Fabrication of Multilayered Nanofiber Scaffolds with a Highly Aligned Nanofiber Yarn for Anisotropic Tissue Regeneration

Dawei Li, Ling Tao, Ying Shen, Binbin Sun, Xianrui Xie, Qinfei Ke, Xiumei Mo,* and Bingyao Deng*

ABSTRACT: Nanofibrous scaffolds were widely studied to construct scaffold for various fields of tissue engineering due to their ability to mimic a native extracellular matrix (ECM). However, generally, an electrospun nanofiber exhibited a two-dimensional (2D) membrane form with a densely packed structure, which inhibited the formation of a bulk tissue in a three-dimensional (3D) structure. The appearance of a nanofiber yarn (NFY) made it possible to further process the electrospun nanofiber into the desired fabric for specific tissue regeneration. Here, poly(L-lactic acid) (PLLA) NFYs composed of a highly aligned nanofiber were prepared via a dual-nozzle electrospinning setup. Afterward, a noobing technique was applied to fabricate multilayered scaffolds with three orthogonal sets of PLLA NFYs, without interlacing them. Thus the constituent NFYs of the fabric were free of any crimp, apart from the binding yarn, which was used to maintain the integrity of the noobing scaffold. Remarkably, the highly aligned PLLA NFY expressed strengthened mechanical properties than that of a random film, which also promoted the cell adhesion on the NFY scaffold with unidirectional topography and less spreading bodies. In vitro experiments indicated that cells cultured on a noobing NFY scaffold showed a higher proliferation rate during long culture period. The controllable pore structure formed by the vertically arrayed NFY could allow the cell to penetrate through the thickness of the 3D scaffold, distributed uniformly in each layer. The topographic clues guided the orientation of H9C2 cells, forming tissues on different layers in two perpendicular directions. With NFY as the building blocks, noobing and/or 3D weaving methods could be applied in the fabrication of more complex 3D scaffolds applied in anisotropic tissues or organs regeneration.

1. INTRODUCTION
Tissue engineering is considered as a potential technique to regenerate functional organs and tissues, which can provide suitable substitutes to repair the defects in tissue and organs.1 Electrospun nanofibers have attracted a wide range of interests due to their large specific area, high porosity, and their interconnected pore structure in the field of tissue engineering.2,3 Nanofibers with a diameter in the scale of tens of nanometer to several micrometers are on the same order of biological nanofibers, constructing the extracellular matrix (ECM) of animal tissues.4,5 Using various materials and methods, nanofibrous scaffolds resembling different tissues in physical structure, biochemical composition, and mechanical properties have been prepared.

Nevertheless, conventional electrospun nanofibrous scaffolds always exhibited two-dimensional (2D) constructs with densely packed nanofibers that limited the cell infiltration into the scaffolds. Generally, they are used in the original form, depositing on the collector. The lack of reprocessibility in the structure limits the application in the repair of bulk tissue defects.6−8 Moreover, the randomly deposited nanofiber film has relatively low mechanical strength, which also cannot guide the growth of a desired specific tissue. The appearance of nanofiber yarns (NFYs) with a highly aligned nanofiber could address these problems and they can be processed into a fabric with various textures via textile methods, including weaving, knitting, braiding, etc.9−11

Electrospun NFYs were first prepared with several winding and twisting drums.12 Later, a variety of devices were invented to collect the nanofiber into nanofiber bundles with or without additional twists, including spinning onto dual collecting rings or parallel electrodes with subsequent rotating,13−15 spinning the nanofiber into a water or nonsolvent reservoir collector and drying later,16 combining the water reservoir collector with the water vortex, which would twist the nanofiber yarn,17−19 introducing specially customized rotating collectors,20,21 coating a nanofiber on the surface of a microfiber yarn,22,23 using rotating plates24 or a funnel target as a collector accompanied...
with a single spinning nozzle\textsuperscript{25,26} or dual spinning nozzles oppositely positioned and charged positively and negatively, respectively.\textsuperscript{37−39} In particular, a dual-nozzle electrospinning system attracts lots of attention due to the continuity and stability in the fabrication of uniform NFYS.\textsuperscript{29} Also, it does not require a solvent-based receptor, which makes it suitable for almost all polymers. Furthermore, the twists in the yarns generated by the rotation of the funnel do improve the mechanical properties, allowing it to meet the requirements for textile processing.\textsuperscript{28,29}

More efforts were made to study the potential of NFYS prepared by a dual-nozzle system with anisotropy texture in tissue engineering. The cells’ behavior on highly aligned NFYS is studied using lined up yarns, which could guide the spreading and orientation of cells along the axis of the yarn.\textsuperscript{31,32} Furthermore, to fabricate 3D scaffolds with electrospun NFYS, methods in conventional textile including weaving, knitting, and braiding were utilized to process the yarns into 3D scaffolds with tunable macroscale structures.\textsuperscript{9,10,33−35}

Up to now, scaffolds prepared by textile methods with NFYS have been studied for various applications including muscle,\textsuperscript{36,37} tendon,\textsuperscript{9,31,38} heart valve,\textsuperscript{10} nerve,\textsuperscript{39} cardiac tissue,\textsuperscript{35,36,42} vessel\textsuperscript{11,35} and tracheal tissue\textsuperscript{43} regeneration. However, conventional textile techniques could only form a plain fabric with a limited thickness (2−3 times of the diameter of the yarns), which may meet the needs of single-layer tissue regeneration, such as skin tissue, small vessel,\textsuperscript{34} tendon, and ligament tissue (bundle of yarns preferred).\textsuperscript{9} As for bulk tissue defects, like myocardial infarction, cartilage and bone necrosis, and tumor removal defects, scaffolds with a certain volume and thickness are urgently required.\textsuperscript{44,45} Moreover, controlling cellular organization in the bulk 3D scaffolds remains a challenge.\textsuperscript{35}

