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Layer-by-layer micromolding of natural biopolymer scaffolds with intrinsic microfluidic networks

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Abstract

A three-dimensional (3D) microfluidic network plays an important role in engineering thick organs. However, most of the existing methods are limited to mechanically robust synthetic biomaterials and only planar or simple microfluidic networks have been incorporated into soft natural biopolymers. Here we presented an automatic layer-by-layer micromolding strategy to reproducibly fabricate 3D microfluidic porous scaffolds directly from the aqueous solution of soft natural biopolymers. Process parameters such as the liquid volume for each layer and contact displacement were investigated to produce a structurally stable 3D microfluidic scaffold. Microscopic characterization demonstrated that the microfluidic channels were interconnected in 3D and successfully functioned as a convective pathway to transport a polymer solution. Endothelial cells grew relatively well in the porous microfluidic channels. It is envisioned that this method could provide an alternative way to reproducibly build complex 3D microfluidic networks into extracellular matrix-like scaffolds for the fabrication of soft vascularized organs.

1. Introduction

Native organs (e.g. liver, heart) commonly possess complex vascular systems, which consistently supply primary cells with nutrients and oxygen to maintain their viability and metabolic functions. Hence, it becomes extremely important to mimic such vascular networks in biomaterial scaffolds for the fabrication of thick vascularized organs in vitro, which has been confirmed by the recent progress in generating an artificial liver and lung using a decellularized organ matrix [1–4]. However, due to inherent complexity of natural vasculature, tissue engineers sought to incorporate microfluidic network into porous scaffolds or hydrogels to mimic the biological functions of native blood vessels, which was considered as an effective way to overcome diffusion limitations in generating large organs [5, 6].

Currently, various techniques have been developed to generate microfluidic channels in tissue engineered scaffolds. Rapid prototyping (RP) is one of the most widely used techniques to build three-dimensional (3D) microfluidic networks through a layer-by-layer manner. Kim et al [7] employed 3D printing to fabricate synthetic polymer scaffolds with interconnected channel networks and hepatocytes inside showing improved viability under dynamic culture conditions. Huang et al [8] utilized selective laser sintering to fabricate 3D porous scaffolds with branched flow-channel network, which was crucial for cell growth and function. In addition, microfabrication techniques (e.g. photolithography) have shown great potential in generating multiscale microvascular system with the resolution of capillary size [9–11].
Kaihara et al [12] employed silicon micromachining to engineer branched vascular channels for liver fabrication. Du et al [13] recently reported a sequential assembly strategy to fabricate 3D cell-laden hydrogels with vascular-like channels. However, most of these direct fabrication techniques are mainly limited to synthetic or specifically modified natural biopolymers, which are different from the extracellular matrix (ECM) of soft organs.

Micromolding techniques were thus employed to enhance the flexibility of these advanced techniques in fabricating microfluidic network in various biopolymers. Borenstein et al [14, 15] used micromolding to fabricate complex vascular network for solid organ engineering applications. Bettinger et al [16] developed a material-specific process to fabricate silk fibroin microfluidic devices for hepatocyte culture. Ryu et al [17] combined micromolding and solvent-vapor bonding to construct 3D multi-layer scaffolds with microfluidic channels and micro-porous structures. We previously combined micromolding and freeze drying to fabricate monolayer microfluidic porous scaffolds and then manually rolled or stacked these scaffolds together to form a 3D microfluidic structure [18–20]. However, the major limitation associated with these micromolding techniques is to assemble the micromolded planar structures into a stable 3D microfluidic construct in a controllable and reproducible manner.

Here we took advantage of the RP and micromolding techniques to present an automatic layer-by-layer micromolding process for the fabrication of 3D microfluidic porous scaffolds. Silk fibroin was chosen since it is one of the widely used natural biopolymers for tissue engineering scaffolds. Silk fibroin solution was prepared as previously described [21]. Briefly, *bombyx mori* raw silk (Yixian Textile Company, Hebei, China) was treated in 0.5 wt% boiled sodium bicarbonate solution and thoroughly washed with distilled water. The dried silk was dissolved in 9.3 M LiBr aqueous solution and then dialyzed against distilled water to obtain an optically clear solution. Triton X-100 (Sigma) was added so that the silk fibroin solution could automatically flow into the microstructures of the hydrophobic polydimethylsiloxane (PDMS) mold. The contents of silk fibroin and Triton X-100 in the final solution were adjusted at 5% (w/w) and 1% (v/v) respectively. All other reagents were local products without specific mention.

