

Fabrication of Highly Anisotropic and Interconnected Porous Scaffolds to Promote Preosteoblast Proliferation for Bone Tissue Engineering

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Electronic Supplementary Information

Abstract Mimicking the complex structure of natural bone remains a challenge for bone tissue scaffolds. In this study, a novel processing strategy was developed to prepare the bone-like scaffolds that are featured by highly oriented and fully interconnected pores. This type of biomimetic scaffolds was evolved from solid phase stretching of immiscible polycaprolactone (PCL)/poly(ethylene oxide) (PEO) blends with co-continuous structure and the pore morphology was inherited from selective extraction of water soluble PEO phase. The pore anisotropy was readily tuned by varying the stretching strain without loss of interconnectivity. Significant promotion in preosteoblast proliferation, alkaline phosphatase activity and osteogenic gene expression was observed in the oriented porous scaffolds compared to the isotropic porous counterpart. The oriented architecture provided a topographical cue for aligned growth of preosteoblasts, which activated the Wnt/ β -catenin signaling pathway. The proposed strategy enriches the toolbox for the scaffold design and fabrication for bone tissue engineering.

Keywords Porous scaffold; Pore anisotropy; Polymer blend; Co-continuous structure; Tissue engineering

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INTRODUCTION

Bone damage resulting from arthritis, trauma or disease has been long prevailing in clinical orthopedics.^[1] In the past few decades, biodegradable polymer scaffolds are becoming preferred alternatives for critical-sized defects to surmount the protruding complications of autografts and allografts.^[2–4] Porous scaffolds not only provide a three-dimensional structural support, but also serve as temporary extracellular matrices (ECMs) to guide cell proliferation and differentiation.^[5–7] Human bone tissues have a structural anisotropy with high porosity, which is essential for cell migration, bone growth and nutrients transport.^[8,9] Thus, constructing biomimetic architecture is of great significance to endow the scaffolds with complex biofunctions for bone regeneration.

Extensive efforts have been undertaken to engineer three-dimensional (3D) scaffolds with oriented pores for the purpose of reproducing structure features of natural bone.^[10] An ice-templating technique has been used for 3D scaffolds with uniaxial microchannels.^[11] The unidirectional temperature

gradient drives the solvent crystallized from the bottom to form a cylindrical template, which renders the polymer component with honeycomb-like anisotropy. The morphology of the scaffold channels can be controlled by polymer concentration, temperature gradient, etc.^[9,12] The proliferation and growth of osteoblasts were enhanced by the oriented microchannels. The ammonia-induced method was also proposed to fabricate bone-repair scaffolds with anisotropic tubular pores.^[13] Due to the strong interaction between ammonia and water molecules, the tube-like pores are obtained by the permeation of ammonia from the top surface. The pore diameters can be regulated by crosslinking degree and composition of the matrix, ammonia concentration, etc.^[14] However, it still faces a technological challenge to simultaneously construct oriented pores and high interconnectivity in 3D scaffolds.^[15–17] Pore interconnectivity plays a primary role in the transportation of nutrients and metabolites, cell seeding and migration, as well as new bone ingrowth.^[18–20]

A co-continuous structure available by blending two immiscible polymers exhibits spatially intertwined and continuous networks throughout the bulk.^[21–23] The individual phase region ranges from a few microns to even hundreds of microns.^[24–26] By selectively extracting one phase, the remaining part provides a desired structural basis for the scaffold, since the pores not only has an appropriate size for cell

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growth but also are fully interconnected.^[27–29] Previous studies have demonstrated that the microstructural features of the scaffolds derived from polymer blends were slickly controlled by phase components and phase coarsening.^[30–32] The combination of binary polymer blends includes poly(lactic acid) (PLA)/PCL, polypropylene (PP)/poly(ethylene oxide) (PEO), PLA/PP and polystyrene (PS)/poly(ethylene glycol) (PEG).^[33–35] While the phase coarsening is spontaneous during annealing. Relevant viscosity, annealing time and temperature are the keys to predicting the phase coarsening. Because of their strong designability, the employment of immiscible binary polymer blends for tissue engineering applications has attracted particular attention.

Using the above motif, we proposed a feasible and scalable approach to fabricating biomimetic scaffolds with oriented and interconnected pores through the uniaxial drawing followed by selective extraction. To this end, a co-continuous structure was built based on the immiscible PCL/PEO blend. Then, uniaxial solid-phase stretching was performed to allow phase deformation along the elongation direction. Finally, by aqueous extraction of the PEO phase, the 3D scaffolds with highly oriented and fully interconnected pores were obtained, showing a close architectural resemblance with natural bone. We demonstrated that the pore anisotropy could be readily manipulated by control over the stretching strain without the loss of connectivity. The prepared biomimetic scaffolds promoted the proliferation and differentiation of preosteoblasts significantly. The expression of osteogenic proteins and the signal pathway were examined to illuminate the inherent mechanism.

