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Using Multilayered Hydrogel Bioink in Three-Dimensional Bioprinting for Homogeneous Cell Distribution

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TITLE:

Using Multilayered Hydrogel Bioink in Three-Dimensional Bioprinting for Homogeneous Cell Distribution

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KEYWORDS:

Tissue engineering, 3D bioprinting, bioink, interfacial retention, cell sedimentation, gelatin methacryloyl

SUMMARY:

Here, we developed a novel multilayered modified strategy for liquid-like bioinks (gelatin methacryloyl with low viscosity) to prevent the sedimentation of encapsulated cells.

ABSTRACT:

During the extrusion-based three-dimensional bioprinting process, liquid-like bioinks with low viscosity can protect cells from membrane damage induced by shear stress and improve the survival of the encapsulated cells. However, rapid gravity-driven cell sedimentation in the

reservoir could lead to an inhomogeneous cell distribution in bioprinted structures and therefore hinder the application of liquid-like bioinks. Here, we developed a novel multilayered modified strategy for liquid-like bioinks (e.g., gelatin methacryloyl with low viscosity) to prevent the sedimentation of encapsulated cells. Multiple liquid interfaces were manipulated in the multilayered bioink to provide interfacial retention. Consequently, the cell sedimentation action going across adjacent layers in the multilayered system was retarded in the bioink reservoir. It was found that the interfacial retention was much higher than the sedimental pull of cells, demonstrating a critical role of the interfacial retention in preventing cell sedimentation and promoting a more homogeneous dispersion of cells in the multilayered bioink.

INTRODUCTION:

Three-dimensional (3D) bioprinting has been a promising method to manufacture complex architectural and functional replicas of native tissues in biofabrication and regenerative medicine[1,2,3]. The common strategies of bioprinting, including inkjet, extrusion, and stereolithography printing, have pros and cons from different perspectives[4]. Among these techniques, the extrusion procedure is most commonly used due to its cost-effectiveness. Bioink plays a key role in the process stability of extrusion bioprinting. The ideal cell-laden bioink should not only be biocompatible but also be suitable for mechanical properties[5]. Bioinks with low viscosity are typically presented as a liquid-like state. These bioinks can be easily and quickly deposited and avoid cell membrane damage induced by high shear stress during extrusion. However, in complex cases requiring long-term printing periods, low viscosity often gives rise to the inevitable sedimentation of the encapsulated cells in the bioink reservoir, which is usually driven by gravity and leads to an inhomogeneous cell dispersion in the bioink[6,7]. Consequently, a bioink with inhomogeneous cell dispersity hampers the in vitro bioprinting of a functional tissue construct.

Several recent studies focusing on bioinks have reported the promotion of homogenous dispersity of encapsulated cells. A modified alginate bioink based on dual-stage crosslinking was used for extrusion bioprinting[8]. An alginate polymer was modified with peptides and proteins in this study. Cells presented a more homogeneous distribution in this modified alginate than in the commonly used alginate due to the attachment sites provided by the peptides and the proteins. Alternatively, blended bioinks have been utilized to solve the sedimentation of cells in bioink. A blended bioink containing polyethylene glycol (PEG) and gelatin or gelatin methacryloyl (GelMA) with improved mechanical robustness was used in another study[9]. The encapsulated cells presented a homogeneous distribution mainly because the viscosity of the blended bioink was improved. In general, there are several factors influencing the dispersity of the encapsulated cells in the bioink, such as the viscosity of the bioink, the gravity of the cells, the density of the cells, and the duration of the working period. Among these factors, the gravity of cells plays a critical role in promoting sedimentation. The buoyancy and friction provided by the viscous bioink have been investigated as the main forces against gravity to date[10].

Herein, we developed a novel strategy to promote homogeneous dispersity of the

encapsulated cells in bioink by manipulating multiple liquid interfaces in the bioink reservoir. These liquid interfaces created by the multilayered modification of bioink can not only provide interfacial retention, which retards the sedimentation of cells, but also maintain a suitable biocompatibility and rheological behavior of the bioink. In practice, we modified aqueous GelMA solution (5%, w/v) with silk fibroin (SF) in a multilayered manner to longitudinally produce four interfaces, providing interfacial tensions in the blended bioink. As a result, the gravity loading on the cells was offset by the man-made interfacial tension, and a nearly homogeneous dispersion of the encapsulated cells in the bioink was obtained due to less sedimentation across the adjacent layers of cells. No similar protocol to slow down the sedimentation of encapsulated cells by manipulating interfacial retention in liquid bioinks has been reported to date. We present our protocol here to demonstrate a new way to solve cell sedimentation in bioprinting.

PROTOCOL:

1. Preparation of cell-laden SF-GelMA

1.1. Sterilize all the materials by using 0.22 µm syringe filter units. Perform all the steps in a biological safety cabinet.

1.2. Warm 1x PBS to 50 °C, and dissolve gelatin in the heated 1x PBS with stirring. The final concentration of gelatin in PBS should be 10% (w/v).