2. Materials and methods

2.1. Materials

The layer-by-layer micromolding process for 3D microfluidic porous scaffolds is shown in figure 1. Dried ice was placed into a metal container and distilled water was added in a flat PDMS mold. When the PDMS mold moved up to make the metal container contact the distilled water, the water would be rapidly frozen into an ice template. The PDMS mold moved down to detach the ice template. The silk fibroin solution was subsequently added into a microstructured PDMS mold. When the ice template was in contact with the silk fibroin solution, the surface of the ice template was slightly melted and rapidly re-frozen with the bottom of the silk fibroin solution together to form a stable bonding. By repeating this micromolding process in a layer-by-layer manner, a 3D microfluidic ice structure could be fabricated. To facilitate the detachment of the micromolded ice structure from the metal container, the first layer was always sacrificial ice template derived from distilled water, which functioned as a cold source to sequentially freeze the biopolymer solution and bond the micromolded ice structures in a layer-by-layer manner. The water in the biopolymer solution was frozen into microscale ice crystals. During the subsequent freeze-drying process, the
Figure 2. Development of a layer-by-layer micromolding system for the automatic assembly of 3D microfluidic scaffolds. (a) Computer-aided design of the layer-by-layer micromolding platform, (b) photograph of the developed layer-by-layer micromolding platform.

Figure 3. Computer-aided design of microfluidic network in micromolded scaffolds. (a) The scaffold layer with predefined microfluidic channels, (b) 3D microfluidic network in virtually assembled scaffold with an inlet and an outlet.

ice template, as well as the ice crystals, would sublimate and the micropores were produced in the microfluidic scaffolds.

To implement the abovementioned process accurately and reproducibly, an automatic layer-by-layer micromolding system was developed by modifying a commercial UV-based stereolithography (SL) system (UV SCPS350B, Shaanxi Hengtong Intelligent Machine Co., Ltd, China). As shown in figure 2(a), a four-channel syringe pump (TS-2A, Baoding Longer Precision Pump Co., Ltd, China) was fixed on the slider of the X-axis beam in the X-Y workbench. The metal container was mounted at the bottom of the X-axis beam in a push-pull manner, which aimed to facilitate the addition of dried ice. PDMS mold and the liquid container was mounted on the Z-axis elevator platform. Figure 2(b) shows the physical configuration of the automatic layer-by-layer micromolding system, which was controlled by a computer program. For the fabrication of each layer, the syringe pump firstly drew a certain amount of silk fibroin solution from the liquid container and then injected it into the PDMS mold. The metal container with dried ice moved overhead of the PDMS mold. The Z-axis elevator platform slowly moved up to make the metal container contact the solution in the PDMS mold. After the solution was completely frozen, the Z-axis elevator platform moved down to detach the ice template. A 3D frozen microfluidic structure was produced by repeating this process in a layer-by-layer manner.

2.3. Computer-aided design of a 3D microfluidic porous scaffold

A 3D microfluidic scaffold mainly consisted of scaffold layers and interface layers, which were designed in commercial software (Pro/Engineer, PTC, USA). Figure 3(a) shows the microfluidic channels designed in the scaffold layer. Culture media or blood was expected to enter the scaffold layer from the six peripheral veins, pass through the interconnected microfluidic channels and finally flow out from the central vein. Around the microfluidic channels, there were highly porous zones that aimed to facilitate nutrient and oxygen diffusion. The peripheral and central veins in each scaffold layer were spatially interconnected via vertical through holes. The interface layer was specifically designed to provide an inlet and an outlet for the whole 3D scaffold. Figure 3(b) shows the virtually assembled 3D scaffolds with predefined microfluidic network. The height of the 3D scaffold can be flexibly modulated by altering the number of scaffold layer.
2.4. Fabrication of microstructured PDMS molds