The noobing technique is a unique nonwoven 3D fabric-forming process, where a set of linear yarns arrayed in either uniaxial or multiaxial orientation were bonded/tied using required sets of binding yarns.\textsuperscript{40} Multilayer scaffolds with regular pores and anisotropic texture were fabricated by noobing strips of an aligned nanofiber membrane, inducing the uniform distribution of cells in each layer even under static culture conditions, which provide a new route for the fabrication of 3D nanofibrous scaffolds.\textsuperscript{35} However, the discontinuity and nonuniformity of stripes could not adapt to the mechanical process, also the e discontinuity and nonuniformity of stripes could not adapt to the mechanical process. Also, the electric field among the two nozzles and the funnel. Also, the electrostatic attraction between the nanofibers carrying a positive charge and a negative charge would result in aggregation and bonding, along with the neutralization. Nanofibers deposited on the funnel would form a thin film. A plastic stick was then placed at the center of the film, which would attract part of the fibers attached on the stick. The act of dragging the stick away from the funnel, toward the winding up roll, caused the formation of a hollow cone between the stick and the funnel edge. During the electrospinning process, subsequent nanofibers were then deposited on the nanofibrous cone, which supplied a constant source of the nanofiber yarn. The rotating funnel would twist the nanofibers at the conical tip of the nanofibrous cone, forming NFYS. Afterward, the well-formed NFYS were withdrawn from the forming zone and wound up by the winding roller.

The key to generating uniform and continuous NFYS lies in the formation of a stable fibrous cone. The in and out balance of nanofibers in the cone zone was achieved, which means the quantity of freshly deposited nanofibers equaled that of nanofibers forming NFYS and being drawn away. For the optimization of NFY preparation, several parameters during electrospinning were adjusted, including the feed rate of the PLLA solution, the applied high voltage, position of two nozzles, rotating speed of the funnel, and the winding up speed.

The feed rate of a polymer solution and the applied high voltage would affect the morphology of a PLLA nanofiber, as well as the income quantity in a cone zone. Also, the negative and positive high voltage affected the electric field in the spinning zone. The nozzle charged with a very high voltage would attract the nanofiber generated from another nozzle,
drawing the nanofibers onto the metal needle and less nanofibers would deposit on the grounded funnel. As for the position of two spinning nozzles, they are placed oppositely beside the cone zone. The distance between two nozzles is larger than that of the nozzle to the funnel, avoiding the miscapture of nanofibers from one nozzle by another. Besides, the axis of the nozzle and the syringe is vertical to the surface of the nanofibrous cone, which favors the collection of nanofibers to the maximum.

The rotation of the funnel was the core movement for the formation of NFYs, which generated twists in the yarns and maintained the nanofibrous cone. At a rotating rate lower than 100 rpm, the insufficient twisting would lead to a prolonged cone and thin nanofibrous film. The nanofiber tended to lie parallelly alongside the axis of the NFYs with insufficient binding. Increasing the rotating rate would drive the cone apex to move toward the funnel. Meanwhile, the increasing twist would skew the nanofiber and magnify the twist angles.

Moreover, the winding speed affects the process, the final twist angle of nanofibers in the yarn, and the diameter of NFYs. A slow winding speed always results in more twists in a certain length, which means increased twist angles.29 Also, more nanofibers were collected by the nanofibrous cone, increasing the thickness of the nanofiber film on the cone as well as the dragging force and diameter. The NFYs formed earlier would have suffered from the increased force, which usually caused the failure of NFYs. As the winding speed exceeds the threshold, less nanofiber is collected to form the nanofibrous cone, which caused the nanofiber film in the cone to be too thin to support an intact nanofiber cone. Thus, the yarn–cone system breaks down. Here, the optimum PLLA NFYs were obtained when the flow rates in the two nozzles were 1.0 mL/h, the voltage was +10 and −10 kV, respectively, the distance of nozzles to funnel was 10 ± 3 cm, with the angle between nozzles and the yarn being 50 ± 5°, and the rotating rate of the funnel was 300 rpm with a winding up speed of 1 m/min.

Figure 2. SEM image of the surface (a), (b) and the cross section (d), (e) of the PLLA nanofiber yarn. Distribution of diameters of nanofibers (c) and NFYs (f). The confocal image of rhodamine B labeled PLLA NFYs (g, h). The SEM image of PLLA NFY after the tensile-strain test (j, k). The stress–strain curve (i) and value (l) of PLLA NFY and PLLA films.
Figure 3. Schematic of the noobing NFY scaffold with three layers (a, b, c, d). Photos of the noobing NFY scaffold on the frame (e, f, g, h), corresponding to the four steps. Photo of the noobing fabric with 11 and 3 layers (i: front view, j: cross-sectional view). The stress–strain curve (k) and stress and strain at break (l) of the noobing fabric in two directions (** indicates statistical difference for p < 0.01. Photos were taken by Dawei Li).