1.3. Add methacrylic anhydride into the gelatin solution (weight ratio of methacrylic anhydride to gelatin of 0.6 to 1) slowly with stirring, and mix the complex for at least 1 h (50 °C). Typically, prepare 200 mL of 10% gelatin solution with 12 g of methacrylic anhydride; the volume depends on the needs of the study.

1.4. Transfer the mixed solution containing gelatin and methacrylic anhydride to a 50 mL sterile tube.

1.5. Centrifuge the mixed solution at 3,500 x *g*. It usually takes 3-5 min to obtain two layers. Collect the upper layer (GelMA) and discard the bottom layer (unreacted methacrylic anhydride).

1.6. Dilute the upper layer solution obtained in step 1.5 with two volumes of deionized water (40−50 °C).

1.7. Dialyze the solution obtained in step 1.6 with a 12-14 kDa molecular weight cutoff dialysis membrane against deionized water for 5−7 days (40−50 °C). Change the water twice every day.

1.8. Collect and freeze the GelMA solution at −80 °C overnight.

1.9. Lyophilize the GelMA solution for 3−5 days in a freeze dryer with the temperature set to

-45 °C and the pressure set to 0.2 mbar.

1.10. Dissolve the lyophilized GelMA (degree of substitution of approximately 75%) in 1x PBS containing 10% FBS (fetal bovine serum, v/v), 25 mM HEPES (N-2-hydroxyethylpiperazine-N-ethane-sulfonic acid), and photoinitiator (0.5%, w/v) to obtain the GelMA bioink preparation.

NOTE: The degree of substitution of GelMA can be calculated by a ninhydrin assay[3].

1.11. Mix the GelMA solution (10%, w/v) with different volumes of initial SF solution (5%, w/v) and different volumes of 1x PBS to obtain SF-GelMA bioinks with different concentrations of SF. The proportion per 1 mL of GelMA and SF solution in the different bioinks is presented in **Table 1**.

NOTE: All bioinks must contain a final concentration of 5% (w/v) GelMA, but the concentration of SF varies: 0.5, 0.75, 1.0, 1.25, and 1.5% (w/v) in the different SF-GelMA bioinks. Use SF-M-Layered-GelMA to term the GelMA bioink modified with SF in a layer-by-layer manner. Use SF-X-GelMA to term those GelMA modified with SF in a homogeneous manner (e.g., GelMA with modification of 1% SF was termed SF-1-GelMA).

1.12. Sonicate all bioinks for 10-20 min.

1.13. Grow NIH3T3 cells using DMEM (Dulbecco's modified Eagle medium) containing 10% FBS and 1% penicillin-streptomycin in an incubator (37 °C with 5% CO_2). Passage the cells at a ratio of 1:3 when the density reaches 80%.

1.14. Centrifuge the suspension of NIH3T3 cells at 1500 rpm for 5 min. Remove the supernatant with suction and resuspend the cell pellet with fresh bioink solution in a 15 mL sterile tube.

NOTE: The concentration of the encapsulated cells in the bioink should be 1 x 10^6 cells/mL, and the concentration needs to be calculated with a cytometer.

1.15. Use 2 mL of different SF-GelMA bioinks to suspend the cell pellets to obtain various cell-laden bioinks.

2. **Loading, reheating and bioprinting of the SF-M-Layered-GelMA**

2.1. Load 0.4 mL of cell-laden SF-0.5-GelMA into the bottom layer of the syringe.

NOTE: Use a 2 mL syringe as the bioink reservoir in this study. Load different SF-GelMA bioinks into the syringe in a layer-by-layer manner.

2.2. Place the syringe in ice water bath (0 °C) for 5 min to cause the bottom-layer bioink to

transform into a gel state.

2.3. Load 0.4 mL of cell-laden SF-0.75-GelMA bioink above the bottom layer.

2.4. Place the syringe with the two layers of bioinks in an ice water bath (0 °C) for 5 min to cause the two layers of bioinks to transform into a gel state.

2.5. Cycle the loading and cooling steps another 3 times with the remaining 3 kinds of bioinks (SF-1-GelMA, SF-1.25-GelMA, SF-1.5-GelMA) to obtain a multilayered bioink system with different concentrations of SF in the different layers. The volume used for the loading of all bioinks should be 0.4 mL.

2.6. Reheat the multilayered bioink by placing the syringe in an incubator (37 °C) for 30 min prior to bioprinting.

2.7. Use a 2 mL syringe as the bioink reservoir with a 27 G printing nozzle. Set the speed of flow at 50 µL/min, the moving speed of the nozzle at 2 mm/s, and the height of the nozzle at 1 mm. Perform the bioprinting procedure at room temperature (approximately 20 °C).

2.7.1. Print the tissue construct in an extrusion manner using a custom-made bioprinter under the parameters adapted from our previous studies[11,12].

2.8. Use ultraviolet light (365 nm, 800 mW) for 40 s to crosslink the bioprinted tissue construct.

2.9. Culture the crosslinked tissue construct in DMEM (Dulbecco's modified Eagle medium) containing 10% FBS and 1% penicillin-streptomycin in an incubator (37 °C with 5% CO_2). The medium was changed every 8 h during the first 2 days and every 2-3 days thereafter.