Since each scaffold layer was replicated from PDMS molds, the structural organizations within a final 3D scaffold were mainly dependent on the microstructures of the PDMS molds. In this study, PDMS molds were flexibly fabricated using a previously developed procedure [16]. Briefly, the computer models of the scaffold layer as well as the interface layer were loaded into the SL system to fabricate the resin molds. PDMS precursor was uniformly mixed with curing agent (mass ratio = 10:1) and cast onto the resin molds under vacuum conditions. After solidification, the resin molds were peeled off and the PDMS molds with negative patterns were obtained.

2.5. Fabrication of 3D microfluidic porous scaffolds

A 3D microfluidic scaffold was fabricated by automatically and sequentially stacking scaffold and interface layers, which were replicated from PDMS molds. The accurate replication of predefined microstructures from the PDMS mold to monolayer scaffold is important for the fabrication of 3D microfluidic channels. To evaluate replication precision, the microstructures of the scaffold layer, corresponding to the resin and PDMS molds, were characterized using laser confocal microscopy (OLS-4000, Olympus). The size of the predefined microfluidic channels was measured in ImageJ software.

During the layer-by-layer micromolding process, the reliable bonding of two neighbor layers is critical for the fabrication of an integral 3D microfluidic scaffold. As mentioned above, when the top frozen structure was in contact with the polymer solution added in the bottom PDMS mold, the surface of the top frozen layer would be slightly melted and then rapidly re-frozen together with the bottom polymer solutions to form a stable bonding. The micromolding and layer assembly were simultaneously completed in the same process. The bonding quality of two neighboring layers was mainly determined by the area of overlapped regions between the top frozen layer and the bottom polymer solution, which was subsequently affected by the liquid volume added into the PDMS mold for each layer as well as the contact displacement (shown in supplementary figure S1 available at stacks.iop.org/BF5/025002/mmedia). To investigate these parameters on bonding properties, 3D porous samples with five scaffold layers were fabricated under different liquid volumes (80 µl, 85 µl, 90 µl and 100 µl) and contact displacements (50 µm, 100 µm and 150 µm). The microscopic morphologies at bonding interfaces were observed with scanning electron microscopy (SEM, S-3000N, Hitachi). The tensile strength of the 3D micromolded scaffolds was measured in a tensile mechanical tester (CMT6503, MTS). The suitable liquid volume and contact displacement were determined for the fabrication of 3D scaffolds.

2.6. Characterization of internal microstructures in 3D micromolded scaffolds

The 3D micromolded scaffolds were sectioned in horizontal and vertical directions to expose internal microfluidic channels and porous structures, respectively. The microscopic images of the cross-sections were observed with SEM. To investigate the spatial interconnectivity of predefined microfluidic channels in 3D, the micromolded scaffolds were visualized with a micro-computerized topography (µCT, YXLON, Japan). Since pure silk fibroin porous scaffold exhibited negligible gray value under x-ray irradiation, it is difficult to directly observe the internal microstructures. To enhance the development of the porous scaffolds in µCT, a thermally crosslinkable polymer solution was injected into the 3D scaffolds from the inlet, flowed through the whole scaffold along the microfluidic network and finally flowed out from the outlet. The polymer solution could penetrate into the surrounding porous zones. After solidification, the polymer-infiltrated scaffold was characterized with µCT, which can virtually section the samples from top to bottom or from left to right to reveal internal microstructures.

2.7. Biocompatibility of the micromolded scaffolds for endothelial cell culture

Since Triton X-100 was added into the silk fibroin solution to facilitate microstructure replication, it is necessary to remove this surfactant from the micromolded scaffolds for future cell culture. To achieve this goal, the scaffold was first treated in methanol for 2 h to improve its water stability and then immersed in distilled water for 3 days. Distilled water was gently stirred with a magnetic bar and changed every 12 h. Triton X-100 was expected to dissolve into the distilled water.