Figure 2a,b shows the scanning electron microscopy (SEM) image of the PLLA NFYs with different magnifications. It can be clearly noticed that the PLLA nanofibers in the NFYs were free from beads and normally exhibited circular cross section. This could also be identified in the cross-sectional view, as shown in Figure 2d,e. The SEM image (Figure 2a) demonstrates that the NFYs are composed of aligned nanofibers along the axis of NFYs with an inclined twist angle. Also, a small amount of a disordered nanofiber can be found at the surface of the yarn. The average diameter of a PLLA nanofiber and PLLA NFYs is 558.2 ± 157.8 nm and 69.06 ± 19.37 μm, as shown in Figure 2c,f. Figure 2d,e illustrates the SEM image of the cross section of PLLA NFYs. It can be seen in Figure 2d that most yarns exhibit a columniform body, which was generated by the rotating funnel. Gaps and holes can be observed in the cross section of the yarn. Also, in Figure 2e, the ribbon-like and convex structure can also be distinguished, which was constructed by two to six threads of nanofibers. It was suggested that nanofibers cohered together before they were twisted into the NFYs. Generally, polymer jets charged with a high voltage from one nozzle would carry the same charge (positive charge or negative charge), which would generate an electrostatic repulsion force that pushes them in the opposite direction. Most likely, the fibers in each bundle that come from different nozzles carry a positive charge and a negative charge, respectively. Once nanofibers approach each other in the cone zone, the electrostatic attraction force would draw them together, neutralizing the charge and forming fiber bundles.

To further observe the structure of PLLA NFYs, rhodamine B was dissolved in the spinning solution to prepare red-labeled PLLA NFYs. The confocal images of rhodamine-loaded PLLA NFYs are shown in Figure 2g,h,j. The parallel texture on the surfaces of NFYs can be easily observed. Also, disordered nanofibers with random loops and curves were also observed. The reason behind this is that some nanofibers deposit on the already formed NFYs outside the cone zone. Limiting the polymer jet deposition in the cone zone would favor a tidy surface texture.

The tensile properties of a fibrous yarn play an integral role in the preparation of fabric through textiles methods. Here, a tensile test was conducted to evaluate the mechanical strength of PLLA NFYs. The stress–strain curve is shown in Figure 2n. The stress and strain at failure of PLLA NFYs is 23 MPa and 27.7%, while that of a PLLA nanofiber film is 4.03 MPa and 54.8%, respectively. It can be noticed that the stress–strain curve is quite different than the PLLA nanofiber film (Figure 2n). Young’s modulus of the NFYs and film is 116.2 and 8.6 MPa, respectively. For the PLLA NFYs, as illustrated in the figure, the stress increases sharply at an early stage, distinct from the slow-rising curve of the PLLA film. On the other hand, the plateau stage of PLLA NFYs is shortened in the range from 10 to 27.7%, narrower than that of the PLLA film, ranging from 5 to 54%. Also, the collapse stage of PLLA NFYs is rather rapid, with a sudden drop in the stress, while the PLLA film goes through a sluggish descent before overall breakage.

To study the reason for the unusual curve, PLLA NFYs after the tensile strength test were observed by SEM, as shown in Figure 2k,m. It can be clearly seen that most of the PLLA nanofibers after stretching exhibited a knobby structure, which means most of the nanofibers in the yarn have suffered an extension and shared the stretching force. Different from the nanofiber film, which was composed of randomly distributed nanofibers, nanofibers in NFYs consisted of continuous filaments through the length of the yarn. While stretching, the nanofibers in the PLLA film would first unbend and then reorient in the stretching direction. Some of the nanofibers
unlock the binding from adjacent nanofibers. The relative movement among nanofibers would reduce the breakage of nanofibers and put off the overall breakage of the film. However, in PLLA NFYS, nanofibers aligned in the direction of the axis of the yarn. Most nanofibers shared the stretching force from the beginning to the breaking ending. The whole body of the nanofiber was elongated and a reduction in the diameter of the nanofibers occurred. Along the plateau stage, more nanofibers broke down (red arrow in Figure 2m), resulting in the fluctuation in tensile strength. At the breaking stage, the rest of the nanofibers reached the critical elongation, causing a sudden rupture.

2.2. Three-dimensional (3D) NFY Scaffold via Noobing. In the textile industry, weaving and knitting are generally used to prepare a plain fabric with a limited thickness. Also, the interlacing and knitting generate crimp and loops in the yarn. Here, the fabrication of a 3D NFY scaffold was conducted by a noobing technique via using plied NFYS. Single PLLA NFY was too fragile to handle and process into a fabric, thus the 4-time folded NFY bundle was twisted into a strand yarn. Noobing is different from weaving, knitting, and braiding. In the noobing fabric, a set of linear yarns is bound or tied using a required set of binding yarns to produce a corresponding multilayer 3D fabric, comprising three orthogonal sets of yarns. Yarns in the noobing fabric do not interlace, interloop, and intertwine. The fabric is inextensible due to the yarns being linear or crimless, which is capable of producing a 3D fabric with profiled cross sections. It also facilitates the fabrication of 3D scaffolds with a desired structure. The schematic of the three-layer noobing fabric is shown in Figure 3a–d. The image of well-prepared PLLA NYF scaffold through noobing is illustrated in Figure 3e–h. It could be clarified that the crimp in red binding yarns can be eliminated as the layers increase.

Unlike the weaving fabric, the process of binding yarns occurs only on the surface. As shown in Figure 3ij, the noobing fabric with 11 layers illustrates larger thickness than that of 3 layers. The binding yarn in the fabric of 11 layers tends to go through a longer distance in the depth direction. Thus, in the middle layers, red binding yarns are set vertically to the plain layers of weft and warp yarns. Furthermore, without crimps and shedding, yarns could be pulled out of the fabric easily, meaning an edge banding process is needed in an actual application. Heat melting was conducted to fix the edge of the PLLA NFY scaffold.

