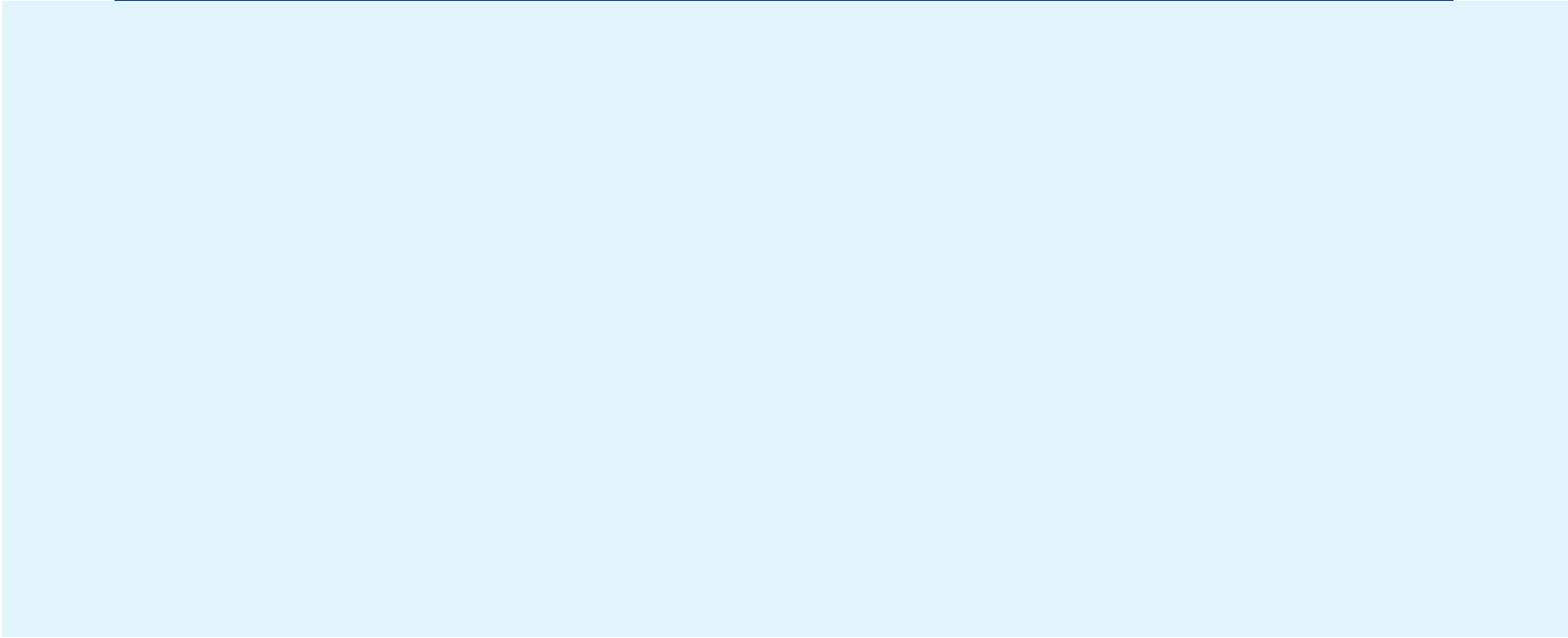


One-Step Generation of Aqueous-Droplet-Filled Hydrogel Fibers as Organoid Carriers Using an All-in-Water Microfluidic System

