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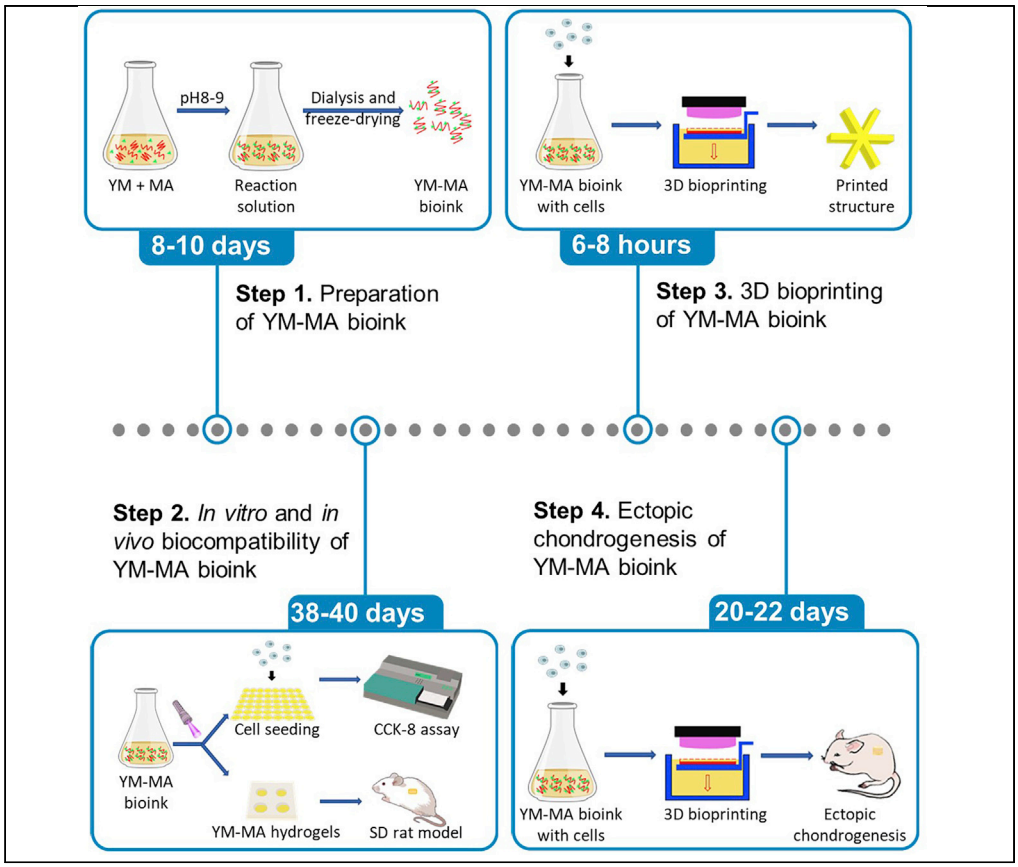
Protocol

3D bioprinting of modified mannan bioink for tissue engineering



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Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Preparation of a modified water-soluble polysaccharide polymer YM-MA bioink

Optimized parameters for 3D printing of YM-MA bioink

YM-MA bioink has good cytocompatibility and histocompatibility

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3D bioprinting of modified mannan bioink for tissue engineering

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SUMMARY

This protocol details the steps for preparation of a recently developed bioink, named YM-MA, which is based on methacrylate anhydride-modified yeast mannan. A light-assisted 3D bioprinting is performed to analyze the printability of YM-MA bioink. We describe how cell experiments, animal models of subcutaneous implantation in a Sprague Dawley rat model, and nude mice are used to evaluate the cytocompatibility, histocompatibility, and chondrogenesis of YM-MA bioink. This protocol provides a versatile strategy to develop bioinks of polysaccharides with chemical modification sites such as hydroxyl group.

For complete details on the use and execution of this protocol, please refer to Huang et al. (2021).

BEFORE YOU BEGIN

Institutional permissions

All animal experiments in this study were approved by the Animal Care and Ethics Committee of Hunan Academy of Chinese Medicine.

General laboratory preparation

Timing: 1 h

1. Prepare the 10×phosphate buffer saline (PBS) solution and store it at 4°C with a shelf-life of about 6 months.
2. Prepare the 1×PBS buffer (pH7.4) by diluting 10×PBS buffer with ddH_2O.
3. Prepare the 1×PBS buffer (pH8.0) by adjusting 1×PBS buffer (pH7.4) with 0.5 M NaOH solution.
4. Use a 0.22 μm bottle top vacuum filter to filter the 10×PBS buffer, 1×PBS buffer (pH7.4), and 1×PBS buffer (pH8.0) to obtain sterilized buffers.

Note: Store the buffers at 4°C after sterilization. Preheat the buffers at 37°C before use for cell experiments and animal models.

Preparation of stock solutions for methacrylate anhydride modified yeast mannan

Timing: 10 min

5. Prepare yeast mannan (YM) solution by dissolving 1 g YM into 50 mL 1×PBS buffer (pH7.4) via magnetic stirring at 20°C–25°C for 10 min.
6. Prepare methacrylate anhydride (MA) emulsion with 0.8 mL MA and 4.2 mL 1×PBS (pH7.4) by vortex oscillation for 1 min at 20°C–25°C in darkness in a fume hood.

Note: In view of the instability of MA, prepare the MA emulsion before use.

⚠ CRITICAL: MA is a liquid acidic organic matter at 20°C–25°C, which is harmful by inhalation, irritating to respiratory system and skin, and risk of serious damage to the eyes. Therefore, when performing step 6, do it in a fume hood.

7. Prepare 0.5% (w/v) lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) solution by dissolve 5 mg LAP into 1 mL 1×PBS buffer (pH7.4).