The tensile behavior of the noobing fabric in the weft and warp direction is shown in Figure 3k,l, it can be noticed that, in both directions, the tensile strength shows no significant difference. However, the elongation at break in the warp direction (or noobing direction) is higher than that of the weft direction. This could be attributed to the zigzag route of the binding yarn. In the weft directions, weft yarns were all laid in a straight way with less crimp. In the warp direction, the warp yarns were also straight, while the binding yarns had a curved path. During the tensile test, the straight warp yarns are stretched, along with the binding yarns being stretched and stretched, resulting in a higher elongation.

2.3. Cell Adhesion. Figure 4 shows the H9C2 cells’ adhesion behavior during the scheduled time. It can be noticed that all of the pseudopodia spread along the whole fiber in the first 20 min on both scaffolds (Figure 4a,f). After 40 min, more cells can be captured in one view of the CLSM camera. The red dots noticed beside the cell body indicate the extended pseudopodia (Figure 4b,g). No obvious change was observed for the cells on the PLLA film. Most of them were still spherical with a small pseudopodium holding onto the nanofiber (Figure 4b). In comparison, cells on NFYS started to
spread around, indicated by the irregular cells with a trident form and a fork shape oriented in the direction of NFYs, as shown in Figure 4g (red arrows). This can also be found in the SEM image of Figure 4g′. One H9C2 cell is embedded in the groove formed by two parallel nanofibers, which also guides the spreading of four pseudopodia stretched from that cell. After 60 min, more irregular cells can be observed on the film scaffold with an enlarged project area (Figure 4c). Flattened cells can also be seen in the SEM image (Figure 4c′). On the NFYs, more cells oriented themselves along the yarn direction with an increasing length (Figure 4h,h′). After 120 min of seeding, round shaped cells could still be observed on the film of the scaffold, only with an increased perimeter (Figure 4d,d′). However, on the NFY scaffold, the pseudopodia spread out and became in touch with adjacent cells, linking up into a single stretch (Figure 4i,i′). Cell bodies line up into several

Figure 5. (a) Project area of H9C2 cells on the PLLA film and the NFY scaffold during the cell adhesion test. The proliferation rate of L929 cells (b) and H9C2 cells (c) on the PLLA film, the noobing NFY scaffold, and TCP. * indicates statistical difference for $p < 0.05$; ** indicates statistical difference for $p < 0.01$.

Figure 6. SEM images of H9C2 cells on the PLLA film and the noobing NFY scaffolds after they have been cultured on the PLLA film for 1 day (a), 3 days (b), 7 days (c), and 14 days (d) and on the PLLA noobing NFY scaffold for 1 day (e, e′, e″), 3 days (f, f′, f″), 7 days (g, g′, g″), and 14 days (h, h′, h″) at different magnifications. Scale bar: 50 μm.
The situation reverses as more L929 cells are detected on 3D PLLA after 14 days of culture, H9C2 cells on the 3D NFYs, after 40 and 60 min, cells exhibited a faster project area on the PLLA NFYs, after 120 to 240 min, the cell area of all-direction spreading cells on the PLLA equals that of NFYs, which then surpasses that of NFYs after 240 min of adhesion. However, from 120 to 240 min, the cell area of NFY stays constant, which is far below the one on the PLLA.

To study the cell behavior on both scaffolds, H9C2 cells cultured on both scaffolds after 1, 3, 7, and 14 days were fixed and observed by SEM and CLSM. Figures 6 and 7 illustrate the SEM images and confocal pictures of H9C2, respectively. It can be found that the PLLA film can support the adhesion and spreading of cells over the surface of the scaffold. The boundary of H9C2 always fuses with several nanofibers as well as around a single NFY, until the whole surface of the yarn was covered.

2.5. Cell Morphology and Distribution in Scaffolds.

To assess the proliferation capacity of cells on the multilayered NFY scaffolds, L929 cells and H9C2 cells were cultured on the noobing NFY scaffold, with the PLLA film and TCP as control. As shown in Figure Sb,c, both cells show a steady increase through the culture period, indicating that the scaffolds had good biocompatibility for cells colonization. It can also be noticed that, in the first 7 days, no significant difference in the proliferation rate on the PLLA film, 3D NFY scaffold, and TCP was found. The L929 cells on the PLLA film even exceed than that on the 3D NFY scaffold at day 7 (Figure 4a). However, after another 7 days, the situation reverses as more L929 cells are detected on 3D NFY scaffold than that on the PLLA film. Similar to the growth of L929, after 14 days of culture, H9C2 cells on the 3D NFY scaffold show a higher proliferation rate. It could be concluded that the 3D structure of noobing scaffolds favored the colonization and proliferation of cells in a long term culture period. As for the PLLA nanofiber film, the pores in the surface were too small for cell spreading and penetration into the scaffold. Thus, the cells were inhibited from spreading over the surface, which would be covered completely in a short time. However, in a 3D noobing scaffold comprised of NFYs, grids formed by NFYs would facilitate the cell penetration into the scaffold and provide more available inner surface for cell colonization. Cells could spread and immigrate alongside the texture of aligned nanofibers as well as around a single NFY, until the whole surface of the yarn was covered.
two different layers (Figure 7h), which means that the migration of cells could cross the gap between the two layers, favoring the colonization of cells throughout the whole 3D scaffold.

