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Author: PT, JB, VK. Version: 3

Bioprinting Protocol

GelMA Bioink

This is a suggested procedure, please adjust according to your experimental needs.

Protocol aim

The aim of this protocol is to provide instructions for bioprinting with the GelMA Bioink using the BIO X. This document covers pre-print mixing with cells, actual 3D bioprinting and post-print processes of crosslinking through photocuring. This protocol was optimized for bioprinting and photocrosslinking of GelMA 10% w/w concentration with a LAP as a photoinitiator at the concentration of 0.25%, using the Temperature-controlled Printhead and cooled print bed at the BIO X. Changing the concentration of a photoinitiator or bioink to cell suspension ratio changes the photocrosslinking time. Refer to *Photocrosslinking Optimization Protocol* to adjust and determine these numbers.

Materials needed

- GelMA Bioink*
- UV shielding cartridges, 3cc*
- Sterile conical bioprinting nozzles, 22-27G*
- BIO X* 3D Bioprinter
- 365/405 LED modules for photocuring
- Petri dish* or well plate
- Mesenchymal stem cells + cell culture medium
- 3 ml syringes with luer lock connections
- Female/female Luer lock adaptor*
- CELLMIXER*

*The product can be purchased in the CELLINK store at *www.cellink.com/store/*.

KEEP THE BIOINK PROTECTED FROM LIGHT IF TRANSFERRED FROM THE ORANGE UV PROTECTED CARTRIDGES TO AVOID CROSSLINKING BEFORE PRINTING. WORK WITH 3D PRINTERS IN DARK MODE. THE PHOTOINITIATOR IS SENSITIVE TO REPEATED OR PROLONGED EXPOSURE TO HEAT.

Arvid Wallgrens Backe 20
413 46 Gothenburg
SWEDEN

100 Franklin St,
Boston, MA 02110
USA

Med-Pharm Collaboration
Building, 46-29 Yoshida-Shimo
Kyoto, JAPAN

www.cellink.com

Protocol

GelMA Bioink has been optimized for bioprinting with the BIO X system. While the bioink can be used with the INKREDIBLE+ system due to its ability to heat the bioink, secondary steps are necessary to cool the printed structure to pre-gel it prior to crosslinking. Clogging may still occur due to lack of temperature control at the nozzle. Therefore, it is not recommended to use the bioink with the INKREDIBLE system since the bioink will not perform as expected and resulting filament characteristics may be inconsistent.

Step	Title	Material	Description
1	Prepare bioink	- GelMA Bioink	- Heat up the GelMA Bioink in a cartridge to 35°C until the GelMA is liquid. This can be tested by flipping the cartridge and observing if air bubbles move freely. The heating of the GelMA can be performed in an incubator. - Set the Temperature-controlled Printhead to 26°C. Pre-cool the print bed to 9°C.
2	Mix GelMA with cells	- Cell suspension - CELLMIXER - Female/female Luer lock adaptor - 3 mL syringes with Luer lock connections - Prewarmed GelMA	If not printing with cells move directly to step 3. At this point, mix ten parts of the bioink with one part of a cell suspension without introducing air bubbles to the mixture. For detailed instructions see the *Mixing Cells Protocol GelMA Series*. - Transfer the cell suspension to the 1 mL cell syringe (PART 1) using a female/female Luer lock adaptor. - Transfer GelMA to the 12 mL syringe (PART 2) using a female/female Luer lock adaptor. - Clip both syringes to the Dispensing unit (PART 3). - Connect the two syringes to the Mixing unit (PART 4), then connect the Empty cartridge (PART 5) to the Mixing unit's other side. - Apply gentle pressure onto the Dispensing unit to mix the content of both syringes into the empty cartridge. Note: To avoid an air gap when mixing the bioink and the cell suspension, carefully pre-fill the Luer lock adaptor with GelMA before attaching the syringe with the cell suspension. If preparing for quantities <2 mL of GelMA, it is recommended to connect two 3 mL Luer lock syringes and mix back and forth between the syringes until homogeneous.

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SWEDEN

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USA

Med-Pharm Collaboration
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3	Cool and load the cartridge	- UV shielding cartridges, 3cc loaded with GelMA Bioink - Sterile conical bioprinting nozzles, 22-27G	- Cap the cartridge with a 22-27G bioprinting nozzle. - Place the GelMA cartridge in the Temperature-controlled Printhead and wait for 10-20 min until the GelMA reaches 26°C. Refer to Figure 1 for viscosity-temperature dependence for the GelMA Bioink.
4	Printing	- BIO X 3D bioprinter	- Bioprint structures with parameters according to Table 1. If printability is not as desired, adjust the pressure up/down by 1 kPa to extrude more/less material. Note: If waiting too long between extrusions, the bioink can dry in the nozzle causing it to clog. If this occurs, take a sterile tweezer and remove the dried GelMA part at the edge of the nozzle or replace with new nozzle. Note: Over time the GelMA becomes more solid. If printing is paused for 15-20 min and this happens, replace the nozzle. If extrusion does not occur, repeat Step 1 and 3 to 'reset' the cartridge.