Preparation of stock solutions and mediums for cell experiments

Timing: 1 h

8. Prepare penicillin-streptomycin solution, then use a 0.22 μm syringe filter to filter penicillin-streptomycin solution to obtain sterilized penicillin-streptomycin solution, and store it at 4°C with a shelf-life of about 3 months.
9. Prepare cell culture medium and store it at 4°C with a shelf-life of about 2 weeks.
10. Prepare 0.5 M ethylenediaminetetraacetic acid disodium salt (EDTA) solution and store it at 4°C with a shelf-life of about 6 months.
11. Prepare trypsin solution and store it at 4°C with a shelf-life of more than 1 month.
12. Prepare fluorescein diacetate (FDA) stock solution and store it away from light at −20°C with a shelf-life of about 3 months.
13. Prepare and use 4% (w/v) paraformaldehyde solution in a fume hood and store it at 4°C with a shelf-life of 12 months.

Note: All solutions and mediums for cell experiments and animal models should be preheated at 37°C before use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Collagen II antibody	Santa Cruz	Cat# sc-52658
Anti-Mouse Secondary Antibody (1:100 dilution)	Servicebio	Cat# G1216-200T
Chemicals, peptides, and recombinant proteins		
YM	Zhao's lab	YM50 (44.7KDa)
MA (94%)	Macklin	Cat# M812900
Lithium phenyl-2,4, 6-trimethylbenzoylphosphinate (LAP)	Shanghai Yuchang New Materials Co., Ltd.	N/A
FDA	Shanghai Yisheng Biotechnology Co., Ltd.	N/A
CCK-8	Biosharp	Cat# BS350A
FBS	Gibco	Cat# 10270-106
DMEM/high glucose	Gibco	Cat# 12100046
EDTA	SinaPharm	Cat# 10009717
Acetone	SinaPharm	Cat# 1000428

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NaOH	SinaPharm	Cat# 100197008
NaCl	SinaPharm	Cat# 10019318
KCl	SinaPharm	Cat# 10016308
$Na_2HPO_4 \cdot 12H_2O$	SinaPharm	Cat# 10020318
KH_2PO_4	SinaPharm	Cat# 10017608
Paraformaldehyde	SinaPharm	Cat# 80096618
Ethanol	SinaPharm	Cat# M1172702
Xylene	SinaPharm	Cat# 10023418
Neutral gum	SinaPharm	Cat# 10004160
Chloral hydrate	SinaPharm	Cat# 30037516
Penicillin G sodium salt	Sangon Biotech	Cat# A600135-100
Streptomycin sulfate	Sangon Biotech	Cat# A100382
Trypsin	Sangon Biotech	Cat# A003702-0010
Iodine	Lircon	Cat# 500ML*1
Critical commercial assays		
Hematoxylin-Eosin (H&E) Staining Kit	Servicebio	Cat# G1005-500ML
Safranin O (SO) Solution	Servicebio	Cat# G1053-1
Experimental models: Organisms/strains		
Sprague Dawley rat (SD rat)	Hunan SJA Laboratory Animal Co., Ltd	Male, 6–8 weeks old, 220–250 g/rat
Balb/c nude mice	Hunan SJA Laboratory Animal Co., Ltd	Male, 6–8 weeks old, 20–25 g/mouse
Articular cartilage chondrocytes from 1 week old rabbit	Chen et al. (2020)	N/A
Other		
Millex-GP Syringe Filter Unit, 0.22 μm, polyethersulfone, 33 mm, gamma sterilized	Millipore	Cat# SLGP033RB
0.22 μm bottle top vacuum filter	Corning	Cat# 430626
Dialysis membrane (MWCO 3500 Da)	Sangon Biotech	Cat# F132592-0001
Centrifugal tube-15 mL	Greiner	Cat# 188261
Centrifugal tube-50 mL	Greiner	Cat# 210261
48-well cell culture plate	Greiner	Cat# 677180
10 μL tip	Greiner	Cat# 771291
200 μL tip	Greiner	Cat# 739290
1000 μL tip	Greiner	Cat# 686290
Pipetman P10, 1–10 μL	Gilson	Cat# F144802
Pipetman P100, 20–100 μL	Gilson	Cat# F123615
Pipetman P1000, 200–1000 μL	Gilson	Cat# F123602
Ophthalmic scissors	Shanghai JZ shoushuqixie	Cat# J21010
Sterile scalpel	Shanghai JZ shoushuqixie	Cat# 11#
Suture	Shanghai Jinhuan	Cat# R413
Electric razor	POVOS	Cat# PS2203
Flashlight with wavelength at 405 nm	EngineeringForLife	Cat# EFL-LS-1600-405
Petri dish	Corning	Cat# 430293
Digital light processing micro 3D printing system	BMF Material Technology Inc.	Cat# nanoArch S140
Enzyme label plate	Thermo Fisher Scientific	Cat# 446469
Microplate reader	Thermo Fisher Scientific	Cat# ThermoK3
Desktop refrigerated centrifuge	Thermo Fisher Scientific	Cat# ST16R
CryoTube	Thermo Fisher Scientific	Cat# 375418
Ultra-low temperature freezer	Thermo Fisher Scientific	Cat# Forma 700 series
HAAKE MARS III photo-rheometer	Thermo Fisher Scientific	Cat# 379-0340
Fluorescence microscope	Nikon	Cat# TS2R-FL
Vertical pressure sterilizer	BXM	Cat# BXM-30R
Biosafety cabinet	Heal Force	Cat# HF-1200

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Freeze dryer	Scientz	Cat# SCIENTZ-10N
UV protective glasses	UVATA	Cat# 1PCS UV protective glasses
Nuclear magnetic resonance spectrometer	Bruker	Cat# Bruker 400 MHz Advance

MATERIALS AND EQUIPMENT

Preparation of 10× PBS solution		
10×PBS	Final concentration	Amount
NaCl	1.37 M	24 g
KCl	26.8 mM	0.6 g
$Na_2HPO_4 \cdot 12H_2O$	0.1 M	10.74 g
KH_2PO_4	17.6 mM	0.72 g
ddH_2O	N/A	200 mL
NaOH (1 M)	N/A	Bring pH up to 6.8
ddH_2O	N/A	Bring volume up to 300 mL
Total	**N/A**	**300 mL**

Note: Store at 4°C for 6 months.

