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Three-Dimensional Bioprinting of Gelatin Methacryloyl Hydrogel

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TITLE:

Three-Dimensional Bioprinting of Gelatin Methacryloyl Hydrogel

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KEYWORDS:

3D bioprinting, gelatin methacryloyl, GelMA, microsphere, microfiber, digital light processing, DLP, microfluidic chip

SUMMARY:

Presented here is a method for the 3D bioprinting of gelatin methacryloyl.

ABSTRACT:

Gelatin methacryloyl (GelMA) has become a popular biomaterial in the field of bioprinting. The derivation of this material is gelatin, which is hydrolyzed from mammal collagen. Thus, the arginine-glycine-aspartic acid (RGD) sequences and target motifs of matrix metalloproteinase (MMP) remain on the molecular chains, which help achieve cell attachment and degradation. Furthermore, formation properties of GelMA are versatile. The methacrylamide groups allow a material to become rapidly crosslinked under light irradiation in the presence of a photoinitiator. Therefore, it makes great sense to establish suitable methods for synthesizing three-dimensional (3D) structures with this promising material. However, its low viscosity restricts GelMA's printability. Presented here are methods to carry out 3D bioprinting of GelMA hydrogels, namely the fabrication of GelMA microspheres,

GelMA fibers, GelMA complex structures, and GelMA-based microfluidic chips. The resulting structures and biocompatibility of the materials as well as the printing methods are discussed. It is believed that this protocol may serve as a bridge between previously applied biomaterials and GelMA as well as contribute to the establishment of GelMA-based 3D architectures for biomedical applications.

INTRODUCTION:

Hydrogels are thought to be a suitable material in the field of biofabrication[1-4]. Among them, gelatin methacryloyl (GelMA) has become one of the most versatile biomaterials, initially proposed in 2000 by Van Den Bulcke et al.[5]. GelMA is synthesized by the direct reaction of gelatin with methacrylic anhydride (MA). The gelatin, which is hydrolyzed by the mammal collagen, is composed of target motifs of matrix metalloproteinase (MMP). Thus, in vitro three-dimensional (3D) tissue models established by GelMA can ideally mimic the interactions between cells and extracellular matrix (ECM) in vivo. Furthermore, arginine-glycine-aspartic acid (RGD) sequences, which are absent in some other hydrogels such as alginates, remain on the molecular chains of GelMA. This makes it possible to realize the attachment of encapsulated cells inside the hydrogel networks[6]. Additionally, the formation capability of GelMA is promising. The methacrylamide groups on the GelMA molecular chains react with the photoinitiator under mild reaction conditions and form covalent bonds upon exposure to light irradiation. Therefore, the printed structures can be rapidly crosslinked to maintain the designed shapes in a simple way.

Based on these properties, a series of fields utilize GelMA to carry out various applications, such as tissue engineering, basic cytology analysis, drug screening, and biosensing. Accordingly, various fabrication strategies have been also demonstrated[7-14]. However, it is still challenging to carry out 3D bioprinting based on GelMA, which is due to its fundamental properties. GelMA is a temperature-sensitive material. During the printing process, the temperature of the printing atmosphere has to be strictly controlled in order to maintain the physical state of the bioink. Besides, the viscosity of GelMA is generally lower than other common hydrogels (i.e., alginate, chitosan, hyaluronic acid, etc.). However, other obstacles are faced when building 3D architectures with this material[15].

This article summarizes several approaches for the 3D bioprinting of GelMA proposed by our lab and describes the printed samples (i.e., the synthesis of GelMA microspheres, GelMA fibers, GelMA complex structures, and GelMA-based microfluidic chips). Each method has specialized functions and can be adopted in different situations with different requirements. GelMA microspheres are generated by an electroassisted module, which forms extra external electric force to shrink the droplet size. In terms of GelMA fibers, they are extruded by a coaxial bioprinting nozzle with the help of viscous sodium alginate. In addition, the establishment of complex 3D structures is achieved with a digital light processing (DLP) bioprinter. Finally, a twice crosslinking strategy is proposed to build GelMA-based microfluidic chips, combining GelMA hydrogel and traditional microfluidic chips. It is believed that this protocol is a significant summary of the GelMA bioprinting strategies used in our lab and may inspire other researchers in relative fields.

PROTOCOL:

1. Cell culturing

1.1. Prepare Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, used to culture human breast cancer cell (MDA-MB-231) lines and human umbilical vein endothelial cell (HUVEC) lines.

1.2. Prepare DMEM with L-glutamine (DMEM/F-12), supplemented with 10% FBS and 1% penicillin/streptomycin, used to culture bone marrow mesenchymal stem cell (BMSC) lines.

1.3. Set the culturing environment as 37 °C and 5% CO_2. Culture MDA-MB-231, HUVEC, and BMSC, and passage the cells in a 1:2 ratio when 90% confluence is reached.

2. Fabrication of GelMA microspheres

2.1. Print the fixture as **Figure 1A** with polylactic acid (PLA) on a fused deposition modeling (FDM) printer. Place two metal ring electrodes in the fixture.

2.2. Connect the two metal ring electrodes with ground and positive poles, respectively. Place the metal plate connected with the high voltage below the ring electrode and place a Petri dish with silicon oil on the metal plate as a droplet receiver.

2.3. Dissolve freeze-dried GelMA (5% w/v) and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, 0.5% w/v) in Dulbecco's phosphate-buffered saline (DPBS) as the bioink (10 mL). Filter the bioink through a 0.22 µm filter for sterility and heat it in a 37 °C water bath for 15 min.

