Published in final edited form as:

Acta Biomater. 2018 March 01; 68: 237–248. doi:10.1016/j.actbio.2017.12.018.

CO₂-Expanded Nanofiber Scaffolds Maintain Activity of Encapsulated Bioactive Materials and Promote Cellular Infiltration and Positive Host Response

Jiang Jiang^{a,#}, Shixuan Chen^{a,#}, Hongjun Wang^a, Mark A. Carlson^{b,c}, Adrian F. Gombart^{d,e}, and Jingwei Xie^{a,*}

^aDepartment of Surgery-Transplant and Mary & Dick Holland Regenerative Medicine Program, University of Nebraska Medical Center, Omaha, Nebraska 68198, United States

^bDepartments of Surgery and Genetics, Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, Nebraska, 68198, United States

^cDepartment of Surgery, VA Nebraska–Western Iowa Health Care System, Omaha, Nebraska, 68105, United States

^dLinus Pauling Institute, Oregon State University, Corvallis, OR 97331, United States

^eDepartment of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, United States

Abstract

Traditional electrospun nanofiber membranes were incapable of promoting cellular infiltration due to its intrinsic property (e.g., dense structure and small pore size) limiting their use in tissue regeneration. Herein, we report a simple and novel approach for expanding traditional nanofiber membranes from two-dimensional to three-dimensional (3D) with controlled thickness and porosity *via* depressurization of subcritical CO₂ fluid. The expanded 3D nanofiber scaffolds formed layered structures and simultaneously maintained the aligned nanotopographic cues. The 3D scaffolds also retained the fluorescent intensity of encapsulated coumarin 6 and the antibacterial activity of encapsulated antimicrobial peptide LL-37. In addition, the expanded 3D nanofiber scaffolds with arrayed holes can significantly promote cellular infiltration and neotissue formation after subcutaneous implantation compared to traditional nanofiber membranes. Such scaffolds also significantly increased the blood vessel formation and the ratio of M2/M1 macrophages after subcutaneous implantation for 2 and 4 weeks compared to traditional nanofiber membranes. Together, the presented method holds great potential in the fabrication of functional 3D nanofiber scaffolds for various applications including engineering 3D *in vitro* tissue models, antimicrobial wound dressing, and repairing/regenerating tissues *in vivo*.

*Corresponding author: jingwei.xie@unmc.edu (J. Xie).

#These two authors contributed equally to this work.

Authors declare that there is no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Graphical Abstract

Keywords

electrospun nanofiber membranes; expansion; subcritical CO₂; three dimensional; drug delivery; regenerative medicine

1. Introduction

Potential applications for electrospun nanofibers include energy storage, healthcare, biotechnology, environmental cleaning, defense and security [1–3]. Due to their ability to mimic the architecture of the extracellular matrix (ECM) and the size of collagen fibrils in ECM, electrospun nanofibers have been widely used as scaffolding materials for tissue repair and regeneration [3–7]. One limiting factor of conventional electrospinning is that the produced nanofiber mats are composed entirely of densely packed nanofibers only providing a superficial porous structure during the process of the sheet-like assembly [8–10]. Cells incubated with such a nanofiber mat normally results in the formation of a cell monolayer on its surface rather than a three-dimensional (3D) cellular construct throughout the mat [11]. The poor cellular penetration has been attributed to the reduced porosity of nanofiber mats, and the sizes of inter-fiber pores often smaller than the dimensions of individual cells [8]. In addition, the reduced porosity could limit oxygen and nutrient transportation, further hindering cellular infiltration [12]. Therefore, the unfavorable characteristic of conventional electrospun nanofiber mats mainly due to the intrinsic property of the electrospinning technique limits cellular infiltration and growth throughout the nanofiber mats.

A number of methods outlined as follows have been attempted to overcome this major obstacle that inhibits the use of nanofiber mats in regenerative medicine. To increase the pore size of electrospun nanofiber scaffolds, a simple and straightforward way is to modulate fiber diameters [13–16]. Previous studies showed that the diameters of fibers larger than 4 μm could lead to the pore size larger than 20 μm [16]. The issue for this method is that the fibers with a size in micron scale lack the biomimetic property and the interactions between microfibers and cells could be different from the interactions between nanofibers

and cells. Manipulation of electric field with a modified collector during electrospinning was also used to generate 3D cotton-like fluffy nanofiber scaffolds [17]. This approach was limited to the generation of scaffolds made of random nanofibers lacking of nanotopographic cues and the difficulty in the control of porosity. Alternatively, ionic salts that were added to the electrospinning solution could manipulate the electrostatic repulsion between substrates and deposited nanofibers to fabricate sponge like nanofiber matrix [18]. This approach only produced limited thickness of nanofiber matrix and necessitated the use of additives (e.g., ionic salts) that may cause side effects or safety issues during tissue regeneration. Another strategy to increase the porosity is selective removal of sacrificial fibers [19, 20]. This method only generated a limited increase of porosity. Based on the similar principle, ice crystals were used as sacrificial templates to fabricate 3D electrospun nanofiber scaffolds with large interconnected pores [21]. Similarly, salt particles incorporated into electrospun nanofiber scaffolds during the electrospinning process resulted in the formation of macropores $\approx 100 \mu\text{m}$ after leaching [22]. This strategy necessitates the removal of sacrificial templates involving multiple steps. As mentioned above, these approaches were associated with various issues including difficulty to control the thickness, limited to certain materials, restriction to randomly oriented nanofibers, the necessity of additives, time-consuming processing, need for aqueous solutions, insufficient expansion ratios, and/or multiple steps.

Our recent work and others demonstrated the expansion of electrospun nanofiber mats in the third dimension with ordered structures using gas bubbles generated by chemical reactions in an aqueous solution [23–25]. Compared to previous approaches, this methodology overcame some of the above shortcomings and showed some improvement in the generation of 3D electrospun nanofiber scaffolds. Although we demonstrated expansion of nanofiber mats with cellular infiltration and proliferation occurring throughout the whole scaffolds [23, 24], some issues still remained with the expansion procedure: *i*) it was a multi-step, time consuming process, involving gas production process in an aqueous solution followed by freeze-drying; *ii*) there was a risk that NaBH_4 could react with polymers or encapsulated substances; *iii*) there could be a loss of bioactive materials encapsulated in fibers; *iv*) there could be a loss of bioactivities for materials incorporated in the fibers; and *v*) the method was limited to water-insoluble materials.