Alternatives: Commercial ready PBS buffer available in different companies such as Sigma (Cat# P5368-10PAK), Thermo Fisher Scientific (Cat# 14190144), and Servicebio (Cat# G4202-500mL) can also be used.

Preparation of penicillin-streptomycin solution		
Penicillin-streptomycin solution	Final concentration	Amount
Penicillin G sodium salt	100 U/mL	0.3125 g
Streptomycin sulfate	100 μg/mL	0.694 g
1×PBS (pH7.4)	N/A	50 mL
Total	**N/A**	**50 mL**

Note: Store at 4°C for 3 months.

Alternatives: Commercial ready penicillin-streptomycin solution available in different companies such as Procell (Cat# PB180120), Gibco (Cat# 15140-122), and Beyotime (Cat# C0222) can also be used.

Preparation of cell culture medium		
Cell culture medium	Final concentration	Amount
DMEM/high glucose medium	N/A	100 mL
FBS	N/A	10 mL
penicillin-streptomycin solution	N/A	1 mL
Total	**N/A**	**111 mL**

Note: Store at 4°C for 2 weeks.

Preparation of 0.5 M EDTA solution		
0.5 M EDTA solution	Final concentration	Amount
EDTA	0.5 M	8.4052 g
1×PBS (pH8.0)	N/A	50 mL
Total	**N/A**	**50 mL**

Note: Store at 4°C for 6 months.

Alternatives: Commercial ready 0.5 M EDTA solution available in Sigma-Aldrich (Cat# 03690-100 mL) can also be used.

Preparation of 0.25% (w/v) trypsin solution		
0.25% (w/v) trypsin solution	Final concentration	Amount
Trypsin	0.25% (w/v)	125 mg
0.5 M EDTA solution	N/A	100 μL
1×PBS (pH7.4)	N/A	49.9 mL
Total	**N/A**	**50 mL**

Note: Store at 4°C for 1 month.

Alternatives: Commercial ready 0.25% (w/v) trypsin-EDTA solution available in Gibco (Cat# 25200-056) can also be used.

Preparation of FDA stock solution		
FDA stock solution	Final concentration	Amount
FDA	0.5% (w/v)	25 mg
Acetone	N/A	5 mL
Total	**N/A**	**5 mL**

Note: Store at −20°C for 3 months.

Preparation of 4% (w/v) paraformaldehyde solution		
4% (w/v) paraformaldehyde solution	Final concentration	Amount
Paraformaldehyde	4% (w/v)	20 g
1×PBS (pH7.4)	N/A	500 mL
Total	**N/A**	**500 mL**

Note: Store at 4°C for 12 months.

Alternatives: Commercial ready paraformaldehyde fixator available in different companies such as Servicebio (Cat# G1101) and Sangon Biotech (Cat# E672002) can also be used.

STEP-BY-STEP METHOD DETAILS

Preparation of MA modified YM

Timing: 8 days

This step describes how to prepare modified YM by MA. The process is also suitable for other water-soluble polysaccharides to be modified by MA. The protocol was adapted from Huang et al. (2021).

Caution: This process should be done in darkness.

1. Add MA emulsion dropwise into YM solution with magnetic stirring at 100 rpm at 20°C–25°C in darkness in a fume hood. Troubleshooting 1.
2. Keep the reaction solution at pH8-9 by adding 1 M NaOH in the reaction process.

Note: If the addition of MA/1×PBS emulsion causes a large change in the pH of the solution, several milliliter of 5 M NaOH can be used to adjust the pH and then switch to a low concentration of 1 M NaOH to adjust the pH to 8–9.

3. Keep MA reacting with YM in the reaction solution for eight hours at 20°C–25°C in darkness.

Note: Under this reaction condition, the degree of functionalization of YM mainly depends on the feed ratio of MA to YM. The higher degree of substitution is obtained with the more mole ratio of MA to YM.

4. Neutralize the reaction solution by adding 0.1 M HCl.

Alternatives: The reaction can also be stopped by adding 5 times the volume of ddH_2O to dilute the solution.

5. Centrifuge the solution of reaction products to remove insoluble impurities at 3,260 × *g* for 5 min in 15 mL centrifugal tubes.
6. Dialyze the reaction product solution with dialysis membrane against 5–10 times volume of ddH_2O to remove unreacted MA and byproducts for at least 4 days, and then ddH_2O for three times a day.
7. Centrifuge the dialyzed product solution to remove insoluble impurities at 3,260 × *g* for 5 min in 15 mL pointed centrifugal tubes.

Alternatives: If you have a vacuum filter, a filter bottle, a Büchner funnel and filter membranes, you can remove insoluble impurities by pumping as well.

8. Freeze the aqueous product at −80°C more than 6 h.
9. Lyophilize the frozen product for three days.
10. Collect and weigh the freeze-dried product, keep the product dry and away from light. The product is MA-modified YM, also called YM-MA.
11. Analyze the characterization of YM-MA by ^1H NMR spectra.
    a. Prepare YM-MA/D_2O solution by dissolving 10 mg YM-MA into 0.5 mL D_2O.
    b. A nuclear magnetic resonance spectrometer is used to detect ^1H NMR spectra of YM-MA.
    c. Analyze ^1H NMR spectra of YM-MA using MestReNova software.

⚠ CRITICAL: MA is not water-soluble, but can be dispersed as an emulsion in water-based solutions. In order to react MA with YM molecules, MA must be fully dispersed in 1×PBS buffer and then added to the stirring mannan solution drop by drop.

Preparation of YM-MA bioink

Timing: 20 min

This step describes how to prepare YM-MA bioink. The protocol was adapted from Huang et al. (2021).

Caution: Perform all steps described in this section in a sterilized biosafety cabinet.

12. Prepare 10% (w/v) YM-MA bioink by dissolving 1 g YM-MA into 10 mL of 0.5% (w/v) LAP solution.
13. Filter the bioink through a 0.22 μm syringe filter and store at 4°C before use.

Note: In this section, LAP is used as the photoinitiator. As an alternative, I2959 can be also used as a photoinitiator. In this case, an UV light at a wavelength of 365 nm is recommended to support the following photocuring process.