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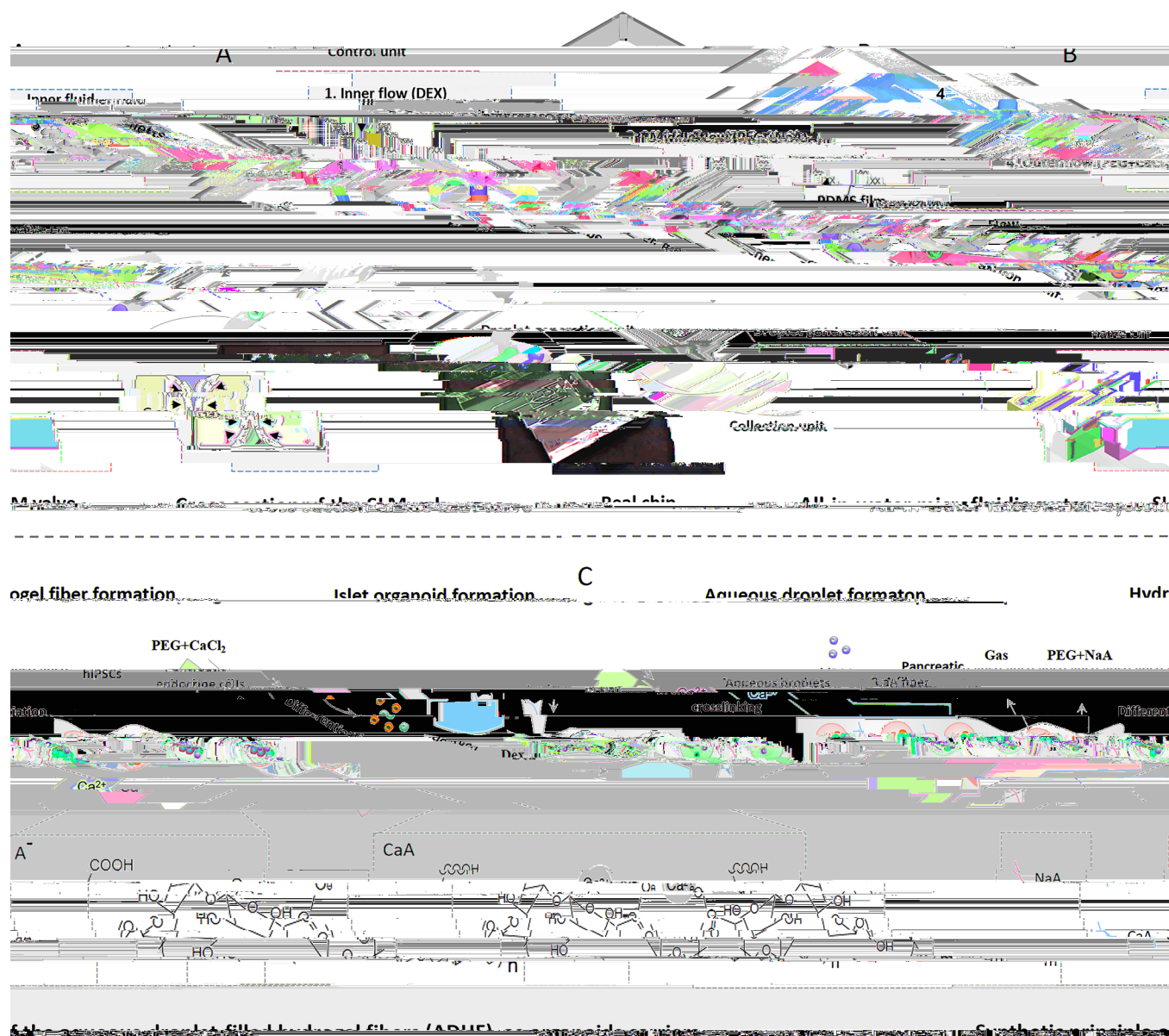


Figure 1. Schematic diagram of the all-in-water microfluidic system to generate aqueous-droplet hydrogel fiber (ADHF) that enables 3D culture of islet organoids. (A) Configuration of the microfluidic chip containing fluid inlets/outlet units, aqueous droplet generation unit, generation unit, and collection unit. (B) Cross section of the SLM (single-layer membrane) valve. (C) Procedures for generation of ADHF serving as carriers for engineering islet organoids derived from human induced pluripotent stem cell (hiPSC).

carrier for biomedical applications. Therefore, the generation of ADHF with high biocompatibility and good functionality is still a huge challenge. To overcome these challenges, the generation of ADHF within an oil-free system is needed. All-in-water microfluidic systems provide a unique oil-free art to meet this demand. To our knowledge, the generation of ADHF using an all-in-water microfluidic system has never been reported before.