Subcritical CO_2 fluid has been used in oil and fragrance extraction and in the processing of polymeric materials, because it is nontoxic, non-flammable, inexpensive, and environmentally friendly [26–31]. Herein, we report for the first time a simple and novel method for processing traditional electrospun poly(ϵ -caprolactone) (PCL) nanofiber mats from 2D to 3D by immersing fiber mats in subcritical CO_2 fluid followed by depressurization. It was expected that CO_2 expanded 3D nanofiber scaffolds could have a similar structure as the one generated using a gas production chemical reaction in an aqueous solution in our previous studies [23, 24]. It was also expected that CO_2 expanded 3D nanofiber scaffolds could better maintain the activity of encapsulated bioactive materials compared to previous approaches due to the low-temperature process. In addition, CO_2 expanded 3D nanofiber scaffolds with arrayed holes could promote cellular infiltration, neovascularization and positive host response compared to traditional 2D nanofiber membranes.

2. Materials and methods

2.1. Fabrication of 2D nanofiber membranes

PCL nanofiber mats were produced utilizing a standard electrospinning setup as described in our previous studies [23, 24]. Briefly, PCL (Mw= 80 kDa) was dissolved in a solvent mixture consisting of dichloromethane (DCM) and N, N-dimethylformamide (DMF) with a ratio of 4:1 (v/v) at a concentration of 10% (w/v). PCL solution was pumped at a flow rate of 0.8 mL/h using a syringe pump. Coumarin 6-loaded PCL fibers were fabricated with 50 µg/mL coumarin 6 in the PCL solution. LL 37 was loaded into PCL fibers by co-axial electrospinning following our previous studies [32]. The core solution was composed of 100 mg/mL pluronic F-127 and 5 mg/mL LL 37 in water. The flow rate was set at 0.08 mL/h. The sheath solution was the same PCL solution as described above. An electrical potential of 15 kV was applied between the spinneret (a 22-gauge needle) and a grounded collector located 20 cm apart from the spinneret. Aligned nanofiber mats were collected on a drum with rotating speeds of 2000 rpm. The fabricated PCL nanofiber mats and coumarin 6-loaded PCL nanofiber mats were about 1 mm thick. LL 37-loaded PCL nanofiber mats were about 100 µm thick. The raw PCL fiber mats were punched by a 1 mm-diameter punch in liquid nitrogen to generate arrayed holes.

2.2. Fabrication of 3D electrospun nanofiber scaffolds

PCL nanofiber mats, coumarin 6-loaded PCL nanofiber mats, LL 37-loaded PCL nanofiber mats, and PCL nanofiber mats with arrayed holes were first cut into 1 cm × 1 cm squares in liquid nitrogen to avoid deformation on the edges. Next, ~ 1 g of dry ice and one piece of nanofiber mat were put into a 30 mL Oak Ridge centrifuge tube. After the dry ice changed into CO₂ fluid, we loosened the cap rapidly and removed the puffed nanofiber scaffold from the tube. This expanding procedure was repeated until the desired thickness was reached. The nanofiber scaffolds were sterilized by ethylene oxide before incubation with bacteria.

2.3. Characterization of 3D nanofiber scaffolds

Based on the volume change of nanofiber scaffolds, we estimated the porosity using the following equation: $\varepsilon = \frac{V - V_0}{V} \times 100\%$ where ε is porosity, $V = L$ (length) $\times$ W (width) $\times$ T (thickness) is the volume of PCL nanofiber scaffold, $V_0 = \frac{m_0}{\rho_0}$ is the calculated volume of the bulk PCL material, m_0 is the mass of the bulk PCL material, and ρ_0 is the density of the bulk PCL materials [23]. PCL nanofiber mats before and after expansion were embedded in deionized water and frozen at -20 °C. Cross sections of nanofiber scaffolds were obtained by a cryostat and then freeze-dried. Scanning electron microscopy (SEM) (FEI, Quanta 200, Oregon, USA) was used to characterize the morphologies of cross sections of nanofiber scaffolds. To avoid charging, nanofiber samples were fixed on a metallic stud with double-sided conductive tape and coated with platinum for 4 min in a vacuum at a current intensity of 10 mA using a sputter coater. SEM images were acquired at an accelerating voltage of 30 kV. The gap distances and layer thicknesses of nanofiber scaffolds after expanding once and twice were measured based on SEM images using the Image J software. At least 250 gaps or layers were analyzed.

2.4. Coumarin 6-loaded 3D nanofiber scaffolds

For comparison, coumarin 6-loaded PCL nanofiber mats were expanded in 1 M NaBH₄ for 1 h. The detailed procedure was described in our previous publications [23, 24]. The top surface of CO₂ expanded coumarin 6-loaded nanofiber scaffolds, NaBH₄ expanded coumarin 6-loaded nanofiber scaffolds, raw coumarin 6-loaded nanofiber mats, and raw PCL fiber mats were observed by a fluorescence microscope with an excitation wavelength at 488 nm and emission wavelength at 530 ± 20 nm and the images were taken by a CCD camera with the same exposure time. The experiments were carried out at least three times. The fluorescent intensity was quantified using Image J software.

2.5. LL 37-loaded 3D nanofiber scaffolds

In vitro release kinetics of LL 37 from nanofiber membranes before and after expansion was evaluated by immersing 5 mg fiber samples in 5 mL PBS at 37 °C. The supernatants were collected at each time point and replaced with fresh PBS solutions. The LL 37 concentrations for all collected samples were determined by an LL 37 ELISA kit according to the manufacturer's instructions.

P. aeruginosa was used to evaluate the anti-bacteria activity of LL 37-loaded fiber membranes before and after expansion. Briefly, *P. aeruginosa* was cultured in liquid Luria-Bertani (LB) medium overnight in a shaking incubator at 37 °C at 220 rpm overnight. Then, 20 µL suspended bacteria were transferred to 4 mL fresh LB medium and incubated for an additional 2 h at 37 °C at 220 rpm. The bacterial suspension was centrifuged at 12000 rpm for 10 min. The cell pellet was re-suspended in 1 mL PBS after removal of supernatant. This procedure was repeated once. The OD₆₆₀ value of bacterial suspension was determined using a NanoDrop (Thermo Scientific, Wilmington, DE). The value of OD₆₆₀ is approximately equal to 1.0×10⁸ CFU bacteria. We diluted the cells into 1.0×10⁵, 1.0×10⁶ and 1.0×10⁷ CFU bacteria with PBS and added 5 mg PCL fibers, unexpanded LL 37-loaded PCL fibers and expanded LL 37-loaded PCL fibers to 5 mL medium containing bacteria, and then placed the culture on a shaker at 37 °C at 220 rpm for 1 h. Then, the culture was then spread on a LB agar plate. After incubation for 12 h at 37 °C, the number of colonies was counted. The counts were repeated with three LB agar plates and averaged.

