Simple Fabrication of Multicomponent Heterogeneous Fibers for Cell Co-Culture via Microfluidic Spinning

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Microfluidic spinning, as a combination of wet spinning and microfluidic technology, has been used to develop microfibers with special structures to facilitate cell 3D culture/co-culture and microtissue formation in vitro. In this study, a simple microchip-based microfluidic spinning strategy is presented for the fabrication of multicomponent heterogeneous calcium alginate microfibers. The use of two kinds of microchip enables the one-step preparation of multicomponent heterogeneous microfibers with various arrangement patterns, including the preparation of one-, two-, and three-component microfibers by a two-layer microchip and preparation of four component microfibers with different arrangement by a membrane-sandwiched three-layer microchip. The obtained microfibers could be used to encapsulate various kinds of cells, such as the human non-small cell lung cancer cell NCI-H1650, the human fetal lung fibroblast HFL1, the normal pulmonary bronchial epithelial cell 16HBE, and human umbilical vein endothelial cells. By adding chitosan to the medium to keep the fibers stable, 3D long-term in vitro cell co-culture has been carried out up to 21 days. This method is very simple and easy to operate, continuously produces spatially well-defined heterogeneous microfibers, has important applications for composite functional biomaterials, and shows great potential in organs-on-a-chip and biomimetic systems.

1. Introduction

As a kind of long, thin, flexible material, fibers can be used to fabricate various functional 3D objects via folding, bundling, reeling, and weaving, and these features facilitate higher-order assemblies such as biomedical materials and tissue function material.[11-7] It is well known that fiber-shaped complex 3D structures are common in the human body, such as vessels,[8,9] trachea, and other lumen-like structures,[10] neural pathways,[11,12] and muscle fibers.[13] Therefore, this fiber-based strategy has also attracted considerable attention for the creation of complex 3D tissues in vitro.

Current methods of preparing fibers mainly include melt spinning,[14] wet spinning,[15,16] and electrospinning.[17-19] However, the presence of high temperatures and volatile solvents in the melt spinning and electrospinning processes limits the encapsulation of cells, microtissues, and other bioactive molecules in fibers.[20] For example, electrospinning microfibers must be removed with organic solvents before they can be used as a substrate for cell culture, which limits the construction of more complex 3D tissues. In contrast, some wet spinning systems include mild conditions, good biocompatibility, and no volatile solvent, such as those to prepare CaA fiber,[21-24] calcium alginate/chitosan composite fiber,[25,26] poly(ethylene glycol) diacylate (PEGDA) fiber,[27,28] and poly(ethylene glycol dimethacrylate) (PEGDMA) fiber.[29] The fibers obtained with these methods directly encapsulate biomolecules and cells and facilitate cell 3D culture/co-culture and microtissue formation in vitro.[30,31] However, it is difficult to prepare microfibers with special structures with traditional wet spinning, and the fibers obtained do not meet the requirements of regularity, controllability, and precision, so new technologies are urgently needed to improve them.

With the ongoing development of micromachining technology, especially the rapid development of soft lithography technology, microfluidic spinning—a combination of wet spinning and microfluidic technology—was recently developed. Some microfibers with special structures such as an anisotropic structure,[32] multiple components,[33,34] hollowness,[35-38] bubbles,[39] a grooved surface,[40,41] or a bamboo-like structure[42] have been prepared and used in cell loading or capsules to carry out 3D cell culture and to construct a cell microenvironment. This indicates that microfluidic spinning plays an important role in the preparation and application of composite functional biomaterials and even has great potential in organs-on-a-chip, biomimetic systems, and intelligent manufacturing.

We present a simple microchip-based microfluidic spinning strategy for the fabrication of multicomponent heterogeneous CaA microfibers. The advantages of this method are: 1) The use of a microchip enables the one-step preparation of two-component, three-component, and four-component heterogeneous...
microfibers with various arrangement patterns. 2) The microfibers thus obtained could be used to encapsulate various kinds of cells to achieve long-term 3D cell co-culture in vitro up to 21 days. 3) We developed a novel method to carry out three kinds of cells co-culture, which facilitate the study of more cell-to-cell interactions, especially during tumor development.