To investigate the biocompatibility of the treated scaffolds, human umbilical vein endothelial cells were gently provided by the first affiliated hospital of Xi’an Jiaotong University in China and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM). The cells were trypsinized to obtain a cell suspension with cell density of $1.0 \times 10^6$ ml$^{-1}$. The scaffolds were sterilized with 75% ethanol and rinsed with culture medium five times. $100 \mu l$ endothelial cell suspension was seeded onto the microfluidic scaffolds. The scaffolds with methanol treatment but without water immersion were used as negative control. After cell attachment for 2 h, the cell-loaded scaffolds were transferred into a new 24-well culture plate with 1 ml fresh culture medium added. After being statically cultured for 2 days, the cell-scaffold constructs were retrieved and stained with Live/Dead assay to facilitate the visualization of cell morphology with a regular fluorescent microscopy (Nikon Ti-5, Japan).

3. Results

3.1. Characterization of monolayer microfluidic scaffolds

Figures 4(a)–(c) shows the macroscopic images of resin mold, PDMS mold and monolayer scaffold. It can be seen that the developed micromolding system could successfully transfer predefined microscale features from PDMS mold to monolayer silk fibroin scaffold. Since the fabrication processes such as biomaterial solution casting, monolayer micromolding
Figure 4. Fabrication precision of the presented micromolding technique for monolayer scaffolds. (a)–(c) Photographs of the microstructured resin mold, PDMS mold and micromolded scaffold, (d)–(f) 3D microscopic profiles of resin mold, PDMS mold and micromolded scaffold, (g)–(h) quantification of the channel size of resin mold, PDMS mold and micromolded scaffold.

3.2. Effect of liquid volume and contact displacement on bonding property of 3D scaffolds

The bonding of two neighbor layers was affected by the area of overlapped regions between the top frozen structure and the bottom biopolymer solution in the PDMS mold, which was regulated by liquid volume and contact displacement. The ideal liquid volume to fill the PDMS mold is about 80 μl. However, it was found that two neighboring layers were not fully bonded at the interface (shown in figure 5(a)), which was mainly caused by the uneven liquid levels. With the increase of liquid volume, the biopolymer solution could gradually enter the microfluidic channels of the top frozen layer, rapidly freeze and finally integrated with the top structure. After freeze-drying, the bonding of two scaffold layers was achieved as shown in figures 5(b)–(d). The tensile strength of the micromolded 3D microfluidic scaffolds was significantly improved from 3.0 ± 0.99 KPa to 35.4 ± 1.79 KPa (shown in figures 5(e)). When the liquid volume was over 90 μl, the tensile strength was not found to increase further. In addition, the through holes designed for peripheral and central veins in the scaffold...
layer were easily blocked, which would affect the spatial interconnectivity of predefined microfluidic network. To achieve relatively high tensile strength with smaller liquid volume, contact displacement was employed to promote interface integration. As shown in figures 6(a)–(c), when the liquid volume was kept constant at 80 μl while the contact displacement increased from 50 μm to 150 μm, the biopolymer solution in the bottom layer gradually penetrated into the microfluidic channels of the top layer, and the corresponding tensile strength was improved from 3.9 ± 1.00 KPa to 14.5 ± 5.60 KPa. Figures 6(d)–(f) show the interface morphologies between two scaffold layers when the liquid volume was 85 μl and the contact displacement increased from 50 μm to 150 μm. The tensile strength was improved from 17.3 ± 4.57 KPa to 30.4 ± 1.59 KPa. However, it was found that when the contact displacement was 150 μm, there was too much biopolymer solution in the bottom layer penetrated into the microfluidic channels of the top layer, which might affect fluid flow in the final 3D scaffolds. Together these results suggested that liquid volume of 85 μl and contact displacement of 100 μm should be chosen to produce a structurally robust and interconnected 3D microfluidic scaffold.