2.6. Fabrication and Subcutaneous implantation of 3D nanofiber scaffolds with arrayed holes

Electrospun nanofiber membranes were first frozen at temperatures lower than glass transition temperatures (e.g., immersed in liquid nitrogen) to make them brittle and the holes in a square array through nanofiber membranes were created by a 1 mm punch. The punched samples were treated with subcritical CO₂ two times followed by ethylene oxide gas sterilization prior to implantation. This animal study has been approved by IACUC at University of Nebraska Medical Center (UNMC). Briefly, 9-week old Sprague Dawley (SD) rats (250–300 g) were anesthetized using 4% isoflurane in oxygen for approximately 2 min. Rats were placed on a heating pad to maintain their body temperature. An area of 8 × 4 cm² on the back of each animal was shaved, and povidone-iodine solution was applied three times on the exposed skin. Each rat received 4 implants. A treatment group had three rats. There were totally 12 implants for each group. Subcutaneous pockets were made through 1.5

cm incisions at 4 supraspinal sites on the dorsum. Each sample was compressed to ~1.5 mm and inserted into a subcutaneous pocket by a tweezer, and then the skin incisions were closed with a stapler. Rats were euthanized by CO₂ at 1, 2, and 4 weeks post-implantation. Each explant with surrounding tissue was gently dissected out of its subcutaneous pocket, and then immersed in formalin for at least 3 days prior to histology analysis. The data presented was the mean with standard deviations.

2.7. Histological and immunohistochemical analysis

Fixed samples were dehydrated in a graded ethanol series (70%–100%), embedded in paraffin, and then sectioned (4 µm). Samples were performed with either hematoxyline and eosin (H & E) or masson's trichrome staining. Immunohistochemical staining was performed to characterize macrophage phenotypes responding to expanded nanofiber scaffolds with arrayed holes. Slides were deparaffinized followed by antigen retrieval in heated citrate buffer for 10 min (10 mM citrate, pH 6.0 at 95–100 °C). Peroxidase was blocked by incubating sections in 3% H₂O₂ for 5 min. Non-specific antibody binding was prevented with blocking solution (2% normal goat serum, 0.1% triton X-100 in PBS; 1 h at room temperature). Sections were decanted and incubated with primary antibodies diluted 1:200 in blocking solution, overnight at 4 °C. Primary antibodies against the pan-macrophage marker CD68, M1 macrophage marker CCR 7 and M2 macrophage marker CD206 were used.

2.8. Histomorphometric and macrophage phenotype quantification

Microscopic images of H & E staining, Masson's trichrome staining, and immunohistochemical staining of CD68, CCR7, and CD206 were all obtained with a Ventana's Coreo Au slide scanner, and edited with Ventana image viewer v. 3.1.3. The magnifications were set at 4×, 10×, and 40×, and then snapshots were taken at three random locations on each sample. The number of blood vessels was measured using Ventana image viewer. The number of blood vessels was converted into counts per mm². All the foreign body giant cells in each specimen were quantified by masson's trichrome staining images. *In vivo* experimental data was obtained from three independent experiments. Images were captured with a Ventana's Coreo Au slide scanner. Three sections were evaluated for each implant. A total of 6 snapshots of CD68, CCR7 and CD163 positive immunohistochemical staining images at 40× magnification were randomly collected on each tissue section. The number of positive cells in each snapshot was quantified.

2.9. Statistical analysis

Each data point represents the mean of three replicates. The statistical analysis was performed on the means of the data obtained from at least three independent experiments. All the results were given as means, and were compared using an analysis of variance (ANOVA) followed by LSD post hoc assessment for evaluating statistical intra- and inter-individual differences, with significance set at $p < 0.05$.

3. Results

3.1. Fabrication and characterization of 3D electrospun nanofiber scaffolds

For the fabrication methodology, we first generated electrospun PCL nanofiber mats and cut the mats into desired sizes (e.g., 1 cm × 1 cm) as described in our previous studies [23, 24]. Then, we put the nanofiber membranes in a centrifuge tube in the presence of dry ice at room temperature and tightened the cap. After the dry ice changed into liquid, the CO₂ fluid was rapidly depressurized, resulting in the formation of 3D nanofiber scaffolds (Fig. 1a, Video S1). Based on the calculation (see Supporting Information and Fig. S1), the CO₂ liquid is in a subcritical state. We can tailor the thickness of 3D nanofiber scaffolds by increasing the number of CO₂ fluid processing times (Fig. 1b). The thickness of nanofiber mats increased from 1 mm to 2.5 mm and further to 19.2 mm after the first and second treatment with subcritical CO₂ fluid. The stress-strain curves along different planes were shown in Fig. S2, indicating that the compressive modulus was in the order of Y-Z plane > X-Z plane > X-Y plane.

The porosity of aligned PCL nanofiber scaffolds increased with increasing the number of processing times, which corresponded with the increased thickness (Fig. 1c). The porosity of the nanofiber scaffolds increased from 78.5% for the raw nanofiber mats to 92.1% and 99.0% after the first and second treatment with subcritical CO₂ fluid (Fig. 1d). The sizes showed a similar distribution for the nanofibers before and after CO₂ expansion (Fig. S3). To maintain the integrity of the nanofiber scaffolds following expansion, the scaffolds were embedded in ice from frozen water and sectioned by a microtome to expose the x-y, y-z plans and then freeze-dried using a lyophilizer. To reveal the detailed structure, the sectioned samples were examined by scanning electron microscopy (SEM). Prior to expansion, aligned electrospun PCL nanofiber mats were composed of densely packed fibrillar structures (Fig. 1, e and f), which agreed with our previous studies [23]. In contrast, nanofiber scaffolds expanded by subcritical CO₂ fluid displayed layered structures with preserved nanotopographic cues rendered by aligned nanofibers (Fig. 1, g and h), which was critical for regeneration of tendon, muscle, and nerve tissues and akin to the nanofiber scaffolds expanded in NaBH₄ solutions [23, 24]. It was confirmed that 3D nanofiber scaffolds expanded by subcritical CO₂ fluid had a similar structure as the ones generated using a gas production chemical reaction in an aqueous solution in previous studies [23, 24]. The distributions of gap distances of one and two treatments were almost the same. Most of gap distances were around 30 μm for both treatments. However, the distributions of layer thickness were different. There were many layers over 90 μm after one treatment. But 90 μm or bigger layers are rarely seen after second treatment, which meant second treatment could help expanding some un-expanded layers after first treatment. Intriguingly, we further demonstrated the expansion of nanofiber membranes made of water-soluble polymers (e.g., polyvinylpyrrolidone (PVP)) (Fig. 2), which could not be achieved using previous methods [23–25, 33, 34].