2.4. Detach MDA-MB-231 cells with 3 mL of 0.25% trypsin-0.02% EDTA solution for 3 min at 37 °C. Centrifuge cells in a 15 mL centrifugal tube at 100 x *g* for 5 min to obtain a cell pellet.

2.5. Remove the supernatant. Mix the cell pellet with 1 mL of prepared bioink by slowly pipetting it to prevent the production of bubbles.

2.6. Place 1 mL of bioink (MDA-MB-231) into a 3 mL sterile syringe. Feed the bioink by the force of compressed air (~0.5 kPa). Put the syringe on the fixture.

NOTE: The printing environment should be strictly controlled at a temperature of 30 °C and humidity of 50%.

2.7. Switch on the high voltage power and set the voltage as 0–4 kV. Simultaneously, turn on the 405 nm wavelength light to crosslink the GelMA droplets in 5 mL of silicon oil.

2.8. Pour the most part of the silicon oil away by decanting the Petri dish. Transfer the remained silicon oil and the GelMA microspheres into a 15 mL centrifugal tube using a spoon.

2.9. Add 5 mL of DPBS and shake the mixture uniformly. Centrifuge the tube at 100 x *g* for 5 min and remove the supernatant fluid.

2.10. Repeat step 2.9 3x.

2.11. Take out the GelMA microspheres with a spoon and culture them in DMEM in a Petri dish at 37 °C and 5% CO_2 for 3 days.

2.12. Discard the medium and wash the microspheres with DPBS. Fix with 2 mL of 4% paraformaldehyde (PFA) for 30 min at room temperature (RT).

2.13. Discard the PFA and wash the microspheres with DPBS. Permeabilize with 2 mL of 0.5% nonionic surfactant (i.e., Triton X-100) for 5 min at RT.

2.14. Discard the nonionic surfactant and wash the microspheres with DPBS. Stain them with 2 mL of tetramethylrhodamine (TRITC) phalloidin for 30 min in darkness at RT.

2.15. Discard the TRITC and wash the microspheres with DPBS. Stain them with 2 mL of 4',6-diamidino-2-phenylindole (DAPI) for 10 min in darkness at RT.

2.16. Discard the DAPI and wash the microspheres with DPBS. Capture the morphology with a confocal fluorescence microscope.

3. Fabrication of GelMA fibers

3.1. Prepare a coaxial nozzle as shown in **Figure 2A**. Fix an inner nozzle (25 G, OD = 510 µm, ID = 250 µm) and outer nozzle (18 G, OD = 1200 µm, ID = 900 µm) with soldering. Connect a glass tube (length = 50 mm, inside diameter = 1.2 mm) to the end of the coaxial nozzle.

3.2. Dissolve sodium alginate (Na-Alg) powder that is sterilized under ultraviolet (UV) light for 30 min in deionized water at 2% (w/v).

3.3. Prepare a sterile bioink solution following step 2.3. Heat the GelMA bioink and Na-Alg solution in a 37 °C water bath for 15 min.

3.4. Detach BMSCs cells with 3 mL of 0.25% trypsin-0.02% EDTA solution for 3 min at 37 °C. Centrifuge cells in a 15 mL centrifugal tube at 100 x *g* for 5 min to obtain a cell pellet.

3.5. Remove the supernatant fluid. Mix the cell pellet with 2 mL of prepared GelMA bioink by slowly pipetting it to prevent the production of bubbles.

3.6. Place 2 mL of bioink (BMSCs) into a 10 mL syringe. Place 2 mL of Na-Alg solution into another syringe (10 mL). Feed them with two syringe pumps, respectively (here, bioink at 50 µm/min and Na-Alg solution at 350 µm/min).

NOTE: The printing environment should be strictly controlled at a temperature of 30 °C and humidity of 50%.

3.7. Turn on the 405 nm wavelength light to irradiate the transparent tube to crosslink the GelMA fibers. Use a Petri dish with DPBS to receive the fibers.

3.8. Take out the GelMA fibers with a spoon from DPBS and culture them for 3 days in the prepared DMEM/F-12 in 37 °C and 5% CO_2.

3.9. Follow steps 2.12–2.16 to prepare the GelMA fibers for morphological observation with a confocal fluorescence microscope.

4. Fabrication of complex 3D GelMA structures

NOTE: **Figure 3A** shows the fabrication sketch of the complex 3D GelMA structures.

4.1. Wipe the DLP bioprinter (**Figure 3E**) with 75% alcohol and expose it to the UV irradiation for 30 min for sterility.

4.2. Dissolve the freeze-dried GelMA (10% w/v) and LAP (0.5% w/v) in DPBS. Add magenta edible pigment into the solution (3% v/v) to improve the printing accuracy.

4.3. Filter the solution through a 0.22 µm filter for sterility and heat it in a 37 °C water bath for 15 min.

4.4. Build the 3D models with computer-aided design (CAD) software. Import the model documents to the upper software (EFL) of the applied DLP bioprinter.

4.5. Add 10 mL of prepared bioink into the trough of the DLP bioprinter.

4.6. Set the printing parameters in the upper software as follows: light intensity = 12 mW/cm^2, irradiation duration = 30 s, and slice height = 100 µm. Start printing.