3.3. Characterization of internal microstructures in 3D micromolded scaffolds

Figures 7(a)–(b) show the microstructures of longitudinal sections within 3D micromolded scaffolds fabricated with liquid volume of 85 μl and contact displacement of 100 μm. The micromolded scaffolds were structurally stable and the predefined microfluidic channels were well fabricated. As shown in figure 7(c), the microporous structures were oriented in the longitudinal direction, which was mainly caused by the unidirectional freezing during the micromolding process. When the 3D scaffolds were transversely sectioned along the bonding interface, two distinguished zones were observed as shown in figure 7(d)–(e). The bonding zones were non-porous and mainly responsible for the connection of two neighbor layers. The microfluidic zones were highly porous and mainly responsible for cell attachment and nutrient transport (shown in figure 7(f)). It was interesting to find that the porous microfluidic zone in monolayer section was
optically transparent and could be clearly observed with regular inverted optical microscopes (figure 7(d)), which might facilitate cell morphology characterization.

To fully understand the interconnectivity of the predefined microfluidic network within 3D micromolded scaffolds, a thermally crosslinkable polymer, as a developing enhancer, was injected into the 3D scaffold through the microfluidic network and only the polymer penetrated area could develop in μCT. Figure 8(a) schematically shows ten typical sections to observe the polymer-penetrated scaffolds. Figure 8(b) illustrates the μCT images corresponding to five transverse sections (numbered with 1, 2, 3, 4 and 5) in the top-down direction. Figure 8(c) illustrates the μCT images corresponding to five longitudinal sections (numbered with 6, 7, 8, 9 and 10) in the left-right direction. The dark regions in these images indicate microfluidic channel zones or porous zones without polymer penetration while the bright white regions indicate polymer-penetrated zones. These results indicate that the predefined microfluidic network in 3D micromolded scaffolds was spatially interconnected and has successfully functioned as a fluid pathway to transport polymer solution through the whole porous scaffolds.

3.4. Biocompatibility of the micromolded scaffolds for endothelial cell culture

To test the biocompatibility of the micromolded scaffolds, endothelial cells were cultured in the microfluidic porous scaffolds with or without water immersion. As shown in figures 9(a)–(b), few living cells (indicating with green dots) were observed in the scaffolds without water immersion. Together with SEM observation (figure 9(c)) this indicated that the residual of Triton X-100 negatively affected cell attachment and growth. In comparison, endothelial cells grew well in the microfluidic channels when the scaffolds were pre-immersed in distilled water for 3 days (figures 9(d)–(e)), and the pores around the microfluidic channels were filled with cells as well as their ECM (figure 9(f)). This indicated that water immersion could effectively remove Triton X-100 from the micromolded scaffolds. In future studies, these microfluidic channels could be potentially used as a fluid pathway to seed cells, deliver nutrients and oxygen as well as facilitate endothelialization and vascularization.

4. Discussion

Microfluidic scaffolds or hydrogels have been considered as one of the most effective strategies to overcome diffusion limitations for the fabrication of thick vascularized organs [6, 22]. However, most of the existing methods to fabricate 3D complex microfluidic network were mainly limited to mechanically robust synthetic or specifically modified natural biopolymers [23, 24]. For natural biopolymers such as collagen, gelatin and silk fibroin, they are more similar to organ-specific matrix, but were prone to dissolve in water to form aqueous solutions and have poor processability. To build microfluidic network in these soft natural biopolymers, micromolding techniques were commonly employed but mainly limited to simple or planar channel structures [25, 26]. Although 3D scaffolds or hydrogels with relatively complex microfluidic network could be generated by stacking or rolling, less control over the manual process might decrease fabrication precision and reproducibility.

In the present study, we developed a layer-by-layer micromolding process to automatically and reproducibly
fabricate 3D tissue-engineered scaffolds with predefined microfluidic network by integrating layered fabrication principle of RP, micromolding and freeze drying techniques. Since the micromolding of monolayer structure as well as the bonding of layers relied on the freezing of the aqueous solution onto ice structures, it was theoretically applied to any aqueous solution of soft natural biopolymers. Relatively low operation temperature might prevent the bioactive molecules in the polymer solution from denaturing. Triton X-100 was added into the polymer solution to facilitate microstructure replication. However, cell culture experiments indicated that Triton X-100 negatively affected cell attachment and growth. Although water immersion could effectively remove Triton X-100 from the micromolded scaffolds, future efforts should focus on replacing Triton X-100 with other cell-friendly surface modification methods such as treating the PDMS mold with oxygen plasma for tiny microstructure replication.