To virtualize the 3D distribution of cell in the noobing NFY scaffolds, Z-stack confocal images were photographed in the depth direction. As shown in Figure 8, the confocal images were taken from top view at different layers with a step size of 20 μm. The merged images of confocal photos at different depth illustrate the spatial distribution of H9C2 cells in the 3D structure (Figure 8a–d). After 1 day, sporadic cells attached on the NFY scaffold and exhibited random orientation with only a few cells oriented alongside the axis of NFYs, which could be verified in the distribution of cell orientation degree. Two blunt peaks were observed at 140 and 320°, belonging to the same direction. As culture time progressed, cells spread and covered more surface space of the NFY scaffold. In Figure 8b,b1, a blank space with parallel edges can be observed on the yarn. Due to the cells’ preference to spread alongside the texture of nanofibers and then migrate around while parallel space was used up. In Figure 8b1, cells were seen on PLLA NFYs in the top, middle, and the lower layers. Figure 8b3 illustrates that cells could be detected across the depth direction from the surface to 500 μm deep. H9C2 cells on the noobing scaffold spread and elongated with a trend to orientate anisotropic, as four orientation peaks belonging to two directions were observed. After 7 and 14 days of culture, the surface of NFY scaffolds have been occupied by H9C2 cells (Figure 8c,d), including the NYF at the lower layer and the binding yarn (labeled by yellow arrows in Figure 8c,d). More cells spread along two perpendicular directions guided by weft and warp NFYs, respectively (Figure 8c,d). Apart from the crimps caused by the binding yarn (labeled by yellow arrows in Figure 8c,d), all cells exhibited a linear arrangement, generating four concentrating peaks of gray value in 0 (360), 90, 180, and 270° (Figure 8b3,c3,d3).

Moreover, the distribution of cells through the thickness direction could also be identified by the amount of fluorescence (Figure 8a₂,b₂,c₂,d₂). Notably, two peaks can be observed in both Figure 8a₂,b₂,c₂, which represent the concentrated colonization of cells on each layer, forming cell layers at different depths. The two equivalent peaks in Figure 8d₂ illustrate the uniformity of cell distribution between different layers. The indiscrimination of proliferated cells through the thickness may favor the regeneration of 3D tissues and organs. At a more macroscopic level, the vertically intersecting texture revealed the possibility of formation of predesigned and well-organized tissues for specific tissue regeneration.

3. CONCLUSIONS

In a nutshell, we successfully developed well-organized 3D scaffolds that are composed of electropsun PLLA NFYs via a noobing method with structural guidance and a regularly porous structure, which may be applied to mimic the native structure of anisotropic soft tissues. The highly oriented PLLA

Figure 8. Distribution of H9C2 cells in the noobing NFY 3D scaffolds after 1 day (a, a1), 3 days (b, b1), 7 days (c, c1), and 14 days (d, d1) of culture. As the first vertical column exhibits the top view of cells on the scaffold (a, b, c, d), while the second column shows the cells in a depth mode with different colors representing different depths (a₂, b₂, c₂, d₂, labeled below each picture). The image in the third vertical column shows the fluorescence amount through the depth direction (a₃, b₃, c₃, d₃). The fourth column shows the orientation degree distribution of H9C2 cells on the NFY scaffolds after 1 day (a₄), 3 days (b₄), 7 days (c₄), and 14 days (e₄) of culture (strips labeled by I and II refer to the yarns directions). The fifth column illustrates the side view of the H9C2 cells on the NFY scaffolds (a₅, b₅, c₅, d₅).
NFYs possessed higher tensile strength while all continuous nanofibers contributed to the tensile strength and shared the stress. The unidirectional NFYs displayed the capacity to facilitate the adhesion, proliferation, and the spreading of both L929 cells and H9C2 cells. Moreover, the 3D NFY scaffolds through the noobing method owned the topographic cues imparted by aligned NFYs in different layers, which could favor the colonization and migration of H9C2 cells in a 3D structure. Z-stack confocal images also revealed the cell distribution in the depth direction in the 3D scaffold. The extensible 3D NFYs scaffolds fabricated via the combination of noobing and 3D weaving techniques in this study would provide a new route to prepare well-designed 3D nanofibrous scaffolds for the regeneration of different types of tissues.

4. EXPERIMENTAL SECTION

4.1. Materials. PLLA (Mn = 500 kDa) was supplied by Daigang Biomaterials Inc. (Jinan, Shandong, China). Hexafluoroisopropanol (HFIP) was purchased from Shanghai Darui Fine Chemical Co., Ltd. (China). Rat cardiac myoblast cell line (H9C2) and mouse fibroblast cells (L929) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The culture medium and other reagents were purchased from Life Technologies Co., Ltd. (Carlsbad, California). Rat cardiac myoblast cell line (H9C2) and mouse fibroblast cells (L929) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The culture medium and other reagents were purchased from Gibco Life Technologies Co., Ltd. All chemicals of analytical grade were used as purchased without further purification.

4.2. Fabrication of PLLA Nanofiber Yarns (NFYs). PLLA was dissolved in HFIP to obtain a uniform solution at the concentration of 7.5%. The setup for electrospun NFYs is shown in Figure 1, which consists of two oppositely positioned steel needles, a rotating metal funnel, a yarn winding device, and 3D power supplies charging high voltage. During electrospinning, the PLLA solution loaded in the two syringes was pumped out through the steel needles with a diameter of 0.8 mm. The flow rate for both nozzles was set as 1 mL/h, which were charged with positive (+12 kV) and negative (−12 kV) high voltages, separately. Nanofibers ejected from two-needle nozzles were first collected on the rotating funnel, forming a thin film of the nanofiber at the end of the funnel. Initial inducing could generate a cone-shaped nanofiber film along the funnel edge, which could be drawn and wound to form a continuous NFY. As a control, a plain PLLA nanofiber film was collected on a grounded aluminum foil. The voltage, collecting distance, and flow rate were set as +12 kV, 15 cm, and 1.0 mL/h, respectively.