REPRESENTATIVE RESULTS:

A schematic of the preparation of cell-laden bioinks is shown in **Figure 1**. After preparation of the different bioinks, loading, reheating and bioprinting were performed (**Figure 2**). To evaluate the distribution of the encapsulated cells in the bioink reservoir, a bioprinting procedure was performed using three different cell-laden bioinks in three 96-well plates (**Figure 3A**). Two control groups (pristine GelMA and SF-1-GelMA bioinks) and the experimental group (SF-M-Layered-GelMA bioink) were used to investigate the dispersity of the encapsulated cells (**Figure 3B**). Sixty microliters of cell-laden bioink was extruded in every well of the three 96-well plates. The total volume of the three kinds of bioink was 2 mL, and as a result, the period of bioprinting was more than 30 min. With the additional incubation prior to printing (30 min), the total working period was more than 1 h and was deemed a suitable fabrication duration for bioprinting large tissues and organs. The number of cells in the target wells, labeled in **Figure 3A**, in the three plates was counted. The counts of the cells in different wells could reflect the dispersity of the encapsulated cells among the different groups. The results showed that as the bioprinting procedure progressed, the density of cells in the target wells decreased in all groups. In the SF-0-GelMA group, approximately 70% of

the cells were deposited in the bottom layer after the total procedure. In the SF-1-GelMA group, approximately 40% of the cells were deposited in the bottom layer, and approximately 5% of the cells were deposited in the top layer. In the SF-M-Layered-GelMA group, the deposition of encapsulated cells was more homogeneous than that of the control groups (**Figure 3C and 3D**).

Figure Legends

Figure 1. Schematic of the SF-GelMA bioink preparation. The SF-GelMA was prepared by mixing the silk fibroin (SF) and GelMA/photoinitiator (PI) complex followed by ultrasonic treatment for 10-20 min. Then, the target cells in the SF-GelMA mixture were suspended to prepare the cell laden SF-GelMA bioink.

Figure 2. The loading, reheating, and bioprinting procedure. The cell laden SF-GelMA, as the bioink (aq), was loaded into the bioink reservoir and transformed into the gel state from the cooling procedure. The loading and cooling procedure was repeated 5 times using the bioinks (aq) with different concentrations of SF (0.5, 0.75, 1.0, 1.25, and 1.5%) to obtain SF-Multilayered-GelMA (SF-M-Layered-GelMA). The gel state SF-M-Layered-GelMA was reheated by placing the bioink in an incubator for 30 min. The gel state bioink turned into a liquid state after incubation. Then, the prepared bioink was used for printing using a 2 mL syringe as the bioink reservoir with a 27 G printing nozzle. The printed tissue construct was crosslinked by using UV light. Aq means aqueous solution.

Figure 3. The bioprinting procedure used three different cell-laden bioinks. (A), Sixty microliters of bioink was extruded into the corresponding 96-well plate per minute, and it took more than 30 min to achieve the bioprinting procedure in the 96-well plate. (B), Three different kinds of bioinks were used to investigate the dispersity of the encapsulated cells in the bioprinting procedure: two control groups (pristine GelMA and SF-1-GelMA bioinks) and the experiment group (SF-M-Layered-GelMA bioink). (C-D), Cell densities of the No. 1, 5, 10, 15, 20, 25, and 30 wells in the three plates were detected with staining, showing the distribution of encapsulated cells in the three bioinks, and the deposition of the encapsulated cells in the SF-M-Layered-GelMA group was more homogeneous.

Table 1. Preparation of SF-GelMA bioinks

DISCUSSION:

The stability of the multilayered system is a key point to perform this protocol successfully. We theoretically calculated the diffusion of SF molecules in the GelMA solution based on Nauman's study[13]. It was found that the diffusion of proteins in solution was related to their molecular weight. The average molecular weight (MW) of bovine serum albumin (BSA) is 66.5 kDa, and its diffusion coefficient is 64-72 $\mu m^2/s$. The average MW of fibrinogen is 339.7 kDa, and its diffusion coefficient is 23-34 $\mu m^2/s$. The average MW of SF molecules in our study is approximately 100 kDa. Based on the results of Nauman's study, the diffusion coefficient of a single SF molecule is between 23-72 $\mu m^2/s$ in water at 25 °C. According to the Stokes–Einstein equation, the diffusion coefficient is defined as follows:

$$D = \frac{KT}{3\eta\pi d}$$

where D is the diffusion coefficient, K is the consistency, T is the absolute temperature, η is the viscosity of the solution, and d is the diameter. It can be concluded that the difference in diffusion coefficient of SF molecules in water and in GelMA solution is determined by the difference in viscosity of these two surrounding solutions. Moreover, the diffusion radius can be calculated as follows:

$$\mathrm{R} = \sqrt{6DT}$$

where R is the diffusion radius, D is the diffusion coefficient, and T is the diffusion time. Pure water presents a viscosity at 8.9 x 10^{-4} Pa·s, and the viscosity of GelMA at zero shear rate in our study was higher than 1000 Pa·s. Hence, the diffusion radius of a single SF molecule at 25 °C is less than 0.66-1.18 μm/hour in the GelMA solution. This indicates that SF can hardly diffuse across the adjacent layers in the SF-M-Layered-GelMA system, which demonstrates the stability of the multilayered system.

