Supporting Information

3D Biofabrication of Microfiber-Laden Minispheroids: A Facile 3D Cell Co-Culturing System

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1. Experimental Section

1.1. Bioink Preparation

Pure GelMA prepolymer solution was prepared by dissolving the freeze-dried GelMA (5% (w/v)) and lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP, 0.5% (w/v)) in Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (DPBS). Then, the solution was filtered through a 0.22μm filter for sterility and stored in 4℃ temperature. For GelMA mixed with sodium alginate, the sodium alginate powder was exposed to the UV light for 30min. Then, place the sterilized sodium alginate and some of the pure GelMA prepolymer solution into a bottle and stirred them with a magnetic mixer to form 5% GelMA mixed with 1% sodium alginate solution.

1.2. Establishing of Fabricating System

Preparation of Two-Layer Co-Axial Nozzle: To fabricate the microfiber-laden minispheroid, a two-layer co-axial nozzle was designed and fabricated. The co-axial nozzle was assembled by inserting a 27G needle (inner needle, OD:400μm; ID:210μm) into a 16G needle (outer needle, OD:1600μm; ID:1160μm) and the connections were fixed using soldering. Then, it was treated with nano-waterproofing spray reagent to ensure the surface hydrophobicity.

Establishment of the Fabricating System: The pure GelMA solution flowing in the outer nozzle and the GelMA mixed with sodium alginate flowing in the inner nozzle were fed by two syringe pumps. The co-axial nozzle was grounded. The metal ring electrode and the metal plate was connected with positive pole. A petri dish with silicon oil was placed on the metal plate as a receiver. The 405nm wavelength light was applied for the crosslinking of the structures.

1.3. Microfiber-Laden Minispheroid Formation Analysis

The fabricating environment was strictly kept as temperature of 30℃ and humidity of 50%. Two kinds of prepared bioink were added into the syringe respectively and fed by their own syringe pump. The frame rate of the high-speed camera was set at 100fps and the exposure time was 3227μs. We set three inner flowrates: 2μl/min, 10μl/min, 20μl/min and three outer flowrates: 50μl/min, 100μl/min, 150μl/min, to carry out orthogonal experiments under continuously changing voltage.

From the videos of the fabricating process under the high-speed camera, the heights

and widths of the droplets around the co-axial nozzle were measured with the ImageJ software and summarized in the X-Y lines. The ratios of the values of heights and widths were calculated and summarized in the scatter diagrams. Furthermore, the frequencies were recorded with the ImageJ software and summarized in the heat maps.

1.4. Effect of Fabricating Parameters on Structure Size

The environment was strictly kept as temperature of 30℃ and humidity of 50%. The diameters of the spheroid and fiber were measured under the microscopy. Here, we fabricated the microfiber-laden minispheroids under different parameters. We set three inner flowrates: 2μl/min, 10μl/min, 20μl/min, three outer flowrates: 50μl/min, 100μl/min, 150μl/min and six voltages: 0kV, 1kV, 2kV, 3kV, 4kV, 4.6kV, to carry out orthogonal experiments.

The morphologies of the structures were captured under the optical microscopy. The spheroid diameter data were summarized with means and SDs, respectively in the bar graphs. The fiber diameter data (measured ignoring two ends of the fiber) were summarized in the form of box-whisker plot. The middle line was the median of the fiber diameter. The two sides of the box were corresponding to the quartiles (Q1 and Q3) of the data. The two lines outside of the box were corresponding to the boundary of outliers (Q3＋1.5IQR and Q1－1.5IQR), the spots outside of which were the outliers of the data.

1.5 Characterization of The Material System

***Degradation Profile Analysis*:** The 5% GelMA and the 5% GelMA mixed with 1% sodium alginate bioink were added into a Ø9mm x 2mm mold and crosslinked respectively. Then, the samples were retrieved from the mold and immersed in the DPBS containing 2U/ml collagenase II in a 1.5ml centrifugal tube. The degradation time was set as 0h, 1h, 3h, 9h, 24h and three samples were utilized for each time. Then, they were placed in the incubator at 37 °C with 5% CO_2. At the planned time, three samples were taken out, removing all liquid in the centrifugal tubes. Then, they were placed into the -80 °C refrigerator. When all the samples were taken out, they were transferred into the vacuum drying machine at -80 °C temperature for 24h. Then, the masses of the dried samples were weighed, respectively. The data were presented as the mean ± SD of measured records.