EXPERIMENTAL

Materials

Biodegradable PCL pellet (Market No: Esun 600C, $M_v=6 \times 10^5$ g·mol⁻¹, $T_m=60.0$ °C) was obtained from Shenzhen Guanghua Weiye Industrial Co., Ltd. Water-soluble PEO powder (Barki, USA, $M_v=5 \times 10^5$ g·mol⁻¹, $T_m=67.8$ °C) was obtained from Guangzhou Ligu Trading Co., Ltd. Deionized water (DIW) and 75% (V/V) alcohol were purchased from Chengdu Kelong Chemical Reagent Factory, China. All chemical reagents were used directly.

Preparation of Oriented Co-continuous Porous Scaffolds

Oriented porous scaffolds were prepared by the combination of uniaxial solid-phase stretching and phase extraction of co-continuous PCL/PEO blends. First, binary PCL/PEO blends were melting mixed with an equal mass ratio in a torque rheometer at 100 °C for 8 min at a rotation speed of 60 r/min to obtain a co-continuous structure. Prior to blending, PCL pellets and PEO powders were vacuum dried for 24 h at 50 °C. The co-continuous blends were compression molded into the base sheet at 100 °C and 10 MPa. The crosshead speed was set as 100% strain/min. Then, the oriented co-continuous blends were prepared by uniaxially solid-phase stretching of the as-prepared scaffolds to different normal strains (75%, 150% and 300%) at 50 °C. Finally, the oriented porous scaffolds were obtained by extracting PEO with DIW at 37 °C. For comparison, the unstretched porous PCL scaffold was also prepared. The

scaffolds were denoted as S0, S75, S150, and S300, respectively, according to the ultimate normal strain.

Characterization

Scanning electronic microscopy (SEM) observation

Porous morphology was characterized using a scanning electron microscopy (SEM, Nova NanoSEM450, FEI, USA) at an accelerating voltage of 5 kV. Prior to observation, the scaffolds were cryogenically fractured parallel and perpendicular to the stretching direction in liquid nitrogen, and sputter-coated with gold. The average pore size was quantified by measuring at least 200 pores via ImageJ software.

Physical parameter measurements

Continuity of the scaffolds was calculated by the equation: Continuity = $(1 - m_i/m_f) \times 100\%$, where m_i and m_f are the weight of PEO phase in the mixture before and after DIW extraction, respectively. The porosity of scaffolds was determined by a gravimetric method using the equation: Porosity = $(1 - V_p/V_m) \times 100\% = (1 - W_m\rho/V_m) \times 100\%$, where V_m and V_p are the apparent and actual volume of PCL scaffolds, respectively, W_m is the weight of PCL scaffolds, and ρ is the density of PCL (1.14 g/cm³). The water absorption of scaffolds was tested by immersing samples into DIW at room temperature for 24 h. The water absorption was calculated using the equation: Water = $(W_1 - W_0)/W_0 \times 100\%$, where W_1 and W_0 represent the dry weight of scaffolds before and after immersing in DIW, respectively.

Water contact angle analysis

Hydrophilicity of the scaffolds was qualitatively determined via measuring the contact angle with a goniometer (DA 30, Krüss, Germany). 4 µL of DIW was dropped on the scaffold and the static contact angle was recorded using a camera equipped. Average value was obtained on five random regions in each sample.

Osteoblasts culture

Osteoblasts (MC3T3-E1 cells) were blown evenly into a cell suspension with a density of 2.0×10^6 . 50 µL of cell suspension was inoculated on the scaffold. After 30 min, the same amount of cell suspension was inoculated. After 1 h, 10 mL of α -men medium containing 3% fetal bovine serum was added and placed at 37 °C. The cells were cultured in 5% CO₂ incubator.

Osteoblasts proliferation

After 7 and 14 days of culture in the incubator, the original culture medium was discarded, washed by PBS for 3 times and then digested with 0.25% trypsin. The cells were collected and counted. The total amount of cells per well was 100 µL and cultured for 6 h. 10 µL of CCK-8 solution was added into each well. The liquid should be slowly injected into the well and incubated for 1 h in a 5% CO₂ incubator at 37 °C. The absorbance was measured at 450 nm wavelength by an enzyme labeled instrument.