3.2. The effect of subcritical CO₂ processing on the encapsulated molecules

3D scaffolds provide not only a substrate for cell attachment and growth but also a local device for delivering therapeutic agents for regulating cellular responses or host immune

response after implantation. To demonstrate the advantages of the current manufacturing approach, we encapsulated both hydrophobic and hydrophilic molecules in the nanofiber scaffolds. We chose coumarin 6, a small hydrophobic fluorescent dye molecule, as a model drug in that a number of small molecules have been examined for use in tissue regeneration [35, 36]. We encapsulated coumarin 6 into PCL nanofiber mats as described in our previous studies [37]. We used a 1 M NaBH₄ solution or subcritical CO₂ fluid to expand the nanofiber mat from 2D to 3D. The green color of coumarin 6 faded after 1 M NaBH₄ solution treatment, which was due to the high reducibility of NaBH₄ (Fig. 3). In contrast, scaffolds expanded by subcritical CO₂ fluid were still in green color (Fig. 3). The top views of each sample were also imaged by fluorescent microscopy (Fig. 3). Pristine PCL fiber samples showed no fluorescence at 488 nm (Fig. 3, bottom right); however, coumarin 6-loaded PCL fiber mats showed the strongest fluorescence (Fig. 3, top right). The fluorescent intensity of NaBH₄ solution expanded samples was much lower than subcritical CO₂ fluid expanded ones. Quantitative analysis of fluorescent intensity using Image J software showed that PCL nanofibers exhibited a very low fluorescent intensity (Fig. 3). The fluorescent intensity of subcritical CO₂ fluid expanded samples was significantly higher than that of NaBH₄ solution expanded samples. The slightly disparity in the fluorescent intensity between coumarin 6-loaded PCL nanofiber mats and subcritical CO₂ fluid expanded nanofiber mats could be due to the structural differences (e.g., different fiber densities).

As for another model drug, we selected an antimicrobial peptide called LL-37. It is a hydrophilic molecule that has been used to treat infections, promote wound healing, enhance angiogenesis, and modulate the immune response [38–40]. LL37 and pluronic F127 (a surfactant) were encapsulated in the core of LL37/pluronic F127-PCL core-sheath fibers using co-axial electrospinning [41]. The resulted LL37-loaded nanofiber mats were expanded using the subcritical CO₂ fluid. The initial drug loading was 5 µg LL 37 per 1 mg PCL nanofibers. *In vitro* release kinetics of LL 37 from both nanofiber mats before and after expansion in subcritical CO₂ fluid were determined using a LL 37 ELISA kit (Fig. 4a). About 75% and 80% LL 37 were released from raw and expanded fiber samples in the first week, respectively. The release rate of expanded fiber scaffolds was slightly higher than unexpanded samples, which may be due to the higher porosity.

To test the retention of bioactivity of encapsulated LL-37 after expansion, we measured the antibacterial performance of LL-37-loaded nanofiber mats before and after expansion (Fig. 4b). We co-incubated 1 mg unexpanded LL37-loaded PCL fiber mats and expanded 3D LL37-loaded PCL fiber scaffolds with 1.0×10^5 , 1.0×10^6 , and 1.0×10^7 CFU *P. aeruginosa* bacteria in 1 ml PBS for 1 h using pristine PCL fiber mats as a control. As expected, pristine PCL fiber mats showed no bacterial killing effect. Both expanded and unexpanded LL37-loaded PCL fiber membranes showed a similar level of bacterial killing effect (Fig. 4b), which indicates that the subcritical CO₂ processing had no influence on the bioactivity of encapsulated antimicrobial peptides. Therefore, it is confirmed that 3D nanofiber scaffolds expanded by subcritical CO₂ fluid better maintained the activity of encapsulated bioactive materials compared to previous approaches [23, 24].

3.3. Fabrication of expanded 3D nanofiber scaffolds with arrayed holes

Transformation of electrospun nanofiber membranes from 2D to 3D increases the thickness and porosity of nanofiber scaffolds. The previous study demonstrated such expanded scaffolds can significantly enhance cellular infiltration from sides after subcutaneous implantation in rats [24]. But cells mainly penetrated scaffolds from sides, in particular along the direction of fiber alignment, instead of from the top and bottom surfaces. Although trans-layer vascularization is not a necessity for tissue regeneration [42], cellular infiltration across different layers could benefit neotissue formation and its integration into surrounding tissues. To overcome this limitation, PCL nanofiber membranes were immersed in liquid nitrogen to make them become brittle and arrayed holes were generated through membranes using a micro-punch under cryogenic conditions [43]. This method showed no damage or deformation to the nanofiber morphology on the surface of punched holes unlike the holes created by laser sintering or punching at room temperature reported in previous studies (Fig. S4 and Fig. S5) [44, 45]. The punched nanofiber membranes were expanded to 3D scaffolds using the subcritical CO₂ fluid. The layered structure was preserved and the surface of holes indicated the fiber morphology remained intact (Fig. S5, c and d). This approach further increased the porosity of expanded 3D nanofiber scaffolds.