***Analysis with Scanning Electron Microscopy (SEM)*:** In order to examine the surface and inner morphology of the microfiber-laden minispheroid, the fabricating structures, 5% GelMA cylinders (Ø6.39mm x 6.24mm) and 5% GelMA mixed with 1% uncrosslinked sodium alginate cylinders (Ø6.39mm x 6.24mm) were fabricated and dried in vacuum drying machine at -80 °C temperature for 24h. Then the samples were treated with a metal spraying machine for capturing under the scanning electron microscopy (SEM).

***Pore Area Analysis*:** The SEM images of the inner morphology of the two applied materials were imported into ImageJ software for pore analysis. The area frequency distribution and the normal distribution data were calculated. After that, the data were plotted as the form of distribution histogram and normal distribution curve.

***Testing of Stress-Strain Curve*:** The 5% GelMA and the 5% GelMA mixed with 1% sodium alginate were added into a Ø9mm x 6.3mm mold and crosslinked respectively. Then, the structures were retrieved from the mold and compressed under a stress-strain curve testing machine. The elasticity modulus was calculated with the stress value at 20% strain and summarized in the bar graph.

1.6. Cell Encapsulation

Cell Culturing: HUVEC, HUVEC (transfected by GFP), MDA-MB-231, MDA-MB-231 (transfected by mCherry) lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). The cell lines were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37℃. The medium was changed every two days. Both of the cell lines were passaged once they reached around 90% confluence.

Fabrication of Microfiber-Laden Minispheroids with cells: The HUVUCs and MDA-MB-231s were detached and resuspended in the prepared GelMA mixed with sodium alginate and pure GelMA bioink respectively to a concentration of $5x10^6$ cells/ml. Three kinds of microfiber-laden minispheroids, HUVECs in the fiber without MDA-MB-231s in the spheroid, MDA-MB-231s in the spheroid without HUVECs in the fiber, HUVECs (transfected by GFP) in the fiber with MDA-MB-231s (transfected by mCherry) in the spheroid, were fabricated with the high voltage electric field. After that, the structures and oil were transferred into the centrifugal tubes and centrifuged at the rate of 1000rpm for 5min for three times to separate the structures from silicon oil. At last, the structures were placed in the DMEM and

cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37℃.

***Cell Activity Analysis*:** Cell viabilities were measured after 1, 5 and 13 days of culturing with the structures encapsulated single type of the cells. The cell viabilities were tested with the cell LIVE/DEAD assay for 40min. After that, the confocal fluorescence microscopy was used to image the encapsulated cells by acquiring two images of each frame: green for live cells and red for dead cells, respectively. The live and dead cell quantifications were performed with ImageJ software and cell viabilities were calculated as the ratio of the number of live cells to the total number of cells.

***Cell Morphology Analysis*:** Co-culturing HUVECs (transfected by GFP) and MDA-MB-231s (transfected by mCherry) morphologies were observed after 1, 5 and 13 days of culturing to examine the cellular spreading inside the microfiber-laden minispheroids. The morphologies of the HUVECs and MDA-MB-231s within the structures were visualized by cell cytoskeleton dyeing, including actin and nucleus dyeing. we carried out actin and nucleus dyeing with TRITC phalloidin and DAPI dyeing solution. At last, the co-culturing structures were imaged with the confocal fluorescence microscopy.

All of the reagents, devices and testing services utilized in this paper were provided by Suzhou Intelligent Manufacturing Research Institute, Suzhou, China.

2. Additional Discussion

2.1. Analysis of Inception Phase of the Fabrication

At the beginning of the fabricating, the outer droplet could not provide enough space for the inner fluid. Thus, the inner fluid had to widen the diameter itself and bend upwards inside the droplet. This process could be complicated and worthy to be discussed as it influenced the formation of the structures. Here, the vertical heights of the inner fiber and outer droplet were analyzed.