2. Results and Discussion

2.1. Chip Design and Microfluidic Spinning Conditions

We used a microchip-based microfluidic spinning strategy for the fabrication of CaA microfibers prepared by a crosslinking reaction between sodium alginate (NaA) and calcium chloride (CaCl₂). Two kinds of microchips were used: a two-layer microchip and a membrane-sandwiched three-layer microchip. The two-layer microchip was prepared with standard soft lithography etching technology and included an upper layer and a polydimethylsiloxane (PDMS) substrate. This chip was used to fabricate type A, A-B, A-B-A, and A-B-C microfibers. The membrane-sandwiched three-layer microchip contained an upper layer, a PDMS membrane with a hollow microchannel, and a bottom layer. This chip was used to fabricate type A/B, A/A-B, A-B/B-A, and A-C/B-D microfibers. Figure 1 includes the microchip design and a schematic diagram of the multicomponent heterogeneous microfibers. All kinds of microchips have the same key sizes, including the main microchannel, the sample microchannel, the sheath flow microchannel, and the Y-shaped liquid junction.

Initially, we prepared type A microfibers with two-layer microchips to optimize the microfluidic spinning conditions. Figure 2A shows a CaA microfiber prepared via microfluidic spinning. The microchip was placed in a petri dish filled with a calcium chloride solution to make the microfibers more stable. In addition, fluorescent dye (Rh-123) was added to the NaA solution to characterize the laminar flow in the channel, as shown in Figure 2B. We studied the morphology and cross-sectional area of the CaA fibers obtained at various velocities of sheath flow and core flow. When the sheath flow velocity was 500 µL min⁻¹, we collected and observed the prepared CaA fibers at core flow velocities of 100, 150, 200, 300, 400, 500, and 600 µL min⁻¹. The fluorescence images are shown in Figure 2C. We also calculated the cross-sectional area of the CaA fiber, as shown in Figure 2D. In the same way, we determined the core velocity of 200 µL min⁻¹ and changed the sheath flow velocity, and the results are shown in Figure 2E. The following results were noted. 1) When the sheath flow velocity remains constant, the cross-sectional areas of microfiber increase as the core flow velocity increases. In contrast, when the core flow velocity is constant, the cross-sectional areas decrease as the sheath flow velocity increases. 2) When the core flow velocity is too low or the sheath flow velocity is too high, the laminar flow in the microchannel is unstable, and the microfiber morphology and edge are irregular. 3) Although the microfluidic channels are square or rectangular, the cross-sections of the CaA

Figure 1. Chip design and schematic diagram of multicomponent heterogeneous microfibers. A: Schematic diagram of two-layer microchip used to fabricate one-, two-, and three-component microfibers by a two-layer microchip, and this chip contained an PDMS upper layer with microchannels and a PDMS substrate. B: Schematic diagram of three-layer microchip to prepare four component microfibers with different arrangement by a membrane-sandwiched three-layer microchip, and this chip contained a PDMS upper layer with microchannels, a PDMS membrane with a hollow microchannel, and a PDMS bottom layer with microchannels. C: Schematic diagram of multicomponent heterogeneous microfibers.
microfiber remain circular or elliptic. By comparing the size, morphology, and operational difficulty of the microfibers obtained, we finally selected the preparation conditions with a core flow velocity of 200 $\mu$L min$^{-1}$ and a sheath flow velocity of 500 $\mu$L min$^{-1}$ for the follow-up experiments. The obtained CaA microfibers were characterized via scanning electron microscopy after drying, as shown in Figure 2F. It can be seen that the fiber was slightly smaller and had a smoother surface after drying.