4.7. Remove the printed structure from the bioprinter and immerse it in DPBS in a Petri dish.

4.8. Detach the MDA-MB-231s cells with 3 mL of 0.25% trypsin-0.02% EDTA solution for 3 min at 37 °C. Centrifuge cells at 100 x *g* for 5 min in a 15 mL tube to obtain a cell pellet.

4.9. Remove the supernatant fluid and mix the cell pellet with 2 mL of DMEM.

4.10. Add 100 µL of cell suspension on the printed structures. Culture them for 3 days in the prepared DMEM at 37 °C and 5% CO_2.

4.11. Follow steps 2.12−2.16 to prepare the complex 3D structures for morphological observation with a confocal fluorescence microscope.

5. Fabrication of GelMA-based microfluidic chips

NOTE: **Figure 4A** shows the fabrication sketch of the GelMA-based microfluidic chip.

5.1. Dissolve the freeze-dried GelMA 10% (w/v) and LAP (0.5% w/v) in DPBS. Filter the GelMA solution through a 0.22 µm filter for sterility.

5.2. Sterilize the gelatin powder under UV light for 30 min and add it to the GelMA-LAP solution prepared in step 5.1 to a final concentration of gelatin of 5% (w/v). Heat the mixture in a 37 °C water bath for 15 min.

5.3. Design a group of molds (**Figure 4B,C**) with CAD software and manufacture them with photopolymer resin on a DLP printer.

5.4. Fill the molds fully with the prepared bioink.

5.5. Put the molds into a 4 °C refrigerator to crosslink the gelatin for 30 min.

5.6. Remove the molds and demold with a blade the partially (physically) crosslinked hydrogel sheets from the molds.

5.7. Combine the two demolded hydrogel sheets and bond them with the help of GelMA by irradiating at 405 nm for 1 min.

5.8. Detach the HUVECs cells with 3 mL of 0.25% trypsin-0.02% EDTA solution for 3 min at 37 °C. Centrifuge cells in a 15 mL centrifugal tube to obtain a cell pellet at 100 x *g* for 5 min.

5.9. Remove the supernatant fluid and mix the cell pellet with 2 mL of DMEM.

5.10. Fill the microchannel fully by injecting the cell suspension with a nozzle and syringe.

5.11. Flip the chip upside down every 15 min during the next 3 h to achieve uniform and complete cell seeding. Culture the chips in the Petri dish for 3 days in the prepared DMEM at 37 °C and 5% CO_2.

5.12. Follow steps 2.12−2.16 to prepare the microfluidic chips for morphological observation with a confocal fluorescence microscope.

REPRESENTATIVE RESULTS:

During the fabrication of GelMA microspheres, the GelMA droplets were separated by the external electric field force. When the droplets fell into the receiving silicon oil, they remained standard spheroid shape without tails. This is because the GelMA droplets were in an aqueous phase, while the silicon oil was in an oil phase. The surface tension that formed between the two phases caused the GelMA droplets to maintain a standard spheroid shape. In terms of the cell-laden microspheres, cells experienced the high voltage electric field force in this process. From the morphology of the stained MDA-MB-231s (**Figure 1B–E**), it was found that the encapsulated MDA-MB-231s maintained its spreading capability, verifying the biocompatibility of this electroassisted fabrication method.

In terms of the GelMA fibers, GelMA and sodium alginate solution flowed in the inner and outer nozzles of the coaxial nozzle, respectively. As the sodium alginate had higher viscosity than GelMA, GelMA was restricted in the sodium alginate solution and maintained a line shape. The irradiation by light (405 nm wavelength) caused the inner GelMA to become crosslinked, forming the GelMA fibers (**Figure 2B**). Besides, BMSCs were encapsulated in the GelMA fibers (**Figure 2C,D**). As shown, the encapsulated BMSCs maintained spreading capability in the GelMA hydrogel networks after the fabrication process (**Figure 2E**).

A DLP bioprinter was chosen to fabricate GelMA structures with more complex shapes. As shown in **Figure 3B–D**, the structures of “nose”, “ear”, and “multichamber” were established. On the surface of the crosslinked GelMA structures, the seeded HUVECs attached to the GelMA materials and spread (**Figure 3F**). This demonstrated the possibility that the establishment of GelMA complex 3D structures with the help of a DLP bioprinter holds great potential in applications in the field of tissue engineering.

Unlike the traditional microfluidic chip that is based on materials without biodegradation properties[16-20] (i.e, resin, glass, polydimethylsiloxane [PDMS], and polymethyl methacrylate [PMMA]), a GelMA-based microfluidic chip was fabricated here using a twice cross-linking strategy. Two components in the bioink were crosslinked successively. Chips with various microchannels were built by designing different molds on demand (**Figure 4B,C**). Besides, it was verified that HUVECs were seeded in the channels and attached to the channel wall, forming the macroscopic vessel shape (**Figure 4D,E**).

FIGURE LEGENDS:

Figure 1: GelMA microspheres. (**A**) Fabrication sketch of the GelMA microspheres. (**B**) Optical microscope image of the GelMA microspheres. (**C**) Optical microscope image of the MDA-MB-231s in GelMA. (**D**) 2D view of the F-actin and nucleus of the encapsulated MDA-MB-231s. (**E**) 3D view of the F-actin and nucleus of the encapsulated MDA-MB-231s.

Figure 2: GelMA fibers. (**A**) Fabrication sketch of the GelMA fibers. (**B**) Optical microscope image of the GelMA fibers (with blue ink). (**C**) Confocal fluorescence microscope image of the GelMA fibers (with green fluorescence particles). (**D**) Optical microscope image of the BMSCs in GelMA fibers. (**E**) The F-actin and nucleus of the encapsulated BMSCs.