Firstly, we assumed that at the beginning stage, the outer droplet was a spherical cap, the cross-section diameter of which was the same with the inner diameter of the outer nozzle (1160 μm). The heights of the outer droplet increased with time going until the height was equal to the half of the inner diameter of the outer nozzle (580 μm). Besides, the inner fiber was assumed to be extruded out of the inner nozzle with the same diameter with the inner diameter of the inner nozzle (210 μm) and in the vertical direction at first.

The volume of the spherical cap was calculated as below:

The equation of a circle was:

$$X^2 + Y^2 = R^2$$

$$X = \pm\sqrt{R^2 - Y^2}$$

where R was the radius of the circle.

And the volume of the spherical cap could be calculated as below:

$$V_{cap} = \int_{R-H}^{R} \pi X^2 dY = \int_{R-H}^{R} (R^2 - Y^2) dY$$

$$V_{cap} = \pi H^2 \left(R - \frac{H}{3}\right)$$

$$R^2 = (\frac{D}{2})^2 + (R - H)^2$$

$$V_{cap} = \frac{\pi H(3D^2 + 4H^2)}{24}$$

where H was the height of the spherical cap, D was the diameter of the cross section of the spherical cap.

In the fabrication process of the microfiber-laden minispheroid, the inner fluid, namely the GelMA mixed with sodium alginate, composed all of the volume of the fiber:

$$V_{in} = V_{fiber}$$

$$q_{in} \cdot t = \frac{\pi H_{in} {D_{inI}}^2}{4}$$

$$H_{in}(t, H_{in}) = 0$$

where V_{in} was the liquid volume from the inner nozzle, V_{fiber} was the volume of the fiber structure, q_{in} was the inner flowrate, t was the duration of the fabricating process, H_{in} was the vertical height of the fiber, D_{inI} was the inner diameter of the inner diameter (210 μm).

For the outer droplet, both the inner and outer fluids consisted the total volume of the spherical cap:

$$V_{out} + V_{in} = V_{cap}$$

$$q_{out} \cdot t + q_{in} \cdot t = \frac{\pi H_{out}(3{D_{outI}}^2 + 4{H_{out}}^2)}{24}$$

$$H_{out}(t, H_{out}) = 0$$

where V_{out} was the liquid volume from the outer nozzle, V_{cap} was the volume of the spherical cap structure, q_{out} was the outer flowrate, H_{out} was the vertical height of the

spherical cap, D_{outI} was the inner diameter of the outer diameter (1160 μm).

Using the calculus and derivation above, the calculation models of inner and outer vertical height in the inception phase of the fabrication were established. We got two implicit functions to be applied to analyze the height differences between inner and outer channels. The relationships between H_{out}(H_{in}) and t were as below:

$$H_{out} = H_{out}(t)$$
$$H_{in} = H_{in}(t)$$

With the MATLAB software, we plotted these two functions with numerical calculation. The inner flowrates were set as 2 μL/min, 10 μL/min, 20 μL/min. The outer flowrates were set as 50 μL/min, 100 μL/min, 150 μL/min. The plotting figure was displayed as Figure S1-A.

From the numerical calculation results, we could find that the flowrate differences would have big effect on the formation of the structures. On the one hand, when the inner flowrate was set as 2 μL/min, the vertical height of the outer spherical cap was obviously higher than the one of the inner fiber all the time. In this situation, the inner fiber tended to be attenuated by the outer liquid. (Figure S1-Bi) On the other hand, when the inner flowrate was set as 10 μL/min or 20 μL/min, the changing rates of the inner fiber could be much faster than the outer spherical cap. Thus, inner liquid had to explore more space to place itself, resulting from accumulating around the nozzle or bend upwards inside the outer droplet. (Figure S1-Bii)

From the numerical analysis, we could conclude that the inner flowrate couldn't be set too high according to the outer flowrate. Inner fluid with too high flowrate would influence the fabrication effect of the fiber-laden shape, even accumulated to be an accumulated block rather than a fiber at the inception phase of the fabrication.

