nature protocols

https://doi.org/10.1038/s41596-026-01341-1

Protocol

Check for updates

# Rapid volumetric bioprinting of pristine protein-based (bio)inks

Maobin Xie[1,2,6], Liming Lian[2,6], Zhenrui Zhang[2], Zeng Lin[2], Emilio Mireles Guajardo[2], Jugal Kishore Sahoo[3], Guosheng Tang[2], Gang Li[3,4], Khoon S. Lim[5], David L. Kaplan[3] & Yu Shrike Zhang[2]

## Abstract

Volumetric bioprinting (VBP) enables the rapid photopolymerization of 3D constructs by modifying the illumination patterns within a build volume. However, only a few unmodified, pristine protein-based bioinks can be used for VBP, making the resulting (bio)printed volumes sometimes incompatible with further modification steps required for extended applications and thus limiting the wider adoption of VBP. We have recently developed new methods for VBP, in which unmodified protein-based (bio)inks with tyrosine groups, including those based on silk, decellularized extracellular matrix (dECM) and gelatin, can be (bio)printed, in their pristine state, by using the tris(2,2-bipyridyl)dichlororuthenium(II) hexahydrate/sodium persulfate photoinitiator system to form sophisticated shapes and architectures. Here, we provide step-by-step instructions to complete the VBP process and include the characterization of these bioinks. After treatment, the volumetrically printed silk sericin constructs show properties including reversible shrinkage and expansion, or shape-memory, whereas the volumetrically printed silk fibroin constructs exhibit broadly tunable mechanical performances ranging from a few hundred pascals to hundreds of megapascals. Both types of silk-based (bio)inks as well as dECM (bio)inks are cytocompatible. We further cover several demonstrations that show the potential uses of volumetrically (bio)printed silk and dECM constructs in clinical and biomedical applications.

## Key points

- The procedure includes the preparation of silk-, dECM- or gelatin-based (bio)inks, followed by volumetric bioprinting and then the embedding of cells within the volume to be bioprinted, with an example of their use for biomedical applications.
- Alternative methods include layer-by-layer bioprinting; however, VBP of unmodified tyrosine-containing protein-based (bio)inks improves the fabrication speed to within ~120 s.

## Key references

Xie, M. et al. *Nat. Commun.* **14**, 210 (2023): https://doi.org/10.1038/s41467-023-35807-7

Lian, L. et al. *Adv. Mater.* **36**, 2304846 (2024): https://doi.org/10.1002/adma.202304846

[1]The Fourth Affiliated Hospital of Guangzhou Medical University, School of Biomedical Engineering, Guangzhou Medical University, Guangzhou, P. R. China. [2]Division of Engineering in Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, USA. [3]Department of Biomedical Engineering, Tufts University, Medford, MA, USA. [4]National Engineering Laboratory for Modern Silk; College of Textile and Clothing Engineering, Soochow University, Suzhou, P. R. China. [5]School of Medical Sciences, Charles Perkins Centre, The University of Sydney, Sydney, Australia. [6]These authors contributed equally: Maobin Xie, Liming Lian. e-mail: yszhang@bwh.harvard.edu

## Introduction

Volumetric additive manufacturing achieves rapid photopolymerization by illuminating dynamic light patterns across the entire volume of a rotating vial containing the (bio)ink to achieve simultaneous cross-linking[1], while volumetric bioprinting (VBP) for fabrication of cell-embedded constructs was concurrently reported[2] (Fig. 1a). The application of VBP spans a broad range of biomedical fields, including but not limited to medical devices[3,4], tissue engineering[5,6] and organoid engineering[7]. However, expanded applications of VBP are limited by the few selections of unmodified, pristine protein-based (bio)inks currently available[8,9], which reduce unnecessary modification steps that are possibly incompatible with certain applications.

### Development of the protocol

Protein-based biomaterials such as those based on silk and decellularized extracellular matrix (dECM) contain abundant native tyrosine residues that could form di-tyrosine cross-links after photooxidation in the presence of the tris(2,2-bipyridyl)dichlororuthenium(II) hexahydrate/sodium persulfate (SPS) (Ru/SPS) system[10,11]. Nonetheless, most protein-based materials used in photocuring biofabrication require chemical modifications, such as the introduction of methacryloyl or norbornene groups[1], to allow light-induced cross-linking. Meanwhile, the high-concentrations needed for these modified protein-based materials pose challenges in using them for further biomedical applications, because of the resulting dense polymer networks that would impede nutrient transport and sometimes be mechanically inhibitive[12]. Moreover, increased photoinitiator concentrations may be needed when using high concentrations of bioinks during photocuring processes, which could be harmful to cells as well[10].

To overcome these challenges, we have developed unmodified protein-based (bio)inks (including silk-based (bio)inks, dECM (bio)inks and gelatin (bio)inks, all formulated separately) that could be (bio)printed in their pristine form to build sophisticated constructs[10,11]. We have identified silkworm silk derived from *Bombyx mori*, a readily available natural protein composed primarily of silk sericin (SS) (the content of tyrosine groups is ~3.38 mol%) and silk fibroin (SF) (the content of tyrosine groups is ~5 mol%)[13,14] (Fig. 1b), as novel (bio)ink candidates. SF is traditionally used in high concentrations (>5%, wt/vol) for extrusion-based or digital light processing–based (bio)printing[15]. We, instead, present the first instance of applying pure SS and SF for VBP. Moreover, compared with high-concentration silk-based (bio)inks used in digital light processing platforms, we have successfully (bio)printed varying concentrations of silk-based (bio)inks in our VBP platform, achieving a minimum concentration of 2.5% (wt/vol)[10].

In addition, our work includes the successful preparation of low-concentration pure dECM (bio)inks[11] (Fig. 1b), which are procured from decellularized tissues, retaining essential components that foster a cell-compatible microenvironment. We have volumetrically (bio) printed sophisticated structures with unmodified heart-derived dECM (h-dECM) (bio)inks (the content of tyrosine groups in h-dECM is ~0.15 mol%) and meniscus-derived dECM (Ms-dECM) (bio)inks (the content of tyrosine groups in Ms-dECM is ~1 mol%), although the inherent mechanical properties of dECM are typically poor for (bio)printing[16–18]. Specifically, we have volumetrically bioprinted the heart-like constructs with rat cardiomyocyte (rCM)-laden h-dECM bioinks and human induced pluripotent stem cell–derived cardiomyocyte (hiPSC-CM)-laden h-dECM bioinks separately, as well as meniscus-like constructs with human mesenchymal stem cell (hMSC)-laden Ms-dECM bioinks. The volumetrically bioprinted heart-like constructs achieved spreading and contracting behaviors, and the volumetrically bioprinted meniscus-like constructs demonstrated appropriate chondrogenic differentiation and gene expressions.

Furthermore, unmodified porcine gelatin has also been successfully (bio)printed by using VBP in this protocol (Fig. 1c), despite the fact that its tyrosine content is <0.5 mol%[19]. The efficiency of this cross-linking process, coupled with the satisfactory (bio)printing performances, underscore the versatility and potential of using a diverse type of unmodified protein-based biomaterials with VBP. Our pioneering efforts in developing a series of pristine, unmodified protein-based (bio)inks (silk, dECM and gelatin as examples) not only expand the

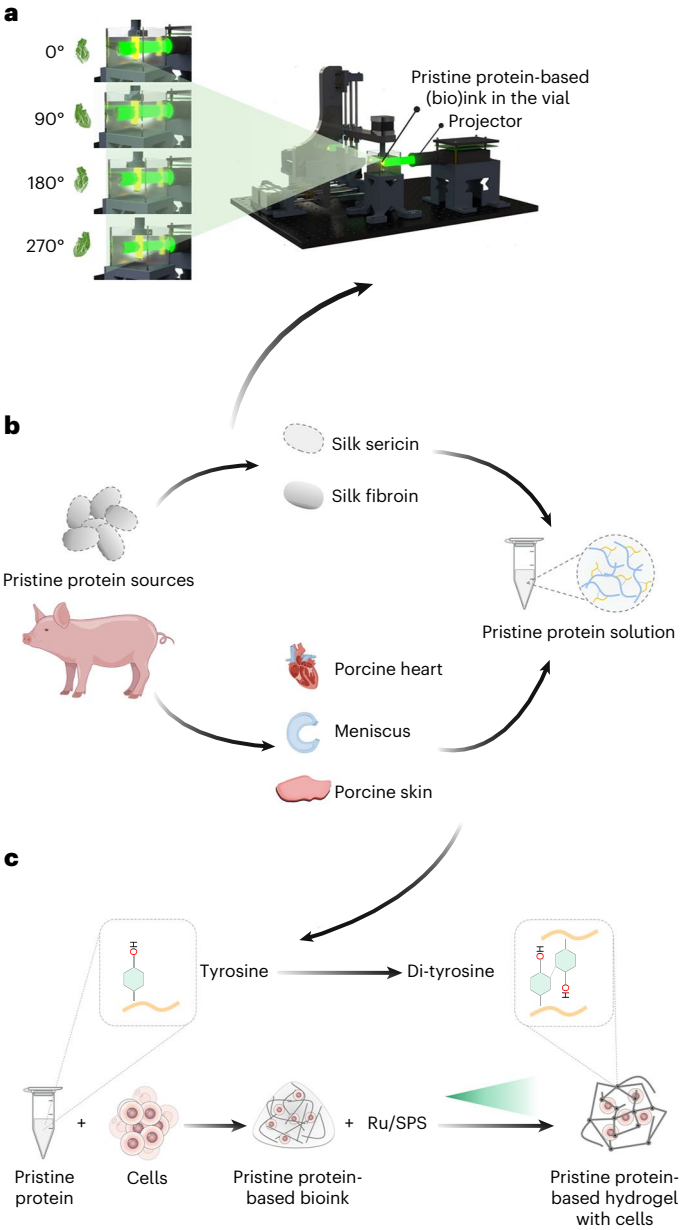



**Fig. 1 | Schematic showing the tyrosine-containing pristine protein-based (bio)ink preparation and VBP process. a**, The customized VBP system, which mainly includes a projector, a vial-rotation motor and a (bio)ink-containing vial. **b**, Extraction and preparation of different types of tyrosine-containing pristine protein biomaterials. **c**, Preparation of cell-laden bioink and the photo-cross-linking mechanism of tyrosine-containing pristine protein-based (bio)ink. Ru/SPS, tris(2,2-bipyridyl)dichlororuthenium(II) hexahydrate/sodium persulfate.

library of photocurable protein-based (bio)inks, but also provide a better understanding for the mechanism of rapid volumetric (bio)printing of these tyrosine-containing biomaterials. Additional possible (bio)ink options may include but are not limited to spider silk, *Antheraea perenyi* silk, and *Antheraea mylitta* silk (Supplementary Table S1). The methodologies for preparing these protein-based (bio)inks and their respective VBP processes are illustrated in the following sections, charting a route for future explorations and applications in VBP.

## Overview of the procedures

The whole procedure includes the following four parts: (i) the VBP method; (ii) the preparation procedures for silk (SS or SF) (bio)inks, dECM (h-dECM or Ms-dECM) (bio)inks and gelatin (bio) inks; (iii) the procedure for VBP of protein-based (bio)inks without embedding cells and their characterizations; and (iv) the procedure for VBP of protein-based bioinks with embedded cells and their biomedical applications.

In Procedure 1, the preparation steps for protein-based (bio)inks are described in detail. We commence with the extraction process of SS and SF from *B. mori* cocoons (Procedure 1, Steps 1–36), followed by the derivation of h-dECM and Ms-dECM solutions from porcine tissues (Procedure 1, Steps 37–55). Then, the protocol describes the preparation processes for silk-based (bio)inks, dECM (bio)inks and gelatin (bio)inks, all formulated separately, alongside the execution steps for the VBP process (Procedure 1, Steps 56–69), which include the optimizations of printing parameters and the evaluations of printing resolutions for all protein-based (bio) inks. In addition, the physicochemical properties of silk-based (bio)inks and dECM (bio)inks are assessed. Specifically, post-printing treatment and additional functional evaluations for the printed silk-based constructs are also presented.

In Procedure 2, the preparation steps for cell-laden silk-based bioinks and dECM bioinks as well as the following VBP process are described (Procedure 2, Steps 1–24). In Procedure 2, NIH/3T3 fibroblasts are encapsulated in silk-based bioinks and dECM bioinks for cytocompatibility tests. Subsequently, the biological applications are described: (i) the volumetrically printed SF screw constructs with double-cross-linked networks induced by immersing in 70% (vol/vol) ethanol aqueous solution with post-seeded hMSCs, (ii) the volumetrically bioprinted heart-like constructs with rCM-laden h-dECM and (iii) the volumetrically bioprinted meniscus constructs with hMSC-laden Ms-dECM.

The versatility of tyrosine-containing protein-based bioinks allows for encapsulation of various cell types, enabling the fast creation of different tissue-like structures through VBP. Therefore, it is believed that a broad utility across diverse tissue-engineering domains could be achieved. Here, we present guidelines for applying these pristine tyrosine-containing protein-based (bio)inks within the field of tissue engineering, with an expectation that they are adaptable to other biomedical applications as well.

## Advantages and limitations of VBP of pristine protein-based (bio)inks

VBP shows outstanding advantages in fast (bio)printing speed, non-contact (bio)printing mode and smooth surface quality compared to layer-by-layer (bio)printing strategies. However, the lack of (bio)inks is a key limitation for expanded biomedical applications of this technology.

The unmodified tyrosine-containing protein-based (bio)inks in this study are composed of photoactive polymers, photoinitiators/co-initiators, without or with cells, ensuring that the intrinsic properties of the proteins are preserved. Notably, in contrast to layer-by-layer (bio) printing methods, VBP of unmodified tyrosine-containing protein-based (bio)inks shows a fast fabrication speed (normally within 120 s); these protein-based (bio)inks could be volumetrically (bio)printed at low concentrations in most cases (down to 2.5% (wt/vol) for SS or SF and 1% (wt/vol) for dECM, with an exception of 10% (wt/vol) for gelatin due to its extra-low tyrosine content) with high fidelity. Furthermore, these protein-based bioinks could facilitate the creation of microenvironments with good cytocompatibility.

However, tissues and organs are intricately assembled systems, comprising different cell types embedded within matrices with varying mechanical properties. Therefore, VBP of a single (bio)ink formulation may not satisfy the complex functional needs for tissues and organs, and methods for multi-material and multi-cellular VBP have been developed[5,20–22]. Moreover, the concentrations of photoinitiators and (bio)inks are important factors that affect (bio)printing resolution and cell viability. In this protocol, we have further evaluated the impact of different photoinitiator concentrations on printability and printing resolution within the range of protein-based (bio)inks that we have showcased.

## Experimental design

### Preparation of silk-based (bio)inks and dECM (bio)inks

For printing the silk-based and dECM solutions, we use Ru/SPS as the photoinitiator complex, which engages with tyrosine residues present in the proteins, catalyzing cross-linking with light exposure[23–25].

For SS, *B. mori* cocoons are cut into pieces and boiled in $Na_2CO_3$ solution to remove SS from SF, and then the SS solution is dialyzed with deionized water (DW). For SF, *B. mori* cocoons are cut into pieces and boiled in aqueous $Na_2CO_3$ solution for varying durations

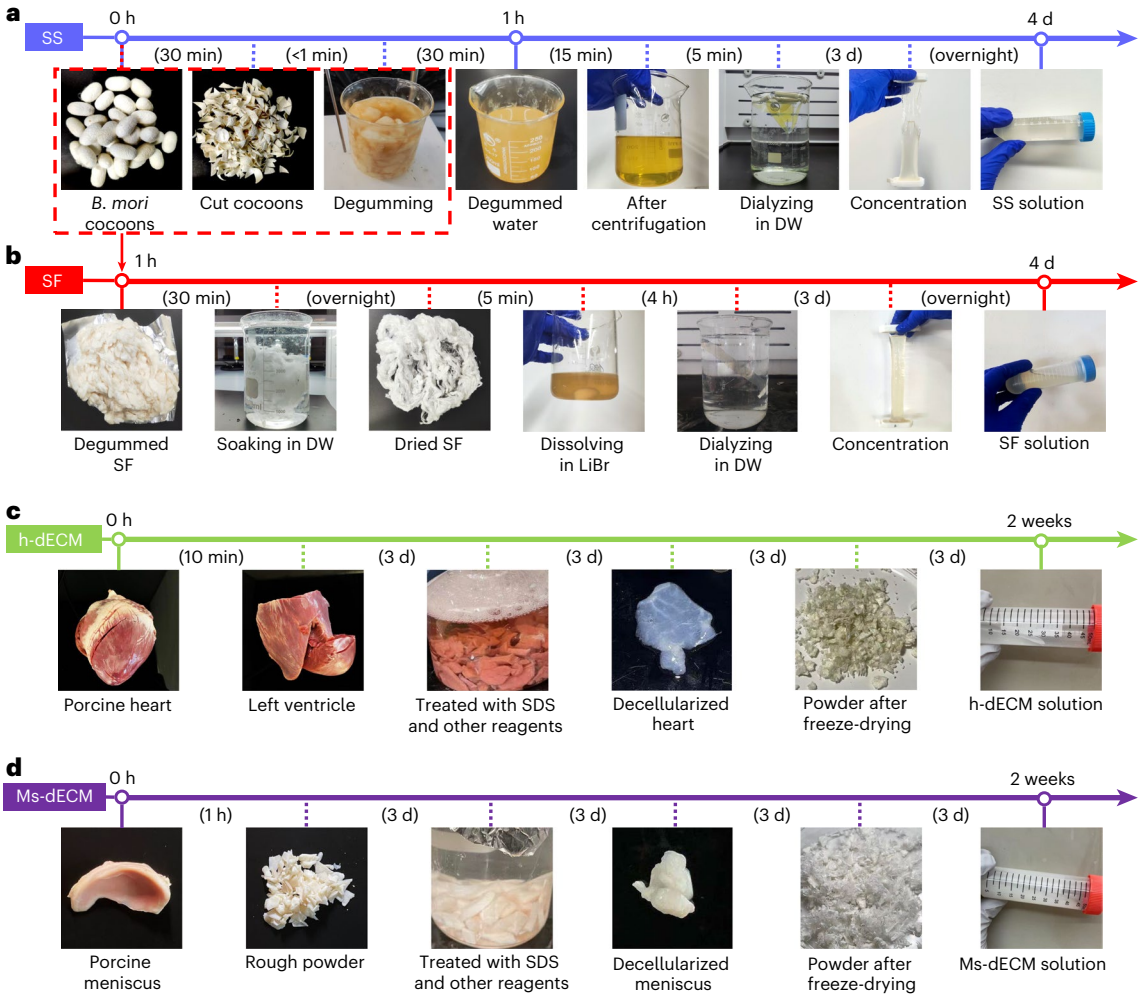



**Fig. 2 | Preparation of silk and dECM solutions. a**, The routine for preparation of SS for silk fibers. **b**, The routine for preparation of SF solutions from silk cocoons. **c**, The routine for processing the porcine heart into h-dECM solution by decellularization and solubilization. **d**, The routine for processing the porcine meniscus into Ms-dECM solution by decellularization and solubilization. *B. mori*, *Bombyx mori*; SDS, sodium dodecyl sulfate. Panels **c** and **d** are adapted with permission from ref. 11, Wiley.

to achieve different molecular weights. Then, the degummed SF is dissolved in lithium bromide (LiBr). After dialyzing the SF/LiBr solution with DW, the solution is centrifuged. Next, the precipitate is discarded, and the supernatant is filtered. The SS and SF solutions are loaded into dialysis bags and placed in the hood for concentrating. The final concentration is calculated by weighing the remaining mass after evaporating the water of the SS or SF solution (Fig. 2a).