14. Use a photo-rheometer with parallel-plate (P20 TiL, 20-mm diameter) geometry and OmniCure Series 2000 (365 nm, 30 mW/cm^2) to measure the photocrosslinking kinetics of YM-MA bioink containing photoinitiator I2959 at 37°C by recording the storage modulus (G′) and loss modulus (G′′) of YM-MA bioink over time.

Biocompatibility of YM-MA bioink

Timing: 38–40 days

This step describes how to prepare photopolymerized hydrogel based on YM-MA bioink and assess the biocompatibility of YM-MA bioink. This protocol requires a flashlight to initiate the bioink curing, cells and SD rats to assess the biocompatibility of the bioink. The protocol was adapted from Huang et al. (2021).

Caution: Perform all steps described in this section in a sterilized biosafety cabinet.

15. Assess cell viability of the YM-MA bioink *in vitro*.
    a. Add 200 μL YM-MA bioink into five wells of a 48-well plate and photocure the bioink by a flashlight of 405 nm for 10 s to prepare YM-MA hydrogel coatings on these wells. Trouble-shooting 2.
    b. Wash the monolayer cultured chondrocytes with 3 mL of 1×PBS buffer (pH7.4), when the cell growth density reaches 80%–90% in petri dish of 100 mm diameter.

    Note: Chondrocytes were isolated from articular cartilage of one-week-old New Zealand white rabbits. Isolation and culture of rabbit chondrocytes were following a previous publication without any modification (Chen et al., 2020).

    Alternatives: Chondrocytes can be purchased available in different companies such as Hefei Wanwu Biological Technology Co., Ltd (Cat# tings-98586).

    c. Remove the used 1×PBS solution and digest the cells by using 1 mL 0.25% (w/v) trypsin solution for 1 min.
    d. Add 4 mL cell culture medium to stop the digestion process and re-suspend the digested cells.
    e. Count cells with a cell counting plate under a microscope.
    f. Dilute the cell suspension with cell culture medium at 10^6 cells per milliliter.
    g. Seed 0.5 mL cell suspension on each YM-MA hydrogel coatings and incubate for 24 h at 37°C in a 5% CO_2 humidified incubator.
    h. Add 50 μL CCK-8 reagent to each well evenly and incubate for 2 h at 37°C.
    i. Measure the supernatant absorbance of the test samples by a microplate reader at 450 nm. The positive control is methylacryloyl modified gelatin (GELMA) bioink (Klotz et al., 2016).
    j. All data are expressed as mean ± standard deviation n = 5. Statistical analysis was performed using the GraphPad Prism 7 software. Statistically significant differences between

groups were detected by one-way analysis of variance (ANOVA) at a confidence interval of 95%. The differences were considered at $p < 0.05$(*), $p < 0.01$(**), and $p < 0.001$(***).

16. Assess histocompatibility of YM-MA bioink *in vivo*.
    a. Add 200 μL YM-MA bioink into each well of a teflon mold (d = 10 mm, h = 2 mm), photocure the bioink by a flashlight of 405 nm for 10 s to prepare photopolymerized hydrogels.
    b. Anesthetize SD rats with 10% (w/v) chloral hydrate by intraperitoneally injecting, 3 mL/kg.
    c. Remove hair from the rats' back by using an electric razor.
    d. Disinfect the back of SD rats with iodine.
    e. Use a sterile scalpel of 11# to cut the skin on the disinfected back of the SD rats to make a wound with 10–15 mm wide.
    f. Use ophthalmic scissors to separate the skin and muscle layers to create space for the implant.
    g. Implant the photopolymerized hydrogel from YM-MA bioink into the created space of the SD rats subcutaneously.
    h. Close the wound with sterilized sutures.
    i. Culture all the test SD rats for 28 days with no dietary or activity restrictions.
    j. Sacrifice the SD rats by intraperitoneally injecting excessive chloral hydrate.
    k. Isolate the samples with surrounding tissue by using a sterile scalpel.
    l. Histological staining of the samples.
        i. Fix the samples in 4% (w/v) paraformaldehyde solution for 48 h.
        ii. Dehydrate the fixed samples with gradient ethanol, which are 50%, 70%, 80%, 90%, 95% and 100% in ddH_2O, for 1 h each.
        iii. Vitrify the dehydrated samples with xylene for three times.
        iv. Embed the vitrified samples with paraffine.
        v. Slice the embed samples into 5 μm thickness with a radial microtome.
        vi. Stain the slices with H&E Staining Kit according to the protocols of the manufacturer (https://www.servicebio.com/G1005-100ML-Hematoxylin-and-Eosin-Joint-Staining-HE-Stain-Solution-pd47001614.html?searchValue=G1005-100ML%20Hematoxylin%20and%20Eosin%20Joint%20Staining%20HE%20Stain%20Solution).
        vii. Seal the stained slices with neutral gum.
        viii. Observe the stained slices with an optical microscope.

Printability of YM-MA bioink

Timing: 0.5–1 day

This step describes how to print the YM-MA bioink by a common light-assisted printer. The protocol was adapted from Huang et al. (2021).

Caution: Perform all the steps described in this section in a clean room.

17. Build 3D computer aided design (CAD) models of designed shapes by solidworks software.
18. Translate the models into slices by BMF 3D slicer software.
19. Wash the monolayer cultured chondrocytes with 3 mL of 1×PBS buffer (pH7.4), when the cell growth density reaches 80%–90% in petri dish of 100 mm diameter.
20. Remove the used 1×PBS solution and digest the cells by using 1 mL 0.25% (w/v) trypsin solution.
21. Add 4 mL cell culture medium to stop the digestion process and re-suspend the digested cells.
22. Count cells with a cell counting plate under a microscope.
23. Dilute the cell suspension with YM-MA bioink at 10^7 cells per milliliter. Troubleshooting 3.
24. Put the YM-MA bioink containing living cells into the tank of the printer. Troubleshooting 4.