Figure 3: GelMA complex 3D structures. (**A**) Fabrication sketch of the complex GelMA 3D structures. (**B**) Optical microscope image of the GelMA "nose". (**C**) Optical microscope image of the GelMA "ear". (**D**) Optical microscope image of the GelMA "multichamber". (**E**) The applied DLP bioprinter. (**F**) The F-actin and nucleus of the seeded MDA-MB-231s.

Figure 4: GelMA-based microfluidic chip. (**A**) Fabrication sketch of the GelMA-based microfluidic chip. (**B**,**C**) Optical microscope images of the GelMA-based microfluidic chip. (**D**) Optical microscope image of the seeded HUVECs on the channel wall. (**E**) The F-actin and nucleus of the seeded HUVECs on the channel wall.

DISCUSSION:

This article describes several strategies to fabricate GelMA 3D structures, namely GelMA microspheres, GelMA fibers, GelMA complex structures, and GelMA-based microfluidic chips. GelMA has promising biocompatibility and formation capability and is widely used in the field of biofabrication. Microsphere structures are suitable for controlled drug release, tissue culturing, and injection into organisms for further therapy[21-25] . Because the viscosity of GelMA solution is low, its formation is challenging. Thus, during the fabrication of the GelMA microspheres, the electrohydrodynamic (EHD) principle was chosen to solve this problem. The voltage applied was relatively low, and the microdroplets were generated one-by-one. To fabricate microspheres of a smaller size, the applied voltage can be increased, and the fluid would be in another state with the Taylor cone[26].

Because of the Coulomb explosion phenomenon, the dropping droplets were further separated by their excessive electric density, resulting in smaller GelMA microspheres. Furthermore, monocomponent GelMA fibers were fabricated with the help of a coaxial nozzle and sodium alginate solution. A coaxial nozzle was applied here. As mentioned above, because of the low viscosity of GelMA, sodium alginate provided resistance to help maintain the shape of fiber. Hydrogel fiber structures are suitable for mimicking the fiber-shaped tissues in vivo (i.e., muscles, vessels, etc.[27-32]). For GelMA fibers with more complicated components, the applied bioprinting nozzle can be further modified. For example, a three-layer coaxial nozzle can be assembled to generate multilayer GelMA fibers.

In the establishment of complex GelMA 3D structures, it was found that the DLP bioprinter breaks through the printing obstacle caused by the low viscosity of GelMA. With the help of CAD software, GelMA 3D structures were fabricated on demand. Finally, a new GelMA fabrication method, the twice cross-linking strategy, was demonstrated and applied to the combination of GelMA and a traditional microfluidic chip. The hydrogels have higher biocompatibility, and researchers can encapsulate cells inside the chip body. The proposed GelMA-based microfluidic chip can be further improved by encapsulating cells in the chips to serve as suitable models in vitro for drug screening, cellular interaction studies, etc. We believe that the methods for fabrication of GelMA described here will increase the rate of development in this field and can be applied in further biomedical research.

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DISCLOSURES:

The authors have nothing to disclose.

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FIG1

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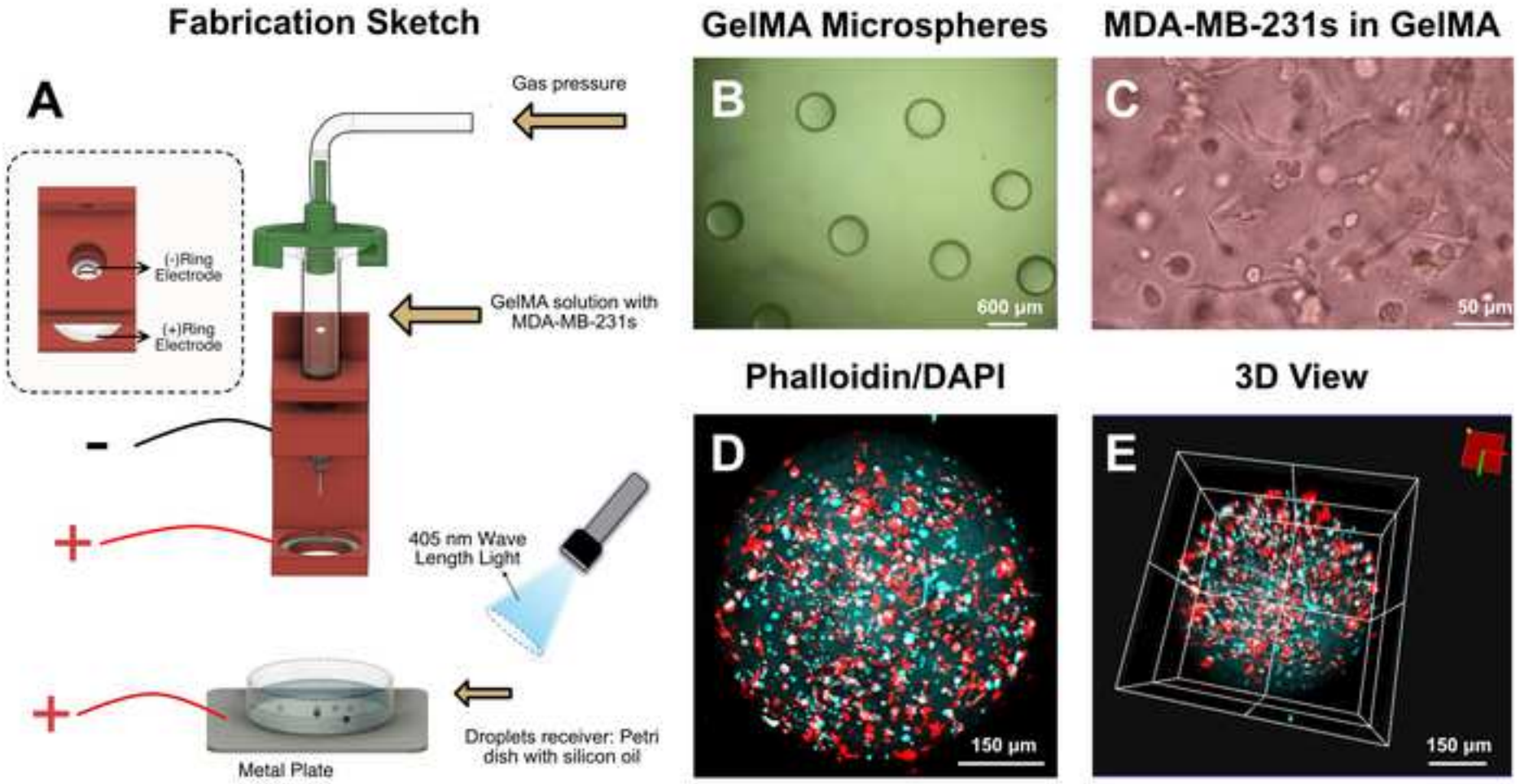