For h-dECM, the fresh left ventricle is separated from the porcine heart (obtained from a local butcher, exempt from institutional review board approval). The tissue should be subjected to decellularization by using a solution of sodium dodecyl sulfate (SDS) in PBS. After treating decellularized tissue with Triton X-100 in PBS, the tissue is stirred in isopropyl alcohol and rinsed with PBS to remove all the residual detergent (Fig. 2b). For Ms-dECM, the fresh porcine meniscus is harvested from fresh knee joints of pigs (obtained from a local butcher, exempt from institutional review board approval). The meniscus is cut into thin slices of 1-mm thickness, followed by freezing using liquid nitrogen and then grounding into rough powder. Then, the meniscus powder is decellularized in SDS solution in PBS. After treating decellularized tissue with trypsin-EDTA solution in PBS, the sample is rinsed in PBS further to remove the residual

chemicals. Both h-dECM and Ms-dECM powders can be dissolved completely in a pepsin acetic acid solution at room temperature (RT) (Fig. 2c).

The silk-based, dECM and gelatin (bio)inks are prepared by mixing each separately with the Ru/SPS photoinitiator system in the dark at RT.

### VBP system

Silk, dECM and gelatin constructs are volumetrically (bio)printed with a customized volumetric (bio)printer[10,11]. The minimum printable volume with our VBP system is ~0.1 $cm^3$, and the maximum volume is ~1 $cm^3$. The highest resolution achieved by using these (bio)inks is ~50 µm in the X,Y direction and ~25 µm in the Z direction, depending on the bioink composition and photoinitiator concentration. The Radon transform algorithm, iterative algorithm and filtered back-projection algorithm are used in VBP. All projection algorithms are controlled via custom MATLAB scripts and programmed to output patterned intensity-modulated images by using the green light (525-nm) channel.

### Physical property characterizations of protein-based constructs

To evaluate the printability of the various protein-based (bio)inks, different formulations for each (bio)ink to print different constructions (screw for SF (bio)inks, temple for SS (bio)inks and cubes for both dECM (bio)inks and gelatin (bio)inks) are used (Fig. 3a–j). The photographs at different angles of the volumetrically printed constructs are taken by using the digital camera to obtain the printability maps (Fig. 3k–o). To evaluate the penetration depth ($D_p$), we recommend using UV-visible spectrophotometry at 525-nm wavelength to assess the light-absorption profiles of (bio)inks with different formulations (Fig. 4a,b). We recommend using a computer-aided design (CAD) model of a solid rod with bars featuring a set of threads of different thicknesses (from 1 to 101 µm) and a CAD model of a radially arranged array of cubes to quantify the printing resolution; meanwhile, dosage assay and other commonly used test models, such as spikes, tips and pillars for positive features and channels, pores and voids for negative features[26,27], are also recommended (Fig. 4c–e).

To test the physicochemical properties of the volumetrically printed protein-based constructs, we recommend characterizing the mechanical properties by using a universal testing machine (Fig. 5a–e) and checking the surface and internal morphologies by scanning electron microscopy (SEM) (Fig. 5f,g); the degradability of the volumetrically printed protein-based constructs is suggested to be evaluated as well (Fig. 5h–k). These procedures are based on previously described methods[10,11].

In addition, for SS and SF, which show major alterations in physical properties (such as volume, morphology, mechanical strength and resolution), after post-printing treatment (such as ethanol treatment), we recommend characterizing and comparing their physical property changes before and after post-printing treatment (Fig. 6).

### Bioprinting and cytocompatibility

Furthermore, the optimal printing concentrations of these bioinks (2.5% and 5% SS, 2.5% and 5% SF, 1% h-dECM and 1% Ms-dECM) are selected for biological evaluations (Fig. 7). For silk-based proteins, myoblasts (C2C12) are embedded within the SS and SF bioinks. The screw model (volume of the model: ~0.5 $cm^3$; 45 s of printing time for SS+C2C12, 57 s of printing time for SF+C2C12), the C60 model (volume of the model: ~0.5 $cm^3$; SS+C2C12, 57 s of printing time) and the channel-in-cube model (volume of the model: ~0.45 $cm^3$; SS+C2C12, 45 s of printing time) are volumetrically bioprinted with the parameters of 0.5/5 mM Ru/SPS for SS and 0.25/2.5 mM Ru/SPS for SF, $5 \times 10^6$ cells $ml^{-1}$ and 3 mW $cm^{-2}$ of light intensity measured at the printing volume. The metabolic activities of embedded cells are measured by the PrestoBlue or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent.

For dECM proteins, NIH/3T3 fibroblasts are embedded within the dECM bioinks. The ear model (volume of the model: ~0.3 $cm^3$; 30 s of printing time for 1% (wt/vol) NIH/3T3-laden Ms-dECM), the heart model (volume of the model: ~0.5 $cm^3$; 45 s of printing time for 1% (wt/vol) NIH/3T3-laden h-dECM) and the screw model (volume of the model: ~0.5 $cm^3$; 30 s of printing

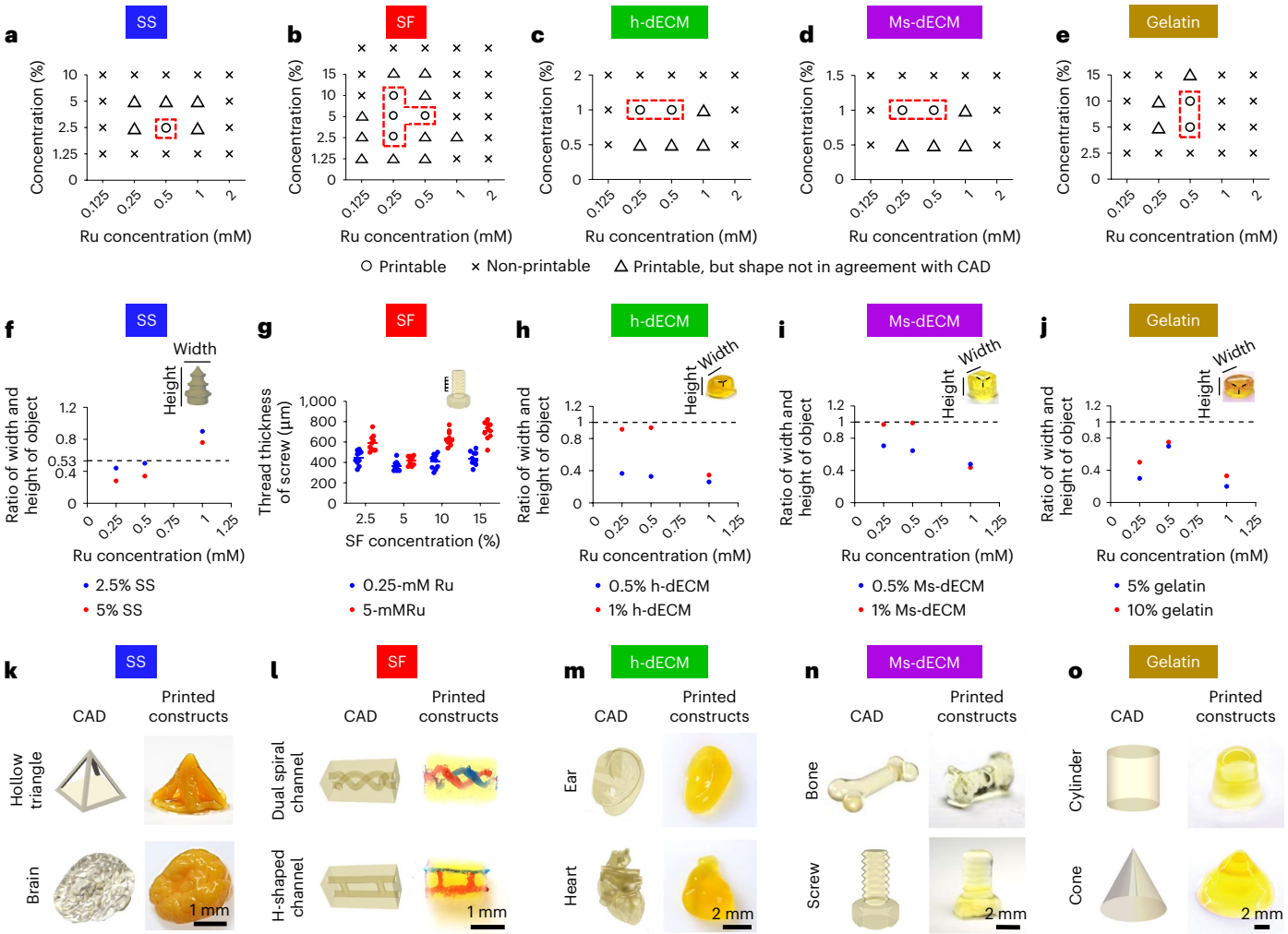



**Fig. 3 | Printing performances of tyrosine-containing protein-based (bio) inks. a**–**e**, Printability maps of SS (bio)ink, SF (bio)ink, h-dECM (bio)ink, Ms-dECM (bio)ink and gelatin (bio)ink with different formulations. **f**, Quantified ratios of the width to height of the volumetrically printed SS temple construct with different formulations. The value close to 0.53 indicates matching with the designed computer-aided design (CAD) model. **g**, Values of thread thickness of the volumetrically printed SF screw construct with different formulations. The smaller values indicate better resolutions. **h**–**j**, Quantified ratios of the height to width of the volumetrically printed cubic construct using h-dECM (bio)ink, Ms-dECM (bio)ink and gelatin (bio)ink with different formulations. The value close to 1 indicates matching with the designed CAD model. **k**, CAD models and photographs of volumetrically printed SS objects with printing parameters: 2.5% (wt/vol) SS with 0.5 mM Ru/5 mM SPS; light intensity measured at the printing volume: 3 mW $cm^{-2}$. The hollow triangle construct (60 s of printing time) and a brain-like construct (65 s of printing time). **l**, CAD models and photographs of volumetrically printed SF objects under 3 mW $cm^{-2}$ of light intensity measured at the printing volume. Construct with dual spiral channel construct: 5% (wt/vol) SF with 0.25 mM Ru/2.5 mM SPS, 57 s of printing time; construct with H-shaped channel: 2.5% (wt/vol) SF with 0.25 mM Ru/2.5 mM SPS, 117 s of printing time. **m**, CAD models and photographs of volumetrically printed objects using 1% (wt/vol) h-dECM with 0.5 mM Ru/5 mM SPS (light intensity measured at the printing volume: 3 mW $cm^{-2}$). The ear construct was printed in 30 s, and the heart construct was printed in 30 s. **n**, CAD models and photographs of the volumetrically printed object using 1% (wt/vol) Ms-dECM with 0.25 mM Ru/2.5 mM SPS (light intensity measured at the printing volume: 3 mW $cm^{-2}$). The bone construct was printed in 45 s, and the screw structure was printed in 30 s. **o**, CAD models and photographs of the volumetrically printed object using 10% (wt/vol) gelatin with 0.5 mM Ru/25 mM SPS (light intensity measured at the printing volume: 15 mW $cm^{-2}$). The cylinder construct was printed in 150 s, and the cone structure was printed in 150 s. In panel **a**: O, the shape was not in agreement with CAD; X, non-printable; √, printable. Panels **a**, **b**, **f**, **g**, **k**, and **l** are adapted from ref. 10, CC BY 4.0. Panels **c**, **d**, **h**, **i**, **m**, and **n** are adapted with permission from ref. 11, Wiley.

time for 1% (wt/vol) NIH/3T3-laden Ms-dECM) are volumetrically bioprinted with the parameters at 0.25/2.5 mM Ru/SPS and 3 mW $cm^{-2}$ of light intensity measured at the printing volume. Similarly, the metabolic activities of embedded cells are measured by the PrestoBlue and MTS reagents. Of note, the printing time does not necessarily scale with the volume of print in VBP[28].

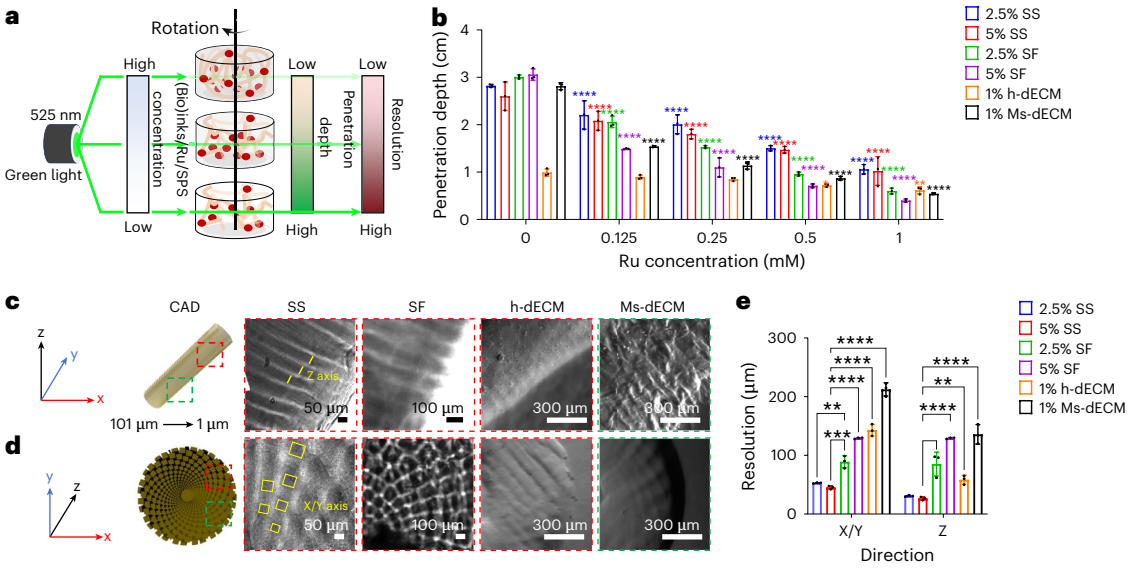



**Fig. 4 | $D_p$ and printing resolution of tyrosine-containing pristine protein-based (bio)inks. a**, Schematic showing the effect of Ru/SPS concentration and protein-based (bio)ink concentration on printing resolution. **b**, $D_p$ of SS, SF, h-dECM and Ms-dECM with different formulations. **c**, Left to right: CAD image of the solid bar; thicknesses of the threads from 1 to 101 µm, microscopic images of the printed solid bar of 2.5% (wt/vol) SS, 2.5% (wt/vol) SF, 1% (wt/vol) h-dECM and 1% (wt/vol) Ms-dECM. The printing parameters were as follows: 0.5 mM Ru/5 mM SPS for SS, h-dECM and Ms-dECM; 0.25 mM Ru/2.5 mM SPS for SF; light intensity measured at the printing volume: 3 mW $cm^{-2}$. **d**, Left to right: CAD image of the radially arranged array of cubes, microscopic images of the printed arrays of cubes of 2.5% (wt/vol) SS, 2.5% (wt/vol) SF, 1% (wt/vol) h-dECM and 1% (wt/vol) Ms-dECM. The printing parameters were as follows: 0.5 mM Ru/5 mM SPS for SS, h-dECM and Ms-dECM; 0.25 mM Ru/2.5 mM SPS for SF; light intensity measured at the printing volume: 3 mW $cm^{-2}$. **e**, Quantified resolutions in the X, Y and Z directions of the printed constructs of different pristine protein-based (bio) inks. The bars in panels **b** and **e** represent the data distribution of at least three replicates. The lines in panel **e** represent the statistical significance comparison between the two groups. Statistical significances are expressed as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ by two-way ANOVA with Tukey's post hoc test; in panel **b**, $n = 3$; in panel **e**, $n = 10$. Panels **c** (CAD, SS and SF) and **d** (CAD, SS and SF) are adapted from ref. 10, CC BY 4.0. Panels **c** (h-dECM and Ms-dECM) and **d** (h-dECM and Ms-dECM) are adapted with permission from ref. 11, Wiley.

### Biomedical applications

The volumetrically printed 10% (wt/vol) SF screw-like constructs, post-treated with 70% (wt/vol) ethanol followed by air drying, are seeded with hMSCs to evaluate cell differentiation. In addition, the volumetrically printed 10% (wt/vol) SF screw-like constructs (without cells) are implanted in an ex vivo porcine femur model (Fig. 8a).

The feasibility of using dECM bioinks for VBP of tissue-specific constructs is demonstrated by two applications, a human heart-like construct and a meniscus-like construct. The h-dECM bioink, composed of rCM-laden h-dECM, is tested with two different concentrations of Ru/SPS. The meniscus constructs laden with hMSCs are volumetrically bioprinted by using 1% (wt/vol) Ms-dECM with 0.25/2.5 mM Ru/SPS (Fig. 8b,c).

## Materials

### Reagents

▲ **CAUTION** When handling the chemicals used in this protocol, always wear suitable personal protective equipment (PPE), including a laboratory coat, nitrile gloves, safety goggles and, where indicated, face shield, respirator (or fume hood) and thermo-insulated gloves. Follow the relevant institutional and governmental safety guidelines and refer to the appropriate material safety data sheets. All synthesis steps should be performed within a chemical fume hood.

- Silkworm (*B. mori*) cocoons (Tajima Shoji; store at RT)
- Sodium carbonate ($Na_2CO_3$; Millipore Sigma, cat. no. 13418-1KG-R; store at RT)
  ▲ **CAUTION** $Na_2CO_3$ can be harmful if inhaled in large quantities, potentially causing irritation or damage to the nasal passages.