Osteogenic differentiation

After 7 and 14 days of culture in the incubator, the original culture medium was discarded and washed by PBS for 3 times. Then, 0.25% trypsin was added for digestion and cells were collected. The culture medium was washed by PBS for 3 times and cell lysate was added and centrifuged to collect the supernatant. According to the instructions, samples, standards

and enzyme labeled reagents were added. Reaction time was 60 min at 37 °C; chromogenic solutions A and B were added; and color reaction was stopped at 37 °C for 15 min. The absorbance of each hole was measured in sequence at 450 nm within 15 min after adding the termination solution. The linear regression equation of the standard curve is calculated by using the concentration and absorbance value of the standard substance. The sample concentration is calculated and multiplied by the dilution ratio, which is the experimental concentration of the measured index of the sample. The experiment is repeated for 3 times.

Expression of Wnt-3 α and β -actin by immunofluorescence

After 14 days, anti-Wnt-3 α , anti- β -actin, anti-Wnt-3 α , anti- β -actin, anti-Wnt-3 α , and anti- β -actin were diluted with 5% BSA in the ratio of 1:200 (V:V), and incubated in dark for 30 min. The excitation wavelengths were measured at 647 and 488 nm. The fluorescence images were observed and recorded by laser confocal microscope.

Statistical analysis

Experiments were performed at least in sextuplicate for each sample ($n > 6$ per group), and all the values were expressed as mean $\pm$ standard deviation. Differences between groups were determined by one-way analysis of variance (ANOVA). A p value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Biomimetic ECM architecture is of great significance to provide the microenvironment conducive to bone tissue engineering.^[36,37] However, current scaffold technologies are limited in simulating interconnected and anisotropic pore features simultaneously. In this study, we developed a feasible and effective approach to fabricating 3D scaffolds with interconnected and anisotropic pores, as schematically depicted in Fig. 1. Considering that the co-continuous structure contains mutual interconnected phases, binary PCL/PEO blends were prepared as in the base material for biomimetic scaffolds. Rheology measurements of PCL and PEO were performed to determine

the optimal composition for the formation of co-continuous structure. As plotted in Fig. S1(a) (in the electronic supplementary information, ESI), the comparative viscosity of PCL and PEO appears at the frequency of ~ 1 rad/s. According to the empirical Cox-Merz rule, the phase inversion point of the blend components is related to the viscosity ratio of PCL to PEO.^[33,38,39] And the PCL/PEO blend with equal weight fraction is selected. Uniaxial solid-phase stretching was used to necessitate the phase deformation of the co-continuous blends. Since the stretching temperature is under the melting point of PCL, phase coarsening is impeded and the co-continuous structure is preserved. After extraction of the hydrosoluble PEO phase, the oriented interconnected porous scaffolds are obtained.

The formation of oriented interconnected pores is evidenced in SEM micrographs (Fig. 2a), where the porous structure parallel and perpendicular to the stretching direction is observed. Compared to round pores in S0, ellipse pores appear along the stretching direction of the oriented scaffolds (S75, S150 and S300). Due to the deformation induced by the elongation flow field, the average pore size along the stretching direction shows a continuous increase with the strain (Fig. 2b). On the other hand, the pores perpendicular to the stretching direction remain round without notable changes in pore size (Fig. 2b). It might be ascribed to the narrowing of the pore wall during solid phase stretching. The orientation index of the pore was defined as the ratio of the long axis to the short axis. Such a morphology feature leads to the substantial increase in orientation index (Fig. 2c), indicating that the pore anisotropy is tunable by simply adjusting the strain.

To probe the internal crystalline structure of porous scaffolds, synchrotron 2D WAXD was performed. Fig. 3(a) illustrates the 2D WAXD patterns of S0 and S300. Two main isotropic diffraction rings corresponding to (110) and (200) reflections are observed in S0, representing a random crystal orientation. In stark contrast, sharp arc-like signals appear in S300, demonstrating the formation of highly aligned lamellae in oriented porous scaffolds. Fig. 3(b) displays the 1D

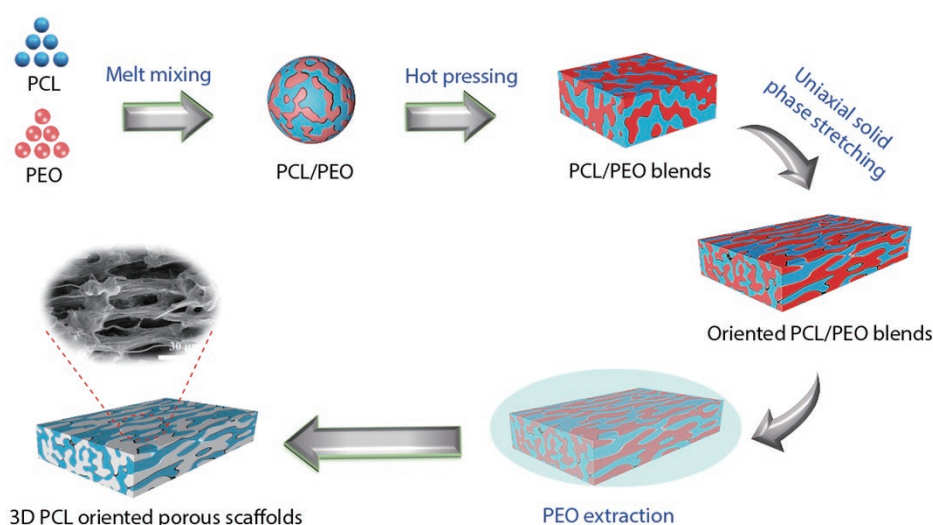


Fig. 1 Schematic illustration of the preparation of 3D oriented porous PCL scaffolds. Blue and red balls represent PCL and PEO, respectively.