25. Use BMF printing system to instruct the light-assisted printer to print layers of models one by one, 10 μm per layer. The exposure intensity is 5 mW/cm^2. The exposure time is 0.5 s per layer. Troubleshooting 5.
26. Observe macroscopic structures of the printed samples by a digital microscope.
27. Wash the printed structures with 1×PBS buffer (pH7.4) for three times.
28. Stain the washed structures with FDA working solution for 10–15 min.

Note: FDA working solution was prepared by diluting FDA stock solution with one hundred times the volume of high glucose DMEM medium.

29. Wash the stained structures with 1×PBS buffer (pH7.4) for three times.
30. Observe the stained printed structures with a fluorescence microscope at 490 nm.

Ectopic chondrogenesis of YM-MA bioink

Timing: 20–22 days

This step describes how to construct tissue engineering cartilage by ectopic implantation in nude mice subcutaneously. The protocol was adapted from Huang et al. (2021).

Caution: Perform all steps described in this section in a sterilized biosafety cabinet.

31. 3D printing YM-MA bioink with chondrocytes into a round pie shape with a diameter of 10 mm and a thickness of 2 mm.
32. Preserve the YM-MA hydrogel samples in culture medium at 37°C in a 5% CO_2 humidified incubator.
33. Disinfect the back of nude mice with iodine.
34. Use a sterile scalpel of 11# to cut the skin on the disinfected back of the nude mice to make a wound with 10–15 mm wide.
35. Use ophthalmic scissors to separate the skin and muscle layers to create space for the implant.
36. Implant the photocured chondrocytes/YM-MA hydrogels into the created space of the nude mice subcutaneously.
37. Close the wound with sterilized sutures.
38. Culture all the test nude mice for 14 days with no dietary or activity restrictions.
39. Sacrifice the nude mice by intraperitoneally injecting excessive chloral hydrate.
40. Isolate the samples by using a sterile scalpel.
41. The samples were fixed, dehydrated, vitrified, embed, and sliced as described in steps 16 l i–v.
42. Stain the slices with Safranin O solution for glycosaminoglycan (GAG) detection according to the protocols of the manufacturer (https://www.servicebio.com/Safranin-O-Fast-Green-Staining-Reagent-For-Bone-Tissue-pd43990414.html?searchValue=Safranin%20O-Fast%20Green%20Staining%20Reagent%20For%20Bone%20Tissue).
43. Stain the slices with immunohistochemical stain for type II collagen according to the protocols of the manufacturer (https://www.servicebio.com/Immunohistochemistry-Kit-HRP-conjugated-Goat-Anti-Mouse-IgG-H-L-pd43814804.html?searchValue=Immunohistochemistry%20Kit%20(HRP-conjugated%20Goat%20Anti-Mouse%20IgG(H%2520L))).
44. Seal the stained slices with neutral gum.
45. Observe the stained slices with an optical microscope.

EXPECTED OUTCOMES

YM is a natural polysaccharide from Saccharomyces cerevisiae, which is the yeast cell wall polysaccharide with the strongest immune function and possesses bioactivities highly valuable to tissue engineering, including biocompatibility, immune-regulation, anti-inflammatory, anti-oxidation,

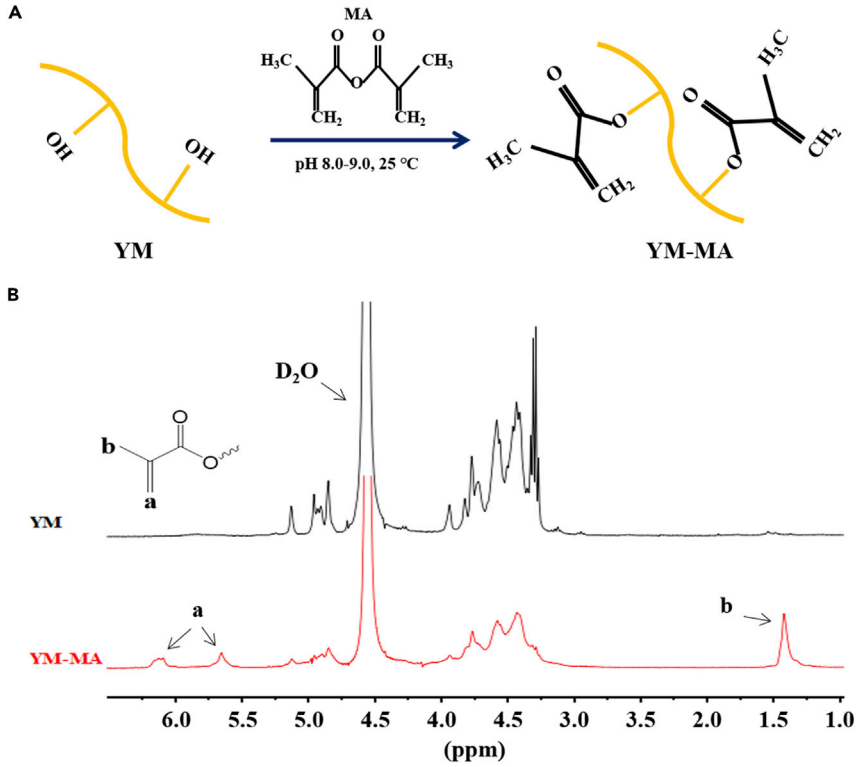

Figure 1. Characterization of YM-MA
(A) Synthesis diagram of YM-MA.
(B) ^{1}H NMR spectra of YM-MA in D_2O.
Adapted from Huang et al. (2021).

anti-tumor and other physiological functions (Singh et al., 2018; Liu and Huang, 2018). Besides, as a typical polysaccharide, YM owns many chemical reaction sites, especially hydroxyl which is conducive to modify YM, and further hydrogels can be prepared by photocrosslinking. Herein, a novel bioink based on modified YM was introduced to 3D bioprinting for tissue engineering applications.