FIG2

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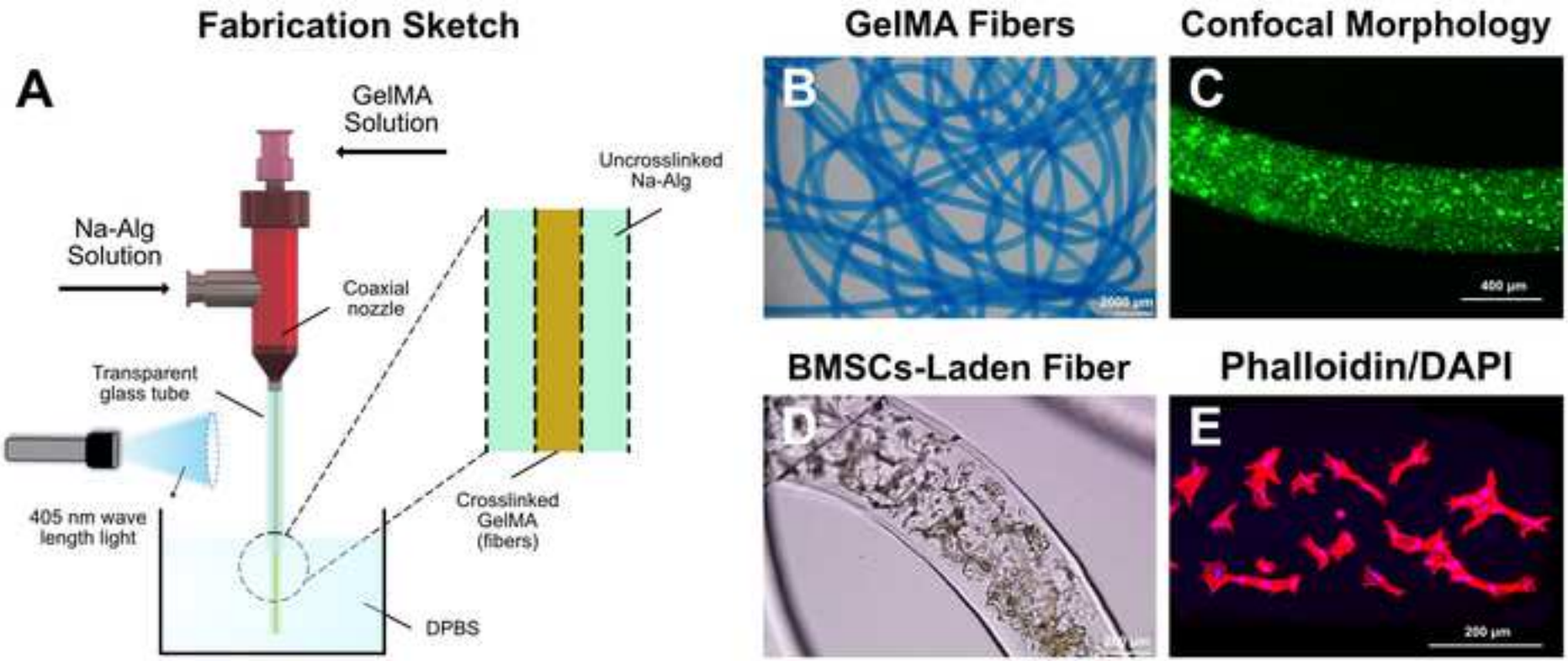