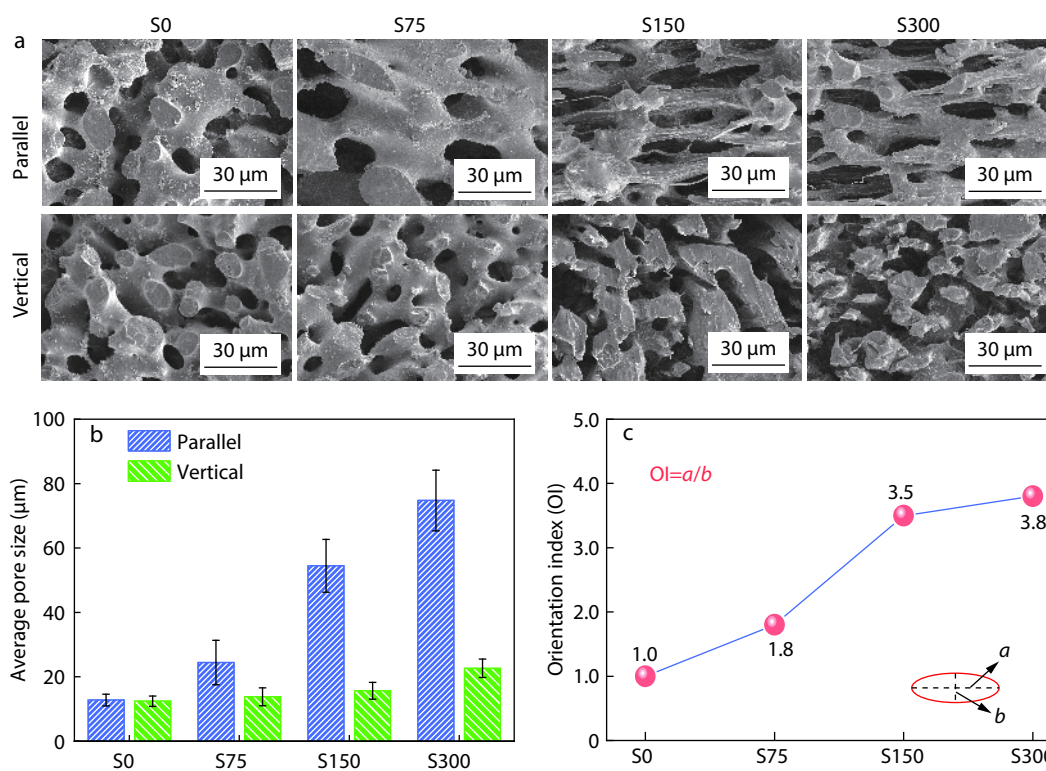


Fig. 2 (a) SEM images of the fractured surface of the scaffolds parallel and perpendicular to the stretching direction. (b) Average pore sizes and (c) orientation index of the scaffolds.