4.3. Characterization. The morphology of PLLA NFYs and the film was sputter-coated with gold primarily and photographed by a digital vacuum scanning electron microscope (SEM, TM 3000, Hitachi, Japan) at the accelerating voltage of 15 kV later. The diameter of nanofibers in the NFYs was determined by the SEM images using the image visualization software ImageJ (National Institutes of Health). Hundred fibers were randomly selected and measured for each sample. The diameter of the yarn was also measured according to the SEM images. Hundred sections were randomly measured for the PLLA NFYs.

When it comes to the stress–strain test, PLLA NFYs were first placed in an incubator with a constant temperature 24 °C and humidity (65%) for 24 h. A universal material tester (HSK-S, Hounsfield, U.K.) with a load cell of 50 N was utilized and the consistent cross-head speed was 10 mm/min. The tensile stress was calculated as the ratio of the record force to the cross section of the yarns, and the strain was recorded as the ratio of the elongated length to the gauge size. After the tensile tests, NFYs were also coated with gold and observed with SEM. The mechanical properties of the noobing fabric were also tested. Briefly, the fabric was cut into strips with a width of 10 mm and a length of 30 mm. The thickness of samples was measured before testing. The testing gauge was set as 10 mm and the stretching rate was 10 mm/min.

4.4. Construction of 3D Scaffold with NFYs. The 3D nanofiber scaffold with anisotropic property and regular pores was fabricated using PLLA NFYs via the “noobing” method, which is a noninterlacing 3D fabric-forming process. Briefly, four threads of PLLA NFY were twisted to form PLLA plied yarn for further process. The plied NFYs were first placed parallelly with a distance of 2 mm to form the layer 1, set as X yarns (green color in Figure 3a). Then, a second layer of PLLA yarns was placed perpendicularly to X yarns with a distance of 2 mm to form the layer 2, set as Y yarns (yellow color in Figure 3b). Subsequently, the layer 3 of the same arrangement as the layer 1 was built upon the layer 2 (green color in Figure 3c). At last, a set of bonding yarns stood vertically to the plane layer at the grids formed by X yarns and Y yarns, which was used to fix the two layers of X yarns in the same direction as Y yarns (red color in Figure 3d). Thus, a three-layer noobing fabric was prepared. Theoretically, adjusting the layers of X and Y yarns could generate a multilayered noobing fabric with different thicknesses.

4.5. In Vitro Experiments. Rat myoblast cells (H9C2 cells) and L929 cells were maintained and expanded in a complete DMEM medium and incubated at a constant atmosphere with 5% CO2 at 37 °C. The culture medium was replaced every 2 days. To allow in vitro cell culturing on the scaffolds, the PLLA NFY scaffolds were cut into a circular column with a diameter of 15 mm and the edges were melt-bonded at the edge to keep the structural integrity, which were then placed in the culture plates and fixed using stainless steel rings.

4.6. Cell Adhesion and Proliferation. To investigate cell adhesion on scaffolds, 8 × 10^4 H9C2 cells per well were seeded on the NFY scaffolds with a PLLA nanofiber membrane as a control. The observation time point was set at 20, 40, 60, 120, and 240 min. At every time point, specimens with cells were rinsed three times with phosphate-buffered solution (PBS) to remove the dissociative cells. Then, cells attached on scaffolds were fixed by 4% paraformaldehyde solution for 30 min at 4 °C. After that, cells were treated with 0.1% Triton X-100 for 10 min. After repeating the rinsing process for another three times, the cytoskeletons and nuclei of cells were stained with 200 µL of rhodamine-conjugated phallolidin (25 µg/mL) and 200 µL of 4′,6′-diamidino-2-phenylindole hydrochloride (10 µg/mL) for 30 and 5 min, respectively. After a thorough washing aimed at removing the residual stain, cells were visualized using confocal laser scanning microscopy (CLSM, Carl Zeiss, LSM 700, Germany).

As for the cell proliferation study, 2 × 10^4 H9C2 cells were seeded on the NFY scaffolds with a PLLA nanofiber membrane and tissue culture plates (TCPs) as a control. The amount of the cells was assessed by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In short, after culturing for 1, 3, 7, and 14 days, the culture medium was removed and samples were washed with PBS for three times. MTT assay was used to determine the amount of viable cells.
on the scaffolds. In brief, the specimens were incubated with 360 mL of the fetal bovine serum-free DMEM medium coupled with 40 mL of an MTT solution (5 mg/mL). After 4 h, the culture media were removed and 400 mL of dimethyl sulfoxide (DMSO) was added, and then incubated in a constant temperature shaker at 37 °C for 30 min to dissolve the crystal. After that, 100 mL of the solution was transferred into a 96-well plate and tested using a microtiter plate reader (Multiskan MK3, Thermo, USA) at the absorbance of 492 nm. For each sample, six individual wells were tested to gain an average value. Mouse fibroblasts (L929) were also utilized for cell proliferation study. Briefly, $2 \times 10^4$ L929 cells were cultured on the PLLA NFY scaffolds with a PLLA nanofiber membrane and TCPs as a control. MTT assay was conducted after 1, 3, 7, and 14 days of culture for cell proliferation assessment.