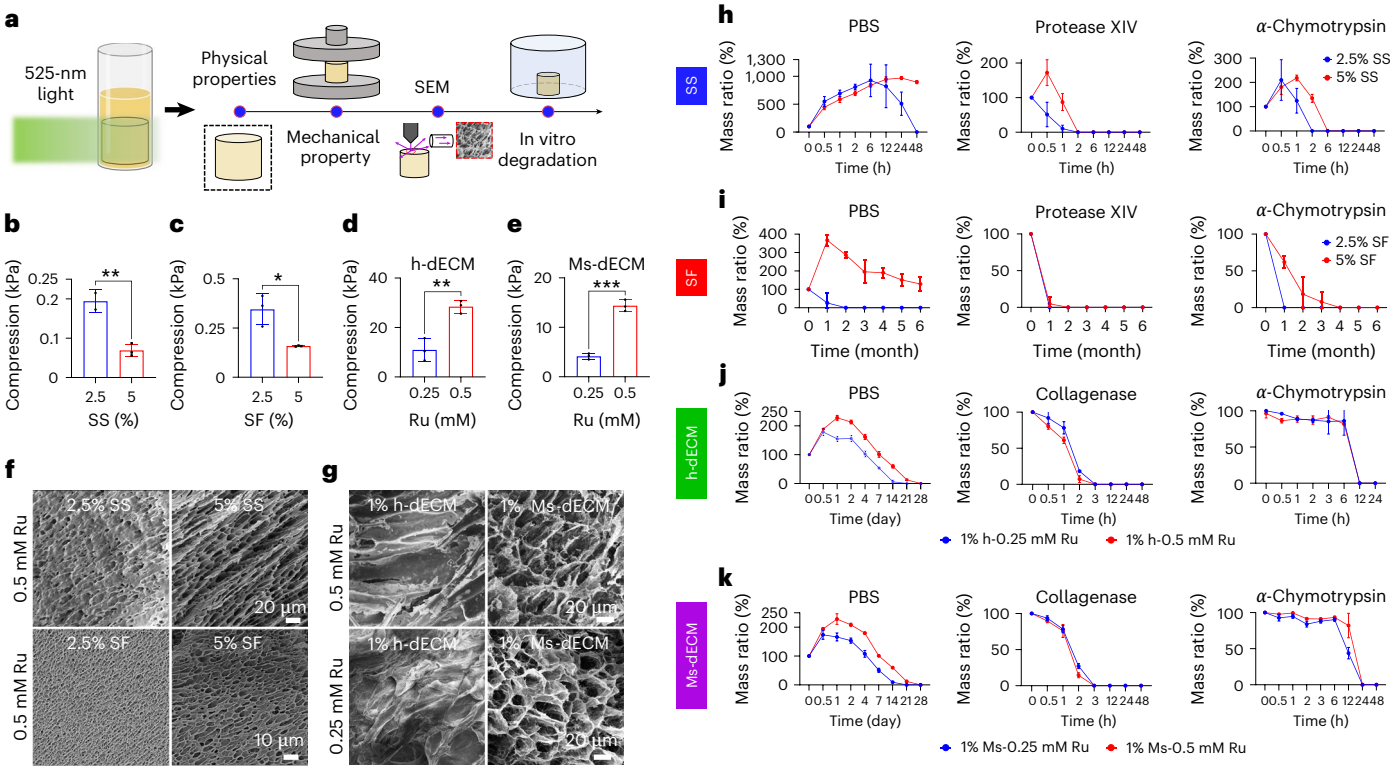
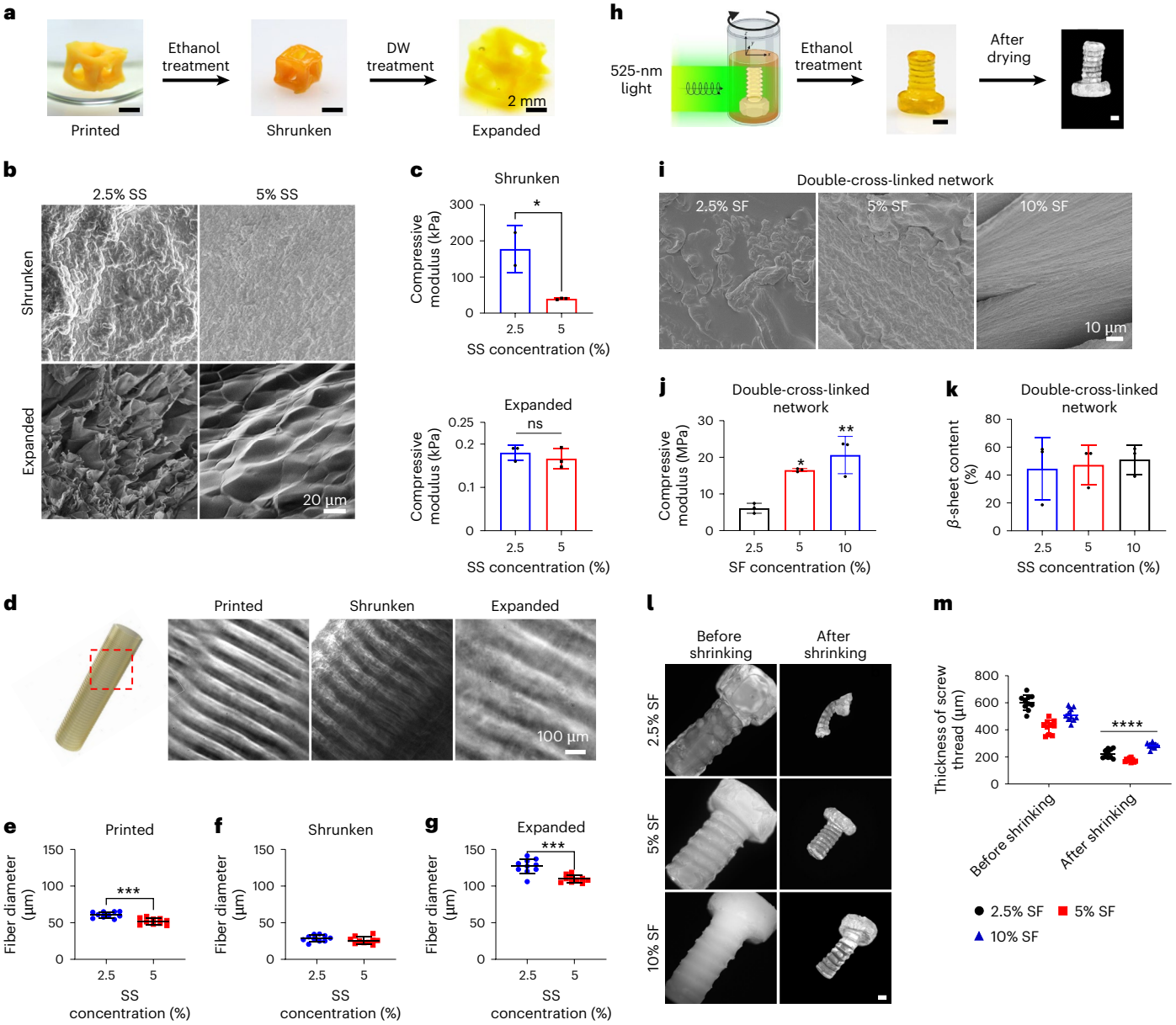



**Fig. 5 | Physical properties of volumetrically printed tyrosine-containing pristine protein-based constructs. a**, Schematic showing the routine of physical properties test with volumetrically printed constructs. **b**, Compressive moduli of constructs volumetrically printed by using 2.5% (wt/vol) and 5% (wt/vol) SS with 0.5 mM Ru/5 mM SPS. **c**, Compressive moduli of constructs volumetrically printed by using 2.5% (wt/vol) and 5% (wt/vol) SF with 0.5 mM Ru/5 mM SPS. **d**, Compressive moduli of constructs volumetrically printed by using 1% (wt/vol) h-dECM with 0.25 mM Ru/2.5 mM SPS and 0.5 mM Ru/5 mM SPS. **e**, Compressive moduli of constructs volumetrically printed by using 1% (wt/vol) Ms-dECM with 0.25 mM Ru/2.5 mM SPS and 0.5 mM Ru/5 mM SPS. **f** and **g**, SEM images of cross-sections of the volumetrically printed constructs of SS (**f**, top), SF (**f**, bottom), h-dECM (**g**, top left and bottom left) and Ms-dECM (**g**, top right and bottom right) with different formulations. **h**, In vitro degradation profiles of the volumetrically printed SS constructs in PBS, 5 U ml$^{-1}$ protease XIV PBS solution and 40 U ml$^{-1}$ α-chymotrypsin PBS solution. **i**, In vitro degradation profiles of the volumetrically printed SF constructs in PBS, 5 U ml$^{-1}$ protease XIV PBS solution and 40 U ml$^{-1}$ α-chymotrypsin PBS solution. **j**, In vitro degradation profiles of the volumetrically printed h-dECM constructs in PBS, 5 U ml$^{-1}$ collagenase IV PBS solution and 40 U ml$^{-1}$ α-chymotrypsin PBS solution. **k**, In vitro degradation profiles of the volumetrically printed Ms-dECM constructs in PBS, 5 U ml$^{-1}$collagenase IV PBS solution and 40 U ml$^{-1}$ α-chymotrypsin PBS solution. The bars in panels **b**–**e** and **h**–**k** represent the data distribution of at least three replicates. The lines in panels **b**–**e** represent the statistical significance comparison between the two groups. Statistical significances are expressed as *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ by two-way ANOVA with Tukey's post hoc test. $n = 3$. Panels **b**, **c**, **f**, **h**, and **i** are adapted from ref. 10, CC BY 4.0. Panels **d**, **e**, **g**, **j**, and **k** are adapted with permission from ref. 11, Wiley.

▲ **CRITICAL** $Na_2CO_3$ absorbs moisture from the air; it is important to keep the container tightly sealed after each use to maintain its integrity.

- Porcine heart and meniscus (local slaughterhouses; store at 4 °C)
- Trypsin-EDTA (Thermo Fisher Scientific, cat. no. 15400-054)
- Triton X-100 (Sigma-Aldrich, cat. no. X100)
  ▲ **CAUTION** Triton X-100 can cause skin and eye irritation. Avoid inhalation and contact with skin or eyes.
- Isopropyl alcohol (Sigma-Aldrich, cat. no. W292912)
- SDS (Sigma-Aldrich, cat. no. L4509)
  ▲ **CAUTION** SDS is toxic; inhalation, ingestion or contact with skin can cause irritation or damage. Use appropriate PPE such as gloves and a mask when handling.
  ▲ **CRITICAL** SDS is hygroscopic; ensure that the container cap is tightly closed after use.
  ▲ **CRITICAL** We recommend using fresh organs for dECM extraction. The quality of the organs has a substantial impact on the printability of the obtained biomaterials.
- SS (from Procedure 1, Steps 1–16; store at 4 °C)




- Ru photoinitiator (Advanced BioMatrix, cat. no. 5246; store at RT)
- SPS photoinitiator (Advanced BioMatrix, cat. no. 5247; store at RT)
  ▲ **CRITICAL** The dry powders of Ru and SPS are stable for >1 year at RT. Once solubilized, use Ru and SPS within 2 weeks.
  ▲ **CAUTION** If inhaled, Ru/SPS may cause skin and eye irritation. When handling, PPE such as rubber gloves, airtight goggles and a full-face shield mask are recommended.
  ▲ **CRITICAL** Do not mix Ru and SPS together in advance. It will initiate a rapid redox reaction even in the absence of light[29].
- Degummed silk (from Procedure 1, Steps 17–36; SF)
- LiBr (Sigma-Aldrich, cat. no. 213225)
  ▲ **CAUTION** LiBr is an anhydrous compound, which may cause skin irritation, severe eye damage and acute toxicity if ingested. Always wear a face shield and respirator. Avoid any direct contact.

**Fig. 6 | Tunable mechanical strengths and secondary structure change of volumetrically printed silk objects before and after post-printing treatment.** **a**, Shrinkage and expansion properties of volumetrically printed SS objects. Photographs of a volumetrically printed hollow square (57 s of printing time) in the as-printed (left), shrunken (center) (induced by immersion in 100% (vol/vol) ethanol for 2 h) and re-expanded (right) (immersion in water for 2 h after shrunken) states. **b**, SEM images of cross-sections of the volumetrically printed structures at different SS concentrations in the shrunken and re-expanded states. **c**, Compressive moduli of volumetrically printed 2.5% (wt/vol) and 5% (wt/vol) SS structures in the shrunken (top) and re-expanded (bottom) states. **d**, Left to right: CAD image of the solid bar; thicknesses of the threads from 1 to 101 µm, microscopic images of the volumetrically printed solid bar of 5% (wt/vol) SS in as-printed, shrunken and re-expanded states (0.5 mM Ru/5 mM SPS, 3 mW $cm^{-2}$ of light intensity measured at the printing volume and 95 s of printing time). **e**–**g**, Fiber diameter profiles of the volumetrically printed solid bar with different SS concentrations in the as-printed, shrunken and re-expanded states. **h**, Left: illustration of the VBP process of an SF screw construct in a rotating vial. **h**, Center: photograph of the same screw after 70% (vol/vol) ethanol treatment for 24 h. Scale bar: 500 µm. **h**, Right: microscopic image of the same screw after evaporating the ethanol solution. Scale bar: 500 µm. **i**, SEM images of cross-sections of the volumetrically printed 2.5% (wt/vol) SF screw, 5% (wt/vol) SF screw, and10% (wt/vol) SF screw with a double-cross-linked network (treated with 70% (vol/vol) ethanol for 24 h followed by 72 h of air-drying). **j**, Compressive moduli of volumetrically printed 2.5% (wt/vol), 5% (wt/vol) and 10% (wt/vol) SF structures with a double-cross-linked network. **k**, $\beta$-sheet contents of 2.5% (wt/vol), 5% (wt/vol) and 10% (wt/vol) SF structures with a double-cross-linked network. **l**, Microscopic images showing volumetrically printed 2.5–10% (wt/vol) SF screws before (i.e., after 24-h ethanol treatment but before air-drying) and after shrinking (i.e., induced by 72 h of air-drying after 24-h ethanol treatment). The printing parameters were 0.25 mM Ru/2.5 mM SPS and 3 mW $cm^{-2}$ of light intensity measured at the printing volume. Scale bar: 500 µm. **m**, Quantified thread thickness changes of the same SF screws before and after post-printing treatment. The bars in panels **c**, **e**, **f**, **j**, **k** and **m** represent the data distribution of at least three replicates. The lines in panels **c**, **e**, **g** and **m** represent the statistical significance comparison between the two groups. Statistical significances are expressed as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ by two-way ANOVA with Tukey's post hoc test. In panels **e**, **f**, **g** and **m**, $n = 10$; in panels **c**, **j** and **k**, $n = 3$. Panels **a**–**m** are adapted from ref. 10, CC BY 4.0.

▲ **CRITICAL** LiBr is extremely hygroscopic. Always minimize exposure to atmospheric air (seal the container with Parafilm) to avoid contamination. It is often best to use an unopened new container to avoid changes in water content.

- h-dECM powder and Ms-dECM powder (from Procedure 1, Steps 37–55; store at −80 °C)
- Pepsin (Sigma-Aldrich, cat. no. P7000)
- Sodium hydroxide (Sigma-Aldrich, cat. no. 655104; store at RT)
- Acetic acid solution (Sigma-Aldrich, cat. no. 45754; store at RT)

  ▲ **CAUTION** Acetic acid and sodium hydroxide can cause severe skin burns and eye damage. Avoid inhalation of vapor, use appropriate PPE and use it in a chemical fume hood.

  ▲ **CRITICAL** Acetic acid and sodium hydroxide are corrosive; ensure that the container cap is tightly closed after use to prevent leakage and contamination.
- Gelatin from porcine skin, type A (Sigma-Aldrich, cat. no. G2656; store at RT)
- SS and SF (bio)inks
- h-dECM and Ms-dECM (bio)inks
- Gelatin (bio)inks

  ▲ **CAUTION** When observing the printing process, wear laser safety glasses to protect your eyes.

  ▲ **CRITICAL** Ru/SPS is easily activated when mixed in solution; always keep the solution away from light. The prepared (bio)inks with Ru/SPS should be used within the same day.
- Protease XIV (from *Streptomyces griseus*) (Pronase E; Merck, cat. no. 9036-06-0; store at −20 °C)
- $\alpha$-Chymotrypsin (Sigma-Aldrich, cat. no. C4129; store at −20 °C)
- Collagenase (Sigma-Aldrich, cat. no. C8051; store at −20 °C)
- Collagenase IV (Sigma-Aldrich, cat. no. C4-28; store at −20 °C)
- Dulbecco's PBS (DPBS) powder (Thermo Fisher Scientific, cat. no. 21600010; store at RT)
- 70% (vol/vol) ethanol (Sigma-Aldrich, cat. no. 493511)
- DMEM (Thermo Fisher Scientific, cat. no. 11965-118)
- FBS (Thermo Fisher Scientific, cat. no. 10437-028)
- Antibiotic-antimycotic (Thermo Fisher Scientific, cat. no. 15240-062)

### Cell lines

- C2C12 myoblasts (American Type Culture Collection (ATCC), cat. no. CRL-1772)
- NIH/3T3 fibroblasts (ATCC, cat. no. CRL-1730)
- MDA-MB-231 breast cancer cells (ATCC, cat. no. HTB-26)
- rCMs (ATCC, cat. no. CRL-1730)
- hMSCs (Lonza, cat. no. PT-2501)
- hiPSC-CMs (FUJIFILM, cat. no. R1092)