FIG3

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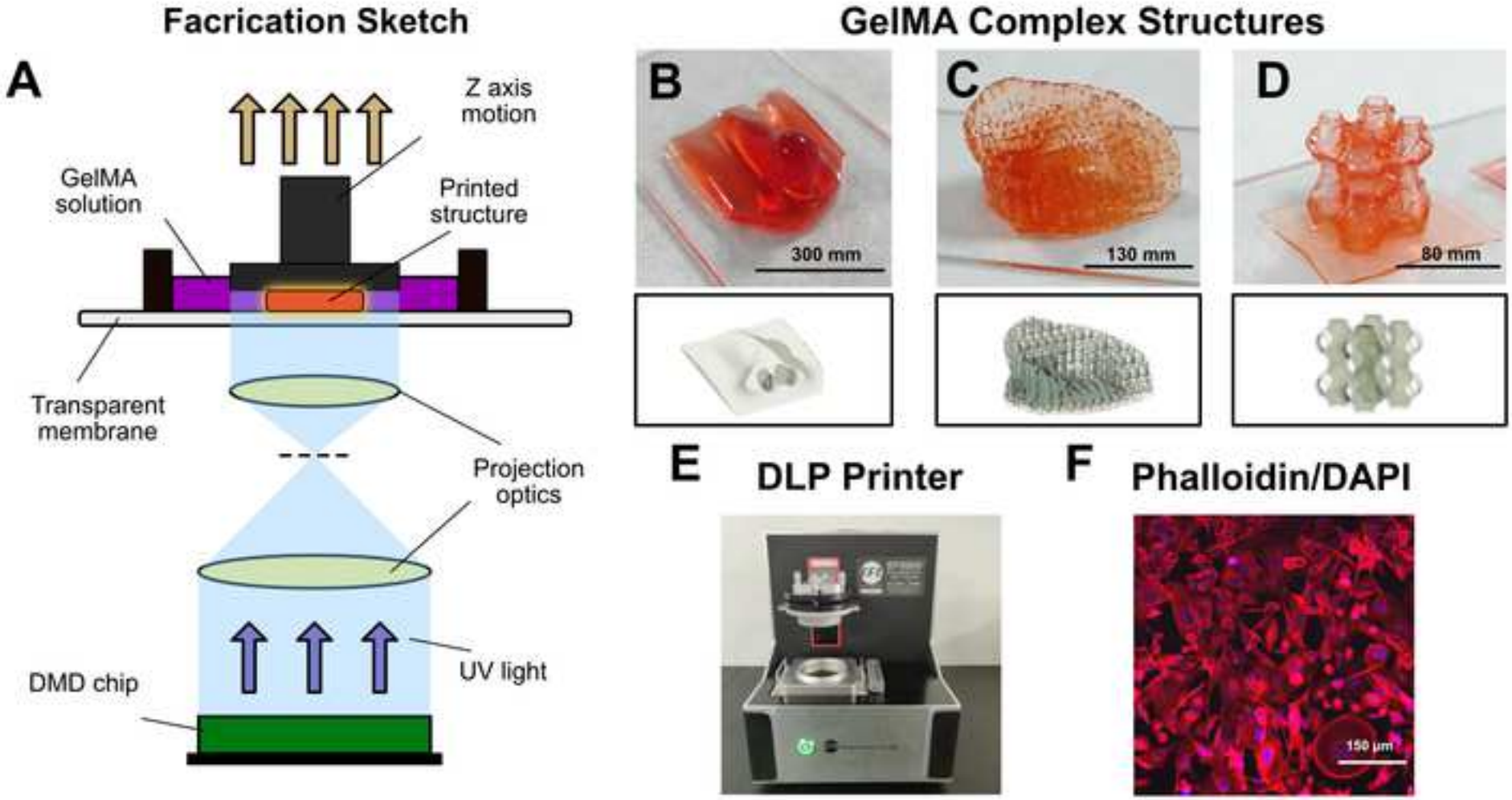

FIG4

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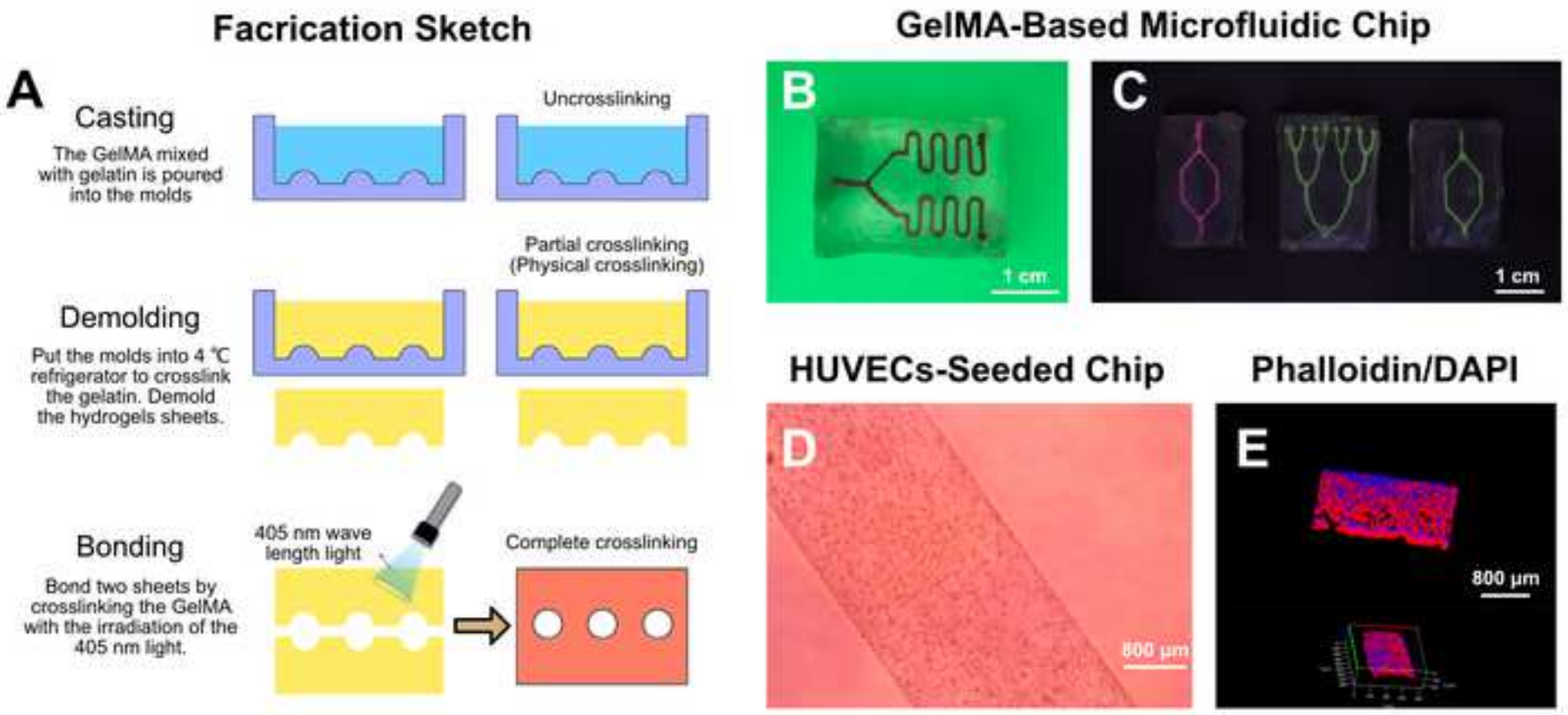