Here, we provided a novel methodology to generate ADHF with aqueous droplets arrayed in calcium alginate (CaA) based on an all-in-water microfluidic system. A pneumatic valve is designed in the flow-focusing microfluidic chip to facilitate the production of ADHF, enhance the controllability, and improve the operability. Stable aqueous droplets are created using the auxiliary pump that separate the active phases of the dextran (Dex) and poly(ethylene glycol) (PEG) in the inner and middle flows followed by the fast reaction of ion

cross-linking between the sodium alginate (NaA) and calcium chloride (CaCl_2) within middle and outer fluids. The morphology of the ADHF, such as the size and interval of droplets, can be flexibly controlled by tuning the three phase flow rates and the valve switch cycle. To demonstrate the possibility of the ADHF as bio-carriers for the 3D culture of organoids, pancreatic endocrine progenitor cells from human induced pluripotent stem cells (hiPSCs) are encapsulated in the droplets of ADHF. The generated islet organoids exhibit good cell viability and high expression of islet-specific genes and sensitive glucose-stimulated insulin secretion (GSIS). The proposed all-in-water microfluidic approach is controllable and flexible, paving a new way to fabricate multifunctional bio-carriers for micro-tissues and organoid engineering.

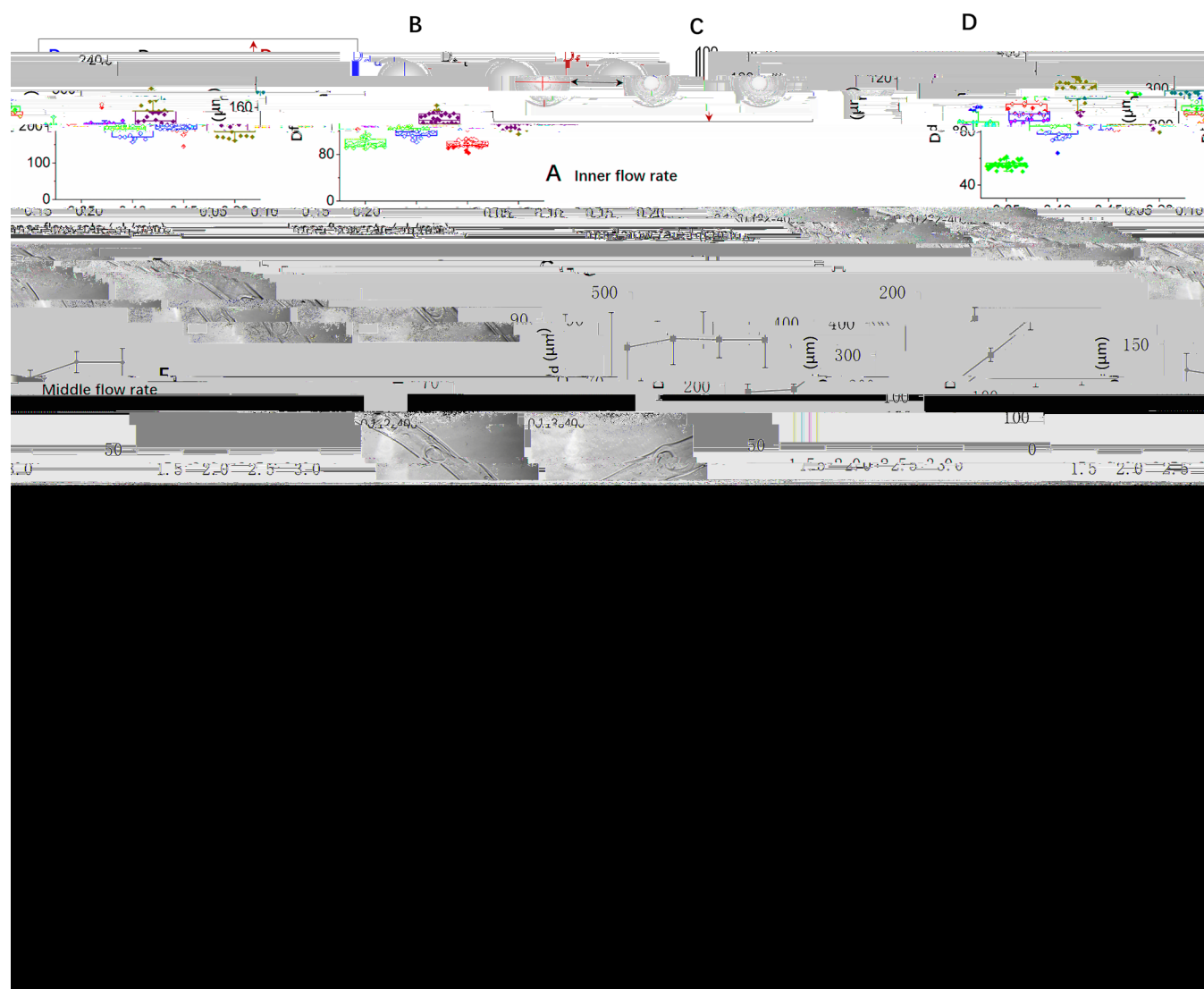


Figure 2. Effects of three-phase flow rates and the valve switch cycle on the droplet diameter (D_d), droplet distance (D_l), and droplet number (D_n). (A) Bright-field images of ADHF fabricated under variable rates of inner-phase flow (0.05 and 0.2 L/min). (B–D) Effects of the inner-phase flow rate on D_d , D_l , and D_n from 0.05 to 0.20 L/min. The middle-phase and the outer-phase flow rates were fixed to 2 and 