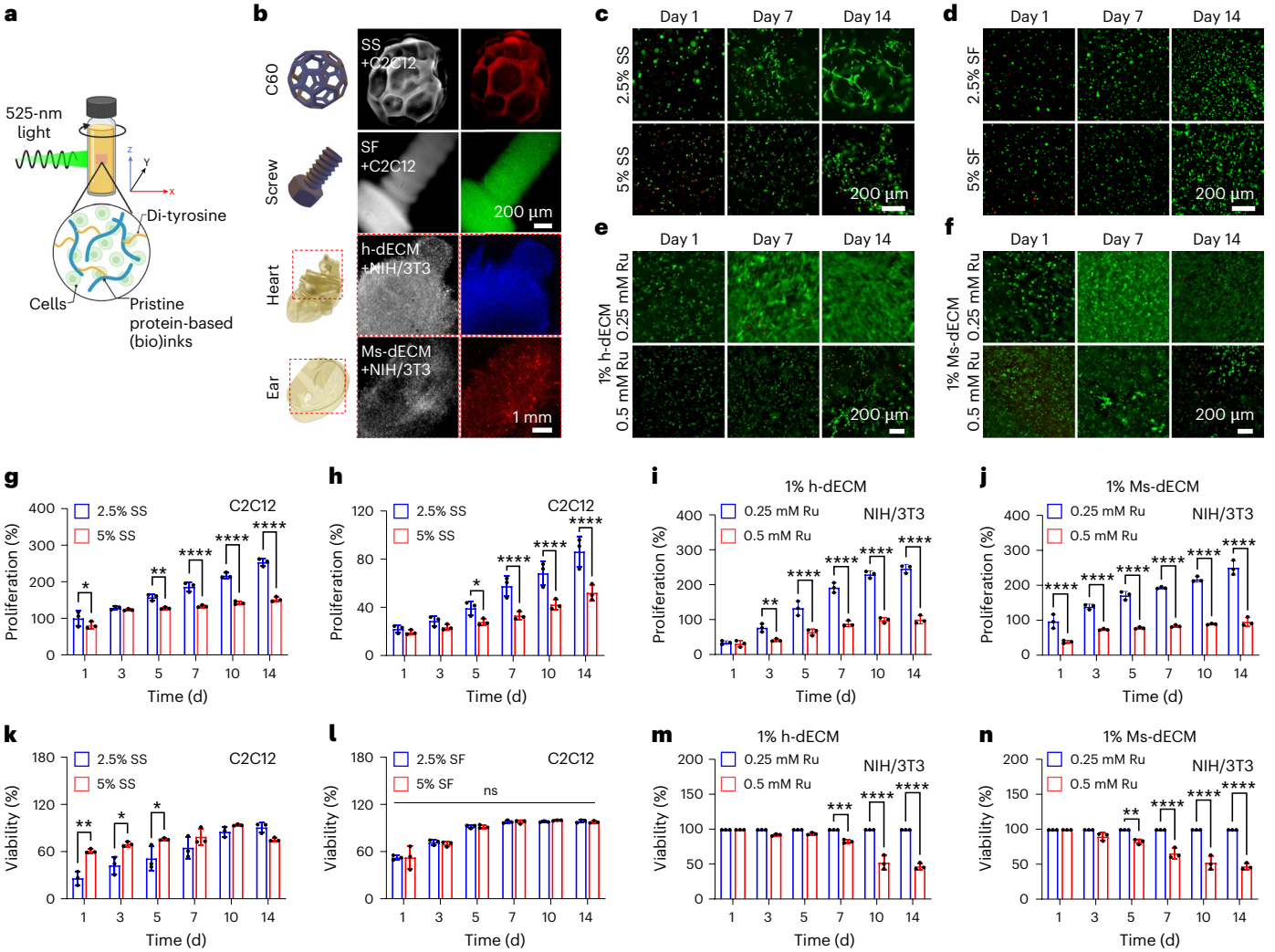



**Fig. 7 | Cytocompatibility of cell-laden tyrosine-containing pristine protein-based bioinks. a**, Schematic showing the VBP process with cell-laden tyrosine-containing pristine protein-based bioinks. **b**, CAD models and microscopic images of the structures volumetrically bioprinted with C2C12-laden silk-based bioinks and NIH/3T3 fibroblast-laden d-ECM-based bioinks. The C60 structure was bioprinted with 2.5% (wt/vol) SS and 0.25/2.5 mM Ru/SPS in 57 s. The screw structure was bioprinted with 5% (wt/vol) SF and 0.25/2.5 mM Ru/SPS in 45 s. The heart structure was bioprinted with 1% (wt/vol) h-dECM and 0.25/2.5 mM Ru/SPS in 45 s. The ear structure was bioprinted with 1% (wt/vol) Ms-dECM and 0.25/2.5 mM Ru/SPS in 30 s. Cells were all stained with CellTracker. **c** and **d**, Live (green)/dead (red) images of C2C12 cultured within the volumetrically bioprinted SS and SF constructs at different concentrations with 0.25/2.5 mM Ru/SPS. **e** and **f**, Live (green)/dead (red) images of NIH/3T3 fibroblasts cultured within 1% (wt/vol) h-dECM and 1% (wt/vol) Ms-dECM constructs at different concentrations of Ru/SPS. **g**–**j**, Proliferation profiles of cells cultured within the volumetrically bioprinted SS, SF, h-dECM and Ms-dECM constructs. **k**–**n**, Viability profiles of cells cultured within the volumetrically bioprinted SS, SF, h-dECM and Ms-dECM constructs. The bars in panels **g**–**n** represent the data distribution of least three replicates. The lines in panels **g**–**n** represent the statistical significance comparison between two groups. Statistical significances are expressed as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ by two-way ANOVA with Tukey's post hoc test. $n = 3$; ns = not significant. Panels **b** (top two panels), **c**, **d**, **g**, **h**, **k** and **l** are adapted from ref. 10, CC BY 4.0. Panels **b** (bottom two panels), **e**, **f**, **i**, **j**, **m** and **n** are adapted with permission from ref. 11, Wiley.

### Cytocompatibility of silk-based and dECM constructs

- 10% (wt/vol) formaldehyde (Sigma-Aldrich, cat. no. HT501128)
- BSA (Sigma-Aldrich, cat. no. A4612)
- Alexa Fluor 488-phalloidin (Thermo Fisher Scientific, cat. no. A12379)
- DAPI (Millipore Sigma, cat. no. D9542-10MG)
- Live/dead viability/cytotoxicity kit (Thermo Fisher Scientific, cat. no. L3224; ethidium homodimer and calcein-AM are included in this kit)
- MTS assay kit (Thermo Fisher Scientific, cat. no. 50-221-5358)
- PrestoBlue (Thermo Fisher Scientific, cat. no. A13261)

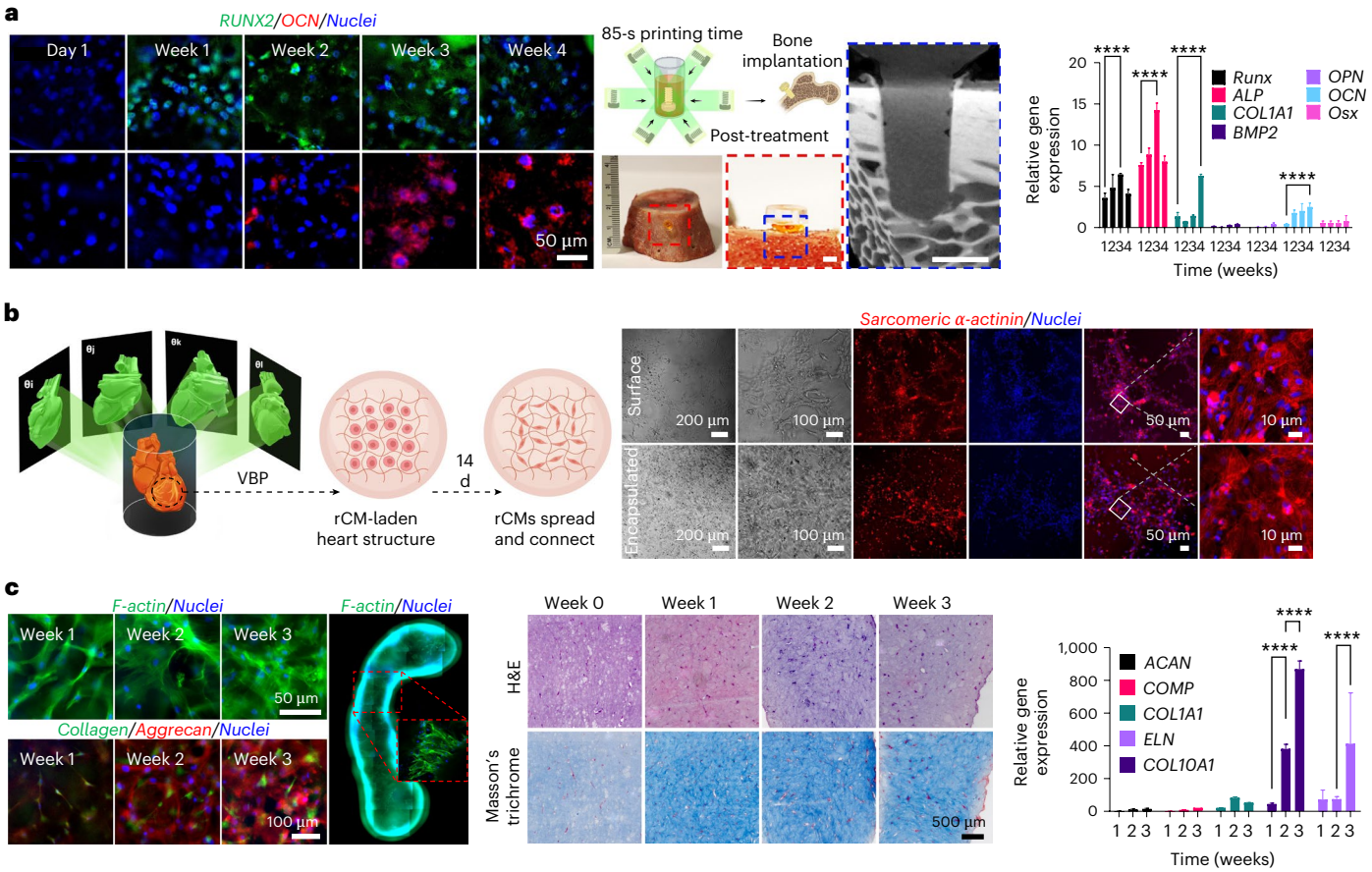



**Fig. 8 | Proof-of-concept applications for volumetrically bioprinted tyrosine-containing pristine protein-based constructs. a**, Left: hMSC immunostaining images showing *RUNX2* and *OCN* expressions. **a**, Center: schematic, photographs and micro-CT scanning image showing an ex vivo implantation test in a porcine femur of the volumetrically printed 10% (wt/vol) SF screws with a double-cross-linked network induced by immersion in 70% (wt/vol) ethanol for 24 h followed by 72 h of air-drying. Scale bars: 1.5 mm. **a**, Right: expression levels of representative genes indicating osteoblast-formation during the 4-week culture period. **b**, Left: schematic showing the VBP process of the heart-like structure and the process of rCM spreading. **b**, Center and right: brightfield (center) and fluorescence (right) micrographs showing immunostaining of hiPSC-CMs both on the surface and encapsulated in the heart-like constructs volumetrically bioprinted with 1% (wt/vol) h-dECM and 0.25/2.5 mM Ru/SPS, reaching a stretching morphology and expressing sarcomeric-α-actinin after culturing for 10 d. **c**, Left: micrographs showing F-actin staining and immunostaining of hMSCs in the meniscus constructs volumetrically bioprinted with 1% (wt/vol) Ms-dECM and 0.25/2.5 mM Ru/SPS over 3 weeks of differentiation. **c**, Center-left: low-magnification micrograph showing F-actin staining of hMSC-ladened meniscus tissue volumetrically printed with 1% (wt/vol) Ms-dECM and 0.25/2.5 mM Ru at 3 weeks of differentiation. The inset shows a confocal image of the high-density differentiated chondrocytes. **c**, Center-right: histology images of H&E and Masson's trichrome for the meniscus constructs volumetrically bioprinted with 1% (wt/vol) Ms-dECM and 0.25/2.5 mM Ru/SPS containing hMSCs after 3 weeks of chondrogenic differentiation. **c**, Right: quantified expression levels of representative genes indicating chondrogenic differentiation status in 3 weeks. The bars in panels **a** and **c** represent the data distribution of at least three replicates. The lines in panels **a** and **c** represent the statistical significance comparison between the two groups. Statistical significances are expressed as ****, $P < 0.0001$ by two-way ANOVA with Tukey's post hoc test. $n = 3$. *ACAN*, aggrecan; *ALP*, alkaline phosphatase; *BMP2*, bone morphogenetic protein 2; *COL10A1*, type X collagen; *COL1α1*, collagen type I alpha 1 chain; *COMP*, cartilage oligomeric matrix protein; *ELN*, elastin; F-actin, filamentous actin; *OCN*, osteocalcin; *OPN*, osteopontin; *Osx*, osterix; *Runx*, runt-related transcription factor. Panel **a** is adapted from ref. 10, CC BY 4.0. Panels **b** and **c** are adapted with permission from ref. 11, Wiley.

## Cell differentiation and immunostaining

- hMSC growth medium (Lonza, cat. no. PT-3001)
- hMSC osteogenic differentiation medium (Lonza, cat. no. PT-3002)
- hMSC chondrogenic differentiation medium (Lonza, cat. no. PT-3003)
- Mouse anti-RUNX2 antibody (Invitrogen, cat. no. NP0326BOX)
- Mouse anti-osteocalcin antibody (Abcam, cat. no. ab198228)
- iCell cardiomyocytes media kit (FUJIFILM, cat. no. R1151; cardiomyocyte medium is included in this kit)
- Goat anti-mouse IgG H&L (Alexa Fluor 488) (Abcam, cat. no. ab150113)

- Anti-sarcomeric-α-actinin antibody (Abcam, cat. no. ab137346)
- Mouse anti-aggrecan (Abcam, cat. no. ab3778)
- Rabbit anti-collagen II antibody (Abcam, cat. no. ab34712)

**RNA isolation and real-time reverse-transcription qPCR**
- QuantiTect reverse transcription kit (Qiagen, cat. no. 205311)
- SYBR Green qPCR master mix (Thermo Fisher Scientific, cat. no. A25742; store at −20 °C)
- RNeasy mini kit (Qiagen, cat. no. 74104)
- hMSC chondrogenic differentiation medium BulletKit (Lonza, cat. no. PT-3003)

**Ex vivo implantation of SF screw-like constructs**
- Volumetrically printed SF screw-like constructs (Procedure 2, Steps 1–6)
- Fresh porcine femur (local supermarket)

**Micro computed tomography (Micro-CT) analyses**
- The SF screw-implanted porcine femur from Procedure 2, Step 58.

**Histological evaluations**
- Formalin (Sigma-Aldrich, cat. no. R03379)

## Equipment
- Sterile 5-, 10- and 25-ml serological pipettes (SPL Life Science, cat. nos. 91005, 91010 and 91025, respectively)
- Sterile 15- and 50-ml conical tubes (SPL Life Sciences, cat. nos. 50015 and 50250, respectively)
- Pipette aid (Drummond Scientific, cat. no. 4-000-201)
- 50-ml, 2-liter and 5-liter glass beakers (DWK Life Science, cat. nos. 14000-50, 211066301 and 211067306, respectively)
- Sterile 3- and 10-ml syringes (Kovax-Syringe Korea Vaccine, cat. no. 22G 11/4″)
- Microplate reader (Biotek, EON microplate reader)
- 10-, 100-, 200- and 1,000-µl micropipettes and appropriate sterile tips (Eppendorf)
- 0.22-µm syringe filters (Sigma, cat. no. Z741969)
- Volumetric bioprinter (see VBP setup)
- Printing vials (12-mm diameter; DWK Life Science, cat. no. W225122)
- Centrifuge (Eppendorf, model. no. 5810R)
- Refrigerators set to 4 °C and −20 °C (Thermo Fisher Scientific, model. nos. TSX1205GA and TSX2320FD, respectively)
- Deep freezer set to −80 °C (Thermo Fisher Scientific, model. no. 902GP-ULTS)
- Dialysis bags (12–14-kDa molecular weight cutoff; Thermo Fisher Scientific, cat. no. Spectrum S432706)
- Freeze-dryer (Operon, model. no. FDCF-12003)
- Balance (AS ONE, model. no. AXA5003)
- Aluminum foil (AS ONE, cat. no. CP-1850-01)
- 1.7-, 2.0- and 5.0-ml conical microcentrifuge tubes (Axygen, cat. nos. MCT-175-C and MCT-200-C; and Eppendorf, cat. no. 0030119401, respectively)
- 6-, 48- and 96-well plates (Thermo Fisher Scientific, cat. nos. 07-200-83, 07-200-86 and 14-245-71, respectively)
- Incubator (AS ONE, model. no. CC-2559-01)
- Spatula (AS ONE, cat. no. 6-524-01)
- UV-visible spectrophotometer (Molecular Devices, model. no. SpectraMax M3)
- Digital camera (Canon, model. no. 70D)
- Inverted fluorescence microscope (Nikon, model. no. Ti-E)
- Mechanical tester (Instron, model. no. 6800 SERIES)
- Confocal Raman spectrometer (Horiba Scientific, model. no. XploRA plus)
- Fourier-transform infrared (FTIR) spectrometer (JASCO, model. no. 6200)

- Scanning electron microscope (Carl-Zeiss, model. no. Ultra 55 field-emission scanning electron microscope)
- Screwdriver (SATA, model. no. 08008)
- Micro-CT X-ray imaging system (version X-Tek HMXST 225)
- UV-visible spectrophotometer (Molecular Devices, model. no. SpectraMax M3)

### Software

- For CAD: SolidWorks, 3ds Max, AutoCAD and Unigraphics NX
- For VBP control: MATLAB and Python
- For data analyses: Origin, GraphPad and SPSS
- For image analyses: ImageJ

### VBP setup

- *Volumetric bioprinter*. The volumetric bioprinter is built and customized within our laboratory[10,11]. The specifications of our customized bioprinter are as follows: (i) a light-emitting diode (LED) light engine with resolution of 50-μm pixel size (912 × 1,140 pixels; Wintech, PRO4500), (ii) maximum output power of the LED light engine of 1,100 mW, (iii) a lens module (focal length: 40 mm; working distance: 230 mm; and field of view: 20 × 12.5 $mm^2$) and (iv) a transparent glass vial with a diameter of 12 mm, with a rotation mount at a 6.3° $s^{-1}$ of rotation rate. The building area of the transparent glass vial is 51.5 × 350 (radius × height) $mm^3$. Detailed information on VBP system designs, including algorithms, software, scripts, hardware and related specifications, is available on the open resource https://github.com/computed-axial-lithography/CAL-software-Matlab, which is readily accessible. The STL files of the main parts used for the VBP setup are provided as Supplementary Data 1–6. The workflow for the VBP process is shown in Supplementary Fig. S1.
  ▲ **CRITICAL** In this protocol and previous studies on VBP published by our laboratory[10,11], we built the VBP system instead of using any commercial ones. Although this protocol may be readily adaptable to commercial bioprinters, the parameters probably need to be adjusted.

## Procedure 1

### Preparation of SS

● **TIMING 3 d**

▲ **CAUTION** Wear appropriate PPE such as a laboratory coat, gloves and safety goggles.

▲ **CAUTION** Perform all functionalization steps in a chemical fume hood.

▲ **CRITICAL** Ensure that all laboratory equipment and glassware are clean by rinsing with DW and drying them properly to avoid chemical contamination during degumming, dialysis and lyophilization.