4.7. Cell Morphology Observation. Meanwhile, after 1, 3, 7, and 14 days of culture, cells on the scaffolds were fixed and dried according to the method reported previously. In short, cells fixed by 4% paraformaldehyde were dehydrated with gradient ethanol-aqueous solutions (concentrations of ethanol solutions: 30, 50, 70, 80, 90, 95, 100%), followed by drying with air flow at room temperature for 12 h. Then, the dried samples with cells were sputter-coated with gold and photographed by SEM.

To visualize the cells’ distribution and morphology on the scaffolds, CLSM was also applied to observe the nuclei and f-actin. At given intervals of the culture time (1, 3, 7, and 14 days), the collected specimens were processed according to the method in 2.6, followed by observation using CLSM. Z-stack mode was also utilized so as to scan the cells at different depths. To assess the orientation of H9C2 cells on the noobing NFYS scaffolds, confocal images were first converted into monochrome mode and then reversed by Adobe Photoshop CC 2018. The imported images were processed into fast Fourier-transform (FFT) spectrum using ImageJ, based on which the gray value was calculated by the plugin of the oval profile in the mode of radial sums. The gray value could represent the orientation of edge and pseudopodium of cells in confocal images.

4.8. Statistical Analysis. Statistical analysis was performed with one-way analysis of variance using Origin 8.0 (OriginLab Inc.). The statistical difference between two sets of data was considered when *$p < 0.05$ and **$p < 0.01$. All of the data were obtained at least in triplicate and all values were presented as the mean and standard deviation (SD).

## Author Information

### Corresponding Authors

Xiumei Mo – State Key Lab for Modification of Chemical Fibers & Polymer Materials, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, China; orcid.org/0000-0001-9238-6171; Email: xmm@dhu.edu.cn

Bingyao Deng – Key Laboratory of Eco-Textiles, Ministry of Education, Jiangnan University, Wuxi, Jiangsu 214122, China; orcid.org/0000-0001-5652-3503; Email: bydeng@jiangnan.edu.cn

### Authors

Dawei Li – Key Laboratory of Eco-Textiles, Ministry of Education, Jiangnan University, Wuxi, Jiangsu 214122, China; State Key Lab for Modification of Chemical Fibers & Polymer Materials, College of Chemistry, Chemical Engineering and Biotechnology and Engineering Research Center of Technical Textiles, Ministry of Education, College of Textiles, Donghua University, Shanghai 201620, China

Ling Tao – State Key Lab for Modification of Chemical Fibers & Polymer Materials, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, China

Ying Shen – Key Laboratory of Eco-Textiles, Ministry of Education, Jiangnan University, Wuxi, Jiangsu 214122, China

Binbin Sun – State Key Lab for Modification of Chemical Fibers & Polymer Materials, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, China

Xianrui Xie – State Key Lab for Modification of Chemical Fibers & Polymer Materials, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, China

Qinfei Ke – Engineering Research Center of Technical Textiles, Ministry of Education, College of Textiles, Donghua University, Shanghai 201620, China; Shanghai Institute of Technology, Shanghai 201416, China

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c02554

### Author Contributions

This paper was written through contributions of all authors. All authors have given approval to the final version of the paper.

### Notes

The authors declare no competing financial interest.

### Acknowledgments

This research was supported by the Natural Science Foundation of China (No. 51908246 and 31470941) and the Fundamental Research Funds for the Central Universities (No. JUSRP52007A). This work was also supported by the National Key Research Program of China (No. SQ2019YFC190007, No. 2019YFC1904502).

### References

1. Li, M.; Mondrinos, M. J.; Gandhi, M. R.; Ko, F. K.; Weiss, A. S.; Lelkes, P. I. Electrospun protein fibers as matrices for tissue engineering. *Biomaterials* 2005, 26, 5999–6008.

2. Boland, E. D.; Matthews, J. A.; Pawlowski, K. J.; Simpson, D. G.; Wnek, G. E.; Bowlin, G. L. Electrospinning collagen and elastin: preliminary vascular tissue engineering. *Front. Biosci.* 2004, 9, 1422–1432.

3. Lannutti, J.; Reneker, D.; Ma, T.; Tomasko, D.; Farson, D. Electrospinning for tissue engineering scaffolds. *Mater. Sci. Eng., C* 2007, 27, 504–509.

4. Smith, L. A.; Liu, X.; Ma, P. X. Tissue Engineering with Nano-Fibrous Scaffolds. *Soft Matter* 2008, 4, 2144–2149.

5. Jang, J. H.; Castano, O.; Kim, H. W. Electrospun materials as potential platforms for bone tissue engineering. *Adv. Drug Delivery Rev.* 2009, 61, 1065–1083.

6. Ghasemi-Mobarakeh, L.; Prabhakaran, M. P.; Morshed, M.; Nasr-Esfahani, M. H.; Ramakrishna, S. Electrospun poly(epsilon-caprolactone)/gelatin nanofibrous scaffolds for nerve tissue engineering. *Biomaterials* 2008, 29, 4532–4539.

7. Hong, J. K.; Madhally, S. V. Three-dimensional scaffold of electrospayed fibers with large pore size for tissue regeneration. *Acta Biomater.* 2010, 6, 4734–4742.