2.2. Preparation and Characterization of Multicomponent Microfibers

After optimizing the conditions of microfluidic spinning, we synthesized multicomponent CaA microfibers. First, the two-layer microchip was used to fabricate type A-B microfibers. The results are shown in Figure 3A, and the synthesized video is included in Supporting Information 1. The fluorescent dyes, Rh-123 and Rh-B, were added to sodium alginate solution and were used to characterize the laminar flow in the microchannel and the various components of the obtained microfibers. The total core flow velocity was 200 $\mu$L min$^{-1}$, so when we changed the ratio of the flow rates of components A and B, we easily obtained A-B fibers with various component ratios, such as 1:1 ($Q_{c1} = 100$ $\mu$L min$^{-1}$; $Q_{c2} = 100$ $\mu$L min$^{-1}$), 3:1 ($Q_{c1} = 150$ $\mu$L min$^{-1}$; $Q_{c2} = 50$ $\mu$L min$^{-1}$), and 1:3 ($Q_{c1} = 50$ $\mu$L min$^{-1}$; $Q_{c2} = 150$ $\mu$L min$^{-1}$). The fluorescent distribution in laminar flow images and in microfiber cross-sectional images both prove this point. We also used the same method to fabricate type A-B-A and A-B-C microfibers. The results are shown in Figure 3B, and the laminar flow video is included in Supporting Information 2. We set the flow rates for the three components of the microfibers to 60, 80, and 60 $\mu$L min$^{-1}$. The three components in the microfibers were clearly distributed from the scanning images of the microfiber cross-sectional. In addition, sodium alginate solution with a concentration of 2% w/v was also used as component B to prepare A-B_high-A microfiber, which had a higher concentration than the other groups. Because the viscosity of the sodium alginate solution increased as the concentration increased, the fluorescence images of laminar flow and fiber cross-section showed that the distribution of component B was different to that of the A-B-A microfiber. The cross-sectional areas of component B increased. Furthermore, regardless of whether it was single-component, two-component, or three-component microfiber, neither the total core flow velocity nor the sheath flow velocity changed, so no significant differences were seen in the cross-sectional areas of the fibers. When the total core flow velocity was 200 $\mu$L min$^{-1}$ and the total sheath flow velocity was 500 $\mu$L min$^{-1}$, the cross-sectional areas were about 0.05 mm$^2$.

On the basis of these experiments, we also fabricated type A/B, A/A-B, and A-B/B-A by membrane-sandwiched microchip. Because the membrane-sandwiched microchip had exactly the same upper and bottom microchannels, and the microchannels of each layer were the same as those of the previous two-layer microchip, the spinning conditions obtained from the previous optimization were used for each layer. The results are shown in Figure 4. Figure 4A shows the microfiber preparation by membrane-sandwiched microchip. The fluorescent dyes Rh-123 and Rh-B were also added in component A and B to characterize the...
Figure 3. A) Fluorescence images of type A-B microfibers with various velocity ratios. B) Fluorescence images of type A-B-A and A-B-C microfibers. Flow images are fluorescence images of the laminar flow obtained with an inverted fluorescence microscope. Microfiber images are fluorescence images of microfibers obtained with a scanning laser confocal microscope. Z-axis images correspond to the position of the yellow line.

Figure 4. A) Image of multicomponent microfibers achieved via microfluidic spinning. B) Schematic diagrams of the cross section of the microfiber and corresponding fluorescence images of the obtained multicomponent microfibers. C) Histogram of the cross-sectional area of each component in the multicomponent fibers.
microfibers. Figure 4B shows a schematic diagram of a cross-section of microfiber and the corresponding fluorescence images of the multicomponent microfibers. As can be seen, the interfaces of various components of the prepared microfibers were clear and accurate. We also calculated the cross-sectional area ratios of each component via fluorescence images (Figure 4C). For type A/B, A/A-B, and A-B/B-A microfibers, the theoretical ratios of component A and component B were 1:1, 3:1, and 1:1:1:1 respectively, and the actual results completely matched. In addition, because the multilayer design with membrane-sandwiched microchip was adopted, the total cross-sectional areas of the obtained microfibers also increased relative to the microfiber obtained from the two-layer microchip (nearly 0.07 mm²).

### 2.3. Multicomponent Microfibers as Cell Scaffolds for Cell Culture/Co-Culture

On this basis, we studied the ability of multicomponent microfibers to act as cell scaffolds for cell culture. Type A-B-A microfibers with cells in the core were obtained by adding NCI-H1650 cells into component B (Figure 5A and Supporting Information 3). To maintain the microstructure of the microfibers for long-term cell culture, we added 0.1% chitosan to the cell culture medium to prevent fiber swelling.[25,26] After several days of culture, we found that the cells had continuously proliferated and spread toward the fiber edge. After 7 days, the cells basically occupied the entire microfiber. It indicated that the cells had good activity. And then for further verification, Calcein-Am/PI staining was carried out to determine cell viability at day 7 (Figure 5B and Supporting Information 4). Calcein-Am/PI staining was mainly used for the characterization of living cells/dead cells. The results showed that only very few cells were labeled as dead cells with red color, accounting for about 5% of the total cells, while majority cells were alive with bright green color. This indicated that the cells were in good survival conditions and microfibers could provide a good condition for cell 3D culture.