The gravity-driven sedimentation of the encapsulated cells is inevitable when bioprinting with liquid-like bioinks. In our study, we developed a novel modification of low viscosity GelMA bioink with cyclic loading-cooling to retard the sedimentation of cells by creating interfacial retention. The multilayered interfacial retention is supposed to offset the action of gravity of the encapsulated cells and consequently prevent the sedimentation of the encapsulated cells across the adjacent layers in the bioink reservoir. The interfacial retention was presented longitudinally in the bioink reservoir between each adjacent layer due to the difference between the rheological behavior of different bioinks loaded in different layers. These interfacial tensions began to work as soon as the encapsulated cells sedimented to the interface. As a result, the gravity pull was offset, and sedimentation was stopped. Although this protocol is novel, more applications utilizing this protocol in other liquid-like bioink systems should be studied for promotion and optimization.

DISCLOSURES:

The authors have nothing to disclose.

ACKNOWLEDGMENTS:

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Figure 1

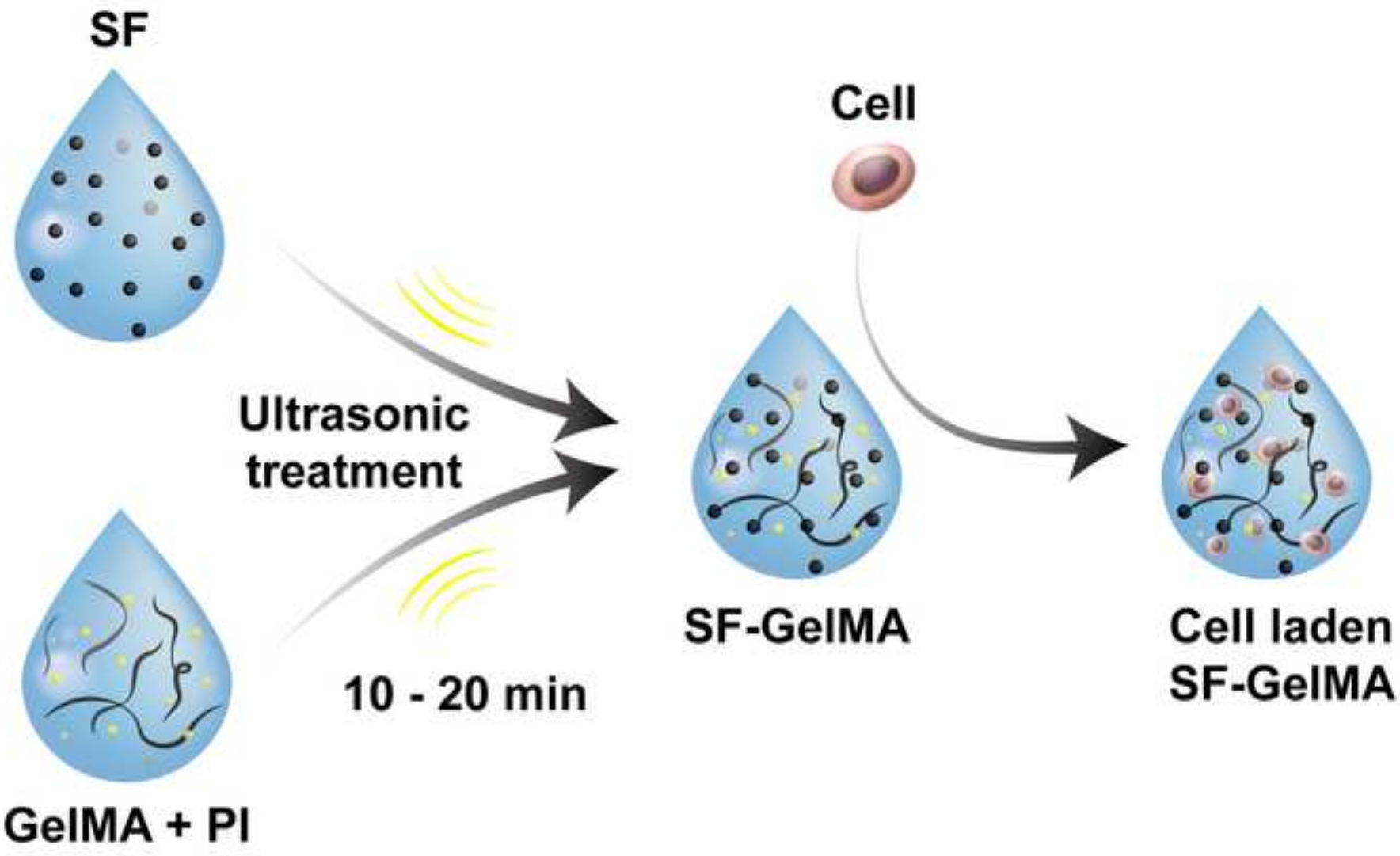

Figure 2

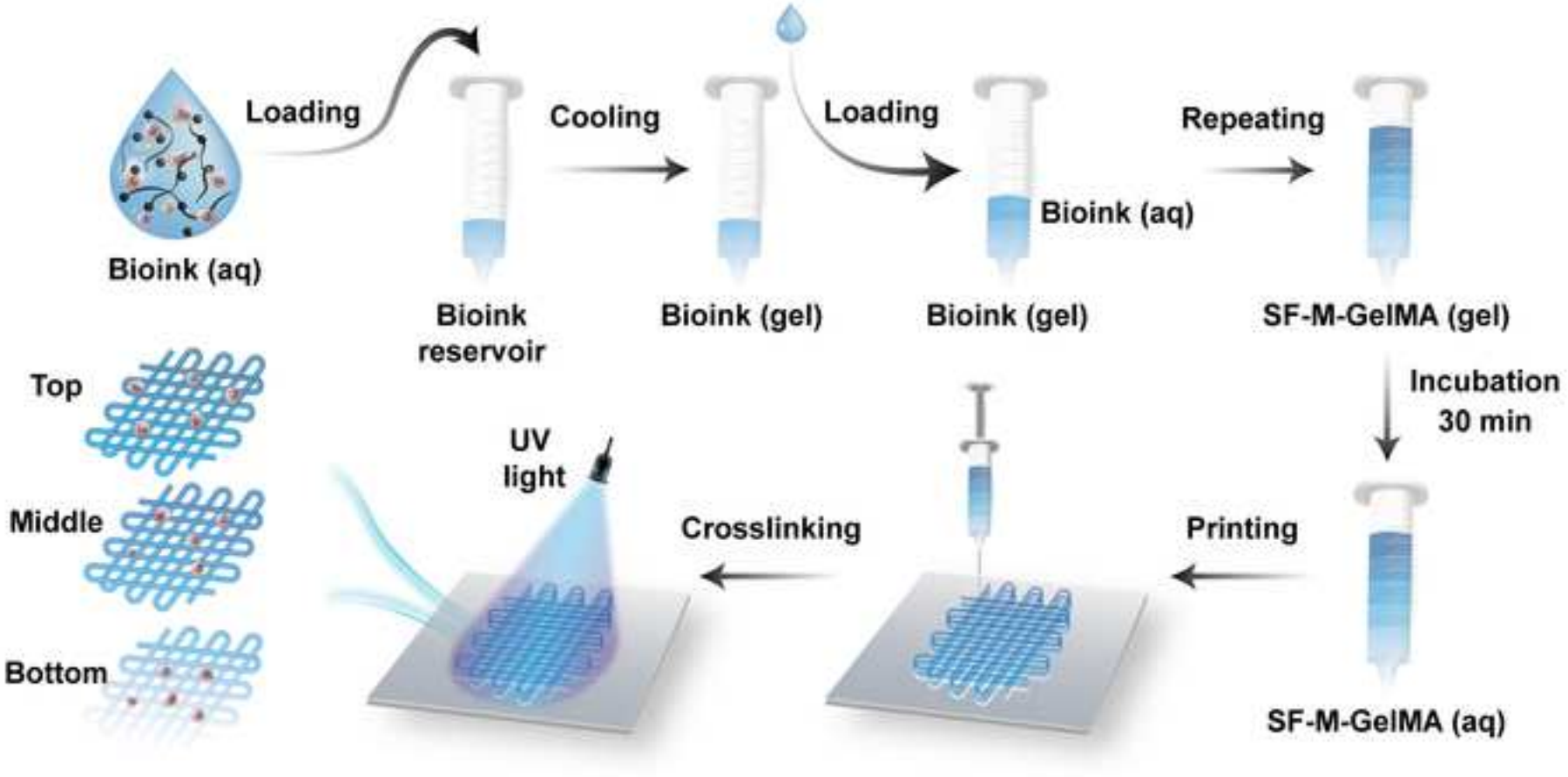

Figure 3

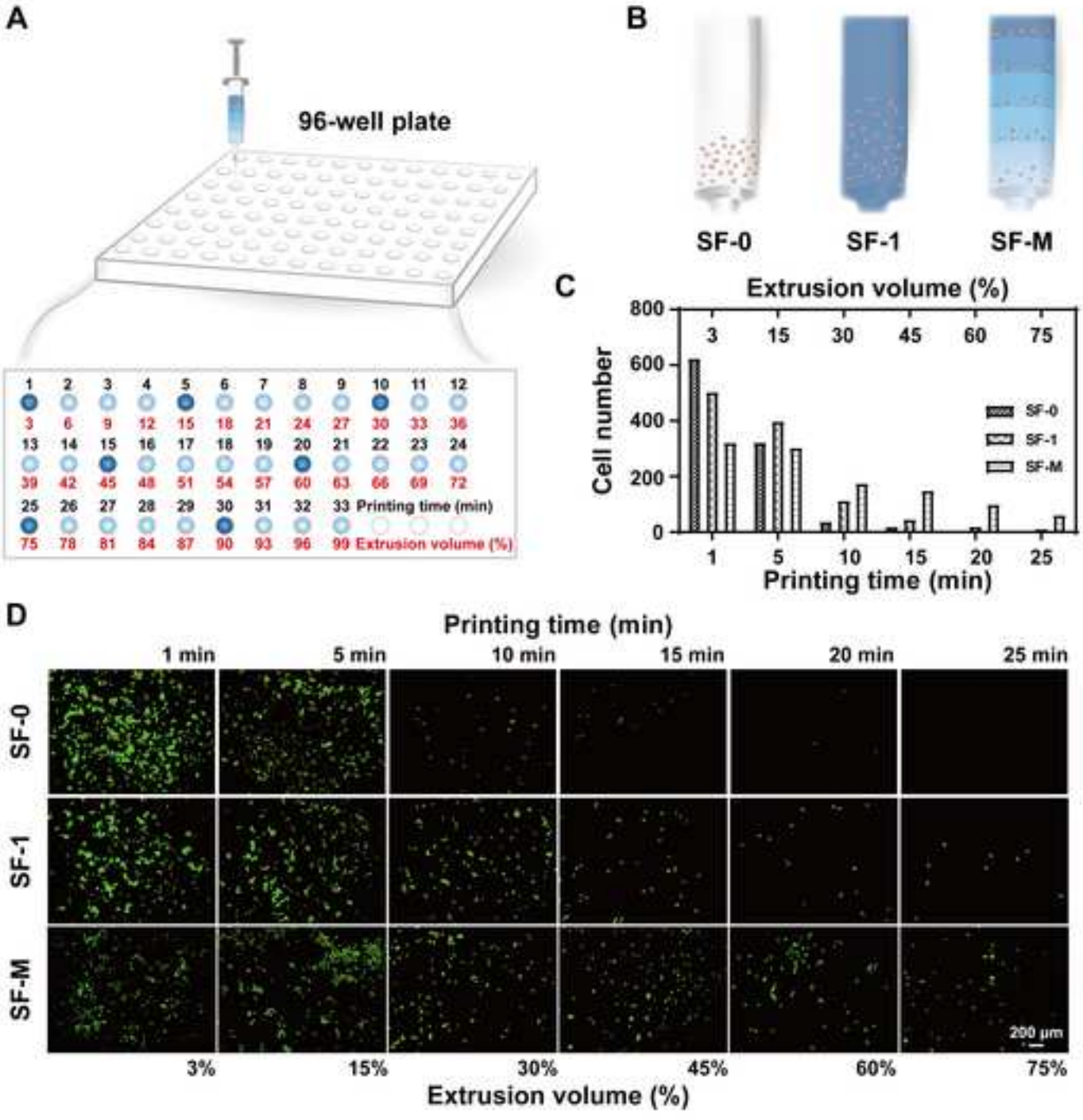

Table 1

Bioinks	Materials (ml)		
	GelMA	SF	PBS
SF-0.5-GelMA	0.5	0.1	0.4
SF-0.75-GelMA	0.5	0.15	0.35
SF-1.0-GelMA	0.5	0.2	0.3
SF-1.25-GelMA	0.5	0.25	0.25
SF-1.5-GelMA	0.5	0.3	0.2

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (PI2959)	TCI	M64BK-QD	
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Gibco	15630080	
Dulbecco's modified Eagle's medium (DMEM)	Gibco	10569044	
fetal bovine serum (FBS)	Gibco	10091	
Gelatin	Sigma-Aldrich	V900863MSDS	
Methacrylic anhydride (MA)	Sigma-Aldrich	276685MSDS	
Penicillin–streptomycin antibiotics	Gibco	15140163	
Phosphate-buffered saline (PBS)	Gibco	10010049	
Silk fibroin	Advanced BioMatrix	5154	

Dear Doctor Nguyen,

We are very grateful for the detailed comments from the editor. We have carefully addressed the editor's concerns in the revised manuscript and listed our improvements point-by-point in the following pages.

Following is a brief list of our revision:

1. We revised the manuscript and added more information on the protocol of this study.
2. We corrected all the mistakes according to the request of revision and improved English language in the revised manuscript with the help from the AJE group.
3. All the revisions have been highlighted in the revised manuscript.

Point-by-point responses are listed as following. Please feel free to contact me if anything else is needed.

Best regards,

Kai Zhu, MD & PhD

Department of Cardiac Surgery and Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University

1. The title was revised into “Using Multilayered Hydrogel Bioink in Three-Dimensional Bioprinting for Homogeneous Cell Distribution” according to editorial comments in *Line 2-3*.