1. Cut dry cocoons with scissors into dime-sized pieces and remove any silkworms in the cocoons. Weigh out 20 g of cocoon pieces into a large weighing dish.
2. Prepare a 500-ml glass beaker filled with 200 ml of DW, cover it with a lid and heat it up until boiling.
   ▲ **CAUTION** Do not leave the beaker unattended during heating.
3. Weigh out 4.24 g of $Na_2CO_3$ (to prepare 0.04 M $Na_2CO_3$) into the boiling water and let it completely dissolve.
   ▲ **CAUTION** Ensure that you are wearing PPE correctly and keep adding $Na_2CO_3$ slowly to avoid boiling over (sputtering from the boiling beaker).
4. Add the cocoon pieces into the beaker and continue boiling for 30 min. Poke with two glass rods every 10 min to promote dispersion of the silk (Fig. 9a).
   ▲ **CRITICAL STEP** For reproducibility, boil for exactly 30 min every time.
   ▲ **CRITICAL STEP** Add sufficient DW to make sure that the silk can be immersed entirely during the boiling process.
   ◆ **TROUBLESHOOTING**

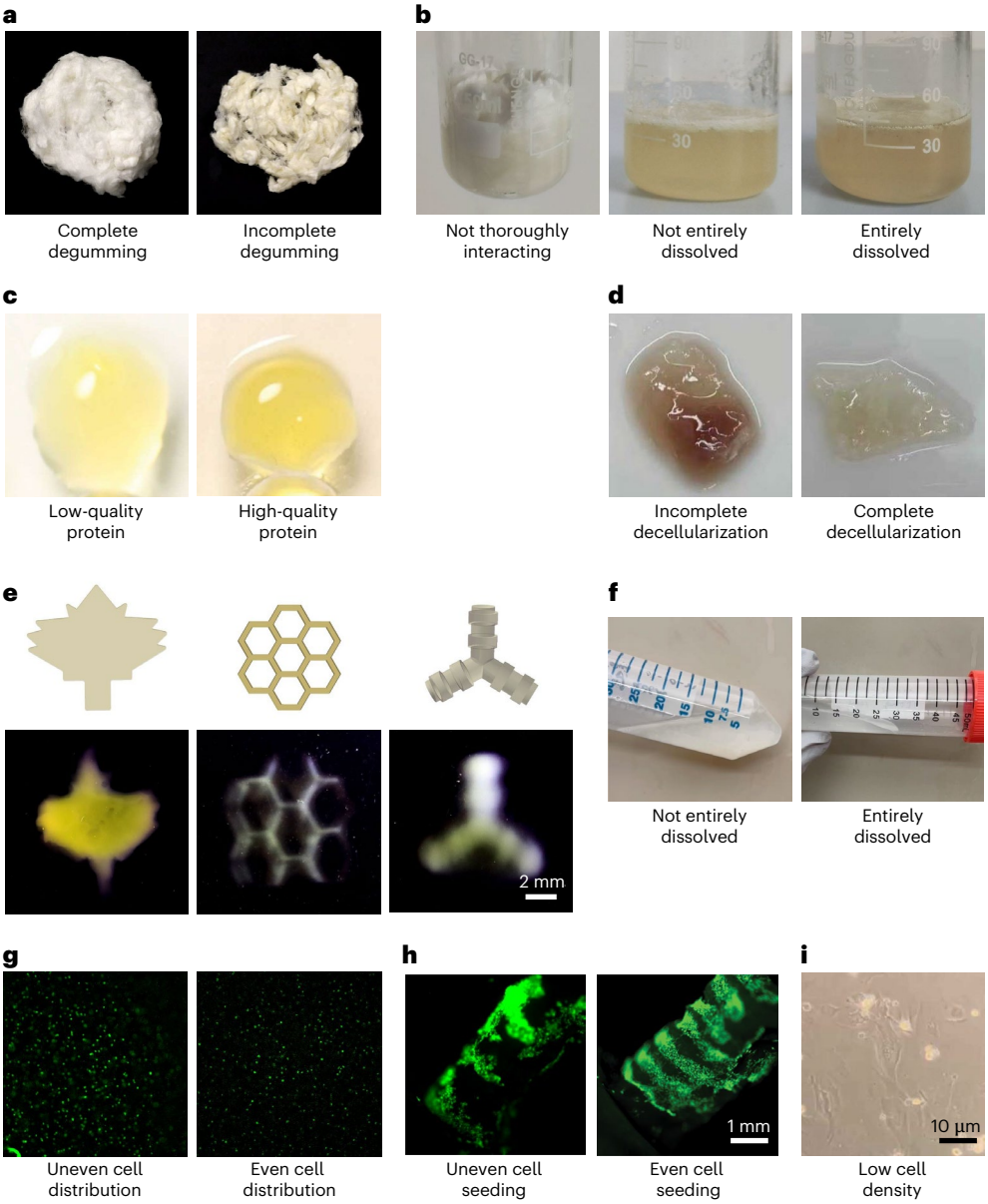



**Fig. 9 | Representative images corresponding to the troubleshooting Table 1. a**, Complete and incomplete degumming of cocoons (Table 1; Procedure 1, Step 4). **b**, Degummed cocoons not thoroughly interacting with the LiBr solution (Table 1; Procedure 1, Step 26). **c**, Low-quality and high-quality proteins with the quick photo-cross-linking test (Table 1; Procedure 1, Step 35). **d**, Incomplete and complete decellularization of h-dECM (Table 1; Procedure 1, Step 40). **e**, Printing results with low-quality SS (Table 1; Procedure 1, Step 58). **f**, Not entirely dissolved and entirely dissolved Ms-dECM (Table 1; Procedure 1, Step 61). **g**, Uneven and even cell distributions after bioprinting with cell-laden bioink (Table 1; Procedure 2, Steps 9 and 24). **h**, Cell seeded evenly or unevenly on the printed screw structure (Table 1; Procedure 2, Step 41). **i**, Low cell density of hiPSC-CMs (Table 1; Procedure 2, Step 89). Panels **c**, **f** and **i** are adapted with permission from ref. 11, Wiley. Panel **h** is adapted from ref. 10, CC BY 4.0.

5. Pick up and move the silk with stainless-steel tongs. Squeeze excess DW out of the silk with your hands while wearing Teflon gloves. Cool the SS and $Na_2CO_3$ solution.
   ▲ **CAUTION** The SF fibers and SS solution will be hot; hand protectors such as Teflon gloves should be used.
6. Purify the cold SS solution by centrifuging at 3,000*g* for 10 min at 4 °C to remove impurities.
7. Hydrate four 200-mm-long 12–14-kDa dialysis bags in DW for 5 min.

8. Double-knot one end of the membrane and introduce 50 ml of DW through the other end of the membrane.
9. While holding the tied end of the dialysis bag with one hand, use the index and middle fingers of your other hand to close the open end of the tube. Invert the dialysis bag containing water five times to rinse the inside of the membrane before discarding the water.
   ▲ **CRITICAL STEP** Check for any leakage in the membrane by gentle squeezing before proceeding to the next step. A fine spray of liquid indicates a hole in the membrane; discard and use a new membrane if any leaks are identified.
10. Insert 50 ml of the solution from Step 6 into each dialysis bag via a plastic funnel.
11. Expunge all air from the dialysis bag and clip the open end of the membrane at >110 mm above the solution.
12. Dialyze with ≥4 liters of DW to remove $Na_2CO_3$ in a 5-liter glass beaker at RT. Change the water three times a day (e.g., at 10:00, 16:00 and 22:00 or at 09:00, 16:00 and 21:00 daily) for 3 d.
    ▲ **CRITICAL STEP** The aliquot volume in the membrane will increase during the dialysis process; ensure firm clipping to avoid spillage of the aliquot into the dialysis water.
13. Separate the dialyzed solution from Step 12, centrifuge at 3,000*g* for 20 min at 4 °C to discard the precipitate and filter the supernatant with a 0.22-μm filter.
14. Insert 100 ml of the solution from Step 13 into the dialysis bag via a plastic funnel and place it in the hood for concentrating.
15. The final concentration (Z%) of SS solution is calculated by weighing the remaining mass (M, in grams) after evaporating the water (N, in grams) as follows:

$$\frac{M}{M+N} \times 100\% = Z\% \text{ of SS}$$

▲ **CRITICAL STEP** It is recommended to dilute the final concentration on the basis of the desired hydrogel concentration (e.g., 2.5% (wt/vol) or 5% (wt/vol)).

16. Store the solution from Step 15 at 4 °C.
    ▲ **CRITICAL STEP** SS will be denatured if it stays over 1 week, which potentially influences the printability. The method for evaluating the storability and stability of SS is provided in Supplementary Fig. S3, whereas the method for detecting the denatured SS is illustrated in Supplementary Fig. S4.
    ◆ **TROUBLESHOOTING**
    ■ **PAUSE POINT** The SS solution can be stored at 4 °C for ≤1 week.

### Preparation of SF

● **TIMING** 3 d

▲ **CAUTION** Wear appropriate PPE such as a laboratory coat, nitrile gloves and safety goggles.

▲ **CAUTION** Perform all functionalization experiments in a chemical fume hood.

▲ **CRITICAL** Ensure that all laboratory equipment and glassware are clean by rinsing with DW and drying them properly to avoid chemical contamination during degumming, dialysis and lyophilization.

17. Perform Procedure 1, Steps 1–5.
18. Place SF in a 5-liter beaker filled with 5 liters of DW.
19. Place SF in DW for 15 min, squeeze it by hand and discard the DW.
20. Repeat Procedure 1, Steps 18 and 19.
    ▲ **CAUTION** Squeeze until there are no bubbles to ensure complete removal of $Na_2CO_3$ from SF.
21. After the third wash, pick up the SF fibers, squeeze them well and then spread them out on clean pieces of large aluminum foil.
22. Allow the SF fibers to dry overnight in a chemical fume hood.
    ■ **PAUSE POINT** Dry SF fibers can be stored almost indefinitely at RT. For long-term storage, wrap them in clean aluminum foil. Be sure to indicate the date and weight on the label.
23. Calculate the amount (X, in grams) of LiBr needed to prepare a 9.3 M LiBr stock as follows:

$$\left(86.85\frac{g}{mol}\right)\left(9.3\frac{mol}{L}\right)\left(\frac{\text{LiBr volume}}{1,000\text{ ml}}\right) = X\,(g) \text{ of LiBr}$$

24. To prepare a 28-ml LiBr solution, dissolve 27.3 g of LiBr in a beaker containing 18 ml of DW. Stir gently with a stainless-steel spatula until completely dissolved and then fill the volume to 28 ml with DW to produce the 9.3 M LiBr solution.
▲ **CAUTION** The dissolution of LiBr in DW is exothermic; be mindful of the heat generated. We recommend adding DW slowly and wearing protectors such as Teflon gloves.
▲ **CRITICAL STEP** LiBr has a low density, and its volume should be considered while preparing the solution. Therefore, ensure that LiBr is dissolved completely before making the solution up to the final required volume.
25. Soak 7 g of SF fibers in the beaker containing 28 ml of the LiBr solution to prepare a 25% (wt/vol) SF solution. Cover the beaker first with cling film and then aluminum foil and transfer the beaker into an oven for 4 h of incubation at 60 °C.
▲ **CRITICAL STEP** Ensure that SF is immersed completely in the LiBr solution. Use a 50-ml beaker that can hold both SF and the LiBr solution without spillage so that the LiBr solution will eventually cover the SF fibers. If a liquid overflow occurs or SF fibers cannot be completely covered, tear the SF fibers into multiple small pieces and add them successively.
▲ **CRITICAL STEP** Add a magnetic rotor to the LiBr solution or use glass rods to mix the solution every 10 min to accelerate the dissolution of SF fibers.
26. Allow the SF fibers to dissolve completely before proceeding to the next step. The obtained SF solution should be viscous and transparent amber in color, without a trace of any intact fiber (Fig. 9b).
◆ **TROUBLESHOOTING**
27. Hydrate a 200-mm-long 12–14-kDa dialysis bag in DW for 5 min.
28. Double-knot one end of the bag and introduce 50 ml of DW through the other end of the bag.
29. While holding the tied end of the dialysis bag with one hand, use the index and middle fingers of your other hand to close the open end of the tube. Invert the dialysis bag containing water five times to rinse the inside of the bag before discarding the water.
▲ **CRITICAL STEP** Check for any leakage in the bag by gentle squeezing before proceeding to the next step. A fine spray of liquid indicates a hole in the bag; discard and use a new bag if any leaks are identified.
30. Insert the SF solution into the dialysis bag.
31. Expunge all air from the dialysis bag and clip the open end of the bag at >110 mm above the solution.
32. Dialyze with ≥4 liters of DW to remove LiBr in a 5-liter glass beaker at 4 °C. Change the water three times a day (e.g., at 10:00, 16:00 and 22:00 or at 09:00, 16:00 and 21:00 daily) for ≥3 d.
▲ **CRITICAL STEP** The aliquot volume in the bag will increase during the dialysis process; ensure firm clipping to avoid spillage of the aliquot into the dialysis water.
33. Separate the dialyzed solution, centrifuge at 9,000*g* for 20 min at 4 °C to discard the precipitate and filter the supernatant with a 0.22-μm filter.
34. Insert 50 ml of the solution into the dialysis bag via a plastic funnel and place it in the fume hood for concentrating.
35. The final concentration (Y%) of SF solution is calculated by weighing the remaining mass (M, in grams) after evaporating water (N, in grams) as follows (Fig. 9c):

$$\frac{M}{M+N} \times 100\% = Y\% \text{ of SF}$$

▲ **CRITICAL STEP** It is recommended to dilute the final concentration on the basis of the desired hydrogel concentration (e.g., 2.5% (wt/vol) or 5% (wt/vol)).
◆ **TROUBLESHOOTING**
36. Store the SF solution at 4 °C.
▲ **CRITICAL STEP** SF will be self-cross-linked if it stays over 1 week, which potentially influences the printability. The method for evaluating the storability and stability of SF is provided in Supplementary Fig. S3.
▲ **CRITICAL STEP** Using SF and SS together would cause an immunogenic reaction and should be cautioned against, especially for in vivo applications.
■ **PAUSE POINT** The SF solution can be stored at 4 °C for ≤1 week.

### Preparation of h-dECM

● **TIMING 2 weeks**

37. Separate the left ventricle from the whole porcine heart.
    ▲ **CRITICAL STEP** Select the freshly sourced tissues carefully, adhering to the standardized protocol that we refined specifically for the VBP purpose[11].
38. The separated left ventricles are sliced into 1-mm thickness and stirred in 5 liters of DW for 1 h to remove the blood.
39. Weigh out 50 g of SDS into 5 ml of 1× PBS solution (to prepare 1% (wt/vol) SDS/PBS) and let it completely dissolve.
    ▲ **CAUTION** SDS can cause respiratory tract irritation, including coughing, shortness of breath and sore throat. Repeated exposure can lead to allergic respiratory reactions or decreased pulmonary function. Keep SDS away from skin, eyes and clothing. Appropriate PPE should be worn when weighing it.
40. Add the tissues into 1% (wt/vol) SDS/PBS in a tissue-to-solution ratio of 1:16 (wt/vol) for 72 h (Fig. 9d).
    ▲ **CRITICAL STEP** The SDS solution should be refreshed every 24 h.
    ◆ **TROUBLESHOOTING**
41. The tissues should then be removed and treated with 1% (wt/vol) Triton X-100 in 1× PBS for 1 h.
    ▲ **CRITICAL STEP** The well-decellularized tissues will turn to a white color. Remove the pieces that are still red or brown in color.
42. Stir the tissue in isopropyl alcohol for 2 h.
43. The decellularized tissues need to be immediately rinsed with 1× PBS for 72 h to remove all the residual detergent.
    ▲ **CRITICAL STEP** The PBS solution should be refreshed every 24 h.
44. Transfer the decellularized tissues to 50-ml tubes and freeze at −80 °C overnight.
45. Lyophilize the frozen tissue until entirely dried (~3 d).
46. Submerge the material in liquid nitrogen for 2 min, ground the meniscal tissues into fine powders and store them at −80 °C.
    ▲ **CAUTION** Wear PPE such as a laboratory coat, thermal gloves and goggles when using liquid nitrogen.
    ▲ **CRITICAL STEP** It will take multiple rounds of grinding to obtain fine powders, which will be helpful for the following digestion process.
    ▲ **CRITICAL STEP** To minimize variability across experiments, we recommend preparing h-dECM in large quantities by following the optimized preparation method at one time.
    ◆ **TROUBLESHOOTING**
    ■ **PAUSE POINT** The prepared h-dECM powder can be stored at −80 °C for ≥2 months.

### Preparation of Ms-dECM

● **TIMING 2 weeks**

47. Harvest the porcine meniscus from fresh knee joints of pigs.
    ▲ **CRITICAL STEP** Select the freshly sourced tissues carefully, adhering to the standardized protocol that we refined specifically for the VBP purpose[11].
48. Cut the meniscus into thin slices at 1-mm thickness and clean in 5 liters of DW for 1 h.
49. Freeze the meniscus pieces at −80 °C for 2 h and ground them into rough powders.
50. Add the meniscus powders in 1% (wt/vol) SDS in PBS solution for 72 h.
    ▲ **CAUTION** SDS can cause respiratory tract irritation, including coughing, shortness of breath and sore throat. Repeated exposure can lead to allergic respiratory reactions or decreased pulmonary function. Keep SDS away from skin, eyes and clothing. Appropriate PPE should be worn when weighing it.
    ▲ **CRITICAL STEP** The SDS solution should be refreshed every 24 h.
51. The meniscus powder should be treated with 0.1% (wt/vol) EDTA solution in PBS for 24 h.
    ▲ **CRITICAL STEP** The well-decellularized tissues will turn to a transluscent whitish color. Remove the pieces that are still red or brown in color.

52. The treated tissue should be rinsed in 1× PBS for 72 h to remove the residual chemicals.
▲ **CRITICAL STEP** The PBS solution should be refreshed every 24 h.
53. Transfer the decellularized tissues to 50-mL tubes and freeze them at −80 °C overnight.
54. Lyophilize the frozen tissue until entirely dried (~3 d).
55. Submerge the material in liquid nitrogen for 2 min, ground the meniscal tissues into fine powders and store them at −80 °C.
▲ **CAUTION** Wear PPE such as a laboratory coat, thermal gloves and goggles when using liquid nitrogen.
▲ **CRITICAL STEP** It will take multiple rounds of grinding to obtain fine powders, which will be helpful for the following digestion process.
▲ **CRITICAL STEP** To minimize variability across experiments, we recommend preparing Ms-dECM in large quantities by following the optimized preparation method at one time.
■ **PAUSE POINT** The prepared Ms-dECM powder can be stored at −80 °C for ≥2 months.
◆ **TROUBLESHOOTING**

### Preparation of silk-based (bio)inks

● **TIMING 30 min–1 h**

56. Dissolve 7.396 mg of Ru in 500 µl of PBS to prepare a 20-mM Ru stock solution, and dissolve 23.875 mg of SPS in 500 µl of PBS to prepare a 200-mM SPS stock solution.
▲ **CRITICAL STEP** Ru/SPS can be activated by light, leading to premature gelation of the (bio)ink. Hence, always block light when preparing photocurable (bio)ink (e.g., cover the container with aluminum foil or work in a dark room).
▲ **CRITICAL STEP** Ensure that Ru/SPS dissolves completely; Ru and SPS should be added to (bio)inks at a fixed concentration ratio of 1:10 without premixing. Insufficient dissolution will lead to poor VBP quality.
▲ **CRITICAL STEP** Use PBS to dissolve Ru/SPS for maintaining a biologically compatible environment for cell-laden bioinks and following biomedical applications. It is also worth noting that the concentrations of Ru/SPS used here are way below the solubility limits.
57. Dilute the high concentration of the above SS/SF solution with PBS to make 2.5–10% (wt/vol) SS or 2.5–5% (wt/vol) SF (bio)ink.
▲ **CRITICAL STEP** The oxygen content within the SS or SF solution without adding photoinitiators should be equilibrated before printing by storing in a container for up to several days at 4 °C.
58. By adding different volumes of Ru/SPS stock solution into each SS or SF solution, different (bio)inks with gradient Ru/SPS concentrations from 0.125/1.25 mM to 2/20 mM are obtained (Fig. 9e).
◆ **TROUBLESHOOTING**
59. Remove air bubbles in the SS or SF (bio)ink by vortex and centrifuge for 30 s at 2,000*g*.
■ **PAUSE POINT** The prepared (bio)ink can be stored for 2–4 h in the dark at RT or 4 °C.

### Preparation of dECM (bio)ink

● **TIMING 3 d**

60. Dissolve h-dECM powder in pepsin/0.5 M acetic acid solution at 15 mg $ml^{-1}$ at RT for 72 h. The ratio of pepsin/h-dECM is 1:10 (wt/wt).
61. Dissolve Ms-dECM powder in pepsin/0.5 M acetic acid solution at 15 mg $ml^{-1}$ at RT for 72 h. The ratio of pepsin/Ms-dECM is 1:10 (wt/wt) (Fig. 9f).
▲ **CRITICAL STEP** The above solutions need to be placed on a shaker to ensure full dissolution of the material.
◆ **TROUBLESHOOTING**
62. Centrifuge the solution at a speed of 3,000*g* for 20 min to remove the undigested parts of the material or other impurities.
63. Collect the transparent dECM supernatant from the centrifuged solution and store at 4 °C.
▲ **CRITICAL STEP** The oxygen content within the dECM solution without adding photoinitiators should be equilibrated before printing by storing in a container for several days at 4 °C.