40 L/min, respectively. (E) Bright-field images of ADHF generated under variable rates of middle-phase flow (1.5 and 3 L/min). (F–H) Effects of the middle-phase flow rate on D_d , D_l , and D_n from 1.5 to 3.0 L/min. The inner-phase and the outer-phase flow rates were fixed to 0.1 and 40 L/min, respectively. (I) Bright-field images of ADHF generated under variable rates of outer-phase flow (35 and 65 L/min). (J–L) Effects of the outer-phase flow rate on D_d , D_l , and D_n from 35 to 65 L/min. The inner-phase and the middle-phase flow rates were fixed to 0.1 and 2.5 L/min, respectively. (M) Bright-field images of ADHF generated under variable valve switch cycles (0.3 and 0.5 s). Effects of valve switch cycles on D_d , D_l , and D_n from 0.2 to 0.5 s. The inner-phase, middle-phase, and the outer-phase flow rates were fixed to 0.1, 2, and 40 L/min, respectively. * $p < 0.05$ and ** $p < 0.001$. Scale bars: 100 μm .

RESULTS AND DISCUSSION

In order to generate ADHF in a controllable and stable way, we designed and established a novel all-in-water microfluidic system. The proposed system combined the droplet microfluidic technology with the wet-spinning approach. As shown in Figure 1, the microfluidic device comprises a series of key units, including the inlet/outlet unit, aqueous droplet generation unit, oil droplet generation unit, and collection unit (Figure A). In the system, the aqueous two-phase solutions containing Dex and PEG served as templates for the generation of ADHF. Specifically, 15% (w/w) Dex solution, 17% PEG solution containing 1% (w/w) NaA, and 17% (w/w) aqueous droplet generation and increase the stability and controllability of arranging the droplets into the ADHF. The cross section of the SLM valve and the schematic diagram are shown in Figure B. The pneumatic valve facilitated the