3. Supporting Figures

3.1. The Analysis of Inception Phase of the Fabrication.

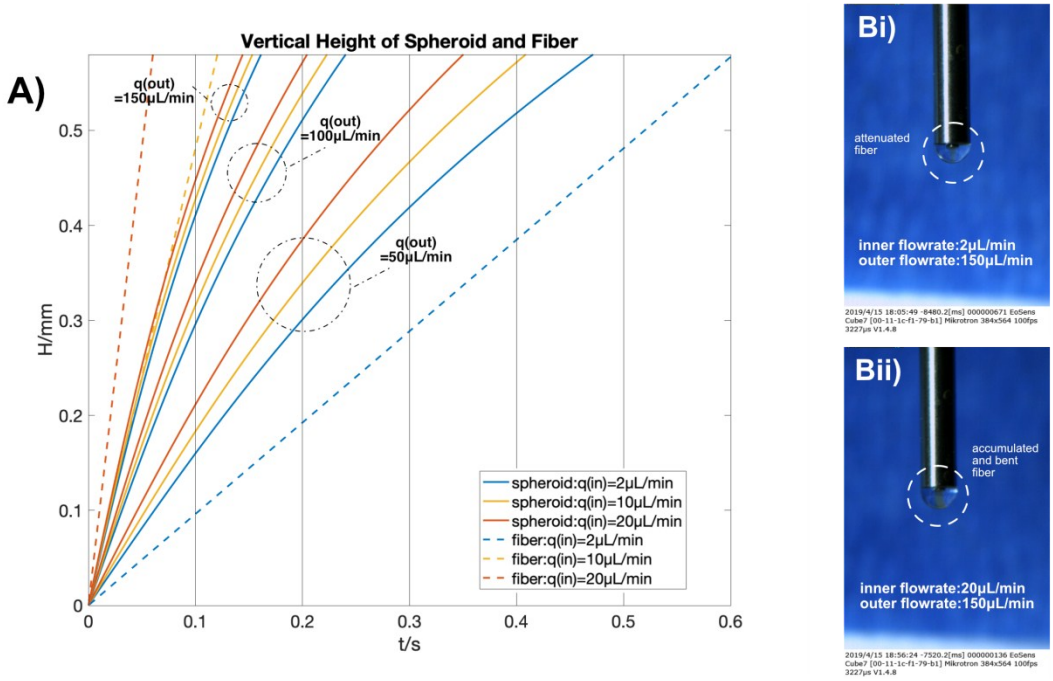

Figure S1. The Analysis of Inception Phase of the Fabrication. A) Vertical Height of spheroid and fiber. The numerical calculation was carried out with the MATLAB software. The inner flowrates were set as 2 μL/min, 10 μL/min, 20 μL/min. The outer flowrates were set as 50 μL/min, 100 μL/min, 150 μL/min. The circle in the figure presented a group data with the same outer flowrate. B) The images under high speed camera with different ratios of the inner and outer flowrates.

3.2. Images of Different Fabricating Stages Recorded by High-speed Camera

In order to observe the different fabricating stages, a high-speed camera was utilized to record the fabricating process. The frame rate of the high-speed camera was set at 100fps and the exposure time was 3227μs.