64. The dECM solution should be neutralized (pH 7.4) by using 10-N sodium hydroxide solution in the ice box.
▲ **CRITICAL STEP** The neutralized dECM materials are thermosensitive, which means that they may self-cross-link at RT, and therefore the materials should be kept in the ice box before the VBP process.
◆ **TROUBLESHOOTING**
65. Add Ru/SPS stock solution into different concentrations of dECM solutions according to the predetermined formulations.
▲ **CRITICAL STEP** Ru/SPS can be activated by light, leading to premature gelation of the (bio)ink. Hence, always block light when preparing the (bio)ink (e.g., cover the container with aluminum foil or work in a dark room).
▲ **CRITICAL STEP** Ensure that Ru/SPS dissolves completely; Ru and SPS should be added to the (bio)ink at a fixed concentration ratio of 1:10 without premixing. Insufficient dissolution will lead to poor VBP quality.
66. Remove air bubbles in the dECM (bio)ink by vortex and centrifuge for 30 s at 2,000*g*.
■ **PAUSE POINT** The prepared dECM (bio)ink can be stored for 2–4 h in the dark at RT or 4 °C.

### Preparation of gelatin (bio)inks

● **TIMING 30 min**

67. Dissolve porcine gelatin powder (type A) in PBS.
▲ **CRITICAL STEP** After adding PBS to the porcine gelatin powder, it should be kept in the 50 °C water bath for >10 min to ensure complete dissolution.
▲ **CRITICAL STEP** The porcine gelatin solution should be kept in a 37 °C water bath before printing.
▲ **CRITICAL STEP** The oxygen content within the gelatin solution without adding photoinitiators should be equilibrated before printing by storing in a container for several days at 4 °C.
68. Add Ru/SPS stock solution at a fixed concentration ratio of 1:50 into different concentrations of gelatin solutions according to the predetermined formulations.
69. Remove air bubbles in the gelatin (bio)ink by vortex and centrifuge for 30 s at 2,000*g*.
■ **PAUSE POINT** The prepared gelatin (bio)ink can be stored for 2–4 h in the dark at RT or 4 °C.

### $D_p$ assessment

● **TIMING 1.5 h**

70. Prepare 500 µl of (bio)inks with different formulations by adding gradient Ru/SPS from 0/0 to 1/10 mM.
71. Add 100 µl of each formulation of the (bio)ink to a well of a transparent 96-well plate with three replicates.
▲ **CRITICAL STEP** The volume height of the tested (bio)ink in the well is critical for calculating $D_p$.
72. Measure absorbance of (bio)inks by using UV-visible spectrophotometry at a wavelength from 400 to 700 nm with a step size of 5 nm.
73. The $D_p$ of 525-nm projected light into silk-based or dECM (bio)ink with or without Ru/SPS is calculated as follows:

$$D_p = \frac{1}{\alpha}$$

$$\alpha = \ln(10) \times (A/t)$$

▲ **CRITICAL STEP** $D_p$ is defined as the depth at which the intensity of the radiation inside the material falls to $1/e$ (roughly 37%) of its original value at (or more properly, just beneath) the surface.
▲ **CRITICAL STEP** $A$ is the absorbance value of materials at the 525-nm wavelength, and $t$ is the height of the tested material.

### VBP of silk-based (bio)inks

● **TIMING 30–120 s**

▲ **CRITICAL** It should be noted that for VBP, the printing time does not scale with the size of the print[28].

74. Launch the printer and open the software.
75. Import the standard triangle language (STL) file of the CAD model into the software, adjust the image, slice the STL file and save it as a .mat file.
    ▲ **CRITICAL STEP** Ensure that the printer is connected before clicking on the 'print' icon.
76. Set the printing parameters (such as printing time and LED power; see Procedure 1, VBP setup).
    ▲ **CRITICAL STEP** The wavelength of the printer is set at 525 nm but can be changed to other wavelengths depending on the photoinitiator used.
77. Gently transfer the silk-based (bio)ink into the printing vial.
    ▲ **CRITICAL STEP** The volume of (bio)ink loaded into the printing vial is typically 1.5 ml.
    ▲ **CRITICAL STEP** Use a 1-ml pipette to gently remove any bubbles at the surface of the (bio) ink or inside the (bio)ink in the printing vial.
78. Place the printing vial onto the printing stage of the VBP system.
79. After printing, remove the printing vial from the printing stage and gently rinse the printed constructs twice with PBS to remove the un-cross-linked (bio)ink.

### VBP of dECM (bio)inks

● **TIMING 30–120 s**

80. Perform Procedure 1, Steps 74–76.
81. Gently transfer the dECM (bio)ink into the printing vial.
    ▲ **CRITICAL STEP** The volume of (bio)ink loaded into the printing vial is typically 1.5 ml.
    ▲ **CRITICAL STEP** Keep the dECM (bio)ink in an icebox in the dark. Remove any bubbles in the vial.
82. Place the printing vial onto the printing stage of the VBP system.
83. After printing, remove the printing vial from the printing stage and gently rinse the printed constructs twice with PBS to remove the un-cross-linked (bio)ink.

### VBP of gelatin (bio)inks

● **TIMING 30–120 s**

84. Perform Procedure 1, Steps 74–76.
85. Gently transfer the gelatin (bio)ink into the printing vial.
    ▲ **CRITICAL STEP** The volume of (bio)ink loaded into the printing vial is typically 1.5 ml.
    ▲ **CRITICAL STEP** Use a 1-ml pipette to gently remove any bubbles at the surface of the (bio) ink or inside the (bio)ink in the printing vial.
86. Place the printing vial onto the printing stage of the VBP system.
87. After printing, remove the printing vial from the printing stage and gently rinse the printed constructs twice with PBS to remove the un-cross-linked (bio)ink.
    ▲ **CRITICAL STEP** Troubleshooting for the VBP process can be found in Table 1.
    ◆ **TROUBLESHOOTING**

### Printability assessment

● **TIMING 3 h**

88. Print samples with different shapes under optimal conditions by using a desired concentration of silk-based, dECM or gelatin (bio)ink prepared in advance.
89. Use the inverted fluorescence microscope at the suitable focal plane to take images, and use a customized Python program to calculate the Jaccard similarity index to measure the similarity of printed construct compared to the CAD model[30].
    ▲ **CRITICAL STEP** Jaccard similarity index ranges from 0% to 100%, and a higher percentage means higher similarity of printed construct compared to the CAD model, indicating better printability (Supplementary Fig. S2). The scripts (run by MATLAB) for printability assessment are provided in Supplementary Script. The CAD models (STL files) used for VBP in this protocol are provided in Supplementary Data 7–24.

### Printing resolution assessment

● **TIMING 3 h**

90. Design a 3D model of a solid bar featuring a set of separated parallel threads of different thicknesses (from 1 to 101 μm) for the Z-axis resolution test, and design a 3D model of a radially arranged array of cubes with different diameters for measuring the X-Y resolution (Fig. 4).
91. Print the above constructs and take images by using an inverted fluorescence microscope at the suitable focal plane to measure the smallest feature by using ImageJ.

### Mechanical property measurement

● **TIMING 2–3 h**

92. Load the compression force at a displacement rate of 5 mm $min^{-1}$ until the specimen (e.g., a hydrogel disk with a diameter of 10 mm and a height of 5 mm) breaks, to acquire the stress (pascals) and strain (percent) values from the compressive stress-strain curve at the point of failure.
    ▲ **CRITICAL STEP** Ensure that the hydrogel surface is flat and parallel to the bottom of the movable platform.
93. Examine the tensile strength on the UTM QMESYS with tensile jigs at a stretch velocity of 5 mm $min^{-1}$.
94. Use a dumbbell-shaped column hydrogel (16 × 7 × 2 (length × width × height) $mm^3$) to obtain the readings.
    ▲ **CRITICAL STEP** To prevent drying out, all mechanical tests should be conducted at RT and a humidity of 80% when possible.

### Raman spectroscopy

● **TIMING 2–3 h**

95. Freeze the volumetrically printed hydrogels at −80 °C for ≥12 h.
96. Lyophilize the frozen hydrogel until entirely dried (~3 d).
    ▲ **CAUTION** Contact of liquid nitrogen with the skin or eyes may cause serious frostbite injury. Avoid any direct contact and wear appropriate PPE.
97. Raman spectra are obtained by using a confocal Raman spectrometer.
98. The Raman spectra are calibrated by using a silicon wafer with a characteristic peak at 520 $cm^{-1}$ and are post-processed via the LabSpec 6 software.
99. Place the black cap (a standard Raman spectrometer sample-holder cap with a central aperture designed for focusing the laser beam onto the specimen) with the small hole in the middle on the Raman probe head and the slice of dried hydrogel on top of it.
100. Initiate the Raman spectrometer and perform the spectral acquisition.
    ▲ **CAUTION** Too long of an integration time can lead to complete saturation of the detector.
    ▲ **CRITICAL STEP** For materials that exhibit weak Raman signals, a longer integration time is needed.
    ▲ **CRITICAL STEP** Collect multiple spectra from each sample.

### FTIR spectroscopy

● **TIMING 3 h**

101. Freeze the volumetrically printed hydrogels at −80 °C for ≥12 h.
102. Lyophilize the frozen hydrogel until entirely dried (~3 d).
103. Break the sample into small pieces of ~3 mm in diameter.
104. FTIR analyses should be carried out on an FTIR spectrometer.
    ▲ **CRITICAL STEP** Set 32 scans and a resolution of 4 $cm^{-1}$ to record the spectrum for each test.
    ▲ **CRITICAL STEP** Collect multiple spectra from each sample.
105. Test and measure samples in every different experimental condition.
106. The secondary structure contents of SF are determined by performing peak deconvolution over the amide I region (1,720–1,580 $cm^{-1}$) by using the Origin software.

### SEM imaging

● **TIMING 1.5–3 h**

107. Freeze the volumetrically printed hydrogels at −80 °C for ≥12 h.
108. Lyophilize the frozen hydrogel until entirely dried (~3 d).
▲ **CRITICAL STEP** Critical point drying is used very often to better preserve the structures, because freeze-drying sometimes leads to artefacts in pore sizes.
109. Break the sample to obtain a complete cross-section.
▲ **CRITICAL STEP** Because of the difficulty in controlling the cross-section of the material, we recommend preparing multiple replicates and breaking the samples at different positions to obtain multiple cross-sections.
110. Fix carbon tape on a clean sample holder.
111. Paste the hydrogel samples on the carbon tape–fixed sample holder.
▲ **CRITICAL STEP** Lyophilization may result in shrinkage of the surface of the hydrogels. Therefore, exposure of their inner structures for SEM observation is needed.
112. Coat the samples with a thin 5-nm layer of gold/palladium for 15 s at 15-mA discharge current with an ion sputter.
113. Take micrographs from each sample at an accelerating voltage of 1.2–1.3 kV under field-emission SEM.

### Post-printing processing for inducing the double-cross-linked network of printed SF constructs

● **TIMING 3–4 d**

114. The volumetrically printed SF constructs (without cells) with single photo-cross-linked networks should be immersed in 70% (vol/vol) ethanol aqueous solution to induce the formation of $\beta$-sheet conformation within the same SF constructs for 2 h (only for taking photographs after printing) or 24 h (for all other experiments related to induction of $\beta$-sheet conformation) at RT.
115. The SF constructs possessing a double-cross-linked network should be left in a chemical fume hood to allow evaporation of the remaining ethanol for 72 h.
116. Harvest the dried SF constructs and store them at RT.

### Post-printing processing for shrinkage and expansion property evaluations of SS constructs

● **TIMING 2 d**

117. The volumetrically printed SS constructs (without cells) with a single photo-cross-linked network should be immersed in 100% (vol/vol) ethanol aqueous solution to induce the formation of $\beta$-sheet conformation within the same SS constructs for ≤24 h.
118. Measure the dimensional changes in diameter, width and height of the printed SS constructs by using a vernier caliper at different time points.
119. Treat the shrunken SS constructs with DW for ≤24 h.
120. Measure the dimensional changes in diameter, width and height of the re-expanded SS constructs by using a vernier caliper at different time points.

### In vitro water-uptake tests

● **TIMING 1 week**

121. Print cylinder constructs under optimal conditions by using desired concentrations of the prepared silk-based and dECM (bio)inks (see Procedure 1, Steps 74–83).
122. Transfer each printed construct into the well of a six-well plate. Perform this procedure with three replicates.
123. Pipette 10 ml of PBS into each well to immerse the construct and then incubate at 37 °C.
124. Soak the constructs in PBS for predetermined time points; the changes in mass signify water-uptake of the samples.
125. Weigh the harvested constructs to obtain swollen masses.
▲ **CRITICAL STEP** Surface water of the constructs should be removed by gently blotting with filter paper before weighing.

126. The wet mass of each construct is noted as $W_s$.
127. Freeze the constructs overnight at −20 °C and lyophilize for 3 d.
128. The dry mass of the corresponding construct is noted as $W_d$.
129. The water uptake (%) is calculated as follows:

$$\text{Water uptake}\,(\%) = \left(\frac{W_S - W_d}{W_S}\right) \times 100\%$$

### In vitro degradation assay

● **TIMING 6 months for silk, 28 d for dECM**

130. The volumetrically printed silk-based constructs should be transferred into PBS, 5 U $ml^{-1}$ of protease XIV PBS solution or 40 U $ml^{-1}$ of $\alpha$-chymotrypsin PBS solution in the wells of 48-well plates at 37 °C.
131. The volumetrically printed dECM constructs should be transferred into PBS, 5 U $ml^{-1}$ of collagenase IV PBS solution or 40 U $ml^{-1}$ of $\alpha$-chymotrypsin PBS solution in the wells of 48-well plates at 37 °C.
    ▲ **CRITICAL STEP** Perform this procedure with three replicates and measure each sample at least three times to obtain an average value.
132. The incubation solution should be changed with fresh solution every 2 d.
    ▲ **CRITICAL STEP** The samples should be rinsed in DW twice at desired time points.
133. Harvest the samples from the solutions at predetermined time points.
134. Freeze the harvested sample at −80 °C for ≥12 h.
135. Lyophilize the samples until completely dry (usually 3 d).
136. The remaining dry mass of the samples should then be measured. The dry mass of each remaining sample at day n is noted as $Wn$, and the initial dry mass of the corresponding sample at day 0 is noted as $W_0$. The residue mass ratio (%) is calculated as follows:

$$\text{Residue mass ratio}\,(\%) = (W_n/W_0) \times 100\%$$

## Procedure 2

### Preparation of cell-laden SS or SF bioink

● **TIMING 2 h**

▲ **CRITICAL** For cell culture, work in a class II biological safety hood and use sterile instruments. Clean all working surfaces with 70% (vol/vol) ethanol and expose to UV light for ≥20 min before use.

▲ **CRITICAL** Use 0.22-μm syringe filters to filter all reagents before mixing with bioinks. All the reagents used need to be sterile.

1. Dissolve 7.396 mg of Ru in 500 μl of serum-free DMEM to prepare the 20-mM Ru stock solution, and dissolve 23.875 mg of SPS in 500 μl of serum-free DMEM to prepare the 200=mM SPS stock solution. Wrap both with aluminum foil.
    ▲ **CRITICAL STEP** Ru/SPS can be activated by light, leading to premature gelation of the (bio)ink. Hence, always block light when preparing the (bio)ink (e.g., cover the container with aluminum foil or work in a dark room).
    ▲ **CRITICAL STEP** Ensure that Ru/SPS dissolves completely; Ru and SPS should be added to the (bio)ink at a fixed concentration ratio of 1:10 without premixing. Insufficient dissolution will lead to poor VBP quality.
    ▲ **CRITICAL STEP** Use serum-free cell culture medium for preparing the Ru/SPS solution and SS or SF solution instead of DPBS.
2. Filter the 20-mM Ru and 200-mM SPS solutions by using a 0.22-μm syringe filter.
3. Dilute the SS or SF solution with corresponding cell culture medium to make the desired concentration.

▲ **CRITICAL STEP** To obtain sterile materials, the SS or SF solution should be filtered by using the 0.22-μm syringe filter, and preparation of SS or SF solution should be performed in a cell culture hood.

4. Add the Ru stock solution with a pipette to the SS or SF solution and invert the tube three to five times gently; then, add the SPS stock solution with a pipette and invert the tube three to five times gently to mix homogeneously in the dark at RT.
   ▲ **CAUTION** To ensure the effectiveness of bioinks containing the photoinitiator, finish the bioprinting procedure within 1 h after mixing with Ru/SPS.
   ▲ **CRITICAL STEP** To prevent contamination, ensure that the lid is closed and wrap with aluminum foil.
   ▲ **CRITICAL STEP** Make sure that no bubbles are induced when mixing Ru/SPS with the SS or SF solution.
5. Use 0.25% (wt/vol) trypsin-EDTA solution to detach cells at ~80% confluence.
6. Terminate the cell trypsinization process with 10% (wt/vol) FBS in DMEM and transfer the cell suspension to 15-ml conical tubes.
7. Count the cells with a cell counter and prepare a final resuspension at the required cell concentration.
   ▲ **CRITICAL STEP** We recommend $5 \times 10^6$ cells $ml^{-1}$ for C2C12 myoblasts, NIH/3T3 fibroblasts and MDA-MB-231 breast cancer cells. Approximately 1 ml of the cell-laden bioink is needed to print five square samples with a size of 10 ×10 × 2 (width × length × height) $mm^3$.
   ▲ **CRITICAL STEP** The appropriate cell density depends on the type and size of cells used and should be determined experimentally. However, high cell densities (>$5 \times 10^6$ cells $ml^{-1}$) may affect the curing effect of bioink during the VBP process[7].
8. Centrifuge the cells for 3 min at 1,000*g*.
9. Aspirate the medium and resuspend with the silk-based bioink (Fig. 9g).
   ▲ **CRITICAL STEP** For keeping high cell viabilities, print the prepared silk-based bioinks as soon as possible and then transfer the bioprinted silk-based constructs into cell culture medium quickly.
   ◆ **TROUBLESHOOTING**
   ■ **PAUSE POINT** Keep the bioprinted samples in the cell culture incubator before further use.

### Preparation of cell-laden dECM bioinks

● **TIMING 3 d**

▲ **CRITICAL STEP** For cell culture, work in a class II biological safety hood and use sterile instruments. Clean all working surfaces with 70% (vol/vol) ethanol and expose to UV light for ≥20 min before use.

▲ **CRITICAL STEP** Use 0.22-μm syringe filters to filter all reagents before mixing with bioinks. All the reagents used need to be sterile.

10. Prepare the Ru/SPS stock solutions as in Procedure 2, Steps 1 and 2.
11. Place the dECM powder under UV light in the cell culture hood for 2 h.
12. Dissolve h-dECM powder in pepsin/0.5 M acetic acid solution at 15 mg $ml^{-1}$ at RT for 72 h. The ratio of pepsin/h-dECM should be 1:10 (wt/wt).
13. Dissolve Ms-dECM powder in pepsin/0.5 M acetic acid solution at 15 mg $ml^{-1}$ at RT for 72 h. The ratio of pepsin/Ms-dECM should be 1:10 (wt/wt).
    ▲ **CRITICAL STEP** The tube needs to be placed on a shaker to ensure full dissolution of the material.
14. Centrifuge the solution at 3,000*g* for 20 min to remove the undigested parts of the material or other impurities.
15. Collect the transparent dECM supernatant from the centrifuged solution and store at 4 °C.
16. The dECM solution is neutralized (pH 7.4) by using the 10-N sodium hydroxide solution in the ice box.
    ▲ **CRITICAL STEP** The neutralized dECM materials are thermosensitive, which means that they may self-cross-link at RT, and therefore the materials should be kept in the ice box before the VBP process.