Dex,⁴¹ the PEG in the middle phase has a better compatibility with the PDMS channel, which leads to a better coating during cell encapsulation. The key units of the device, including the aqueous droplet generation unit and oil droplet generation unit, are shown in Figure C. The “+” junction geometry of the aqueous droplet generation unit was used to form shear force between two immiscible liquids (Dex and PEG) for the generation of aqueous droplets. Meanwhile, pneumatic solenoid valves located beside the inner-phase and middle-phase inlets were integrated in the aqueous droplet generation unit, which was utilized to improve the operability of the droplet generation and increase the stability and controllability of arranging the droplets into the ADHF. The cross section of the SLM valve and the schematic diagram are shown in Figure B. The pneumatic valve facilitated the

generation of aqueous droplets, enhanced the controllability, and improved the operability^{Figure S1}. This enables the generation of an ADHF containing pre-encapsulated aqueous droplet arrays. In addition, compared to the traditional hydrogel spheres, the ADHF shows superior advantages in terms of the functions and potential applications. The ADHF can be served as uniform carriers with multiple compartments, which can be used to carry a variety of chemical components or cell spheroids and can be easily assembled into higher-ordered structures²³. These fibers can be used for the reconstruction of functional tissues with a fiber-shaped matrix, which can mimic the muscle tissue, blood vessels, or nerve networks²³. Also, in the respect of potential diabetes treatment, the ADHF can be scaled up in the longitudinal direction to a large capacity, which enables easy handling, implantation, and retrieval. This property can minimize the safety risks of transplant and can be rapidly retrieved⁹.

A spinning nozzle was designed at the outlet of the channel for the fiber fabrication. Meanwhile, a traditional dual-focusing structure was designed to manipulate the laminar flow of the middle-phase and outer-phase fluids, which can avoid the clogging of the microchannel induced by the reaction between Ca^{2+} and NaA. Finally, CaA hydrogels with good mechanical strength were formed in channels. The collection unit containing a 17% PEG solution was served to collect the ADHF. Under the supporting effect of the outer fiber, a delicate knotted structure can be realized. Here, the ADHF could serve as carriers for engineering islet organoids derived from human induced pluripotent stem cell (hiPSC). The synthetic principle is shown in Figure C. The aqueous droplets loaded in hydrogel fibers could improve the advantage of this approach in cell biological applications because of all the water environment and fiber structure. The ADHF well avoids the in

microscope and scanning electron microscope (SEM) were used to characterize the ADHF.

The fluorescent microscope was an expedient equipment to intuitively observe the morphology of transparent materials. A schematic diagram of ADHF formation via the all-in-water microfluidic system is shown in Figure 2A. To visualize the structure of ADHF, we added L4655 (green) and L3280 (red) fluorescent polystyrene nanoparticles in the inner phase and middle phase, respectively (Figure 2A). Here, we chose the valve switch cycles to visualize the distance of the droplets in ADHF. The results show that the droplets generated in this work were entire and continuous. The distance of droplets could be easily operated and separated in a controllable way, and corresponding changes can be performed (172.67 ± 7.43 to 468.85 ± 37.25 μm , Figure 2B). To vividly illustrate the well-defined spatial distribution, we showed the droplets' locations, respectively (Figure 2C). A distinct partition of droplets and beads was observed without cross-contamination between them. During the generation of the ADHF, NaA in the middle phase could not enter the inner phase (Figure S3). These results revealed that the ADHF could be due to the co-encapsulation of the diverse components in a controllable spatial arrangement, which could meet many potential requirements and exhibit multiple useful properties with flexible functionalities.

To reveal the droplet structure in the beads, the ADHFs were treated in the lyophilizer for freeze drying in situ, and then they were characterized by using SEM (Figure 2D). As shown in Figure 2D(I), the ADHF was intact, smooth, and continuous, demonstrating that our method could effectively