2. In *Line 50*, the sentence “the extrusion procedure carries the highest appearance rate due to its cost-effectiveness” has been revised into “the extrusion procedure is most commonly used due to its cost-effectiveness”.

3. In *Protocol 1.3., Line 100*, we provided the amount of materials when we generally prepare the GelMA. The volume depends on the needs of different studies.

4. In *Protocol 1.11., Line 127*, we provided Table 1 presenting the proportion of GelMA, SF, and PBS in per 1 ml of different SF-GelMA bioinks.

5. In *Protocol 1.13., Line 144*, we provided the information of the medium using for cell culture.

6. In *Protocol 1.14., Line 148*, we provided the speed for centrifuging the cell suspension of NIH-3T3 cells.

7. In *Protocol 1.15., Line 155*, we provided the volume of bioinks commonly used in our study to resuspend NIH-3T3 cells.

8. In *Protocol 2.1., Line 160, Protocol 2.3., Line 168*, and *Protocol 2.5., Line 173*, we provided the volume of all bioinks loaded in the bioink reservoir.

9. In *Figure 1*, we revised the abbreviation of minutes: min instead of mins.

10. Figure 3A and 3B are schematics. In the schematics, we use different colours to present different bioinks. In practice, the appearance of all bionks is the same (*Line 198*).

11. In *Line 226-228*, the sentence “Re-heat the gel state SF-M-Layered-GelMA in the incubation for 30 min to turn it into liquid state” has been revised into “The gel state SF-M-Layered-GelMA was reheated by placing the bioink in an incubator for 30 min. The gel state bioink turned into a liquid state after incubation” for clarity.

Cover Letter for Revision

Dear Professor Bajaj,

We are very grateful for the detailed comments from the reviewers and the revising instruction from the editor. We have carefully addressed the reviewers' and editor's concerns in the revision and listed our improvements point-by-point in the following pages.

Following is a brief list of our revision:

1. We revised the title of the manuscript to out stand the novelty of our study.

2. We added some description on the novelty of this study in the abstract and introduction.

3. We revised the whole manuscript according to the suggestion of editor and format of JoVE, especially the protocol part, to make it more clearly.

4. We corrected all the mistakes according to the request of revision and improved English language in the revised manuscript.

5. All the revisions have been highlighted in the revised manuscript.

Point-by-point responses are listed as following. Please feel free to contact me if anything else is needed.

Best regards,

Kai Zhu, MD & PhD

Department of Cardiac Surgery and Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University

Point-by-point responses to the editorial comments:

Editorial comments：

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

   Response: We have read the editorial comments carefully and tried our best to revise the manuscript according to the suggestions, and also turned to a native speaker for language improvement.

2. *Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.*

   Response: We have re-formatted the manuscript according to the suggestion and changed the font to Calibri (12 points).

3. *Please provide an email address for each author.*

   Response: We have provided email address for each author in the revision version.

4. *Please provide at least 6 keywords or phrases.*

   Response: “Tissue engineering” has been added as another keywords. In total, we provided 6 keywords or phrases.

5. *Please define all abbreviations during the first-time use.*

   Response: All abbreviations are defined and confirmed during the first time they appeared in the manuscript.

6. *Please include a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"*

   Response: The summary "Here, we present a protocol about the preparation of the cell-laden SF-GelMA bioink and the multilayered bioink system." has been added in the beginning of the protocol section.

7. *Please ensure that the Abstract is between 150-300 words.*

   Response: The length of the Abstract is about 163 words.

8. *Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: igracure, etc*

   Response: All the commercial language have been changed into generic terms. For example, "igracure" has been corrected into "photoinitiator" **(protocal:1.9.).** Besides, All commercial products have been referenced in the Table of Materials and Reagents.

9. *Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).*

Response: All personal terms in Protocol have been removed and modified.

10. *Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.*

    Response: The numbering of the protocol has been corrected following the JoVE instructions.

11. *Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.*

    Response: The sentences of the protocol part have been corrected to imperative tense.

12. *The Protocol should contain only action items that direct the reader to do something.*

    Response: The sentences of the protocol part have been corrected to contain only action items.

13. *The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.*

    Response: We have segmented the statements of the experimental protocol, and less than 2~3 actions were included in each step.

14. *Please use complete sentences throughout.*

    Response: We have gradually revised the full text so that each sentence is complete.

15. *Please include volume and concentrations of all the solutions used.*

    Response: Volume and concentrations of all the solutions described in the manuscript have been added.

16. *Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?*

    Response: We checked the Protocol part carefully and tried to make the statement of experimental steps as detailed as possible.

17. *1a-b: In what ratio? Amount added?*

    Response: The final concentration of gelatin in PBS is 10% (w/v). The ratio of methacrylic anhydride to gelatin is 0.6 to 1, meaning add 0.6g methacrylic anhydride per 1g gelatin. We revised in the manuscript to make it clear.

18. *1d: Do you remove the supernatant after centrifugation? DO you obtain two layers? Please explain all specific details.*

    Response: Centrifuge the mixed solution at 3500 ×g. It usually takes for 3-5 min to obtain two layers. Collect the upper layer (GelMA) and discard the bottom layer (unreacted methacrylic anhydride). We revised in the manuscript to make it clear.