▲ **CRITICAL STEP** The cold condition of dECM would typically not change within 2 min during the VBP process at RT, and thus the dECM materials should be printed once taken out of the ice box.

17. Add 10× DPBS solution into the neutralized dECM solution at a ratio of 1:9 (vol/vol) to make sure that the pH value and osmotic pressure are suitable for cell encapsulation and proliferation.
18. Dilute the dECM solution from Procedure 2, Step 17 with corresponding cell culture medium to prepare the desired concentration (e.g., 1% (wt/vol) dECM is usually prepared).
    ▲ **CRITICAL STEP** To obtain sterile materials, the dECM solution should be filtered by using the 0.22-μm syringe filter, and preparation of the dECM solution should be performed in a cell culture hood.
    ▲ **CRITICAL STEP** To prevent contamination, ensure that the lid is closed and wrap with aluminum foil.
19. Add the Ru/SPS stock solution from Procedure 2, Step 10 into different concentrations of dECM solutions according to the predetermined formulations.
    ▲ **CAUTION** To ensure the effectiveness of bioinks containing the photoinitiator, finish the bioprinting procedure within 1 h after mixing with Ru/SPS.
20. Use 0.25% (wt/vol) trypsin-EDTA solution to detach cells at 80% confluence.
21. Terminate the cell trypsinization with 10% (wt/vol) FBS in DMEM and transfer the cell suspension to 15-ml conical tubes.
22. Count the cells with a cell counter and prepare a final resuspension at the required cell concentration.
    ▲ **CRITICAL STEP** We recommend $1 \times 10^6$ cells $ml^{-1}$ for NIH/3T3 fibroblasts, rCMs and hMSCs. Approximately 1 ml of cell-laden bioink is needed to print five square samples with a dimension of 10 × 10 × 2 (width × length × height) $mm^3$.
    ▲ **CRITICAL STEP** The appropriate cell density depends on the type and size of cells used and should be determined experimentally. However, high cell densities (>$5 \times 10^6$ cells $ml^{-1}$) may affect the curing effect of bioink during the VBP process[7].
23. Centrifuge the cells for 3 min at 1,000*g*.
24. Aspirate the medium and resuspend with the prepared sterilized dECM bioink (Fig. 9g).
    ▲ **CRITICAL STEP** For keeping high cell viabilities, print the prepared dECM bioinks as soon as possible and then transfer the bioprinted constructs into cell culture medium quickly.
    ◆ **TROUBLESHOOTING**
    ■ **PAUSE POINT** Keep the bioprinted samples in the cell culture incubator before further use.

### VBP of silk screw-like constructs

● **TIMING 45 s**

25. Perform Procedure 1, Steps 74–76.
26. Gently introduce 2.5% (wt/vol) silk (bio)ink into the printing vial.
    ▲ **CRITICAL STEP** The volume of (bio)ink loaded into the printing vial is typically 1.5 ml.
    ▲ **CRITICAL STEP** Use a 1-ml pipette to gently remove any bubbles at the surface of the (bio) ink or inside the (bio)ink in the printing vial.
27. Place the printing vial onto the printing stage of the VBP system.
    ▲ **CRITICAL STEP** The screw-like construct is printed by using 2.5% (wt/vol) SF with 0.25/2.5 mM Ru/SPS for 45 s of printing time or 2.5% (wt/vol) SS with 0.5/5 mM Ru/SPS for 45 s of printing time.
28. After printing, remove the printing vial from the stage and rinse the printed construct twice for 2 min with growth medium to remove the un-cross-linked SF (bio)ink.
29. The SF screw-like constructs (without cells) with a single photo-cross-linked network should be immersed in 70% (vol/vol) ethanol aqueous solution to induce the formation of $\beta$-sheet conformation within the same SF prints for 2 h at RT.
30. The same SF screw-like constructs with double-cross-linked networks should be left in a chemical fume hood to evaporate the remaining ethanol overnight for further use.

### Cell seeding and cell proliferation assessments

● **TIMING 1 d (with an additional 7 d of cell proliferation)**

▲ **CRITICAL** The cells are seeded on the surface of printed SF screws, because the double-cross-linked networks of SF screws are induced by immersing in 70% (vol/vol) ethanol aqueous solution after printing, which is fatal to living cells.

31. Use 0.25% (wt/vol) trypsin-EDTA solution to detach hMSCs at 80% confluence from the culture dish.
32. Count the cell number by using a cell counter and prepare a final suspension with hMSC growth medium at the required cell concentration.
33. Place the SF screw-like constructs in the wells of 48-well plates and cover them with hMSC suspension ($1 \times 10^7$ cells $ml^{-1}$). Keep the plates in a cell incubator for 3 h.
34. Use autoclaved tweezers to move the SF screw-like constructs to new wells and turn them over. Add hMSC suspension ($1 \times 10^7$ cells $ml^{-1}$) to seed the cells again. Keep the plates in a cell incubator overnight.
35. Transfer the SF screw-like constructs to new wells and wash twice with DPBS.
36. Add 1 ml of hMSC growth medium and culture for 7 d.
    ▲ **CRITICAL STEP** Change the medium every 24 h.
37. Fix the printed SF screw-like constructs at days 1, 4 and 7 with 10% (wt/vol) formaldehyde solution for 20 min.
38. Permeabilize with 0.1% (wt/vol) Triton X-100 in DPBS for 10 min at RT.
39. After blocking with 1% (wt/vol) BSA in DPBS at 4 °C overnight, the samples should be separately incubated with the Alexa Fluor 488-phalloidin DPBS solution at a ratio of 1:1,000 (vol/vol) at 4 °C overnight.
40. Wash the sample twice and stain with the DAPI DPBS solution at a ratio of 1:5,000 (vol/vol) for 15 min at RT.
41. Capture the fluorescence micrographs by using an inverted fluorescence microscope to check the cell morphologies (Fig. 9h).
    ◆ **TROUBLESHOOTING**

### Cell differentiation and immunostaining

● **TIMING 4 weeks**

42. Change the hMSC growth medium to the hMSC osteogenic differentiation medium after day 7.
43. Fix the SF screw-like constructs at days 1, 7, 14, 21 and 28 with 10% (wt/vol) formaldehyde solution for 20 min and permeabilize with 0.1% (wt/vol) Triton X-100 in DPBS for 10 min at RT.
44. After blocking with 1% (wt/vol) BSA in DPBS at 4 °C overnight, the samples should be separately incubated with the mouse anti-RUNX2 antibody and mouse anti-osteocalcin antibody solutions at 4 °C overnight.
    ▲ **CRITICAL STEP** The mouse anti-RUNX2 antibody DPBS solution should be prepared at a ratio of 1:500 (vol/vol), and the mouse anti-osteocalcin antibody DPBS solution should be prepared at a ratio of 1:1,000 (vol/vol) or according to the manufacturers' instructions.
45. The samples that were stained with primary antibodies should then be stained with the secondary antibody (goat anti-mouse IgG H&L) DPBS solution at a ratio of 1:400 (vol/vol) after washing with DPBS three times.
46. The samples should be incubated with the DAPI DPBS solution at a ratio of 1:5,000 (vol/vol) for 15 min.
47. Capture the fluorescence micrographs by using an inverted fluorescence microscope to check the cell differentiation status.

### RNA isolation and real-time reverse transcription qPCR

● **TIMING 4 weeks**

48. RNAs of differentiated hMSC at days 1, 7, 14, 21 and 28 should be extracted by using the RNeasy mini kit.
49. All the extracted RNAs are then reversed-transcribed into cDNAs by using the QuantiTect reverse transcription kit.
50. qPCR should be performed by using the SYBR Green qPCR master mix.

51. The expressions of each target mRNA relative to GAPDH on different days are calculated on the basis of the threshold cycle (Ct) as $2 - \Delta(\Delta Ct)$, where:

$$\Delta Ct = Ct\,(sample) - Ct\,(GAPDH)$$

$$\Delta(\Delta Ct) = \Delta Ct\,(cells\ undergoing\ osteogenic\ differentiation) - Ct\,(control)$$

52. Quantitative gene-expression levels should be measured from three replicate samples at days 1, 7, 14, 21 and 28.
▲ **CRITICAL STEP** The expression at day 1 is set as the control group for each gene.

### Ex vivo implantation

● **TIMING 1 week**

53. Purchase fresh porcine femur from a local supermarket or grocery store.
54. Remove the meat from the femur.
▲ **CAUTION** When using a knife, be careful to avoid cutting yourself.
▲ **CRITICAL STEP** Make sure that the meat is properly removed and that the bones are exposed to the air.
55. A transparent cap with a notch should be attached onto the head of each printed SF screw-like construct to assist implantation.
56. The femur should be pre-drilled by using a drill bit (1-mm wide) to create holes (1-mm deep) that are slightly smaller than the diameter of the SF screw-like constructs.
57. Use a screwdriver to implant the printed SF screw-like constructs into the femur.
58. The SF screw-like construct-implanted porcine femur should be examined with a micro-CT X-ray imaging system.
59. Perform Micro-CT scanning by using a microfocus reflection tungsten X-ray source with tube voltage of 70 kV and target current of 180 µA (12.6 W).
60. Capture 3,142 projection images per sample on a 16-bit 2,000 × 2,000 flat panel detector with each exposure time at 1.0 s. The achievable voxel resolution is 24 µm.

### VBP of heart-like constructs with cell-laden h-dECM bioink

● **TIMING 45 s**

61. Perform Procedure 1, Steps 74–76.
62. Gently introduce rCM-laden or hiPSC-CM-laden 1% (wt/vol) h-dECM bioink into the printing vial.
▲ **CRITICAL STEP** rCMs or hiPSC-CMs should be embedded at $1 \times 10^6$ cells $ml^{-1}$ or slightly higher.
▲ **CRITICAL STEP** The volume of (bio)ink loaded into the printing vial is typically 1.5 ml.
▲ **CRITICAL STEP** Use a 1-ml pipette to gently remove any bubbles at the surface of the (bio) ink or inside the (bio)ink in the printing vial.
▲ **CRITICAL STEP** To prevent premature thermo-cross-linking of the dECM bioinks at RT, we recommend maintaining the bioinks at a low temperature (~4 °C) by chilling them on ice before the printing process.
▲ **CRITICAL STEP** After thawing, hiPSC-CMs should be centrifuged and immediately mixed with 1% (wt/vol) h-dECM bioink and used for bioprinting without additional culturing.
63. Place the printing vial onto the printing stage of the VBP system.
▲ **CRITICAL STEP** The heart-like construct should be volumetrically bioprinted with 0.5/5 mM Ru/SPS for 45 s of printing time.
64. After printing, remove the printing vial from the stage and rinse the printed construct twice with growth medium to remove the un-cross-linked cell-laden bioink.
◆ **TROUBLESHOOTING**

### Live/dead assay

● **TIMING 1 h, culture ≤2 weeks**

65. The volumetrically bioprinted rCM-laden heart-like constructs should be cultured in the growth medium for 2 weeks.
66. Harvest samples from the growth medium at predetermined time points (e.g., days 1, 3, 5, 7, 10 and 14).

67. The samples should be rinsed twice with DPBS and placed in a 24-well plate.
68. Add the staining solution in DPBS containing 2 µl $ml^{-1}$ of ethidium homodimer and 1 µl $ml^{-1}$ of calcein-AM to the samples in the 24-well plate.
69. Place the samples in the incubator at 37 °C for 30 min.
70. The samples should be rinsed with DPBS twice, and fluorescence images should be taken on an inverted fluorescence microscope.
    ▲ **CRITICAL STEP** The numbers of live and dead cells should be quantified with ImageJ.

### Cell viability assay

● **TIMING 1 h**

71. Export the detected image to ImageJ.
72. Count the number of live and dead cells through ImageJ by clicking on 'Plugins' → 'Analyze' → 'Cell Counter' → 'Initialize'.
73. Calculate the percentage viability (%V) as follows:

$$\%V = \frac{\text{Live cells}}{\text{Total cells}} \times 100\%$$

■ **PAUSE POINT** The samples can be stored in DPBS for ~5 d at 4 °C. However, the fluorescence signal of samples will reduce with time.

### Proliferation assay

● **TIMING 4 h, culture ≤2 weeks**

74. The volumetrically bioprinted rCM-laden heart-like constructs should be cultured in the growth medium for 2 weeks.
75. Harvest samples from the growth medium at predetermined time points (e.g., days 1, 3, 5, 7, 10 and 14).
76. Rinse the samples twice with DPBS and place them individually in the wells of a 24-well plate.
77. Add the staining solution in culture medium containing the MTS reagent into each well.
78. Place the samples in the incubator at 37 °C for 3 h.
79. Set the UV-visible spectrophotometer at 490 nm.
80. Harvest the supernatants of each well to quantify from the UV-visible spectrophotometer.
    ▲ **CRITICAL STEP** Similarly, the metabolic activities of samples can also be measured by the PrestoBlue reagent according to the manufacturer's instructions.

### F-actin staining

● **TIMING 2 d, culture ≤2 weeks**

81. The volumetrically bioprinted rCM-laden heart-like constructs should be cultured in the growth medium for 2 weeks.
82. Harvest samples from the growth medium at predetermined time points (e.g., days 1, 3, 5, 7, 10 and 14).
    ▲ **CRITICAL STEP** The culture medium should be changed every 2 d.
83. Fix the cardiac samples with 10% (vol/vol) formalin for 20 min at RT.
84. The samples should be permeated with the 0.1% (wt/vol) Triton X-100 DPBS solution for 15 min at RT.
85. Block the samples with the 1% (wt/vol) BSA DPBS solution at 4 °C overnight.
86. The samples should be incubated with the Alexa Fluor 488-phalloidin DPBS solution at a ratio of 1:1,000 (vol/vol) at 4 °C overnight.
87. Stain the samples with the DAPI DPBS solution at a ratio of 1:5,000 (vol/vol) for 15 min at RT and use an inverted fluorescence microscope to capture the fluorescence micrographs.

### Maturation and synchronized contractions of hiPSC-CMs

● **TIMING 10 d**

88. Replace the growth medium with cardiomyocyte medium and culture the samples for ≤10 d.
89. Observe the spontaneous beating movements of hiPSC-CMs every day under bright-feild microscopy (Fig. 9i).

▲ **CRITICAL STEP** Because the qualities of hiPSC-CMs in different batches are not consistent, the exact start day of the spontaneous beating could be different. Check all cell clusters in the heart-like construct under a microscope everyday carefully. Usually, the first spontaneous beating movement will happen at approximately day 5.

◆ **TROUBLESHOOTING**

### Immunostaining of differentiated hiPSC-CMs

● **TIMING 2 d**

90. After observing spontaneous beating movements, fix the heart-like construct at day 10 with 10% (wt/vol) formaldehyde solution for 20 min and permeabilize with 0.1% (wt/vol) Triton X-100 in DPBS for 10 min at RT.
91. After blocking with 1% (wt/vol) BSA in DPBS at 4 °C overnight, the samples should be separately incubated with the anti-sarcomeric-$\alpha$-actinin antibody solutions at 4 °C overnight.
    ▲ **CRITICAL STEP** The anti-sarcomeric-$\alpha$-actinin DPBS solution should be prepared at a ratio of 1:500 (vol/vol) or according to the manufacturer's instructions.
92. The samples that were stained with primary antibodies should then be stained with the secondary antibody (goat anti-mouse IgG H&L) DPBS solution at a ratio of 1:400 (vol/vol) after washing with DPBS three times.
93. The samples should be incubated with the DAPI DPBS solution at a ratio of 1:5,000 (vol/vol) for 15 min.
94. Capture the fluorescence micrographs by using an inverted fluorescence microscope to check the cell differentiation status.

### VBP of meniscus-like constructs with Ms-dECM bioink

● **TIMING 30 s**

95. Perform Procedure 1, Steps 74–76.
96. Gently transfer the hMSC-laden 1% (wt/vol) Ms-dECM bioink into the printing vial.
    ▲ **CRITICAL STEP** hMSCs are embedded within 1% (wt/vol) Ms-dECM at $5 \times 10^6$ cells $ml^{-1}$.
    ▲ **CRITICAL STEP** The volume of (bio)ink loaded into the printing vial is typically 1.5 ml.
    ▲ **CRITICAL STEP** Use a 1-ml pipette to gently remove any bubbles at the surface of the (bio)ink or inside the (bio)ink in the printing vial.
    ▲ **CRITICAL STEP** To prevent premature thermo-cross-linking of dECM bioink at RT, we recommend maintaining the bioinks at a low temperature (~4 °C) by chilling them on ice before the printing process.
97. Place the printing vial onto the printing stage of the VBP system.
    ▲ **CRITICAL STEP** The artificial meniscus-like construct should be volumetrically bioprinted with 0.25/2.5-mM Ru/SPS for 30 s of printing time.
98. After printing, remove the printing vial and rinse the printed hydrogel twice with growth medium to remove the un-cross-linked cell-laden bioink.
    ◆ **TROUBLESHOOTING**

### Live/dead assay

● **TIMING 1 h, culture ≤2 weeks**

99. The volumetrically bioprinted hMSC-laden meniscus constructs should be cultured in the growth medium for ≤2 weeks.
100. Perform Procedure 2, Steps 66–70.

### Cell viability assay

● **TIMING 1 h**

101. Export the detected image to ImageJ.
102. Perform Procedure 2, Steps 72 and 73.

### Proliferation assay

● **TIMING 4 h, culture ≤2 weeks**

103. The volumetrically bioprinted hMSC-laden meniscus constructs should be cultured in the growth medium for ≤2 weeks.
104. Perform Procedure 2, Steps 75–80.

### F-actin staining

● **TIMING 2 d, culture ≤2 weeks**

105. The volumetrically bioprinted hMSC-laden meniscus constructs should be cultured in the growth medium for ≤2 weeks.
106. Perform Procedure 2, Steps 82–87.

### RNA isolation and real-time reverse transcription qPCR

● **TIMING 4 weeks**

107. The volumetrically bioprinted hMSC-laden meniscus constructs should be cultured in chondrogenic differentiation medium for 21 d.
108. Harvest the meniscus samples at days 1, 7, 14 and 21 from the chondrogenic differentiation medium.
▲ **CRITICAL STEP** The culture medium should be changed every 2 d.
109. Incubate the meniscus samples with collagenase IV solution at 37 °C for 30 min for digestion of the dECM.
110. Centrifuge the digestion solution at 1,000*g* for 5 min and collect the cells.
111. Use an RNeasy mini kit to extract the mRNAs of the cells according to the manufacturer's instructions.
112. Perform Procedure 2, Steps 48–52.