In order to make YM a bioink, MA was successfully grafted on YM as confirmed in ^{1}H NMR spectra, which provided YM with photocrosslinkability (Figure 1). The result of dynamic time-sweep rheological analysis showed that hydrogels, which are round shape with 20-mm diameter and 1-mm thickness, formed in less than 20 s following exposure to 365 nm UV at the dose of 30 mW/cm^2 (Figure 2). Since excellent biocompatibility of biomaterial is the requirement for tissue engineering, cell experiments *in vitro* and subcutaneous implantation of SD rats *in vivo* were conducted to evaluate the biocompatibility of YM-MA bioink. Compared with GELMA bioink, YM-MA bioink displayed no cytotoxic to cells *in vitro* (Figure 3). In an SD rat model, no macroscopic signs of inflammation or toxicity were evident in host tissue surrounding the implants after four weeks (Figure 4A). Although fibrous tissue was identified surrounding the implants, it was acceptable for host according to documented biomaterials for tissue engineering. Additionally, H&E staining analysis further confirmed that YM-MA bioink implants were encased in fibrous capsules without any inflammatory cells (Figure 4B). Biocompatibility tests suggested that YM-MA bioink was a prospective candidate in 3D printing for tissue engineering.

A sunken light-assisted printer was applied to evaluate printability of YM-MA bioink (Figure 5). In 3D bioprinting tests, YM-MA containing chondrocytes was printed respectively at 5 mW/cm^2 for 0.5 s per 10 μm thickness. The results indicated that printed products with good structural presentation

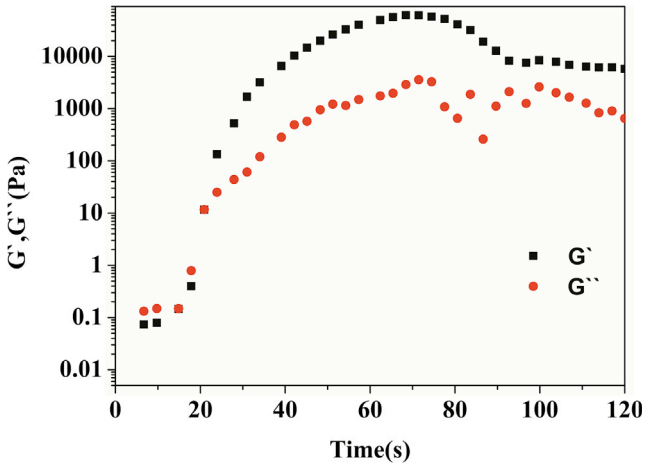

Figure 2. Rheological analysis of YM-MA bioink
Adapted from Huang et al. (2021).

maintained a high degree of consistency with CAD models (Figures 6 and 7). Notably, cells encapsulated in printed structures *in situ* were viable and distributed evenly (Figure 7). It suggested that biocompatible YM-MA bioink owned good printability.

Still, YM-MA bioink with chondrocytes was performed in nude mice model for two weeks to evaluate ectopic chondrogenesis ability of YM-MA bioink. The collected samples were sliced and subjected to the safranin O staining and immunohistochemical staining. The results showed that abundant GAG and type II collagen secretion was identified in test specimens (Figure 8), indicating that the YM-MA bioink could provide a promising hydrogel for cartilage tissue engineering. Hence, the proposed YM-MA is a promising bioink in 3D printing for tissue engineering applications.

Six advantages are obtained from this strategy: (1) the solubility of YM in neutral pH solution was significantly increased by introducing methacryloyl groups, which were injectable with adjustable viscosity, which can be used as an injectable photocrosslinkable hydrogel for minimally invasive tissue repair (Liu et al., 2017); (2) YM-MA is easy to be sterilization and can be stored up for one year at −20°C in darkness; (3) when exposed to blue light radiation, YM-MA bioinks show rapid gelation; (4) by adjusting methacrylation degree of YM-MA, the compression strength of YM-MA hydrogels could be tuned from 27 to 57 kPa (Huang et al., 2021), covering the range of the mechanical properties of various native tissues, including

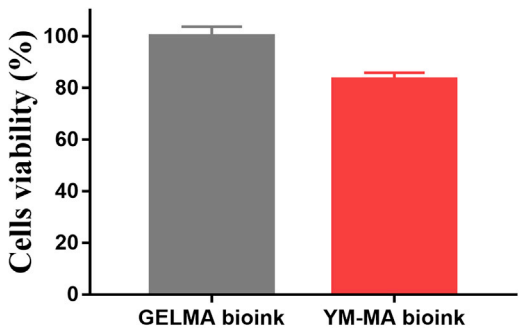

Figure 3. Cell viability in photocrosslinked YM-MA bioink and GELMA bioink
Adapted from Huang et al. (2021).

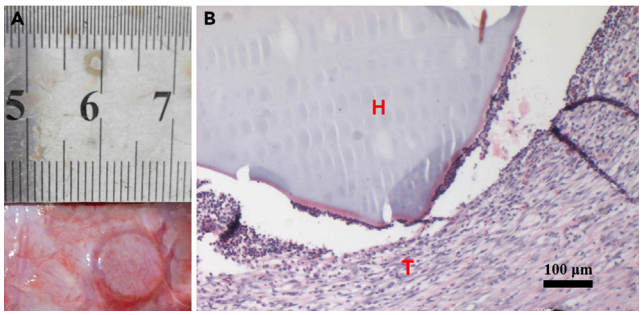

Figure 4. *In vivo* biocompatibility of YM-MA hydrogel

(A and B) Gross morphology examination and (B) H&E staining of implanted YM-MA hydrogel after four-week post-implantation. T: tissue; H: hydrogel. Scale bars, 100 μm.

Adapted from Huang et al. (2021).

cartilage, cardiac muscle and lung; in addition, (5) the gelation method not only provided spatiotemporal control for gelation *in situ*, but also eliminated the need for biotoxic crosslinking agents and did not require temperature changes, thus ensuring biocompatibility and permitting embedding cells alive (Klotz et al., 2016); notably, (6) 3D printing of YM-MA bioink outlined clear boundary consistent with the CAD model, it also achieved ectopic chondrogenesis. These advantages strongly support the notion that YM-MA bioink is a potential 3D printing platform for tissue engineering.