We also used type A-B-C microfibers to achieve long-term 3D cell co-culture. Two kinds of cells were used in this experiment:

**Figure 5.** Three component microfibers for cell culture or co-culture. A) Images of NCI-H1650 cells in type A-B-A microfiber for cell culture at 0, 1, 3, and 7 days. NCI-H1650 cells labelled by Cell Tracker Green CMFDA were added to component B as the core of the microfiber. NCI-H1650, green. Z-axis images show sections of microfiber scanning with a laser confocal microscope. Z-axis images correspond to the position of the yellow line. B) 3D fluorescence images of NCI-H1650 cell stained by Calcein-Am/PI at 7 days in different views. Calcein-Am, green; PI, red. C) Fluorescence images of cell co-culture in type A-B-C microfibers at 0, 7, and 14 days. Green: 16HBE cells marked by Cell Tracker Green CMFDA. Red: NCI-H1650 cells marked by Cell Tracker Red CMTPX. D) Vimentin expression of cells in microfiber after 21 days of co-culture. Green, vimentin; blue, cell nucleus stained by DAPI. E) Fluorescence image of cells co-culture in type A-B-C microfiber at 21 days. Green: 16HBE cells; Red: NCI-H1650 cells. Z-axis image is also a section of microfiber.
NCI-H1650 cells and 16HBE cells. The microfibers for cell co-culture were obtained by adding NCI-H1650 cells and 16HBE cells into components A and C, respectively (Figure 5C). At the beginning, the two kinds of cells were loaded at each side of the microfiber, not in contact. After 7 days of continuous culture, the two kinds of cells came into contact. After 14 days, NCI-H1650 cells had invaded the 16HBE cells, and the number of 16HBE cells had decreased. At the same time, vimentin expression was detected by immunofluorescence staining (Figure 5D). Vimentin is a marker protein that can be used to detect the occurrence of epithelial mesenchymal transformation (EMT). The NCI-H1650 cells and 16HBE cells were all epithelial-derived and expressed almost no vimentin. After 14 days of cell co-culture, some individual cells overexpressed vimentin on the side of the microfiber. From their location and distribution, they were identified as 16HBE cells. They may have undergone EMT after co-culture with tumor cells. After 21 days of co-culture, the NCI-H1650 cells had almost filled the microfibers, and the 16HBE cells had nearly disappeared. Figure 5E shows the fluorescence images.

After cell culture and co-culture of these two kinds of cells, we also developed multicomponent type A-C/B-D microfibers to achieve co-culture of three kinds of cells (Figure 6 and Supporting Information 5). Generally in the physiological state, tumor cells and fibroblasts or tumor cells and endothelial cells have very complex cellular interactions, such as: the tumor cells could induce the death or mesenchymal-epithelial transition (MET) of fibroblasts, or also could possible induce death of endothelial cells during extravasation, or promote vascularization during tumor neovascularization. Therefore, it is of great significance to study their co-culture for the occurrence and development of tumors. In this study, we used three different types of cells, NCI-H1650 cells for tumor cells, HFL1 cells for fibroblasts, and HUVECs for endothelial cells, and co-cultured them to try to study their interactions. Figure 6A shows fluorescence images of the cells immediately after preparation of the fiber. It can be seen that the cells are in their own place. Figure 6B includes 3D images from different views. At the beginning of the co-culture, the three kinds of cells had similar densities and clear boundaries. Fluorescence images were taken after 1, 3, and 5 days of culture (Figure 6C). After 3 days of co-culture, the boundaries between the cells had completely disappeared, and the three types of cells had clearly mixed together. After 5 days, the tumor cells were so powerful that they gradually took over the positions of the other two types of cells. The HFL1 cells were difficult to observe. Figure 6D shows the 3D fluorescence images at day 5. At the same time, the HFL1 cells were difficult to observe. We suspect that the HFL1 cells were induced death by tumor cells. We present a 3D, multi-day co-culture model of three types of cells, which