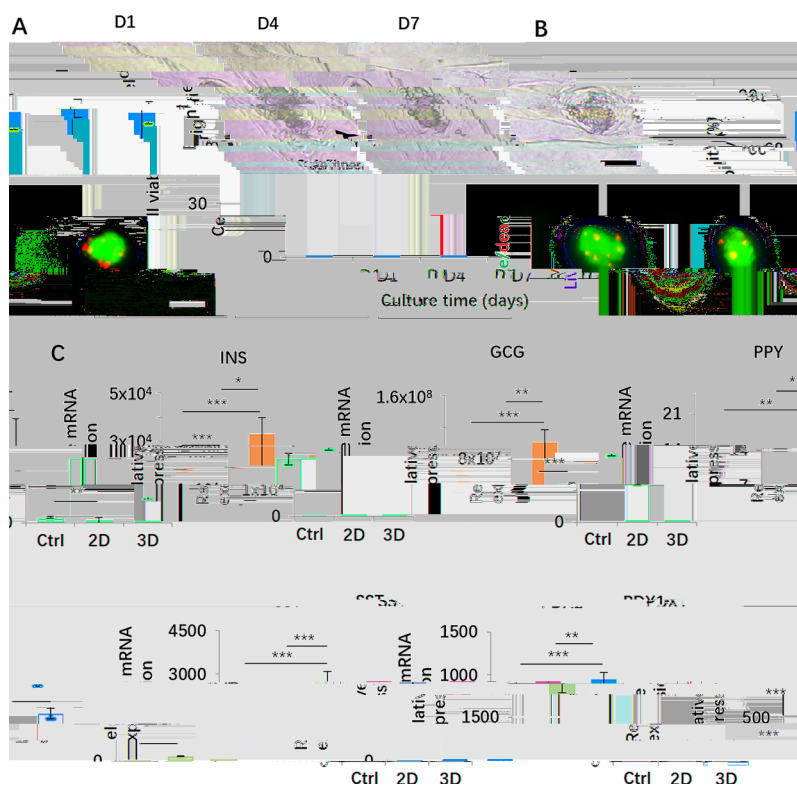


Figure 4. Encapsulation and generation of hiPSC-derived islet organoids in ADHF. (A) Representative brightfield and fluorescent images of the ADHF after encapsulating pancreatic endocrine progenitor cells for 1, 4, and 7 days. The green and red channels represent live and dead cells, respectively (valve cycle: 0.4 s; the inner-phase, middle-phase, and outer-phase were 0.1, 2, and 4 min, respectively). (B) Cell viability of islet organoids is detected at days 1, 4, and 7 in ADHF based on fluorescence quantitative analysis. (C) Comparison of the pancreatic maturity-related gene expression in the undifferentiated hiPSC (Ctrl), differentiated cells cultured in a 2D plate (2D) for 7 days, and differentiated organoids in 3D for 7 days (3D); the results were tested by PCR. The expression values are normalized to the GAPDH. 0.05, ** $p < 0.01$, and *** $p < 0.001$. Scale bars: 50 μ m.