Reliable bonding between layers is critical for the integrity of the micromolded 3D scaffolds. To achieve a reliable bonding of layers, the top frozen layer was slightly melted and rapidly re-frozen together with the bottom biopolymer solution. Liquid volume as well as contact displacement was found to regulate the bonding properties of 3D micromolded scaffolds. When the liquid volume was 85 μl and the contact displacement was 100 μm, a structurally stable 3D scaffold with interconnected microfluidic network could be automatically micromolded in a layer-by-layer manner, which effectively avoided the manual assembly-related issues like poor reproducibility and time consumption. For ideal micromolded or assembled scaffolds, there should be no visible bonding interface among layers.

Figure 8. Characterization of the interconnectivity of the 3D microfluidic network within micromolded scaffolds using μCT. (a) Strategy to characterize the polymer-penetrated 3D micromolded scaffolds using μCT, (b) typical μCT images corresponding to five transverse positions in the top-bottom direction, (c) typical μCT images corresponding to five longitudinal positions in the left-right direction.

Figure 9. Biocompatibility of the micromolded scaffolds for endothelial cell culture. (a)–(b) Fluorescent and (c) SEM images of endothelial cells cultured in the micromolded scaffolds without water immersion, (d)–(e) fluorescent and (f) SEM images of endothelial cells cultured in the micromolded scaffolds with 3-day water immersion.
Although the tensile strength of the micromolded 3D scaffold have been significantly improved by adjusting liquid volume and contact displacement, we still observed the bonding interface in the micromolded scaffolds, which might be eliminated by optimizing the process parameters such as improving the temperature of polymer liquid in the PDMS mold. Besides the predefined microfluidic network, the micromolded scaffolds possess highly porous structures, which were caused by the freeze-drying technique. Since the bottom biopolymer solution was unidirectionally frozen from the top frozen layer, it induced the formation of oriented ice crystals in the micromolded ice construct. When the ice crystals sublimated during the freeze-drying process, oriented porous structures in the longitudinal section were finally obtained in 3D microfluidic scaffolds, which might facilitate nutrient delivery and cell seeding [18]. In addition, the porosity as well as the pore size of the freeze-dried scaffold could be potentially modulated by adjusting the concentration of polymer solution, freezing rate and pre-freezing temperature [16].

A 3D microfluidic scaffold with variable cross-section morphologies can also be fabricated by using our layer-by-layer micromolding process, which was easily achieved by sequentially replicating the microstructures of different PDMS molds. μCT characterization indicated that the obtained microfluidic network, with an inlet and an outlet was interconnected in the 3D and successfully functioned as a convective pathway to transport a polymer solution. The microscale pores within the microchannels accelerated the penetration of the polymer solution to the surrounding porous zones, which enhanced the development of the porous scaffolds in μCT. To further understand the unique properties of 3D microfluidic networks in promoting mass transport as well as facilitating the formation of endothelialized network, co-culture of parenchymal cells with endothelial cells in the microfluidic scaffolds under dynamic culture conditions is required in future studies.

5. Conclusion

Here we presented an automatic layer-by-layer micromolding strategy to reproducibly fabricate 3D microfluidic porous scaffolds directly from the aqueous solutions of soft natural biopolymers. Fabrication precision and process parameters such as liquid volume and contact displacement were investigated to produce a structurally stable and well-defined 3D microfluidic scaffold. The microfluidic channels were found to be interconnected in 3D and successfully functioned as a convective pathway to transport a polymer solution. Endothelial cells grew well in the porous microfluidic channels when Triton X-100 was extracted by immersing the micromolded scaffolds in distilled water for 3 days. Future studies will focus on improving the biocompatibility of the layer-by-layer micromolding process (e.g., treating the PDMS mold with oxygen plasma instead of adding Triton X-100 for tiny microstructure replication) and conducting dynamic co-culture experiments on 3D microfluidic scaffolds. We envision that this method could be potentially used to build more complex, biomimetic 3D microfluidic networks in ECM-like biopolymers to facilitate nutrient delivery and vascularization.

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References

solvent vapor based bonding of micro-molded layers

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