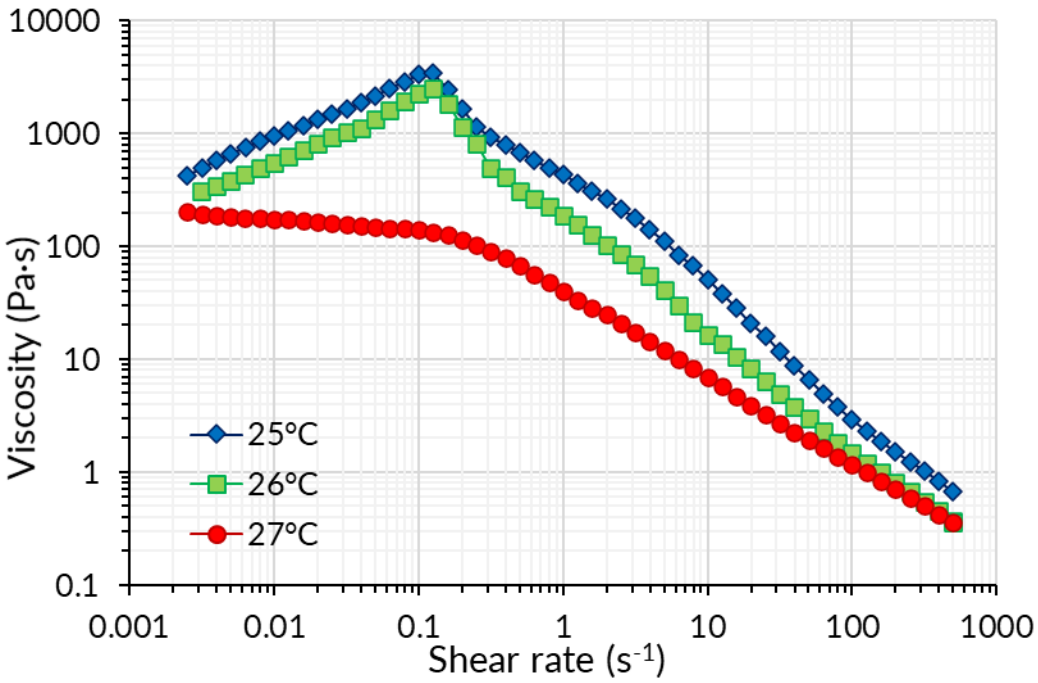

Figure 1. Viscosity change of GelMA Bioink upon applied shear forces at various temperatures over a shear rate range of 0.01 to 500 s^{-1}.

Arvid Wallgrens Backe 20
413 46 Gothenburg
SWEDEN

100 Franklin St,
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USA

Med-Pharm Collaboration
Building, 46-29 Yoshida-Shimo
Kyoto, JAPAN

Table 1. Recommended minimal extrusion pressure** (±2 kPa) for printing continuous filaments at 26°C using $^{diluted}/_{undiluted}$ bioink. 'Diluted' assumes a mixture of one part of PBS to ten parts of bioink, which is the simulation of bioink and cell suspension mixing conditions. For smaller dilutions, the pressure needs to be increased towards the pressure used for undiluted bioink.

Printing speed (mm/sec) → Nozzle size (G) ↓	5	10	15	20
22	8 / 11	13 / 15	16 / 18	14 / 19
25	15 / 18	21 / 25	26 / 30	32 / 32
27	15 / 19	21 / 30	22 / 35	22 / 40

***This is only a recommended reference of starting pressures. The actual pressure needed will vary depending on the preparation procedures (amount of bioink and actual temperature of the bioink) as well as the fitting of the piston in the cartridge and the leveling of the print surface. This table was generated with printhead temperature at 26°C and with a 10:1 bioink dilution.*

Step	Title	Material	Description
5	Crosslinking	- 405/365 UV modules for photocuring	GelMA with LAP can be crosslinked with photoinitiation using either the 405 or 365 nm photocuring module. - See Table 2 below for recommended crosslinking times. Ensure that the bioprinted GelMA construct is thermally gelled after printing by cooling the print bed on the BIO X for 30 s. - If photocrosslinking during bioprinting, set the crosslinking parameters appropriately in the printhead setup page for the BIO X. - Let the structure sit for 3-5 min to allow crosslinking after the light source is turned off. Note: It is recommended to use the 405 nm photocuring module instead of 365 when photocuring GelMA with LAP. Overexposure at the 365 nm wavelength might damage the cells. Note: If crosslinking is unsure, add 37°C media to one printed well to validate that it doesn't dissolve.

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413 46 Gothenburg
SWEDEN

100 Franklin St,
Boston, MA 02110
USA

Med-Pharm Collaboration
Building, 46-29 Yoshida-Shimo
Kyoto, JAPAN

www.cellink.com

Table 2. Recommended time of the construct photocrosslinking***. Distance from a photocuring module to construct was set at 5 cm. If using the INKREDIBLE+ photocuring modules, the time required can possibly be decreased. For crosslinking with other parameters, see *Photocrosslinking Optimization Protocol*. This table was generated using GelMA with mesenchymal stem cells. Don't exceed the exposure time at these conditions for more than 120 s when printing with cells.

	365 nm, LAP 0.25%	405 nm, LAP 0.25%
Construct depth (mm)/time (s)	1/5	1/15
	3/20	3/30

****This is only a recommended reference of crosslinking times to start with. The actual time needed for crosslinking will vary depending on the size and temperature of the constructs as well as the intensity of the photocuring module and the distance to the construct.*

Step	Title	Material	Description
6	Incubation	- Cell culture medium	- After photocrosslinking, add the desired medium to the constructs and place in incubator. - Incubate the constructs in cell culture medium in standard culture conditions (37°C, 5% CO_2 and 95% relative humidity) or according to your application.

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