3.4. In vivo response of expanded 3D nanofiber scaffolds with arrayed holes

To further test the effect of expansion and punched holes on *in vivo* response, we implanted 3D expanded nanofiber scaffolds with arrayed holes subcutaneously in rats for 1 week, 2 weeks and 4 weeks, respectively. It seems that cells grew into the punched holes and then penetrated into the space between thin nanofiber layers within expanded nanofiber scaffolds (Fig. 5). Masson's trichrome staining showed the collagen deposition indicated by green arrows from infiltrated cells in the punched holes and in the gaps between thin nanofiber layers (Fig. 5b). Many blood vessels were also formed within the newly formed tissues in the holes or gaps between thin nanofiber layers (Fig. 5c). Multinucleated giant cells were also present (Fig. 5d). Numbers of blood vessels per mm² were around 39, 66, and 17 at week 1, 2 and 4, respectively (Fig. 5e). More blood vessels formed at week 2 could be attributed to the early inflammatory response. In contrast, no newly formed blood vessels were observed within traditional nanofiber mats (Fig. S6) [24]. Numbers of multinucleated giant cells per implant for expanded 3D nanofiber scaffolds with arrayed holes were 16, 60, and 129 at week 1, 2 and 4, respectively (Fig. 5f). For comparison, the number of multinucleated giant cells per implant for traditional nanofiber mats was close to 16, but decreased to 9 and 6 at week 2 and 4 (Fig. S6) [24]. In addition, expanded nanofiber scaffolds with arrayed holes showed certain cellular infiltration from both the top and bottom surfaces (Fig. S7), which was different from the traditional nanofiber mats.

We further performed the immunohistological staining of 3D nanofiber scaffolds with arrayed holes and the surrounding tissues in order to identify the infiltrated macrophages with different surface makers (Fig. 6), indicating the number of CCR7 positive cells (macrophages in M1 phase which encourage inflammation) decreased while the number of CD206 (macrophages in M2 phase which decrease inflammation and encourage tissue repair) and CD 68 (pan macrophages) positive cells increased with increasing the implantation time. The quantified data for macrophages with different surface markers was

shown in Fig. 7, indicating a dramatic increase of M2/M1 ratio at week 4 after implantation. In contrast, the M2/M1 ratios remained constant from week 1 to week 4 for traditional nanofiber mats (Fig. 7 and Fig. S8) [24]. To reveal the expressing markers and spatiotemporal distributions of multinucleate giant cells, we further analyzed the highly magnified immunostaining images with different surface markers (Fig. 8), indicating multinucleated giant cells were heterogeneous likely expressing CCR7, CD 206, and/or CD68 markers, which may be important for the new blood vessel formation and tissue regeneration [46]. Therefore, it was confirmed that subcritical CO₂ fluid expanded 3D nanofiber scaffolds with arrayed holes could promote cellular infiltration, neovascularization, and positive host response.

4. Discussion

Due to the biomimetic property, electrospun nanofibers have been widely used as scaffolds for regenerative medicine [3]. However, the traditional electrospinning often produce nanofiber membranes/mats with smaller pore size and tight structure, limiting cellular infiltration because of its intrinsic property [23]. Researchers attempted to develop 3D electrospun nanofiber scaffolds making use of sacrificial templates (e.g., hydrophilic fibers, ice, and salts), manipulation of electrical field (e.g., customized collector and additive of ionic salts), noobing/weaving, melt jet writing/printing, and modified gas-foaming [17–25, 47, 48]. These methods are associated with various problems (e.g., time consuming, limited thickness, and necessitate the use of aqueous solution). In this study, we demonstrated a simple and novel approach to generate 3D nanofiber scaffolds *via* depressurization of subcritical CO₂ fluid. Based on the CO₂ phase diagram [49], the CO₂ liquid phase changes to the gas phase when the pressure is reduced rapidly. The CO₂ liquid that permeates fiber matrix changes into CO₂ gas bubbles and greatly expand the fiber matrix. After venting the CO₂ gas, the expanded nanofiber scaffolds can be readily formed without freeze-drying. For this expansion process, the plasma treatment procedure is also eliminated as CO₂ fluid easily penetrates the PCL nanofiber membranes probably due to its non-polar property. Importantly, this method allows us to achieve the expansion of nanofiber membranes in minutes. Compared with previous approaches [23, 24], the method we develop in this study saves time, eliminates the use of an aqueous solution and freeze-drying process, is environmentally friendly, uses low temperature processing, and maintains the aligned nanotopography. In addition, it is possible to expand the nanofiber mat in a custom-designed mold. Alternatively, we can compress the expanded nanofiber scaffold to a desired thickness. Using these two methods, we could achieve the expansion with tunable or desired thickness. Importantly, nanofiber membranes made of water-soluble polymers (e.g., PVP) can be expanded into 3D scaffolds using the subcritical CO₂ fluid (Fig. 2), which was not able to achieve in previous studies [23, 24]. Furthermore, this CO₂ expansion process can maintain the bioactivity of encapsulated molecules to a great extent, which is critical as 3D nanofiber scaffolds often combine with growth factors or other bioactive molecules for use in regenerative medicine.

Our recent studies demonstrated the expansion of electrospun nanofiber membranes in the aqueous solution using a modified gas-foaming technique [23,24]. The resultant 3D scaffolds can facilitate cellular infiltration through the gaps between nanofiber layers. In this

work, the 3D nanofiber scaffolds generated by subcritical CO₂ fluid showed a similar structure as the ones expanded in a NaBH₄ aqueous solution [23,24]. We further created arrayed holes throughout the scaffolds under a cryogenic condition for enhancement of cellular infiltration. Indeed, we observed that cells penetrated to the scaffolds not only from sides but also from the holes (Fig. 5a). On the basis of immunostaining results, we could propose the cellular infiltration and spatiotemporal distributions of M1 macrophages, M2 macrophages, and multinucleated giant cells within the scaffold after implantation for 1, 2, and 4 weeks, which are schematically illustrated in Fig. 9. For traditional nanofiber mats, cells often stay on the surface of nanofiber mats with marginal penetration and form collagen capsules (Fig. 9a). For CO₂ expanded 3D nanofiber scaffolds with arrayed holes, cells infiltrated into the punched holes within 1 week and continued penetrating to the scaffolds through the gaps between nanofiber layers. The infiltration of macrophages showed a similar trend. There were more M1 macrophages at week 1 and 2 but more M2 macrophages at week 4. At week 1, multinucleated giant cells were mostly located on the surface of punched holes. At week 2, some giant cells were formed either on the surface of punched holes or within the infiltrated fiber layers. At week 4, multinucleated giant cells were relatively evenly distributed throughout the infiltrated areas. In addition, the multinucleated giant cells show heterogeneous phenotypes with positive staining of different markers including CCR7, CD208 and CD68 (Fig. 6), indicating the importance for tissue regeneration. This finding is in line with recent studies [45]. Our future studies will be focusing on the use of CO₂ expanded nanofiber scaffolds for regeneration of specific tissues, in particular, those tissues with anisotropic properties such as nerve, muscle and tendon.