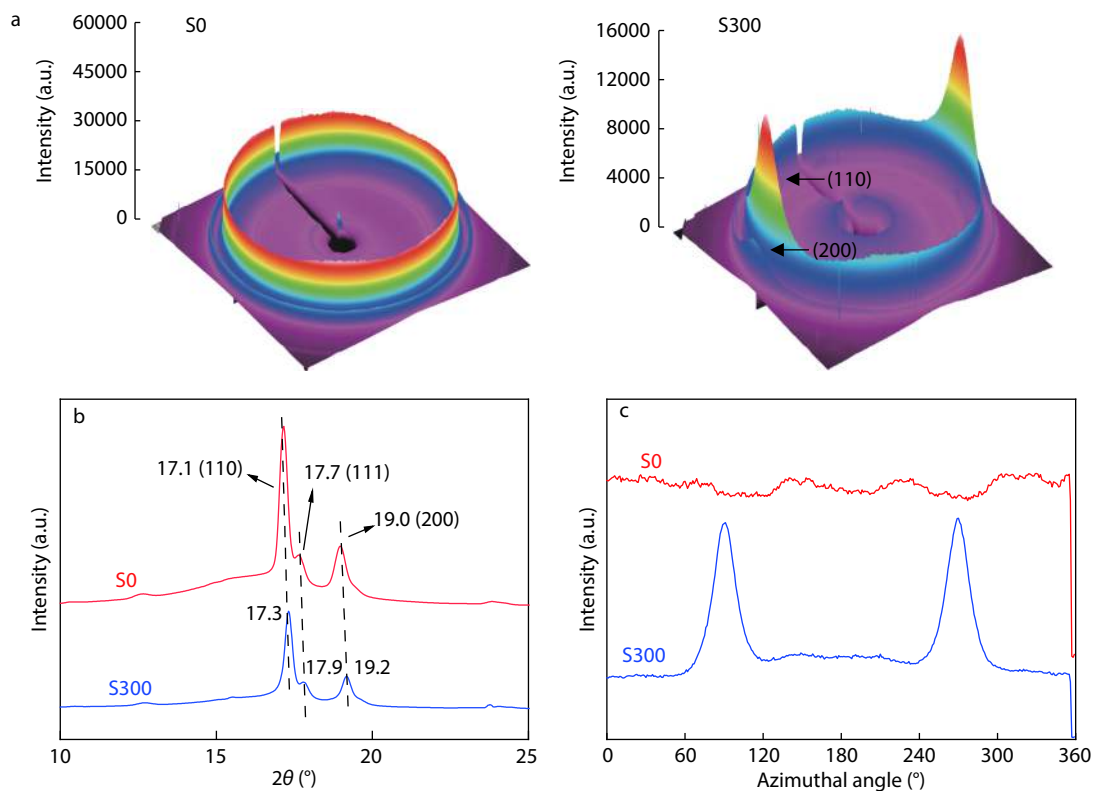


Fig. 3 (a) 2D WAXD patterns, (b) 1D WAXD profiles and (c) azimuth maps of S0 and S300.

WAXD profiles integrated circularly form the 2D patterns. Both S0 and S300 show two main diffraction peaks and a shoulder peak at almost the same position, suggesting no change in the crystalline form of the PCL matrix in oriented porous scaffolds. Following the LaShanda's method,^[40] the Herman's orientation factor (f_H) of PCL lamellae in the porous scaffolds was calculated. The random and complete orientation of lamellae correspond to the f_H values of 0.50 and 1.00, respectively. S300 shows an orientation degree of 0.68, significantly higher than that of the unoriented scaffolds (0.49). It reflects the notable phase deformation induced by uniaxial solid-phase stretching.

Fig. 4(a) exhibits that the continuity of oriented porous scaffolds maintains a high level of ~100%, which are the same with the unstretched porous scaffolds. It is attributed to the formation of co-continuous structure that PCL and PEO phases are both interconnected in the binary blends. Such an architecture is conducive to the interaction of adjacent cells and cellular metabolism.^[41,42] The porosity of the oriented porous scaffolds was obtained *via* a gravimetric method. The overall porosity increases from 48.6% for S0 to ~60% for S300 (Fig. 4b). The narrowing of the pore wall with the strain makes the large volume ratio of pore in the whole 3D scaffolds. The ascending porosity of oriented porous scaffolds can provide more space for cell proliferation and growth.

Surface wettability plays an important role in regulating cell behavior, so the WCA test was conducted to assess the hydrophilicity of scaffolds. It can be observed from Fig. 4(c) that a WCA value of 86.5° appears on S0 due to the inherent hydrophobicity of PCL, while it shows a monotonous decrease with the strain. The WCA of S300 drops to 77.3°. The water absorption related to nutrient permeation was estimated by immersing scaffolds into DIW at room temperature for 24 h. On account of the escalation of porosity and hydrophilicity, the water absorption of the scaffolds increases notably with the strain (Fig. 4d). The S300 (161.5%) presents a nearly 2.1-fold augment compared to S0 (76.7%).

Fig. 5(a) illustrates the proliferation of preosteoblast on the oriented porous scaffold. The absorbance (optical density) was measured after 7 and 14 days of incubation. The architecture of the scaffolds strongly affects the cell proliferation. There is an apparent increase in the cell number on the oriented porous scaffolds with the increasing strain ($p < 0.05$). It demonstrates that the formation of oriented porous structure is beneficial to cell proliferation. As a crucial early differentiation marker, alkaline phosphatase (ALP) activity was detected after culturing preosteoclasts for 7 and 14 days. As presented in Fig. 5(b), oriented porous scaffolds present a stronger positive ALP expression than the isotropic porous scaffold on day 7. The ALP activity of all the scaffolds samples