LIMITATIONS

Although this protocol provides a versatile strategy to develop novel bioinks from natural materials, it is limited to water-soluble polymers containing hydroxyl and amino groups. The reason why is that modification process is based on the acylation of amino and anhydride or the esterification of hydroxyl and anhydride.

In our previous publication (Huang et al., 2021), we prepared three YM-MA, named YM-MA-1, YM-MA-2, and YM-MA-3, respectively. These three YM-MA bioinks were photocrosslinked as hydrogels, named YM-MA-1-H, YM-MA-2-H, and YM-MA-3-H, respectively. Cell viability in these photocrosslinked hydrogels has been previously assessed by CCK-8 assay and no significant differences among ctrl (GELMA hydrogel), YM-MA-1-H and YM-MA-2-H were found, with cell viability more than 80% (Huang et al., 2021). In this protocol, we used YM-MA-2-H, which displays both good cytocompatibility and histocompatibility.

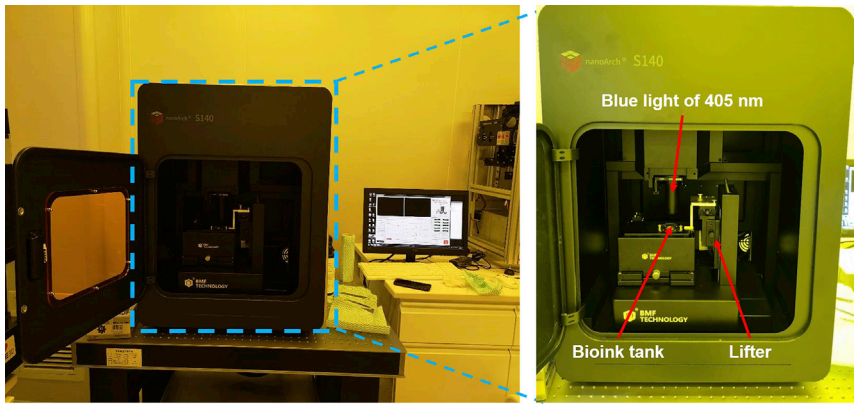

Figure 5. 3D printing of YM-MA bioink by a BMF technology S140 printer

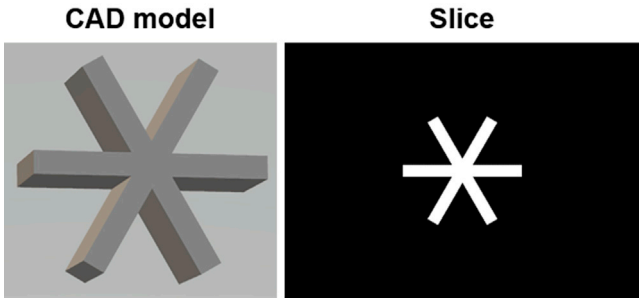

Figure 6. CAD model and slice of printing structures with cells
Adapted from Huang et al. (2021).

TROUBLESHOOTING

Problem 1

The degree of functionalization of YM-MA molecules by MA were lower than expected.

Potential solution

During the modification process, add the homogenized MA emulsion dropwise into YM reaction solution and stir the reaction solution at a certain speed such as 100 rpm to avoid phase separation of the solution. The uniform solution facilitates the full contact of solutes and facilitates the modification process (Figure 1).

Problem 2

Bioink was not photocured by blue light irradiation.

Potential solution

When bioink was not photocured by blue light irradiation, it might be the reasons that photoinitiators LAP was failing or YM-MA was not modified successfully. We recommend replacing the LAP, and using ^{1}H NMR to evaluate whether YM was modified by MA successfully (Figure 1).

Problem 3

Cells encapsulated in photocrosslinked YM-MA bioink were not evenly distributed.

Potential solution

Mix bioink and cells sufficiently before print and increase printing energy to spend a short time in printing (Figure 7).

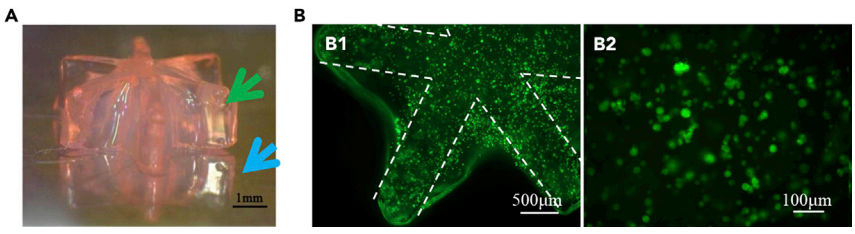

Figure 7. Characterization of 3D printed YM-MA bioink containing chondrocytes
(A) Profiles of YM-MA hydrogel with chondrocytes. The green arrow points to the printed product while the blue arrow points to the reflection of the printed one on the platform.
(B) Live/dead staining of cells in 3D printed structure. The white dotted line represents the outline of the printed structure. Scale bars, 1 mm (A), 500 μm (b1), 100 μm (b2).
Adapted from Huang et al. (2021).

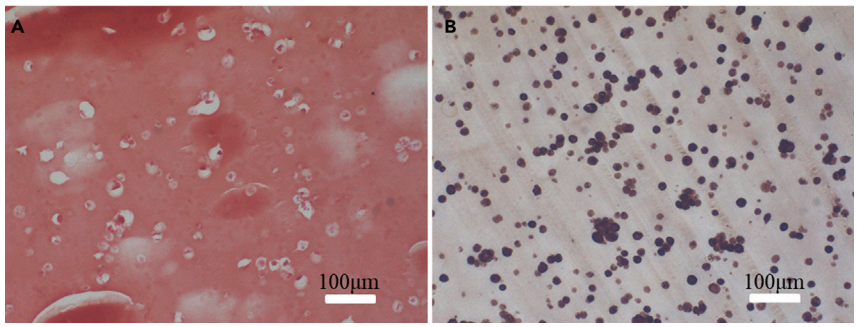

Figure 8. Safranin O staining (A) and immunohistochemical staining of type II collagen (B) on slices of YM-MA hydrogel samples containing chondrocytes after two-week post-implantation

Scale bars, 100 μm.