### Histological evaluations

● **TIMING 24 h, culture ≤3 weeks**

113. The volumetrically bioprinted hMSC-laden meniscus-like constructs should be cultured in the growth medium for 14 d and in the chondrogenic differentiation medium for 21 d.
114. Harvest the samples at day 14 from the growth medium and at days 7, 14 and 21 from the chondrogenic differentiation medium.
▲ **CRITICAL STEP** The culture medium should be changed every 2 d.
115. Fix the meniscus samples in 10% (vol/vol) formalin for 24 h.
116. The fixed samples should b dehydrated and embedded in paraffin wax.
117. The paraffin wax should be serially sectioned (5 mm in thickness) for histological analyses.
118. The sections should be rehydrated and stained with hematoxylin and eosin (H&E), Masson's trichrome or Sirius red.
119. Use a bright-field microscope to take images of the sections.

## Troubleshooting

Troubleshooting advice can be found in Table 1.

**Table 1 | Troubleshooting table**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| **Procedure 1** | | | |
| 4 | Degumming of cocoons is not good. Cocoons are still hard after degumming, and only a little SS is taken off from the cocoons | The cocoons are not entirely immersed in water during the degumming process | Cut the cocoons into smaller pieces and press them into the bottom of the beaker. During the degumming, poke with two glass rods every 10 min to promote good dispersion of cocoons and to make sure that all the cocoons are immersed in the boiling water |

**Table 1 (continued) | Troubleshooting table**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 16, 35, 46, 55 | Protein quality between batches is not consistent | The original cocoons' quality is not the same | For quantifying the nitrogen content of protein, we recommend using the Kjeldahl method[31]<br>For quantifying tyrosine content in extracted proteins, we recommend using UV-visible spectroscopy[32] and high-performance liquid chromatography[33] |
| 26 | SF does not entirely dissolve in LiBr solution after 4 h | SF does not thoroughly interact with the LiBr solution | Before adding the LiBr solution, press SF into the bottom of a 100-ml beaker to remove as much air as possible. Use a glass rod to stir the SF fibers<br>Replace the LiBr solution with a new container in case of water uptake by the salt |
| 40 | A substantial amount of tissue still looks red in the 1% (vol/vol) SDS/PBS buffer after 72 h | The decellularization of heart tissue is not complete | Make sure that the heart tissue slides are not too thick. Increase the tissue-to-solution ratio to 1:24 if necessary |
| 58 | SS (bio)ink cannot be photo-cross-linked | The dialysis process for the SS solution is not completed | Separate dialysis bags into several 5-liter dialysis beakers to make sure that they are well dialyzed |
| 61 | Ms-dECM powders do not entirely dissolve in the pepsin/0.5 M acetic acid solution after 72 h | The sizes of Ms-dECM powders are too large | Remove the large pieces of Ms-dECM after serval grounding rounds |
| 64 | Failure to form a thermal dECM gel | Incorrect pH neutralization (proper pH: 7.2–7.4) | Slowly add 1 M sodium hydroxide solution drop by drop. Once the pH value exceeds 5, switch to using a 2-µl pipette for finer control |
| 74–87 | Uneven cross-linking of the printed construct | The rotation speed of the printing vial is not properly synchronized with the image projection rate, leading to misaligned light exposure during reconstruction | Ensure that the vial rotation speed matches the image-update frequency |
| | Crosslinking occurs on the surface of the printing vial | The concentration of photoinitiator such as Ru/SPS is too high | Test a proper concentration of photoinitiator before VBP |
| | Misalignment or distortion of the printed construct | The concentration of cells embedded in the bioink is too high | Test a proper concentration of cells before VBP |
| **Procedure 2** | | | |
| 9, 24 | Encapsulated cells are not distributed evenly | Insufficient mixing of the bioink and cells, or the bioink is left for too long a time before bioprinting | Mix cell-laden bioink very well by gently and repetitively pipetting and then transfer it into the printing vial immediately |
| 9, 24, 64, 98 | Resolution of bioprinting with cells is low | Printing resolution is reduced with strong light scattering caused by the increasing cell concentration | Reduce the cell concentration, or when high cell densities are needed, introduce cytocompatible refractive index–matching compounds such as iodixanol[7] |
| 41 | hMSCs are not seeded on the screw surface evenly | In the second round of seeding incubation, the side of SF screws has not been changed | Make sure that the side of SF screws is changed and observed by using a microscope and that move the samples are moved carefully and slowly |
| 89 | There are no spontaneous beating movements after 5 d | The culturing time is not sufficient | Culture the samples for ≤2 weeks and observe them daily |
| | | The cell density might be too low | Increase the cell density |

## Timing

### Procedure 1

Steps 1–16, preparation of SS: 3 d
Steps 17–36, preparation of SF: 3 d
Steps 37–46, preparation of h-dECM: 2 weeks
Steps 47–55, preparation of Ms-dECM: 2 weeks
Steps 56–59, preparation of silk-based (bio)inks: 30 min–1 h
Steps 60–66, preparation of dECM (bio)ink: 3 d
Steps 67–69, preparation of gelatin (bio)inks: 30 min
Steps 70–73, $D_p$ assessment: 1.5 h
Steps 74–79, VBP of silk-based (bio)inks: 30–120 s
Steps 80–83, VBP of dECM (bio)inks: 30–120 s
Steps 84–87, VBP of gelatin (bio)inks: 30–120 s

Steps 88–89, printability assessment: 3 h
Steps 90–91, printing resolution assessment: 3 h
Steps 92–94, mechanical property measurement: 2–3 h
Steps 95–100, Raman spectroscopy: 2–3 h
Steps 101–106, FTIR spectroscopy: 3 h
Steps 107–113, SEM imaging: 1.5–3 h
Steps 114–116, post-printing processing for inducing the double-cross-linked network of printed SF constructs: 3–4 d
Steps 117–120, post-printing processing for shrinkage and expansion property evaluations of SS constructs: 2 d
Steps 121–129, in vitro water-uptake tests: 1 week
Steps 130–136, in vitro degradation assay: 6 months for silk, 28 d for dECM

**Procedure 2**
Steps 1–9, preparation cell-laden SS or SF bioink: 2 h
Steps 10–24, preparation of cell-laden dECM bioinks: 3 d
Steps 25–30, VBP of silk screw-like construct: 45 s
Steps 31–41, cell seeding and cell proliferation assessments: 1 d (with 7-d of cell proliferation)
Steps 42–47, cell differentiation and immunostaining: 4 weeks
Steps 48–52, RNA isolation and real-time reverse transcription qPCR: 4 weeks
Steps 53–60, ex vivo implantation: 1 week
Steps 61–64, VBP of heart-like constructs with cell-laden h-dECM bioink: 45 s
Steps 65–70, live/dead assay: 1 h, culture ≤2 weeks
Steps 71–73, cell viability assay: 1 h
Steps 74–80, proliferation assay: 4 h, culture ≤2 weeks
Steps 81–87, F-actin staining: 2 d, culture ≤2 weeks
Steps 88–94, maturation and synchronized contractions of hiPSC-CMs: 12 d
Steps 95–98, VBP of meniscus-like constructs with Ms-dECM bioink: 30 s
Steps 99–100, live/dead assay: 1 h, culture ≤2 weeks
Steps 101–102, cell viability assay: 1 h
Steps 103–104, proliferation assay: 4 h, culture ≤2 weeks
Steps 105–106, F-actin staining: 2 d, culture ≤2 weeks
Steps 107–112, RNA isolation and real-time reverse transcription qPCR: 4 weeks
Steps 113–119, histological evaluations: 24 h, culture ≤3 weeks

## Anticipated results

This protocol outlines a method for rapid VBP of unmodified protein-based (bio)inks with tyrosine groups, including those based on silk, dECM and gelatin, by using an Ru/SPS photoinitiator system to form sophisticated shapes and architectures. We anticipate that these methods will be useful for bioprinting of in vitro models embedded within living cells.

The results in Fig. 7 show that both 2.5% (wt/vol) SS and 2.5% (wt/vol) SF constructs support better proliferation rates and viabilities than constructs with higher silk concentrations (5% (wt/vol) SS, or 5% (wt/vol) and 10% (wt/vol) SF) (Fig. 7g,h,k,l). The cytocompatibility of low-concentration (2.5%, wt/vol) SS and SF bioinks makes them suitable for VBP applications in which living cells are encapsulated. Of note, unlike SF, the SS constructs support desired activities (such as spreading) of encapsulated cells, implying a more promising biomedical potential than SF for cell culture–related applications. The cell proliferation rates and metabolic activities within both 1% (wt/vol) h-dECM and 1% (wt/vol) Ms-dECM with 0.25/2.5-mM Ru/SPS increased over 14 d, whereas the metabolic activities of cells with 0.5/5-mM Ru/SPS remained steady in the late stage (Fig. 7i,j), suggesting that the cells stopped proliferating or reduced their metabolic activities after 7 d. In addition, the viabilities of cells embedded in both 1% (wt/vol) h-dECM and 1% (wt/vol) Ms-dECM bioprinted with 0.25/2.5-mM Ru/SPS were higher than those

for cells in dECM bioprinted with 0.5/5-mM Ru/SPS (Fig. 7m,n), probably because of reduced phototoxcity when a lower concentration of photoinitiator is used.

hMSCs exhibited nearly 100% proliferation and viability over 7 d, indicating the cytocompatibility of the double-cross-linked SF screw-like constructs. Extensive cell spreading was observed on the screw surfaces. These SF screw-like constructs, treated with 70% (wt/vol) ethanol and air-dried, were successfully tightened into the cortical bone without breakage or deformation, demonstrating their potential for bone implantation and device fixation. These observations highlight the feasibility and functionality of double-cross-linked SF screw-like constructs for biomedical applications (Fig. 8a). The protocol further integrated hiPSC-CMs into the bioprinted h-dECM constructs, which demonstrated structural maturation and spontaneous beating, suggesting the functional potential of these bioinks for cardiac tissue engineering (Fig. 8b). High cell viability (~95%) was sustained over 14 d, revealing the cytocompatibility of Ms-dECM. Constructs bioprinted with 0.25/2.5 mM-Ru/SPS showed nearly double the metabolic activities compared to those at 0.5/5 mM. Extensive cell spreading (~100%) was observed in both constructs. Further evaluation revealed successful chondrogenic differentiation, with increased expressions of collagen and aggrecan, supported by immunostaining and qPCR results. Histological staining confirmed the presence and intensification of cartilage extracellular components over time, suggesting potential applications in cartilage tissue engineering (Fig. 8c).

Furthermore, our experience indicates that these protein-based (bio)inks should be prepared and treated very carefully to avoid unsatisfactory results (Fig. 9a–i). Therefore, we recommend keeping biomaterial sources such as cocoons, dECM and gelatin as consistent as possible between batches (or sourcing/preparing a larger batch for all key direct-comparison experiments) and quantifying the critical components in these proteins before use (Table 1; Procedure 1, Steps 16, 35, 46 and 55). There are some challenges that might occur during the VBP process, such as inhomogeneous cross-linking of the (bio)printed construct, cross-linking on the surface of the printing vial, misalignment or distortion of the (bio)printed construct and low resolution of bioprinting with cells, for which some possible solutions have been presented (Table 1; Procedure 1, Steps 74–87; Procedure 2, Steps 9, 24, 64 and 98). Overall, this protocol aims to provide step-by-step instructions to maximize the success of VBP of pristine protein-based (bio)inks that contain tyrosine groups.

Received: 19 February 2024; Accepted: 20 January 2026;
Published online: 13 April 2026

## References

1. Kelly, B. E. et al. Volumetric additive manufacturing via tomographic reconstruction. *Science* **363**, 1075–1079 (2019.
2. Bernal, P. N. et al. Volumetric bioprinting of complex living-tissue constructs within seconds. *Adv. Mater.* **31**, 1904209 (2019).
3. Größbacher, G. et al. Volumetric printing across melt electrowritten scaffolds fabricates multi-material living constructs with tunable architecture and mechanics. *Adv. Mater.* **35**, 2300756 (2023).
4. Peng, S. et al. Ultra-fast 3D printing of assembly-free complex optics with sub-nanometer surface quality at mesoscale. *Int. J. Extreme Manuf.* **5**, 035007 (2023).
5. Ribezzi, D. et al. Shaping synthetic multicellular and complex multimaterial tissues via embedded extrusion-volumetric printing of microgels. *Adv. Mater.* **35**, 2301673 (2023).
6. Falandt, M. et al. Spatial-selective volumetric 4D printing and single-photon grafting of biomolecules within centimeter-scale hydrogels via tomographic manufacturing. *Adv. Mater. Technol.* **8**, 2300026 (2023).
7. Bernal, P. N. et al. Volumetric bioprinting of organoids and optically tuned hydrogels to build liver-like metabolic biofactories. *Adv. Mater.* **34**, 2110054 (2022).
8. He, N. et al. Photoinhibiting via simultaneous photoabsorption and free-radical reaction for high-fidelity light-based bioprinting. *Nat. Commun.* **27**, 3063 (2023).
9. Madrid-Wolff, J. et al. A review of materials used in tomographic volumetric additive manufacturing. *MRS Commun.* **13**, 764–785 (2023).
10. Xie, M. et al. Volumetric additive manufacturing of pristine silk-based (bio)inks. *Nat. Commun.* **14**, 210 (2023).
11. Lian, L. et al. Rapid volumetric bioprinting of decellularized extracellular matrix bioinks. *Adv. Mater.* **36**, 2304846 (2024).
12. Qin, N., Qian, Z. G., Zhou, C., Xia, X. X. & Tao, T. H. 3D electron-beam writing at sub-15 nm resolution using spider silk as a resist. *Nat. Commun.* **12**, 5133 (2021).
13. Kim, S. H. et al. 3D bioprinted silk fibroin hydrogels for tissue engineering. *Nat. Protoc.* **16**, 5484–5532 (2021).
14. Chakraborty, J., Mu, X., Pramanick, A., Kaplan, D. L. & Ghosh, S. Recent advances in bioprinting using silk protein-based bioinks. *Biomaterials* **287**, 121672 (2022).
15. Kim, S. H. et al. Precisely printable and biocompatible silk fibroin bioink for digital light processing 3D printing. *Nat. Commun.* **9**, 1620 (2018).
16. Ali, M. et al. A photo-crosslinkable kidney ECM-derived bioink accelerates renal tissue formation. *Adv. Healthc. Mater.* **8**, 1800992 (2019).
17. Yang, X. et al. A 3D-bioprinted functional module based on decellularized extracellular matrix bioink for periodontal regeneration. *Adv. Sci.* **10**, 2205041 (2023).
18. Brown, M., Li, J., Moraes, C., Tabrizian, M. & Li-Jessen, N. Y. K. Decellularized extracellular matrix: new promising and challenging biomaterials for regenerative medicine. *Biomaterials* **289**, 121786 (2022).
19. Soliman, B. G. et al. Programming delayed dissolution into sacrificial bioinks for dynamic temporal control of architecture within 3D-bioprinted constructs. *Adv. Func. Mater.* **33**, 2210521 (2023).
20. Ribezzi, D. et al. Multi-material volumetric bioprinting and plug-and-play suspension bath biofabrication via bioresin molecular weight tuning and multiwavelength optics. *Adv. Mater.* **37**, 2409355 (2024).
21. Chansoria, P. et al. Synergizing algorithmic design, photoclick chemistry and multi-material volumetric printing for accelerating complex shape engineering. *Adv. Sci.* **10**, 2300912 (2023).

22. Riffe, M. B. et al. Multi-material volumetric additive manufacturing of hydrogels using gelatin as a sacrificial network and 3D suspension bath. *Adv. Mater.* **36**, 2309026 (2024).
23. Zhao, Z., Li, Y. & Xie, M. B. Silk fibroin-based nanoparticles for drug delivery. *Int. J. Mol. Sci.* **16**, 4880–4903 (2015).
24. Lim, K. S. et al. New visible-light photoinitiating system for improved print fidelity in gelatin-based bioinks. *ACS Biomater. Sci. Eng.* **2**, 1752–1762 (2016).
25. Cui, X. et al. Rapid photocrosslinking of silk hydrogels with high cell density and enhanced shape fidelity. *Adv. Healthc. Mater.* **9**, 1901667 (2020).
26. Levaoto, R. et al. Light-based vat-polymerization bioprinting. *Nat. Rev. Methods Primers* **3**, 47 (2023).
27. Rizzo, R., Ruetsche, D., Liu, H. & Zenobi-Wong, M. Optimized photoclick (bio)resins for fast volumetric bioprinting. *Adv. Mater.* **33**, 2102900 (2021).
28. Bernal, P. N. et al. The road ahead in materials and technologies for volumetric 3D printing. *Nat. Rev. Mater.* **10**, 826–841 (2025).
29. Soliman, B. G. et al. Stepwise control of crosslinking in a one-pot system for bioprinting of low-density bioinks. *Adv. Healthc. Mater.* **9**, 1901544 (2020).
30. Tang, M. et al. Evaluating single-cell cluster stability using the Jaccard similarity index. *Bioinformatics* **37**, 2212–2214 (2021).
31. Orellana-Palacios, J. C. et al. Corn silk as an underutilized sustainable source of plant proteins: extraction, fractionation, structure, and techno-functional properties. *Food Hydrocoll.* **158**, 110550 (2025).
32. Murphy, A. R. et al. Modification of silk fibroin using diazonium coupling chemistry and the effects on hMSC proliferation and differentiation. *Biomaterials* **29**, 2829–2838 (2008).
33. Hausken, K. G. et al. Quantitative functionalization of the tyrosine residues in silk fibroin through an amino-tyrosine intermediate. *Macromol. Chem. Phys.* **223**, 2200119 (2022).


## Acknowledgements

This work was performed in part at the Center for Nanoscale Systems (CNS), Harvard University, and supported by the National Institutes of Health (P41EB027062, R01AR070975, R01HL166522 and R01CA282451), a National Science Foundation award (1541959, CBET-EBMS-1936105 and CISE-IIS-2225698), the ARO (W911NF2120130), the AFOSR (FA9550-20-1-0363), the Chan Zuckerberg Initiative (2022-316712 and 2024-347836), the National Research Foundation of Korea (NRF) grant (RS-2024-00403376) funded by the Korean government (MSIT), and the Brigham Research Institute.

## Author contributions

M.X. and L.L. contributed equally to this work. M.X. and Y.S.Z. conceptualized the project; M.X., L.L., Z.Z., Z.L., E.M.G. and J.K.S. performed experiments, and analyzed data; all authors discussed data; K.S.L., D.L.K. and Y.S.Z. supervised the study; M.X., L.L. and Y.S.Z. wrote the original draft; M.X., L.L., K.S.L., D.L.K. and Y.S.Z. reviewed and edited the manuscript.

## Competing interests

Y.S.Z. consulted for Allevi by 3D Systems; consults for PepGel; cofounded, consults for and holds options of Linton Lifesciences; cofounded, consults for, and holds options of Criocore; and sits on the scientific advisory board and holds options of Xellar Biosystems. The relevant interests are managed by the Brigham and Women's Hospital. M.X. consulted for Green Key, which, however, did not participate in or bias the work. The other authors declare no competing interests.

## Additional information

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41596-026-01341-1.

**Correspondence and requests for materials** should be addressed to Yu Shrike Zhang.

**Reviewer recognition** *Nature Protocols* thanks the anonymous reviewers for their contribution to the peer review of this work.