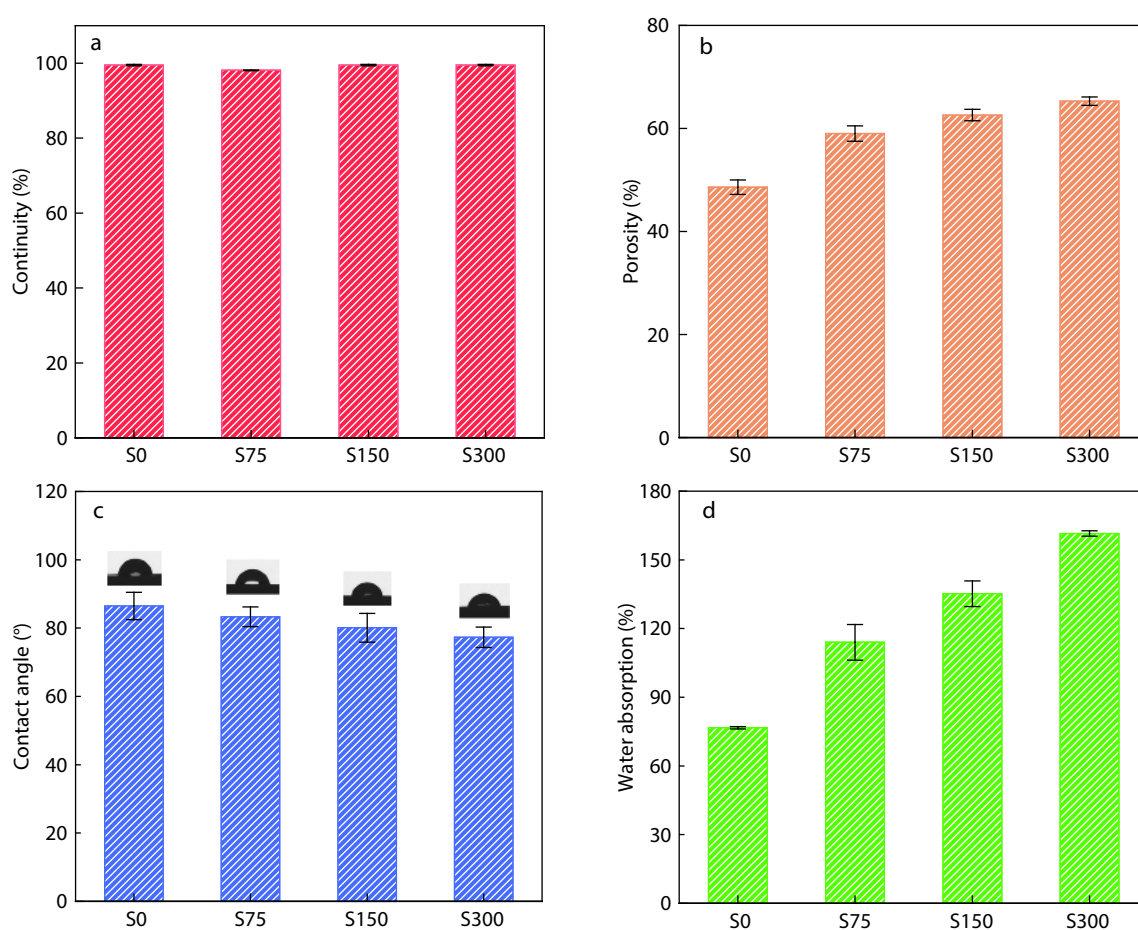


Fig. 4 Physical parameters of porous scaffolds: (a) continuity, (b) porosity, (c) contact angle and (d) water absorption.

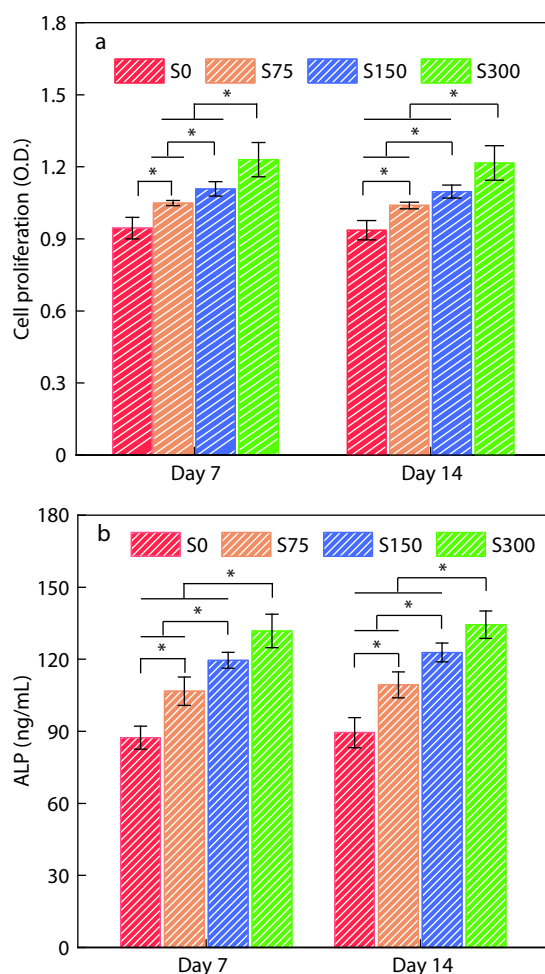


Fig. 5 (a) Proliferation and (b) alkaline phosphatase (ALP) levels of preosteoblasts on the oriented porous scaffolds after cultured for 7 and 14 days.