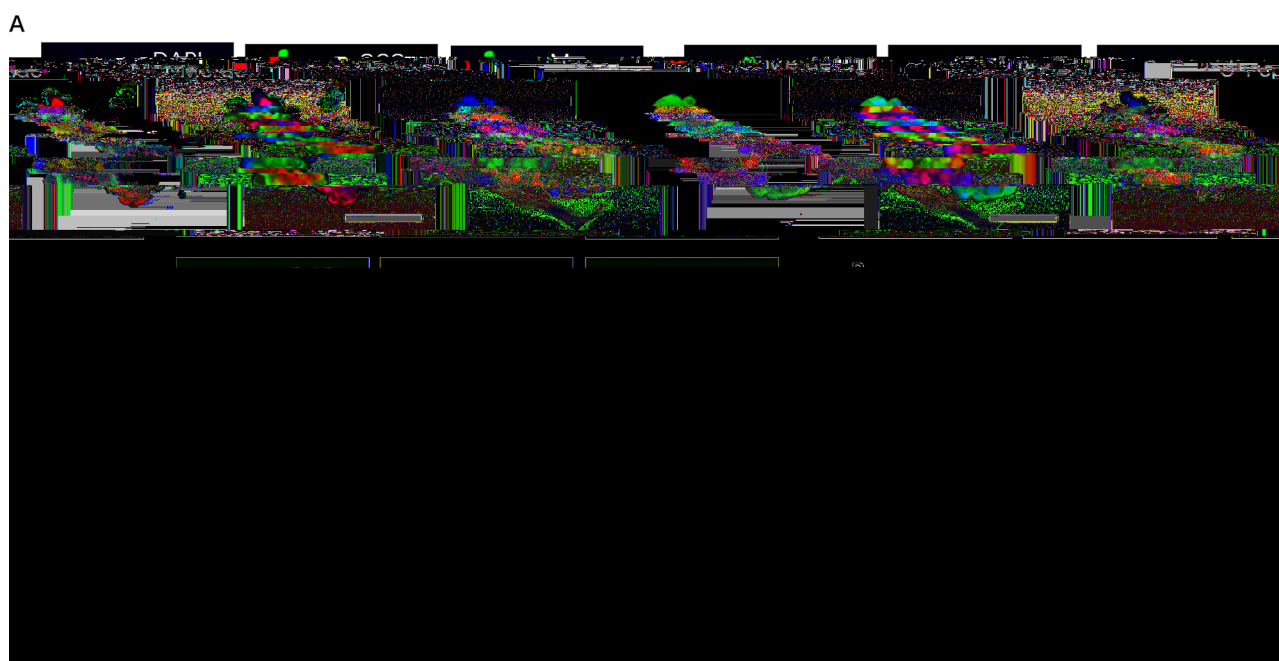


Figure 5. Biofunctions of islet organoids encapsulated in ADHF. (A) Immunofluorescent images of pancreatic endocrine hormone markers INS, GCG, PPY, and PDX1 in islet organoids at 7 days of culture. (B) Insulin secretion of islet organoids exposed to 2.5 and 25 mM glucose for 30 min of incubation within the ADHF. About 100 organoids were measured in each group using ELISA. Scale bars: 50 μ m.

the cells were apoptotic cells in islet organoids, exhibiting favorable cell activity. Also, the proliferation ability of the encapsulated islet organoids was tested using a CCK-8 assay kit during the culture period (Figure S4). To double evaluate the

cellular growth vitality, we measured the size of islet organoids after 7 days of differentiation, which showed a sensitive response to high-glucose stimulation. The islet organoids maintained an average diameter of 606.5 μm after 1 and 7 days of culture in ADHF. Compared to previously reported methods, our approach avoids a multi-step and tedious process in fabrication of cell spheroid-laden droplets. Furthermore, it has feasibility and operability to generate organoid-laden ADHF, which were formed in a flexible and convenient way. As above, the human islet organoids generated within ADHF display good viability and excellent islet-related functions, which prove that the ADHF is a suitable scaffold for the 3D culture of organoids with flexible operability and great biocompatibility. Also, the ADHF could reduce the hypoxia in large implantable scaffolds. These results demonstrated that our platform could generate stable and uniform organoids with controllable distances and sizes with fibers, which were useful for modeling the construction of organoids in vitro. In addition, we have partially addressed the existing challenge in DHF fabrication with an oil-free system. Without the encapsulated oil/water interface, the molecular diffusion and nutrient exchange in the fibers are obviously increased. Owing to the unique structures and proper biocompatibility of ADHF scaffolds, our method may provide an alternative way to translational therapy of diabetes by simultaneously loading growth factors and proteins with stem cells or organoids, which could be readily implanted into the human body.

CONCLUSIONS

To further verify the biocompatibility and potential of the ADHF as 3D scaffolds, we culture the islet organoids in 2D plates and 3D fibers for 7 days. Then, the differentiation and maturation of islet organoids were assessed by using the real-time PCR (Figure C). The results showed that the expressions of insulin secretion-associated marker insulin (INS), α -cell marker glucagon (GCG), pancreatic polypeptide (PPY), β -cell-associated pancreatic duodenal homeobox (PDX1), and somatostatin (SST) were very low in the undifferentiated hiPSCs. After 7 days of differentiation, the expressions of the above markers were upregulated in a 2D plate, which exhibited the feasibility of overdifferentiation protocols. As to the organoids in 3D fibers, the expression of the above islet-related genes further increased remarkably compared to that in the 2D culture. These results indicate the presence of pancreatic cells and β cells in islet organoids, proving the importance of ADHF on the differentiation and maturity of islet organoids under more physiological microenvironments.