Click here to access/download;Table of Materials;60545_Table_of_Materials.xlsx

Name of Material/Equipment

0.22 μm filter membrane
2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI)
3D bioprinter
405nm wavelength light
co-axial nozzle
confocal fluorescence microscope
digital light processing (DLP) bioprinter
DLP printer
Dulbecco’s Phosphate Buffered Saline (DPBS)
Dulbecco's Modified Eagle Medium (DMEM)
Dulbecco's Modified Eagle Medium with L-glutamine (DMEM/F-12)
EFL Software
fetal bovine serum (FBS)
gelatin
gelatin methacryloyl (GelMA)
high voltage power
lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP)
paraformaldehyde
penicillin/streptomycin
sodium alginate (Na-Alg)
TRITC phalloidin
Triton X-100

Company	Catalog Number	Comments/Description
Millipore		
Yeasen Biological Technology Co., Ltd., Shanghai, China		
SuZhou Intelligent Manufacturing Research Institute, SuZhou, China		
SuZhou Intelligent Manufacturing Research Institute, SuZhou, China		
SuZhou Intelligent Manufacturing Research Institute, SuZhou, China		
OLYMPUS FV3000		
SuZhou Intelligent Manufacturing Research Institute, SuZhou, China		
SuZhou Intelligent Manufacturing Research Institute, SuZhou, China		
Tangpu Biological Technology Co., Ltd., Hangzhou, China		
Tangpu Biological Technology Co., Ltd., Hangzhou, China		
Tangpu Biological Technology Co., Ltd., Hangzhou, China		
SuZhou Intelligent Manufacturing Research Institute, SuZhou, China		
Tangpu Biological Technology Co., Ltd., Hangzhou, China		
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Yeasen Biological Technology Co., Ltd., Shanghai, China		
Solarbio Co., Ltd., Shanghai, China		

Rebuttal Letter
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Dear Editors and Reviewers,

Thanks for your letter and for the reviewers' comments concerning our manuscript entitled "A Handbook about 3D Bioprinting of GelMA Hydrogel" (JoVE60545). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction which we hope meet with approval.

Response to Editorial comments:

1. We have checked the spelling and grammar issues of this manuscript.
2. We have checked the signed ALA to my Editorial Manager account.
3. Thanks for your suggestions. The title has been modified.
4. We have modified the keywords as: 3D Bioprinting; Gelatin methacryloyl (GelMA); Microsphere; Microfiber; Digital light processing (DLP); Microfluidic chip.
5. We have added the E-mail of each author.
6. We have added the Summary section.
7. We have adjusted the numbering of the Protocol.
8. We have modified 1000rpm to 100 x g.
9. We have revised the Protocol section to contain only action items.
10. We have added the detailed action in the Protocol section.
11. We have added how to detached the cells in Step 2.6, 3.5, 4.9 and 5.10.
12. We have added the liquid volume and the size of the syringe in Step 2.7, 3.6.
13. We have added the volume of silicon oil and DPBS in Step 2.9-2.11.
14. We have added the method to take out the microspheres and the culturing tool.
15. The microspheres are fixed in the 30℃ environment. (It is introduced in the Step 2.8.)
16. The staining details have been added in Protocol 2-5.
17. The Protocol have been revised.
18. The scalebars have been added and the space between numbers and units have been added.
19. Figure 4 has been referenced in the manuscript.
20. The embedded figures have been removed.
21. The figure legends have been placed together at the end of the Representative Results.
22. The Discussion has been modified.
23. The Disclosures section has been added.
24. The Table of Materials have been made.