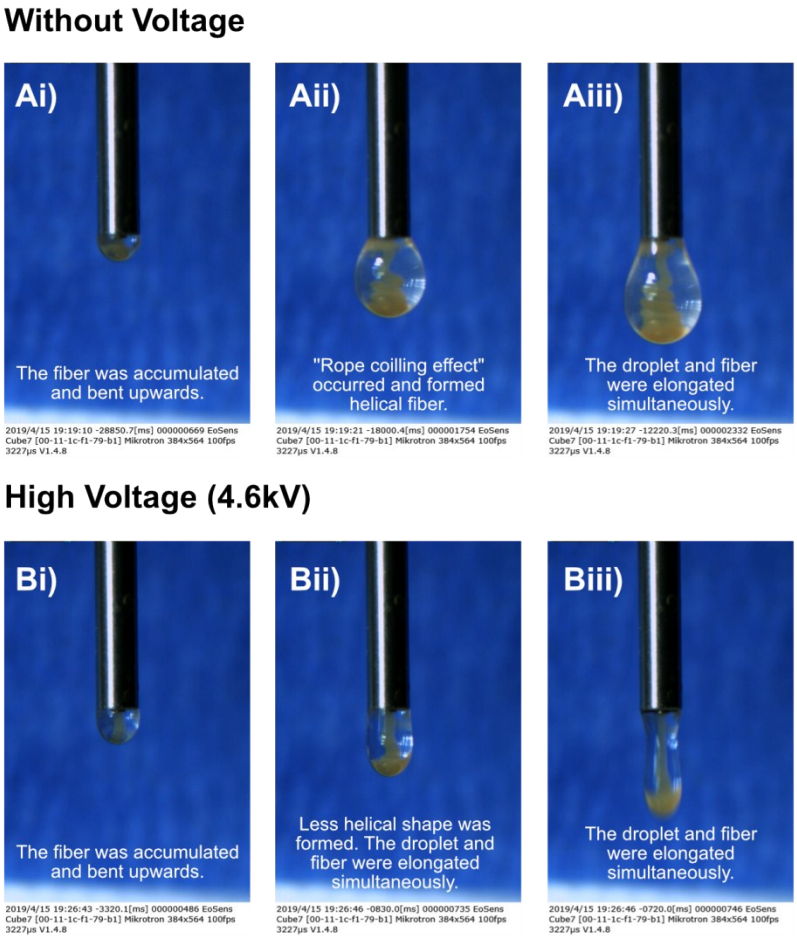

Figure S2. The Images of Different Fabricating Stages. The inner flowrate was 20 μL/min. The out flowrate was 50 μL/min. A) Without voltage. B) With high voltage (4.6kV).

3.3. Optical Morphology of the Materials

Under the optical microscopy, we could find that the pure crosslinked 5% GelMA surface looked plain. By contrast, the crosslinked 5% GelMA with 1% uncrosslinked sodium alginate owned many visible pores.

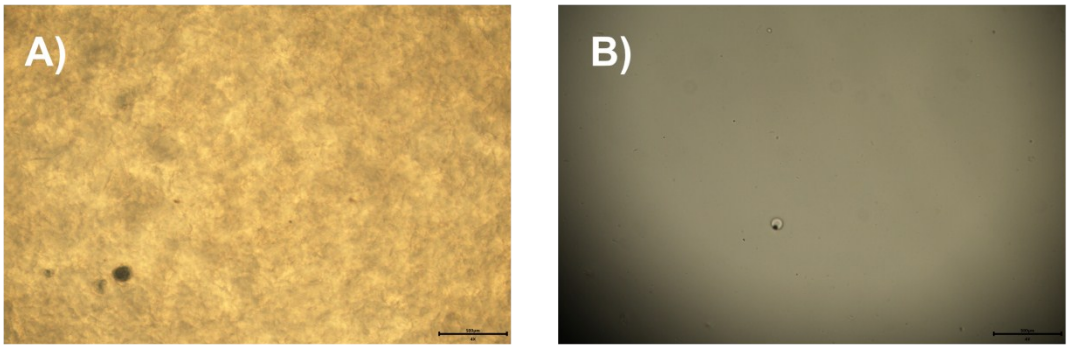

Figure S3. Optical Morphology of the Materials. A) The crosslinked 5% GelMA with 1% uncrosslinked sodium alginate. B) the pure crosslinked 5% GelMA.

3.4. The Testing of Degradation Profile of the Materials

The material degradation experiment was carried out. The samples were fabricated by the syringe mold (φ9mm x 2mm). Bioink was subjected to the 405nm wavelength light.

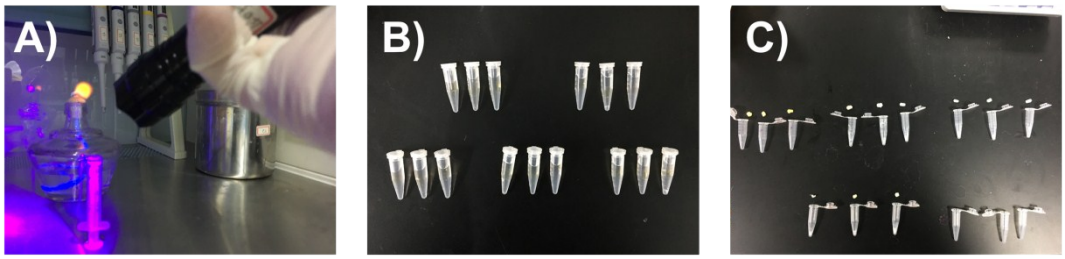

Figure S4. The Testing of Degradation Profile of the Materials. A) The fabrication of the tested samples. B) The samples immersed in the 1.5ml centrifugal tubes with 1ml DPBS containing 2U/ml collagenase II. C) The treated samples after freeze-dried process whose massed were weighed by the balance.