To double check the differentiation of the islet organoids within ADHF, hormone-related markers (INS, GCG, PPY, and PDX1) were evaluated in islet organoids on day 7 as well (Figure A). The immunofluorescent results exhibited significant expression of hormone proteins in organoids, which further reveal the maturity and bio-functionality of islet organoids. As widely known, a vital functional trait of the islets (β -cells) is the ability to implement GSIS. We tested the insulin secretion of about 100 islet organoids within the ADHF in response to the treatments of low/high-glucose at days 1, 4, and 7, respectively (Figure B). The results show that the islet organoids could maintain insulin secretion up to at least 7 days. In addition, there was a significant increase in the insulin secretion in islet organoids with high glucose treatment (25 mM) at 7 days. These results indicated that the generated islet organoids within ADHF displayed the function of insulin secretion, and the islet organoids were more mature and

In this work, we presented a novel all-in-water microfluidic system for one-step generation of aqueous-droplet-laden hydrogel fiber with flexible properties and configurations. By integrating the pneumatic valve, the generation of ADHF could be facilitated with the improved controllability and the enhanced operability. Stable aqueous droplets are created based on the separation of active phases of the dextran and polyethylene glycol as well as the fast cross-linking of the sodium alginate and calcium chloride in the droplet-laden fibers. The ADHF produced in this work are entire, continuous, and repeatable. The size and the distance of the droplets within the ADHF can be flexibly controlled by tuning the flow rates and the valve switch cycle. The uniformly arranged droplets in fibers could increase the capability of the fibers and reduce the hypoxia in large implantable scaffolds. The ADHF with separated droplets can be facilely the co-encapsulation of the diverse components without cross-contamination in a controllable spatial arrangement. Furthermore, we prove that the ADHF fabricated in this work is a suitable scaffold for organoid generation. The hiPSC-derived islet organoids are formed in our ADHF, which exhibit good cell viability and maintain islet-related functions. The proposed all-in-water microfluidic system holds promise for fabrication of multifunctional scaffolds with good biocompatibility and permeability, which could be used to carry various cells, microtissues, and bio-factors.

EXPERIMENTAL SECTION

Materials. Dex (Mw = 500 kDa) and PEG (Mw = 20 kDa) were purchased from Sigma. NaA (55 cps) was bought from Qingdao Hyzlin Biology Development Co., Ltd. Anhydrous CaCl_2 and NaCl were purchased from Tianjin Damao Chemical Reagents Factory. Red (L3280) and green (L4655) fluorescent polystyrene nanoparticles with average sizes of 0.5 and 1 μm , respectively, were purchased from Sigma-Aldrich. FITC-NaA (R-F8801) was purchased from Reibio.

Micro uidic Chip Manufacture. The polydimethylsiloxane (PDMS) micro uidic chip was processed via conventional lithography as follows⁵¹. The chip is composed of two pieces of PDMS layers. The top layer with microchannels was templated from an SU-8 (Microchem, USA) mold and then was bonded to a blank PDMS layer undergoing oxygen plasma cleaning. The height of the channel is 200 μ m. The widths of the inner, middle, and outer channels and the gas channel are 100, 350, 200, and 200 μ m respectively. The width of inner ow channel located between the pneumatic valves is 50 μ m, and the width between the inner ow channel and valve chamber is 45 μ m.

ADHF Generation and Characterization. According to the existing reports^{36,40}, Dex and PEG were chosen in the all-in-water system due to good biocompatibility. The following reagents were injected into the micro uidic chip: (1) a Dex (15% (w/w)) solution was injected into the inner ow channel, (2) a mixed solution that contained PEG (17% (w/w)) and NaA (1% (w/w)) was injected into the middle ow channel, (3) a mixed solution that contained PEG (17% (w/w)) and CaCl₂ (4% (w/w)) was introduced into the outer

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Author Contributions

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Notes

The authors declare no competing financial interest.

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