5. Conclusion

In conclusion, we have demonstrated for the first time the transformation of electrospun nanofiber membranes from 2D to 3D using the subcritical CO₂ fluid. This method provides several advantages over previous approaches such as shortening the processing time, eliminating the use of aqueous solutions and freeze-drying procedures, and avoiding the loss of encapsulated biologics. Most importantly, this method maintains the bioactivity of encapsulated molecules to a greater extent. Further, holes across nanofiber membranes can be generated through a micro-punch under a cryogenic condition to further promote cellular penetration and new blood vessel formation. These transformed 3D nanofiber scaffolds may have great potential for applications in tissue repair/regeneration, engineering 3D tissue models, wound dressing, hemostasis, and topical drug delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institute of General Medical Science (NIGMS) at the NIH (2P20 GM103480-06 and 1R01GM123081 to J.X.), the Otis Glebe Medical Research Foundation and startup funds from the University of Nebraska Medical Center.

References

1. Ramakrishna S, Sujihar K, Teo WE, Yong T, Ma Z, Ramaseshan R. Electrospun nanofibers: solving global issues. *Mater, Today*. 2006; 9:40–50.
2. Sridhar R, Lakshminarayanan R, Madhaiyan K, Amutha Barathi V, Lim KH, Ramakrishna S. Electrospayed nanoparticles and electrospun nanofibers based on natural materials: applications in tissue regeneration, drug delivery and pharmaceuticals. *Chem Soc Rev*. 2015; 44:790–814. [PubMed: 25408245]
3. Xie J, Li X, Xia Y. Put electrospun nanofibers to work for biomedical research. *Macromol Rapid Commun*. 2008; 29:1775–1792. [PubMed: 20011452]
4. Xie J, MacEwan MR, Schwartz AG, Xia Y. Electrospun nanofibers for neural tissue engineering. *Nanoscale*. 2010; 2:35–44. [PubMed: 20648362]
5. Xie J, Li X, Lipner J, Manning CN, Schwartz AG, Thomopoulos S, Xia Y. “Aligned-to-random” nanofiber scaffolds for mimicking the structure of the tendon-to-bone insertion site. *Nanoscale*. 2010; 2:923–926. [PubMed: 20648290]
6. Kennedy KM, Bhaw-Luximon A, Jhurry D. Cell-matrix mechanical interaction in electrospun polymeric scaffolds for tissue engineering: implications for scaffold design and performance. *Acta Biomater*. 2017; 50:41–55. [PubMed: 28011142]
7. Liu W, Thomopoulos S, Xia Y. Electrospun nanofibers for regenerative medicine. *Adv Healthc Mater*. 2012; 1:10–25. [PubMed: 23184683]
8. Mahjour SB, Sefat F, Polunin Y, Wang L, Wang H. Improved cell infiltration of electrospun nanofiber mats for layered tissue constructs. *J Biomed Mater Res A*. 2016; 104:1479–1488. [PubMed: 26845076]
9. Wu J, Hong Y. Enhancing cell infiltration of electrospun fibrous scaffolds in tissue regeneration. *Bioactive Materials*. 2016; 1:56–64.
10. Sun B, Long Y, Yu F, Li M, Zhang H, Li W, Xu T. Self-assembly of a three-dimensional fibrous polymer sponge by electrospinning. *Nanoscale*. 2012; 4:2134–2137. [PubMed: 22344309]
11. Kang D, Kim JH, Jeong YH, Kwak JY, Yoon S, Jin S. Endothelial monolayers on collagen-coated nanofibrous membranes: cell-cell and cell-ECM interactions. *Biofabrication*. 2016; 8:025008. [PubMed: 27186924]
12. Kim G, Kim W. Highly porous 3D nanofiber scaffold using an electrospinning technique. *J Biomed Mater Res B Appl Biomater*. 2007; 81:104–110. [PubMed: 16924612]
13. Sell S, Barnes C, Simpson D, Bowling G. Scaffold permeability as a means to determine fiber diameter and pore size of electrospun fibrinogen. *J Biomed Mater Res A*. 2008; 85:115–126. [PubMed: 17688269]
14. Balguid A, Mol A, van Marion MH, Bank RA, Bouten CV, Baaijens FP. Tailoring fiber diameter in electrospun poly(epsilon-caprolactone) scaffolds for optimal cellular infiltration in cardiovascular tissue engineering. *Tissue Eng Part A*. 2009; 15:437–444. [PubMed: 18694294]
15. Fong FLS, Lamhamedi-Cherradi SR, Burdett E, Ramamoorthy V, Lazar AJ, Kasper FK, Farach-Carson MC, Vishwamitra D, Demicco EG, Menegaz BA, Amin HM, Mikos AG, Ludwig JA. Modeling ewing sarcoma tumors in vitro with 3D scaffolds. *Proc Natl Acad Sci USA*. 2013; 110:6500–6505. [PubMed: 23576741]
16. Pham QP, Sharma U, Mikos AG. Electrospun poly(e-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: characterization of scaffolds and measurement of cellular infiltration. *Biomacromolecules*. 2006; 7:2796–2805. [PubMed: 17025355]
17. Blakeney BA, Tambralli A, Anderson JM, Andukuri A, Kim DJ, Dean DR, Jun HW. Cell infiltration and growth in a low density, uncompressed three-dimensional electrospun nanofibrous scaffold. *Biomaterials*. 2011; 32:1583–1590. [PubMed: 21112625]
18. Jin G, Shin M, Kim SH, Lee H, Jang JH. SPONGE: Spontaneous organization of numerous-layer generation by electrospray. *Angew Chem*. 2015; 54:7587–7591. [PubMed: 25958994]
19. Baker BM, Gee AO, Metter RB, Nathan AS, Marklein RA, Burdick JA, Mauck RL. The potential to improve cell infiltration in composite fiber-aligned electrospun scaffolds by the selective removal of sacrificial fibers. *Biomaterials*. 2008; 29:2348–2358. [PubMed: 18313138]