19. *1e: What is the mixed solution?*

    Response: The mixed solution is the gelatin solution with the combined methacrylic anhydride. We revised in the manuscript to make it clear.

20. *1f: Do you change the water in between any time?*

    Response: The water was changed twice every day. We revised in the manuscript to make it clear.

21. *1i: How do you calculate the degree substitution?*

    Response: The degree substitution of GelMA is calculated by Ninhydrin assay. It has been reported in the reference 3. We have attached the information and reference in the revised manuscript.

22. *1k: How is this done? What is the setting? Do you perform this on ice? Do you have an incubation period?*

    Response: The procedure is performed in a freeze dryer. The temperature is set as -45℃, the pressure is stable at 0.2 mbar. We have attached the information in

the revised manuscript.

23. *1l: What is the amount of NIH3T3 cells used for the experiment prior to centrifuging? How do you calculate the concentration of encapsulated cells in bio-ink?*

    Response: We resuscitated suitable volume of NIH3T3 cells and pass the cells at a ratio of 1: 3 when their density reaches 80%, and then, we centrifuged the suspension of NIH3T3 cells and removed supernatant, and suspended the cell pellets with fresh bioink solution in a 15 mL sterile tube. The concentration of the encapsulated cells in bioink is 1×10^6 cells/mL. We calculated the concentration by a cytometer. We have attached the information in the revised manuscript.

24. *2: Please explain each step to show how it is done in a step wise manner.*

    Response: We did revision in the manuscript to make the protocol clear.

25. *Please explain how bioprinting was performed.*

    Response: Bioprinting was performed using a custom-made bioprintor with a extrusion manner under the parameters adapted from our previous studies. The custom-made bioprinter was controlled by computer-control box, which CAD data can be input. The target pattern was chosen prior to printing. The extrusion of bioink was controlled by a custom-made syringe pump, which can adapt the speed of flow. All parameters for the printing procedure were provided in the manuscript and we chose to attach the references 11&12 which provide more details about the biprinting system.

26. *Here is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable*

content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: The second part of protocol on loading, and re-heating are the essential steps for video.

27. *Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."*

    Response: Any figure in this manuscript has not been published in whole or in part elsewhere.

28. *Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.*

    Response: The Figure Legends have been included together at the end of the Representative Results.

29. *Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate the journal-title.*

    Response: All the reference have been adapted to the recommended format.

30. *Please remove the titles and Figure Legends from the uploaded figures. The information provided in Figure Legends after the Representative Results is sufficient.*

    Response: We have removed the titles and Figure Legends from the uploaded figures.

31. *All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.*

    Response: We have added the figures and their figure legends after the Representative Results of the manuscript text, and uploaded the figures separately according to the suggestion.

Point-by-point responses to the reviewer's comments:

Reviewer 1 comments:

1. *Authors need to make the novelty clearer in the abstract and introduction sections. They need also to modify the title to reflect this.*

   Response:

   1. We have presented the novelty of this protocol more clearly in the revised manuscript in both abstract and introduction section as following:

   "we developed a multilayered-modified strategy, which hasn't been detected in other studies to date, for liquid-like bioinks (gelatin methacryloyl with low viscosity in this study) to prevent sedimentation of encapsulated cells"

   "No similar protocol to slow down the sedimentation of encapsulated cells by manipulating interfacial retention in liquid bioinks has been reported to date. We report our protocol here to put forward a new aspect on solving the cell sedimentation in bioprinting."

   2. The title has been modified to out-stand the novelty of this protocol as following:

   "A novel strategy using multilayered hydrogel bioink in three-dimensional bioprinting promotes homogeneous cell distribution in printed applications"

2. *Please also make sure you define all cited abbreviations such as MITCH, DMEM and FBS.*

   Response: All cited abbreviations have been re-defined in the revised manuscript. MITCH means mixing-induced, two-component hydrogels. These contain a large group of hydrogels. We found that the term MITCH-Alginate is not suitable enough, so we used "modified alginate" instead. More details about this hydrogel can be found in the reference 8.

3. *Do you mean "systems" in "⋯ other liquid-like bioink system"? Please check and correct accordingly*

   Response: We have corrected “system” into “systems”. **(Discussion, line 33)**

Reviewer 2 comments:

1. *Please add the scale bar in figure 3D.*

   Response: The scale bar has been added in figure 3D.

2. *There are typos and grammar mistakes, such as "all the steps was performed in biological safety cabinet" in the protocol 1 note, "The tissue construct were printed" in the protocol 2h, " molecular mass" in the third line of discussion part, please correct them.*

   Response: The sentence "all the steps was performed in biological safety cabinet" has been corrected into "Perform all the steps in biological safety cabinet"，and "The tissue construct were printed" has been corrected into "Print the tissue construct under the parameters adapted from our previous studies", and " molecular mass" has been corrected into "molecular weight".