increased dramatically on day 14. Nevertheless, a higher ALP expression is observed on the oriented porous scaffolds, where S300 shows the greatest ALP expression. It is consistent with the results of cell proliferation (Fig. 5a).

To evaluate the osteoblastic differentiation at the protein level, enzyme-linked immunosorbent assay (ELISA) was used to assess the differentiation at the genetic level by measuring the relative RNA content in preosteoclasts cultured on the scaffolds with different oriented pores. As shown in Fig. 6(a), the higher expression of collagen I (COL-I) on the oriented pore scaffolds suggests that osteoblastic differentiation is motivated by the oriented pores. Osteopontin (OPN) and osteocalcin (OCN) are related to the early and late differentiation of osteoblasts and show a significant higher expression on the oriented porous scaffolds compared to the controls (Figs. 6b and 6c). These results strongly confirm that the anisotropic porous structure promotes the osteogenic differentiation of preosteoblasts.

To further clarify the underlying of proliferation and osteogenic differentiation of preosteoblasts induced by the oriented pores, the Wnt/ β -catenin signaling pathway, a vital pathway that regulates osteogenesis,^[43] was marked by im-

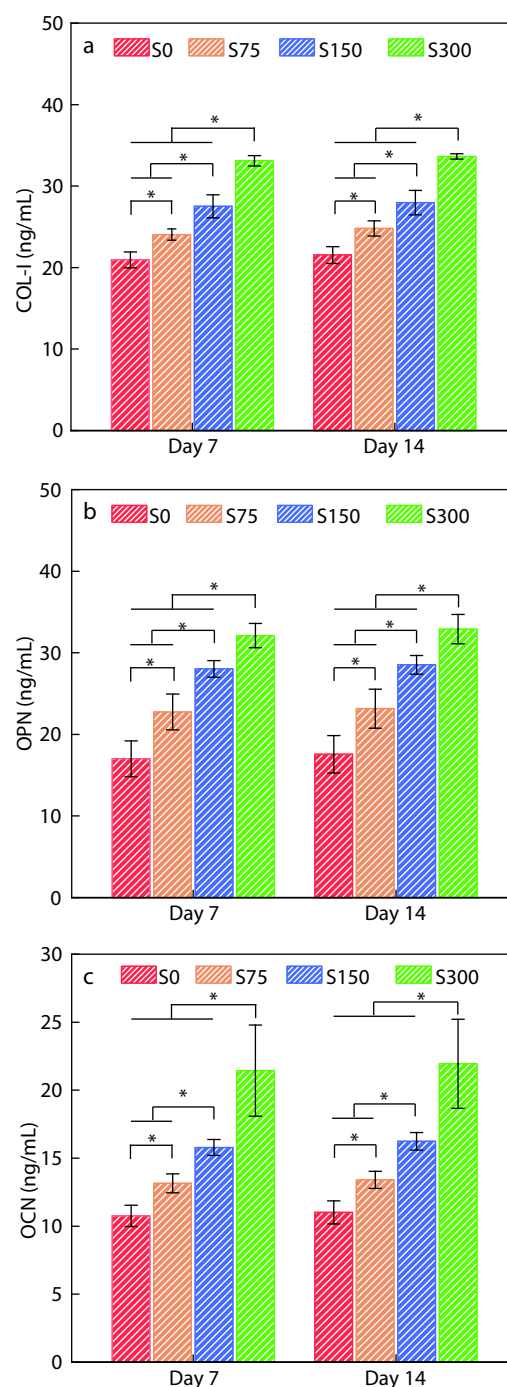


Fig. 6 Protein expression of (a) COL-I, (b) OPN, and (c) OCN of preosteoclasts cultured on the oriented porous scaffolds after 7 and 14 days.

munofluorescence staining. Fig. 7 shows that the level of Wnt-3a and β -catenin is much higher on the oriented porous scaffolds compared to that on the isotropic porous scaffolds, which clearly suggests the activation of Wnt/ β -catenin signaling pathway during the oriented porous structure-induced osteogenesis. And the activation enhances with the orientation degree of anisotropic porous structure.^[44] More interestingly, preosteoblasts on the isotropic porous scaffolds are ran-

