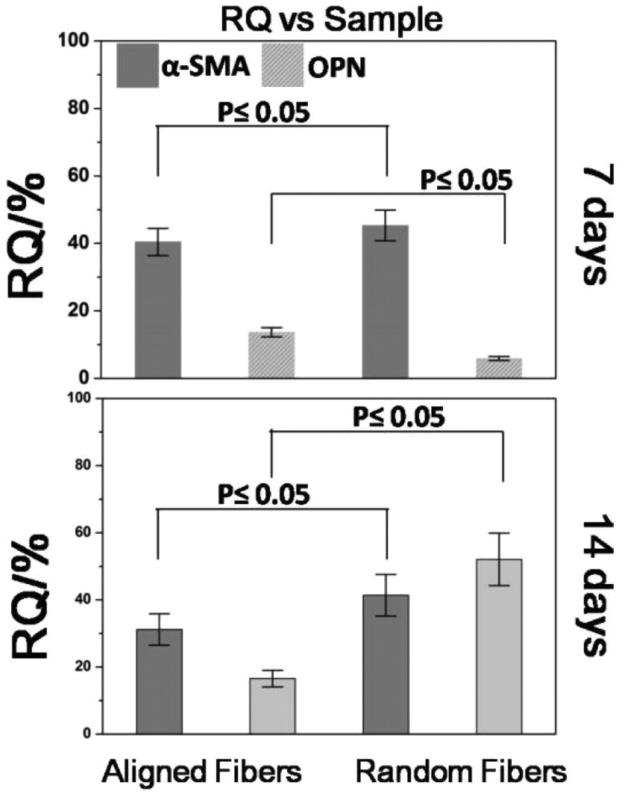

Figure 6. Real-time PCR histogram of α-SMA and OPN of SMCs on different substrates at different culture times. Data are presented as mean ± standard error of the mean.

contractile phenotype, which indicates the composite biocompatible PLLA/PDMS tube with inner aligned surface will be a good candidate for vessel tissue engineering.

CONCLUSIONS

Biocompatible PLLA tubes with circumferentially aligned topology were fabricated by electrospinning technique with assistance of rotating collector and parallel auxiliary electrode. The tubular scaffold was further coated with PDMS to enhance its compressive strength. The obtained composite PLLA/PDMS tubes showed good biocompatibility and various cells could grow into spindle-like configuration along the orientation of the PLLA fibers in the inner side of the tubular scaffolds. This technique can therefore be applied to mimic the healthy vessel tunica media. Future work will include improving mechanical properties of the composite tube, investigating interaction between blood and lumen of tubular scaffold, and growth situation of endothelial cells in the composite tube. All of these will lead to a composite tube that has great potential in the development of vascular grafts.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: niu@mail.ipc.ac.cn.
*E-mail: linyuan@ciac.ac.cn.

Notes

The authors declare no competing financial interest.

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