Figure 6. Multicomponent microfiber type A-C/B-D for three kinds of cell co-culture. A) Images of NCI-H1650, HUVECs, and HFL1 cells in type A-C/B-D microfiber for initial cell co-culture. B) 3D fluorescence images of three kinds of cell co-culture in different views. C) Images of NCI-H1650, HUVECs, and HFL1 cells in type A-C/B-D microfiber for cell co-culture at 1, 3, and 5 days. D) 3D fluorescence images of three kinds of cell co-culture at 5 days. Green: HUVECs marked by Cell Tracker Green CMFDA. Red: NCI-H1650 cells marked by Cell Tracker Red CMTPX. Blue, HFL1 cells marked by Hoechst33342. Z-axis images show sections of microfiber scanning with a laser confocal microscope. Z-axis images correspond to the position of the yellow line.
facilitate the study of more cell-to-cell interactions, especially during tumor development.

3. Conclusions

We present a simple, multiple-laminar-flow microfluidic strategy for the fabrication of multicomponent heterogeneous CaA microfibers with tunable, cell loading, and biocompatibility features. The microfluidic spinning conditions were optimized to obtain two-component, three-component, and four-component microfibers with various arrangements, including type A, A-B, A-B-A, A-B-C, A/B, A-A-B, and A-C/B-D microfibers. By adding chitosan to the medium to keep the fibers stable, we encapsulated various kinds of cells into microfibers and achieved long-term 3D cell co-culture in vitro. Type A-C/B-D microfibers encapsulating three different types of cells were successfully prepared with membrane-sandwiched three-layer microchips to facilitate the successful construction of complex 3D tissues. This method is very simple and easy to operate and can continuously produce spatially well-defined heterogeneous microfibers. We therefore expect these microfibers to have important applications in composite functional biomaterials and may even have potential in organs-on-a-chip and biomimetic systems.

4. Experimental Section

Material: Sodium alginate (NaA) and calcium chloride dehydrate (CaCl₂·2H₂O) were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Chitosan (from shrimp shells, >75% deacetylated), acetic acid, isopropanol, paraformaldehyde, rhodamine 123 (Rh-123), rhodamine B (Rh-B), and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. PDMS (Sylgard 184) was purchased from Dow Corning. SU-8 (3035) photoresist and SU-8 photoresist developer were purchased from Microchem. Fetal bovine serum, Roswell Park Memorial Institute Medium 1640 (1640), and penicillin-streptomycin double antibody were purchased from Microchem. Fetal bovine serum (1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin) was used to culture cells in vitro. All cells were cultured in 1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin.

Cell Culture: Human non-small cell lung cancer cells (NCI-H1650), human fetal lung fibroblasts (HFL1), normal pulmonary bronchial epithelium cells (16HBE), and HUVECs were used in this study. The HUVECs were purchased from BeNa Culture Collection Co., Ltd. (ATCC source), and the other cells were purchased from the Cell Bank/Stem Cell Bank of the Chinese Academy of Sciences Cell (ATCC source). All cells were cultured in 1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin.

Cell Staining: The cells were labeled with fluorescent tracing dye for living cells according to the instructions. Cell Tracker Green CMFDA was used to label H1650 cells or HUVECs. Cell Tracker Red CMTPX was used to label NCI-H1650 cells, and Hoechst 33342 was used to label HFL1 cells. Calcein-Am/PI staining was also carried out to detect cell viability according to the instructions. Immunofluorescence staining was used to detect the EMT-related protein vimentin expression. After 14 days of cell co-culture, microfibers that contained the cells were washed three times in phosphate-buffered saline solution (PBS), fixed in 4% paraformaldehyde for 30 min at room temperature, and blocked with blocking buffer for 1 h to decrease nonspecific hybridization. The cells were then incubated overnight with anti-human vimentin primary antibody at a 1:100 dilution. The cells were washed in PBS and incubated with the secondary antibody. The cell nucleus was stained by DAPI. After sufficient washing in PBS, the fluorescence images were obtained with a laser scanning confocal microscope (Olympus FV3000).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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