Reviewer #1:

In this study Xie et al., provide a wide variety of biomedical applications for the use of GelMA hydrogel. The authors describe in great detail how in their laboratory they are able to fabricate GelMA into microspheres, fibers, 3D complex structures, and microfluidic chips. The provided examples and protocols make it very easy to follow the stated protocols and would be very beneficial for any lab looking to potentially use GelMA in a specific example. Overall, this manuscript is appropriate for the journal and would be a beneficial handbook for the greater scientific community.

Response: We are very honor to have your comments and acceptance recommendation of this manuscript for publication. In terms of the issues you proposed to improve our paper, we have modified the corresponding parts as below.

Major Concerns:

Could you please comment and provide data for the viability of cells encapsulated with the different GelMA structures? Phalloidin and Dapi staining does not give any insight into whether the processing affects the encapsulated cell viability.

Response: The viability has been examined in the corresponding references. Furthermore, the cells spread can be a strong evidence to verify the high viability.

It would be beneficial to show a comparison between your CAD files imported into the DLP Printer and what is eventually printed. How does the resolution compare? Is there some loss of design with this approach? It is hard to tell without seeing an original CAD file. Please also comment on this within the discussion section.

Response: The CAD files have been added in Figure 3. In this part, we can see the structure is well reproduced. The detailed accuracy is not important here because this paper is aimed at the introduction of different printing methods of GelMA.

Please make all the scale bars for the figures more noticeable, as they currently stand, it is hard to read them within your figures making it impossible to fully comprehend the sizing of your different GelMA structures.

Response: The scalebars have been added.

Could you please provide either data or at the very least comment on how your GelMA microfluidic devices compare to a more traditional PDMS microfluidic device in regards to its fluid flow patterns.

Response: The comment on the GelMA microfluidic devices compared to traditional ones has been added in Discussion.

Minor Concerns:

Please remove the graphing background found in all figures

Response: The background has been removed.

Pg 4 line 90- How long do you heat the GelMA and LAP bioink at 37□C?

Response: We have added the heating duration (15min) in all corresponding protocol steps.

Figure 1, image A needs to be made clearer and in reference to the text of the article. Please look into making a flow diagram showcasing the order of things. In the protocol for the "GelMA Complex 3D structures", please direct the reader to the Figure 3 for the diagrams and images from this section of the article. In the protocol for the "GelMA-Based Microfluidic Chips", please direct the reader to the Figure 4 for the diagrams and images from this section of the article.

Response: Actually, in the Figure1, Figure3, Figure 4, the protocol have been showed in detail according to the article. The main devices have been displayed in the figures.

In the Representative Results, when referring to specific results from your figures, please direct the reader to those specific results. For example: "From the morphology of the stained MDA-MB-231s (Figure 1B-D), we could find that the encapsulated MDA-MB-231s remained the spreading capability, verifying the biocompatibility of this electro-assisted fabrication methods."

Response: The text have been modified.

Reviewer #2:

The article is well framed and doesn't have much corrections. It can be accepted.
I read the article and it quite good.

Response: We are very honor to have your comments and acceptance recommendation of this manuscript for publication.

Reviewer #3:

Manuscript Summary:

The manuscript covered about the general protocol for various GelMA 3D printing method, including microspheres, fibers and complicated structure. It also showed the representative result regarding to the fabrication method.

Response: We are very honor to have your comments and acceptance recommendation of this manuscript for publication. In terms of the issues you proposed to improve our paper, we have modified the corresponding parts as below.

Minor Concerns:

1. In the protocol part, the reviewer suggests that the authors supplement the reason why HUVEC and HBMEC were employed in this project for elementary researchers, who refer the article.

Response: Actually, the cells are just used for verifying the biocompatibility of the materials and fabrication methods without special choosing.

2. At the "2. Fabrication of GelMA Microspheres" part, the reviewer also suggests that the author additionally explain how to mix the cell into bioink in which way, the pellet or cell suspension. The encapsulation method can effect on cell viability.

Response: The cells are mixed with pipette. It has been introduced in the corresponding parts.

3. Regarding to the part of "Fabrication of GelMA Fibers", the reviewer suggests that the author refer additional article which is related to co-axial printing method. The article can show the cutting edge of application for co-axial printing method.

- Gao, G., Lee, J. H., Jang, J., Lee, D. H., Kong, J. S., Kim, B. S., ... & Cho, D. W. (2017). Tissue engineered bio- blood- vessels constructed using a tissue- specific bioink and 3D coaxial cell printing technique: a novel therapy for ischemic disease. Advanced Functional Materials, 27(33), 1700798.

Response: The references has been added.

4. The reviewer asks the author add information for cell viability after printing. The reviewer

suggests that the article include live and dead assay result, which can be the reference for the readers of this article.

Response: The viability has been examined in the corresponding references. Furthermore, the cells spread can be a strong evidence to verify the high viability.

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FIG3D.stl

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FIG4B.stl

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FIG4C-1and3.stl

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FIG4C-2.stl

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Title of Article:	Three–Dimensional Bioprinting of Gelatin Methacryloyl Hydrogel
Author(s):	Dr. Mingjun Xie 1,2, Dr. Jing Nie 1,2, Dr. Lei Shao 1,2, Dr. Kang Yu 1,2, Dr. Qing Gao 1,2, Dr. Jingjiang Qiu 3, Prof. Jianzhong Fu 1,2, Prof. Zichen Chen 1,2, Prof. Yong He 1,2,*

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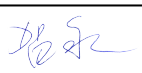
Signature:	
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