3.5. The Actin and Nucleus Morphology of MDA-MB-231s Cultured Alone

The MDA-MB-231s were encapsulated in the microfiber-laden minispheroids and cultured for 13 days. The cells could spread during the culturing.

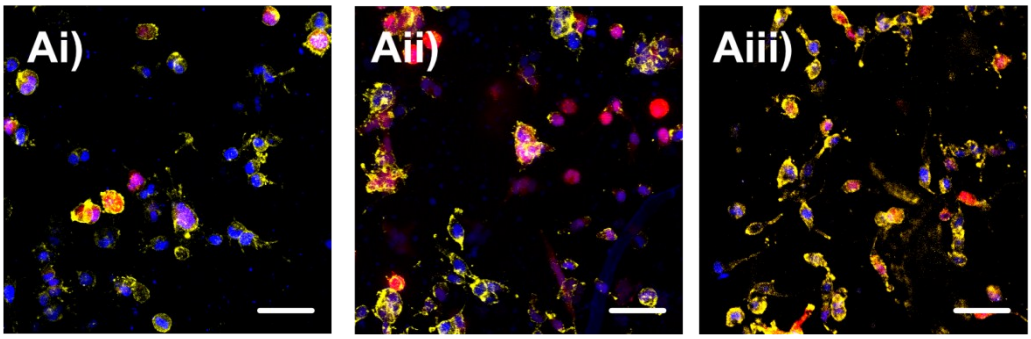

Figure S5. The Actin and Nucleus Morphology of MDA-MB-231s Cultured Alone. A) The general images. Scalebar=500μm. i) Day 1. ii) Day 5. iii) Day 13.

3.6. The Actin and Nucleus Morphology of HUVECs Cultured Alone

The HUVECs were encapsulated in the microfiber-laden minispheroids and cultured for 13 days. The cells could spread during the culturing.

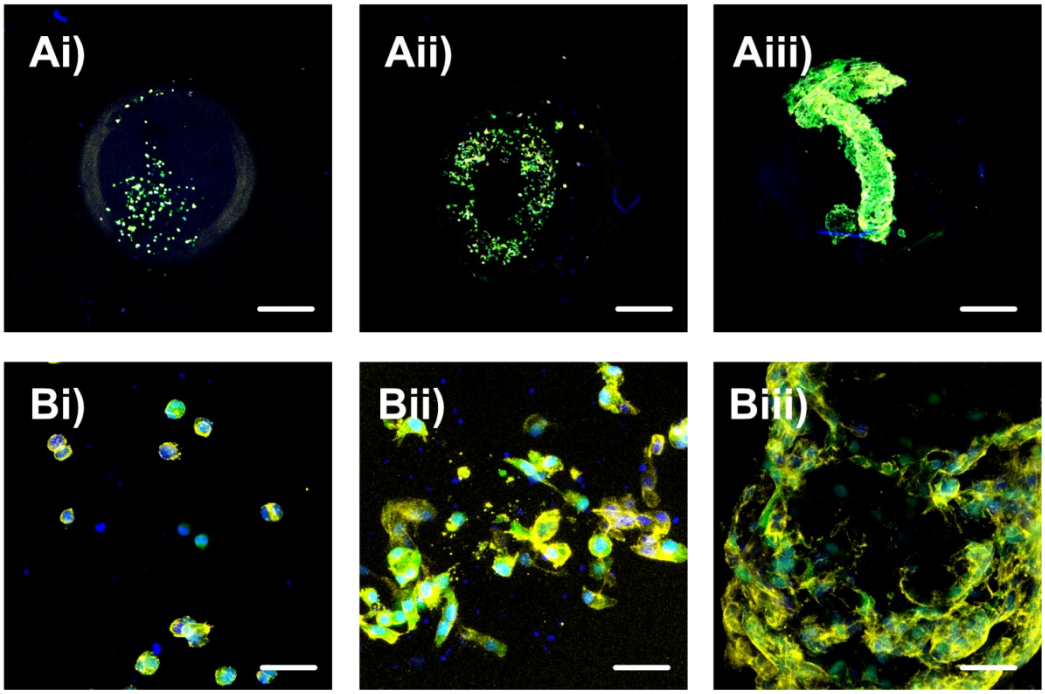

Figure S6. The Actin and Nucleus Morphology of HUVECs Cultured Alone. A) The general images. Scalebar=500μm. i) Day 1. ii) Day 5. iii) Day 13. B) The detailed images. Scalebar=50μm. i) Day 1. ii) Day 5. iii) Day 13.