20. Baker BM, Shah RP, Silverstein AM, Esterhai JL, Burdick JA, Mauck RL. Sacrificial nanofibrous composites provide instruction without impediment and enable functional tissue formation. *Proc Natl Acad Sci USA*. 2012; 109:14176–14181. [PubMed: 22872864]
21. Leong MF, Rasheed MZ, Lim TC, Chian KS. In vitro cell infiltration and in vivo cell infiltration and vascularization in a fibrous, highly porous poly(D,L-lactide) scaffold fabricated by cryogenic electrospinning technique. *J Biomed Mater Res A*. 2009; 91:231–240. [PubMed: 18814222]
22. Nam J, Huang Y, Agarwal S, Lannutti J. Improved cellular infiltration in electrospun fiber via engineered porosity. *Tissue Eng*. 2007; 13:2249–2257. [PubMed: 17536926]
23. Jiang J, Carlson MA, Teusink MJ, Wang H, MacEwan MR, Xie J. Expanding two-dimensional electrospun nanofiber membranes in the third dimension by a modified gas-foaming technique. *ACS Biomater Sci Eng*. 2015; 1:991–1001.
24. Jiang J, Li Z, Wang H, Wang Y, Carlson MA, Teusink MJ, MacEwan MR, Gu L, Xie J. Expanded 3D nanofiber scaffolds: cell penetration, neovascularization, and host response. *Adv Healthcare Mater*. 2016; 5:2993–3003.
25. Joshi MK, Pant HR, Tiwari AP, Kim HJ, Park CH, Kim CS. Multi-layered macroporous three-dimensional nanofibrous scaffold via a novel gas foaming technique. *Chem Eng J*. 2015; 275:79–88.
26. Garland SM, Menary RC. Sub-critical carbon dioxide extraction of the hexane extract of the leaves of *Tasmannia Lanceolata*. *J Essential Oil Res*. 2016; 28:55–63.
27. Taraj K, Delibashi A, Andoni A, Lazo P, Kokalari E, Lame A, Xhaxhiu K, Como A. Extraction of chamomile essential oil by subcritical CO₂ and its analysis by UV-VIS spectrophotometer. *Asian J Chem*. 2013; 25:7361–7364.
28. Rout PK, Naik SN, Rao YR. Subcritical CO₂ extraction of floral fragrance from *Quisqualis indica*. *J Supercritical Fluids*. 2008; 45:200–205.
29. Bhamidipati M, Sridharan BP, Scurto AM, Detamore MS. Subcritical CO₂ sintering of microspheres of different polymeric materials to fabricate scaffolds for tissue engineering. *Mater Sci Eng C Mater Biol Appl*. 2013; 33:4892–4899. [PubMed: 24094202]
30. Wang X, Vipin K, Li W. Low density sub-critical CO₂-blown solid-state PLA foams. *Cellular Polym*. 2007; 26:11–35.
31. Yang Y, Lee LJ, Lu W. Subcritical carbon dioxide assisted polymer nanofabrication at low temperatures. *J Vac Sci Tech B*. 2005; 23:3202.
32. Xie J, Blough ER, Wang CH. Submicron bioactive glass tubes for bone tissue engineering. *Acta Biomater*. 2012; 8:811–819. [PubMed: 21945829]
33. Sheikh FA, Ju HW, Ju JM, Moon BM, Park HJ, Lee OJ, Kim JH, Kim DK, Park CH. 3D electrospun silk fibroin nanofibers for fabrication of artificial skin. *Nanomedicine: NBM*. 2015; 11:681–691.
34. Lee JB, Jeong SI, Bae MS, Yang DH, Heo DN, Kim CH. Highly porous electrospun nanofibers enhanced by ultrasonication for improved cellular infiltration. *Tissue Eng Part A*. 2011; 17:2695–2702. [PubMed: 21682540]
35. Ding S, Schultz PG. Small molecules and future regenerative medicine. *Curr Top Med Chem*. 2005; 5:383–395. [PubMed: 15892681]
36. Lu B, Atala A. Small molecules and small molecule drugs in regenerative medicine. *Drug Discov Today*. 2014; 19:801–808. [PubMed: 24252867]
37. Xie J, Wang CH. Electrospun micro- and nanofibers for sustained delivery of paclitaxel to treat C6 glioma in vitro. *Pharm Res*. 2006; 23:1817–1826. [PubMed: 16841195]
38. Fumakia M, Ho EA. Nanoparticles encapsulated with LL37 and Serpin A1 promotes wound healing and synergistically enhances antibacterial activity. *Mol Pharm*. 2016; 13:2318–2331. [PubMed: 27182713]
39. Chereddy KK, Her CH, Comune M, Moia C, Lopes A, Porporato PE, Vanacker J, Lam MC, Steintraesser L, Sonveaux P, Zhu H, Ferreira S, Vandermeulen G, Preat V. PLGA nanoparticles loaded with host defense peptide LL37 promote wound healing. *J Control Release*. 2014; 194:138–147. [PubMed: 25173841]

40. Durr UHN, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochim Biophys Acta*. 2006; 1758:1408–1425. [PubMed: 16716248]
41. Xie J, Blough E, Wang CH. Submicron bioactive glass tubes for bone tissue engineering. *Acta Biomater*. 2012; 8:811–819. [PubMed: 21945829]
42. Mahjour SB, Sefat F, Polumin Y, Wang L, Wang H. Improved cell infiltration of electrospun nanofiber mats for layered tissue constructs. *J Biomed Mater Res A*. 2016; 104:1479–1488. [PubMed: 26845076]
43. Fu L, Xie J, Carlson MA, Reilly DA. Three-dimensional nanofiber scaffolds with arrayed holes for engineering skin tissue constructs. *MRS Communications*. 2017; 7:361–366.
44. Walthers CM, Nazemi AK, Patel SL, Wu BM, Dunn JCY. The effect of scaffold macroporosity on angiogenesis and cell survival in tissue-engineered smooth muscle. *Biomaterials*. 2014; 35:5129–5137. [PubMed: 24695092]
45. Bonvallet PP, Culpepper BK, Bain JL, Schultz MJ, Thomas SJ, Bellis SL. Microporous dermal-like electrospun scaffolds promote accelerated skin regeneration. *Tissue Eng Part A*. 2014; 20:2434–2445. [PubMed: 24568584]
46. Barbeck M, Motta A, Miglizresi C, Sader R, Kirkpatrick CJ, Ghanaati S. Heterogeneity of biomaterial-induced multinucleated giant cells: possible importance for the regeneration process. *J Biomed Mater Res Part A*. 2016; 104:413–418.
47. Xie J, Ma B, Michael PL. Fabrication of novel 3D nanofiber scaffolds with anisotropic property and regular pores and their potential applications. *Adv Healthcare Mater*. 2012; 1:674–678.
48. Hochleitner G, Jungst T, Brown TD, Hahn K, Moseke C, Jakob F, Dalton PD, Groll J. Additive manufacturing of scaffolds with sub-micron filaments via melt electrospinning writing. *Biofabrication*. 2015; 7:035002. [PubMed: 26065373]
49. Mazzoldi A, Hill T, Colls JJ. CO₂ transportation for carbon capture and storage: sublimation of carbon dioxide from a dry ice bank. *Int J Greenh Gas Con*. 2008; 2:210–218.