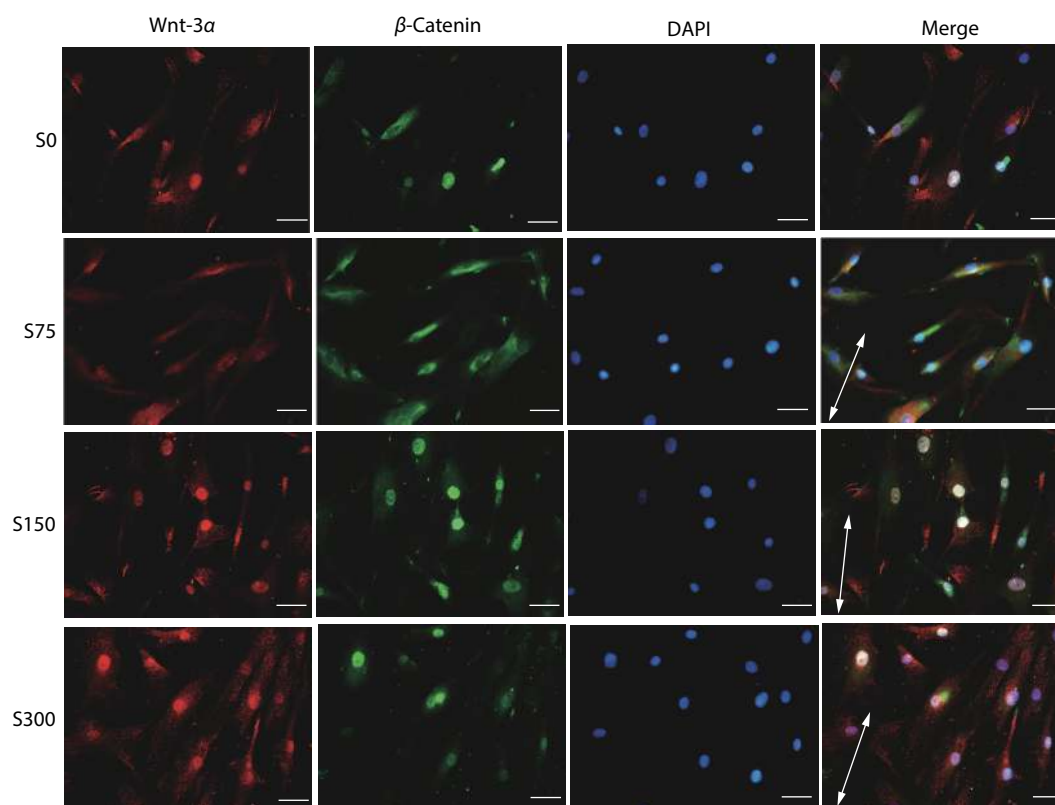


Fig. 7 Expression of the Wnt/ β -catenin of preosteoblasts on isotropic and oriented porous scaffolds. Cells were cultured on the scaffolds for 14 days, and then stained for Wnt-3 α (red), and β -catenin (green). Nuclei were counterstained with DAPI (blue). Double-headed arrows indicate the orientation direction of the scaffolds. Scale bar: 20 μ m.

domly distributed, whereas preosteoblasts are elongated and well-aligned along the oriented direction of anisotropic pores. Previous studies reported that the cells cultured on topographical substrate were sensitive to the physical cues (mechanical stimuli), which would be likely transduced into biochemical signals by morphological changes like cell alignment, and then the biochemical signals were delivered to the nucleus *via* internal cytoskeleton to promote gene expression such as osteogenesis.^[44–46] Hee *et al.* reported that the poly(lactic-co-glycolic acid) (PLGA) aligned pattern can enhance osteogenic activity by inducing cell orientation *via* the mechanical activation.^[47] On the basis of these results, it can be referred that the oriented porous scaffolds promote the osteogenic differentiation of preosteoblasts through the Wnt/ β -catenin signaling pathway, which is activated by the cells directional growth induced by oriented architecture as the physical cue.^[48]

CONCLUSIONS

Biomimetic porous PCL scaffolds were prepared *via* solid phase stretching of cocontinuous PCL/PEO blends followed by selective extraction of the water-soluble PEO phase. Stretching enabled the elongation phase deformation and the pore morphology was a replica of the original PEO phase. It endowed the scaffold with fully interconnected and highly oriented pores, structurally mimicking the feature of nature bone. The oriented scaffold offered an effective guiding architecture for

preosteoblast proliferation and differentiation. It was attributed to the activation of the Wnt/ β -catenin signaling pathway. The above findings open a workable avenue to fabricate bone tissue scaffolds with promising biologic functions.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <https://doi.org/10.1007/s10118-021-2573-4>.

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