3.7. Images of the Structures Fabricated with Different Parameters

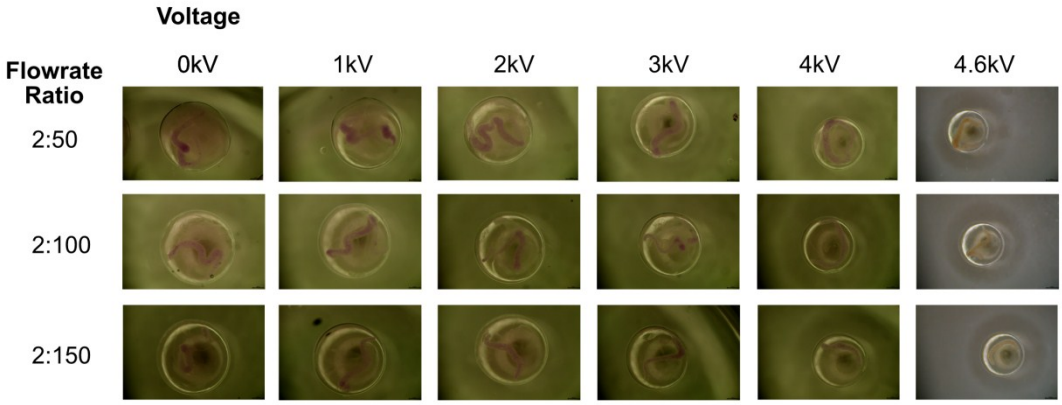

Figure S7. Images of the Structures Fabricated with Different Parameters.

3.8. The CD31 Express of the Encapsulated HUVECs

The HUVECs were encapsulated in the microfiber-laden minispheroids and cultured for 13 days. The CD31 express was tested and captured with the confocal fluorescence microscopy.

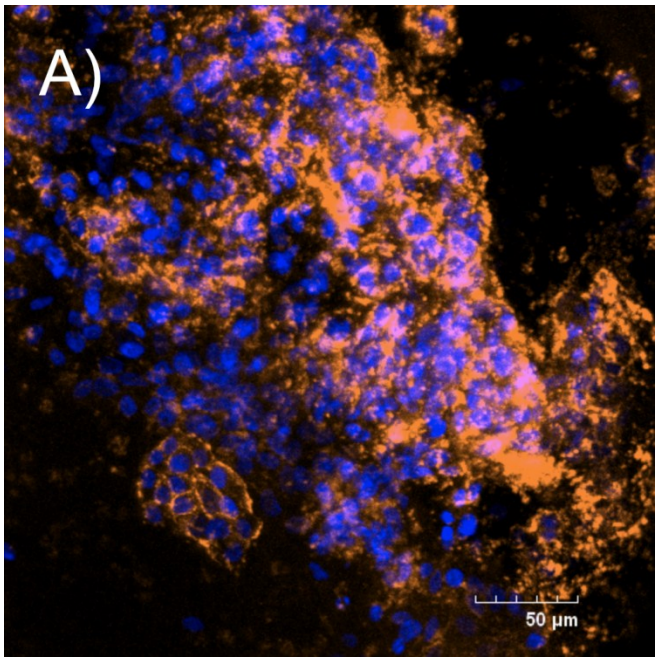

Figure S8. The CD31 Express of the Encapsulated HUVECs.

3.9. Longer-term LIVE/DEAD Test of the MDA-MB-231s And HUVECs

The MDA-MB-231s and HUVECs were encapsulated in the minispheroids and

microfibers respectively and cultured for 18 days. The LIVE/DEAD was tested and captured with the confocal fluorescence microscopy. The results showed high viabilities in 18-day culturing in this co-culturing system.