Statement of Significance

Electrospun nanofibers have been widely used in regenerative medicine due to its biomimicry property. However, most of studies are limited to the use of 2D electrospun nanofiber membranes. To the best of our knowledge, this article is the first instance of the transformation of traditional electrospun nanofiber membranes from 2D to 3D *via* depressurization of subcritical CO₂ fluid. This method eliminates many issues associated with previous approaches such as necessitating the use of aqueous solutions and chemical reactions, multiple-step process, loss of the activity of encapsulated biological molecules, and unable to expand electrospun nanofiber mats made of hydrophilic polymers. Results indicate that these CO₂ expanded nanofiber scaffolds can maintain the activity of encapsulated biological molecules. Further, the CO₂ expanded nanofiber scaffolds with arrayed holes can greatly promote cellular infiltration, neovascularization, and positive host response after subcutaneous implantation in rats. The current work is the first study elucidating such a simple and novel strategy for fabrication of 3D nanofiber scaffolds.

Fig. 1.

Expansion and characterization of aligned nanofiber scaffolds. (a) Photographs of aligned PCL nanofiber mats after the first treatment of subcritical CO₂ fluid (left) and raw PCL nanofiber mats (right). (b) Photographs of aligned PCL nanofiber mats after the second treatment (left) and raw PCL nanofiber mats (right). (c) Thickness of aligned PCL fiber mats after expanding once and twice. (d) The corresponding porosities of aligned PCL fiber mats after expanding once and twice. (e–h) SEM images showing cross-sectional morphologies of aligned PCL fiber mats before (e, f) expansion and after expansion in subcritical CO₂ fluid two times (g, h). The scale bar is 20 μm .

Fig. 2. Photographs of poly(vinylpyrrolidone) (PVP) nanofiber mats before (a, b) and after (c, d) expansion in subcritical CO₂ fluid. Due to the high hydrophilicity of PVP nanofibers, expanded PVP membranes were kept in the capped tube to prevent dissolving from water condensed from the surrounding air (c). The expanded membrane was taken out after the temperature of samples reached the room temperature (d).

Fig. 3.

Expansion of coumarin 6-loaded PCL nanofiber scaffolds. (a) Photographs showing NaBH_4 expanded PCL fiber mats with coumarin 6 loading (NaBH_4) and CO_2 expanded PCL fiber mats with coumarin 6 loading (CO_2), (b) Top view of CO_2 liquid expanded PCL fiber mats with coumarin 6 loading (top left), NaBH_4 expanded PCL fiber mats with coumarin 6 loading (bottom left), PCL fiber mats with coumarin 6 loading (top right) and raw PCL fiber mats (bottom right). Insets: fluorescent images of each sample. (c) The fluorescence intensity quantified by Image J software.

Fig. 4. Expansion of LL37-loaded PCL nanofiber scaffolds using CO_2 fluid. (a) The *in vitro* release kinetics of LL 37 from expanded and unexpanded PCL fiber samples (Initial drug loading: 5 $\mu\text{g}/\text{mg}$). (b) Antibacterial performance of different fiber samples. PCL: unexpanded pristine PCL nanofiber membranes. PCL-LL37: LL37-loaded PCL nanofiber scaffolds.

Fig. 5.

In vivo response of expanded nanofiber scaffolds with arrayed holes and traditional nanofiber mats. (a) H & E staining. Green dots indicate the boundary of cell filtrated area. (b) Masson's trichrome staining. Green arrows indicate collagen deposition. (c) Highly magnified images in (a). (d) Highly magnified images in (a). Green arrows indicate giant cells. (e) Quantification of blood vessel formation per mm². (f) Quantification of giant cells per implant. For comparison, the result for traditional nanofiber mat was adapted from our previous published studies [24].

Fig. 6. Immunohistological staining of 3D expanded nanofiber scaffolds with arrayed holes and surrounding tissues against CD68 - a surface marker for pan macrophages, CD 206 – a surface marker for macrophages in M2 phase, and CCR7 – a surface marker for macrophages in M1 phase. The nanofiber scaffolds were subcutaneously implanted to rats for 1 week, 2 weeks, and 4 weeks.

Fig. 7.

Quantification of immunohistological analysis of 3D expanded nanofiber scaffolds with arrayed holes and traditional nanofiber mats after subcutaneous implantation. a) CD 68, b) CCR 7 (M1), c) CD 206 (M2) immunopositive cells and d) ratio of number of CD163 positive cells (M2)/number of CCR7 positive cells (M1). The values were obtained by measuring six scanning images at 40× (objective lense) magnification for each specimen. For comparison, the result for traditional nanofiber mat was adapted from our previous published studies [24].

Fig. 8.

Multinucleated giant cells after subcutaneous implantation of 3D expanded nanofiber scaffolds with punched holes. The rats were sacrificed at week 1, 2, and 4 after surgery. The multinucleated giant cells were stained against CD68 - a surface marker for pan macrophages, CD 206 - a surface marker for macrophages in M2 phase, and CCR7 - a surface marker for macrophages in M1 phase. Green arrows indicate multinucleated giant cells.

Fig. 9.

The proposed schematic illustrating the cell infiltration and spatiotemporal distributions of M1 macrophages (yellow color), M2 (blue color) macrophages (top panel) and multinucleated giant cells (bottom panel) on the surface of traditional nanofiber mats and within expanded 3D nanofiber scaffolds with arrayed holes after subcutaneous implantation. The cell-infiltrated area is labeled in red. For comparison, the schematic of traditional nanofiber mats was drawn based on previous published results [24].