Adapted from Huang et al. (2021).

Problem 4

Bioinks were gelled before printing.

Potential solution

Always keep bioinks away from light, store them at 4°C or lower temperature. In fact, we recommend preserving the bioink materials in powder form. Prepare aqueous state of bioinks prior to use is expected.

Problem 5

Printed structures did not conform with CAD models because that bioinks were overcured or no gelation was obtained (Table 1 and Figure 9).

Potential solution

When printing results did not match the preset shapes, it usually meant that the printing energy needed to be adjusted. Printing energy could be adjusted by exposure intensity, exposure time and printing layer thickness. When bioinks were overcured, decrease printing energy. When bioinks were not gelled, increase printing energy.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Zheng Zhou, zhouzheng@hnu.edu.cn.

Materials availability

The prepared YM-MA in this study will be made available on request, but we may require a payment and/or a completed Materials Transfer Agreement if it is potentially used for commercial production.

Data and code availability

This study did not generate datasets or codes.

Table 1. 3D printing parameters of YM-MA bioinks without cells

Model	Square	Letters	Porous structure	Character	Hemisphere
Size (mm)	8 (Length)	0.5 (Line diameter)	0.5 (Hole radius)	0.5 (Line diameter)	3 (Radius)
Section numbers (10 μm/layer)	50	200	150	200	300
Exposure intensity (mW/cm^2)	20	10	7	5	4
Exposure time (seconds)	0.5	0.5	0.5	0.5	0.5

Adapted from Huang et al. (2021).

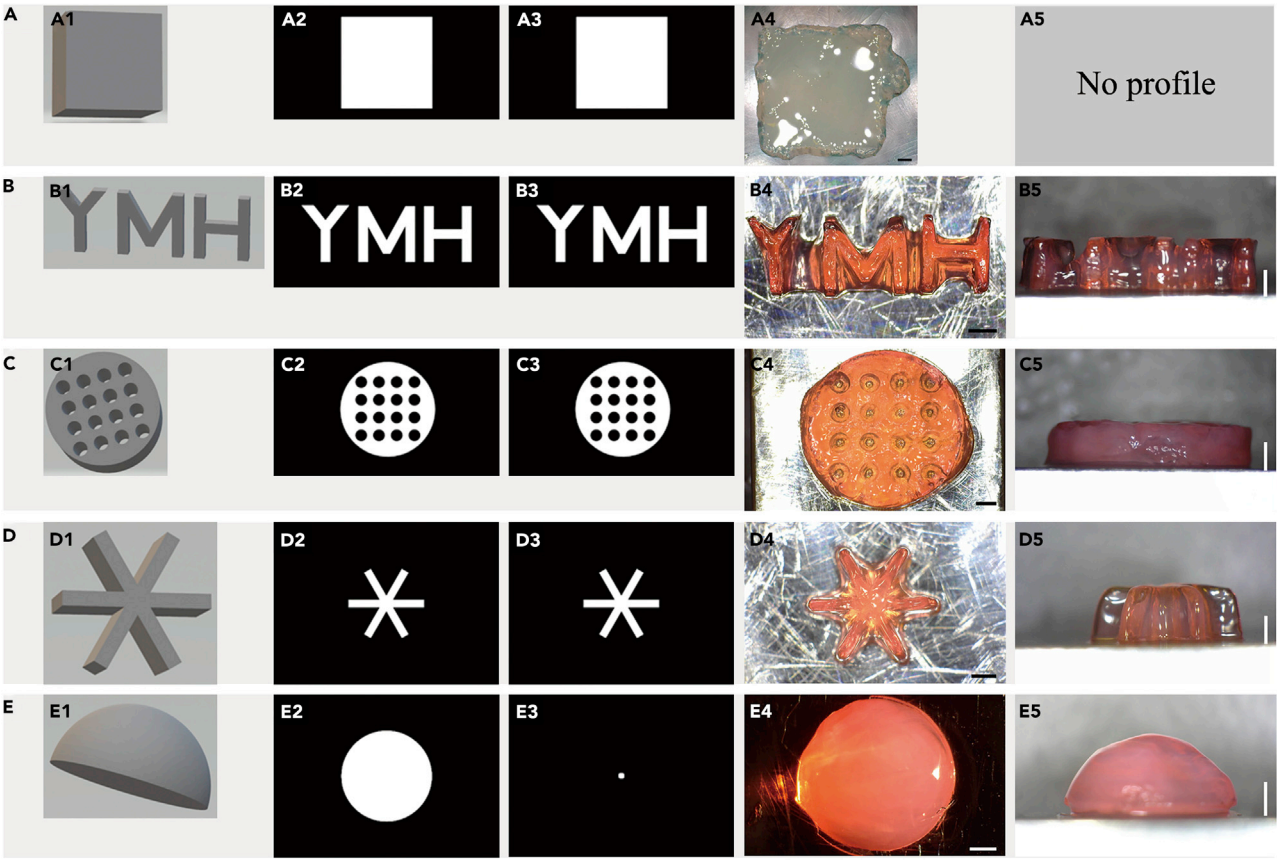

Figure 9. Digital models and general view of 3D printed YM-MA bioinks without cells

(A) Square.

(B) Letters.

(C) Porous structure.

(D and E) Character and (E) hemisphere. From left to right, (a1, b1, c1, d1, e1) 3D computer models, (a2, b2, c2, d2, e2) the first slice of the 3D computer models, (a3, b3, c3, d3, e3) the last slice of the 3D computer models, (a4, b4, c4, d4, e4) top view of printed hydrogels and (a5, b5, c5, d5, e5) side view of printed hydrogels respectively.

Scale bars, 1 mm.

Adapted from Huang et al. (2021).

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AUTHOR CONTRIBUTIONS

Conceptualization and investigation, Z.Z., Y.H., and H.L.; writing – original draft, Z.Z. and Y.H.; writing – review & editing, all authors; visualization, Z.Z. and Y.H.; funding acquisition, Z.Z. and H.L.; supervision, Z.Z., H.L., and G.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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