8. Soliman, S.; Pagliari, S.; Rinaldi, A.; Forte, G.; Fiaccavento, R.; Pagliari, F.; Franzese, O.; Minieri, M.; Di Nardo, P.; Licoccia, S.; Pagliari, S.; 24340 https://dx.doi.org/10.1021/acsomega.0c02554

ACS Omega 2020, 5, 24340−24350
Traversa, E. Multiscale three-dimensional scaffolds for soft tissue engineering via multimodal electrospinning. Acta Biomater. 2010, 6, 1227−1237.

(9) Wu, S.; Wang, Y.; Streuleb, P. N.; Duan, B. Living nanofiber yarn-based woven biotextiles for tendon tissue engineering using cell tri-culture and mechanical stimulation. Acta Biomater. 2017, 62, 102−115.

(10) Wu, S.; Duan, B.; Liu, P.; Zhang, C.; Qin, X.; Butcher, J. T. Fabrication of Aligned Nanofiber Polymer Yarn Networks for Anisotropic Soft Tissue Scaffolds. ACS Appl. Mater. Interfaces 2016, 8, 16950−16960.

(11) Wu, T.; Zhang, J.; Wang, Y.; Li, D.; Sun, B.; El-Hamshary, H.; Yin, M.; Mo, X. Fabrication and preliminary study of a biomimetic tri-layer tubular graft based on fibers and fiber yarns for vascular tissue engineering. Mater. Sci. Eng. C 2018, 82, 121−129.

(12) Ko, F.; Gogotis, Y.; Al, A.; Naguib, N.; Ye, H.; Yang, G. L.; Li, C.; Willis, P. Electrospinning of Continuous Carbon Nanotube-Filled Nanofiber Yarns. Adv. Mater. 2003, 15, 1161−1165.

(13) Dalton, P. D.; Klee, D.; Möller, M. Electrospinning with dual collection rings. Polymer 2005, 46, 611−614.

(14) Yan, H.; Liu, L.; Zhang, Z. Continuously fabricating staple yarns with aligned electrospun polycaprolactone nanofibers. Mater. Lett. 2011, 65, 2419−2421.

(15) Zhou, Y.; Fang, J.; Wang, X.; Lin, T. Strip twisted electrospun nanofiber yarns: Structural effects on tensile properties. J. Mater. Res. 2011, 27, 537−544.

(16) Smit, E.; Böttner, U.; Sanderson, R. D. Continuous yarns from electrospun fibers. Polymer 2005, 46, 2419−2423.

(17) Teo, W.-E.; Gopal, R.; Ramaseshan, R.; Fujihara, K.; Ramakrishna, S. A dynamic liquid support system for continuous electrospun yarn fabrication. Polymer 2007, 48, 3400−3405.

(18) Yousefzadeh, M.; Latifi, M.; Teo, W.-E.; Amani-Tehran, M.; Ramakrishna, S. Producing continuous twisted yarn from well-aligned nanofibers by water vortex. Polym. Eng. Sci. 2011, 51, 323−329.

(19) Wu, J.; Liu, S.; He, L.; Wang, H.; He, C.; Fan, C.; Mo, X. Electrospun nanoyarn scaffold and its application in tissue engineering. Mater. Lett. 2012, 67, 146−149.

(20) Matsumoto, H.; Imaizumi, S.; Konosu, Y.; Ashizawa, M.; Minagawa, M.; Tanioka, A.; Lu, W.; Tour, J. M. Electrospun composite nanofiber yarns containing oriented graphene nanoribbons. ACS Appl. Mater. Interfaces 2013, 5, 6225−6231.

(21) Li, N.; Hui, Q.; Xue, H.; Xiong, J. Electrospun Polycaprolactone nanofiber yarn prepared by funnel-shape collector. Mater. Lett. 2012, 67, 245−247.

(22) Zhou, F.-L.; Gong, R.-H.; Porat, I. Nano-coated hybrid yarns using electrospinning. Surf. Coat. Technol. 2010, 204, 3459−3463.

(23) Bazbouz, M. B.; Stylios, G. K. A new mechanism for the electrospinning of nanoyarns. J. Appl. Polym. Sci. 2012, 124, 195−201.

(24) Hosseini Ravandi, S. A.; Hassanabadi, E.; Tavanai, H.; Abuzade, R. A. Mechanical properties and morphology of hot drawn polycaprolactone nanofibrinous yarn. J. Appl. Polym. Sci. 2011, 5002−5009.

(25) Maleki, H.; Gharehaghi, A. A.; Moroni, L.; Dijkstra, P. J. Influence of the solvent type on the morphology and mechanical properties of electrospun PLLA yarns. Biofabrication 2013, 5, No. 035014.

(26) Afifi, A. M.; Nakano, S.; Yaman, H.; KIimura, Y. Electrospinning of Continuous Aligning Yarns with a ‘Funnel’ Target. Macromol. Mater. Eng. 2010, 295, 660−665.

(27) Wu, S.-H.; Qin, X.-H. Uniaxially aligned polycaprolactone nanofiber yarns prepared by a novel modified electrospinning method. Mater. Lett. 2013, 106, 204−207.

(28) He, J.; Qi, K.; Wang, L.; Zhou, Y.; Liu, R.; Cui, S. Combined application of multinozzle air-jet electrospinning and airflow twisting for the efficient preparation of continuous twisted nanofiber yarn. Fibers Polym. 2015, 16, 1319−1326.

(29) Ali, U.; Zhou, Y.; Wang, X.; Lin, T. Direct electrospinning of highly twisted, continuous nanofiber yarns. J. Text. Inst. 2012, 103, 80−88.