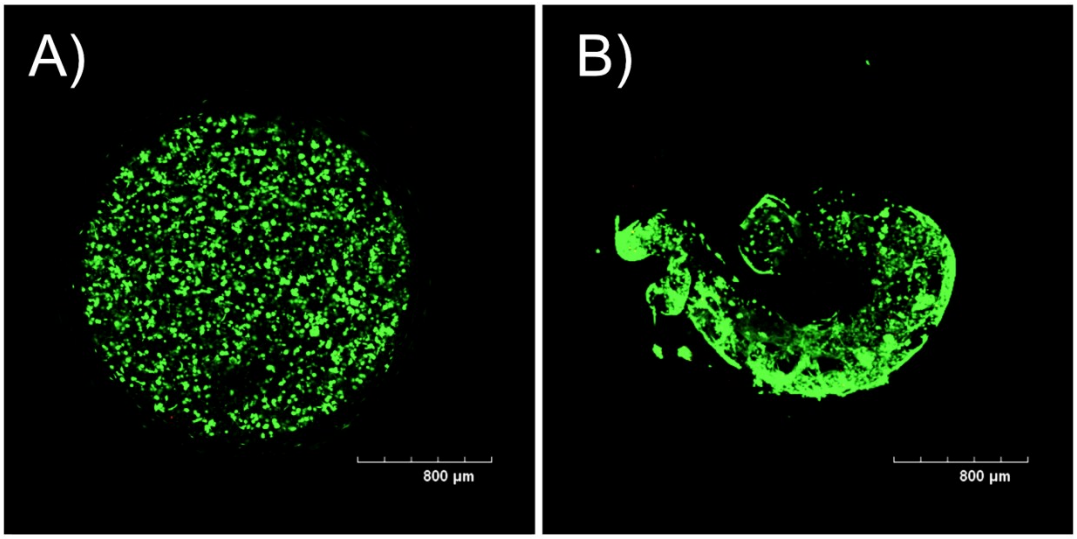

Figure S9. LIVE/DEAD Test of the MDA-MB-231s And HUVECs on the 18th day. A) MDA-MB-231s in the minispheroids. B) HUVECs in the microfibers

3.10. Rheological Property of Bioink

The rheological property of the applied bioink was tested with rheometer. The temperature was set as 30℃. The range of shear rate was set from $0.1s^{-1}$ ~ $500\ s^{-1}$. The testing duration was set as 60s. The cell density was $5x10^6$ cells/ml.

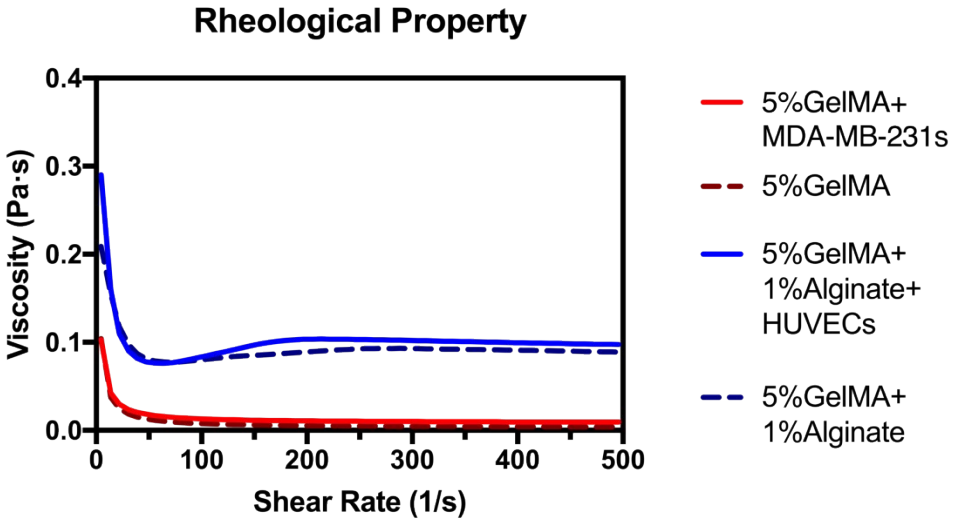

Figure S10. Rheological Property of Bioink