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Author: MSL, VK. Version: 1

RECONSTITUTION AND BIOPRINTING PROTOCOL

PhotoHA® Stiff

This is a suggested procedure, please adjust it according to your experimental needs. To maintain the sterility of the product, work under sterile conditions.

Protocol aim

The aim of this protocol is to provide instructions on how to reconstitute PhotoHA® Stiff (methacrylated hyaluronic acid, also known as HAMA) to your desired concentration using 1x PBS as well as mixing with cells and bioprinting. The addition of a photoinitiator (PI) and the use of 365 or 405 nm LED modules ensures stable and controlled photocrosslinking of PhotoHA constructs for 3D cell culturing. This protocol has been optimized for use with the BIO X and BIO X6 systems.

Materials needed

- PhotoHA® Stiff (100 mg)*
- 1x PBS
- Photoinitiator (PI)*
- Two 3 mL syringes
- 0.22 µm sterile syringe filter
- Two 15 mL Falcon tubes
- Female/female Luer lock adaptors*
- Cell suspension and cell culture medium
- 3cc amber cartridge* with tip cap
- Bioprinter (BIO X or BIO X6)
- 22G conical bioprinting nozzle
- Well plate

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

Protocol

This protocol describes the reconstitution of 100 mg of PhotoHA® Stiff to obtain bioinks of different concentrations. We recommend concentrations from 5 to 30 mg/mL (0.5–3.0%). The reconstitution solution contains the photoinitiator LAP, which allows photocrosslinking of the PhotoHA at 365 or 405 nm to provide various gel stiffness. Different LAP concentrations and light exposure times will directly affect the final stiffness of the bioink. Protect the bioinks from light when mixed with LAP. Employ aseptic practices to maintain the product's sterility throughout the preparation and handling of PhotoHA and other solutions.

1. Preparation of the reconstitution solution with PI

MATERIAL

1x PBS

Photoinitiator (PI), e.g., LAP

20 mL syringe

0.22 µm sterile syringe filter

Two 15 mL Falcon tubes

DESCRIPTION

- Weigh the desired amount of PI in a 15 mL Falcon tube. See Table 1 for suggested LAP concentrations.
- Add 15 mL of 1x PBS to the tube and slightly shake until dissolution.

Note: Remember to protect all PI containing solutions from light. Ensure PI is properly dissolved before proceeding.

- Transfer the PI solution (Reconstitution agent + PI) to a 20 mL syringe and sterile filter it into another sterile 15 mL Falcon tube using a 0.22 µm sterile syringe filter.

Table 1. Suggestions of LAP concentrations for PhotoHA bioink.

LAP concentration in bioink	LAP mass for 15 mL of 1x PBS
0.10% (1 mg/mL)	15 mg
0.25% (2.5 mg/mL)	37.5 mg

2. Reconstitution of PhotoHA® Stiff

MATERIAL

PhotoHA® Stiff

PI solution

DESCRIPTION

- Add the prepared PI solution to the 100 mg PhotoHA® Stiff vial. See Table 2 for suggested bioink concentrations.

Note: If not reconstituting the total mass of the vial, aliquot the necessary amount and store the remaining PhotoHA® Stiff at -20°C. Adjust the PI volume solution accordingly.

- Mix on a shaker table or rotator plate until fully solubilized (30-60 minutes) at 2-10°C. Alternatively, place the bottle in the fridge and gently rotate it a couple of times until complete dissolution.

Note: Solubilization times may vary depending on the desired concentration and volume of PBS added.

- Store the reconstituted PhotoHA solution at 2-10°C if needed.

Table 2. Suggestions of final bioink concentrations for reconstitution of 100 mg PhotoHA® Stiff.

Concentration of PhotoHA bioink (w/v)	Volume of PI solution needed
1% (10 mg/mL)	10 mL
2% (20 mg/mL)	5 mL
3% (30 mg/mL)	2 mL

3. Mixing PhotoHA with cells

MATERIAL

PhotoHA solution

Two 3 mL syringes

Cell suspension

Female/female Luer lock adaptor

3cc amber cartridge with tip cap

DESCRIPTION

- Transfer the PhotoHA solution into a 3 mL syringe using the following procedure: remove a syringe plunger → cap the syringe → transfer the bioink to the syringe → insert the plunger → flip the syringe → release the tip cap to evacuate the air.
- Prepare a cell suspension with the desired number of cells dispersed in cell culture medium. The volume of the cell suspension should be 10% of bioink volume. Transfer the cell suspension to a 3 mL syringe.
- Connect the PhotoHA solution syringe to the syringe with cell suspension using a female/female Luer lock adaptor.
- Mix ten parts of PhotoHA solution with one part of the cell suspension without introducing air bubbles to the mixture. If using other ratios, recommended printing parameters (Table 3) should be adjusted accordingly. For detailed instructions see the *Mixing Cells and Bioink Protocol.*

Note: To avoid an air gap when mixing the bioink and the cell suspension, carefully pre-fill the Luer lock adaptor with PhotoHA solution before attaching the syringe to the cell suspension.

- Mix the bioink with the cell suspension by gently pushing them back and forth between the syringes.
- Transfer the mixture to a 3cc amber cartridge by connecting the syringe to the cartridge using the Luer lock adaptor.
- Add a tip to the amber cartridge containing the mixture and keep it at room temperature.

4. Bioprinting PhotoHA

MATERIAL

Bioprinter (BIO X or BIO X6)

3cc cartridge with PhotoHA mixed with cells

22G conical bioprinting nozzle

Well plate

DESCRIPTION

- Attach the 22G conical nozzle to the 3cc cartridge with the PhotoHA mixed with cells.
- Place the cartridge in the printhead of the bioprinter.
- Calibrate the bioprinter to the correct position of the well plate.
- In the pressure settings, test the flow of the bioink. Start with low pressure and gradually increase until bioink extrudes from the nozzle.

Note: The initial pressure for the liquid to come out of the nozzle is higher than the pressure used during printing. Reduce the pressure at this stage to not lose material with the first droplet during printing.

- Print droplets with the desired size. Use the suggested parameters (see Table 3) as a guide for your printing to achieve droplets of approximately 10-15 µL.

Note: If printability is not as desired, adjust the pressure up/down to extrude more/less material at different extrusion times.

Note: Different nozzle sizes and concentrations of bioink can be used if desired. Adjust the parameters for printing accordingly.

Table 3. Recommended starting parameters for droplet printing of PhotoHA at different concentrations*.

Concentration of PhotoHA (mg/mL)	Pressure for printing (kPa)	Extrusion time (s)	Initial pressure for testing bioink flow (kPa)
10	13	0.8	16
30	17	0.8	22

*Bioprinting parameters were obtained with PhotoHA prepared at a specific concentration and further mixed with cells (10:1). The cell density and different proportionality may affect the final viscosity of the bioink. If changes are made, printing parameters need to be adjusted accordingly.

5. Crosslinking and incubation

MATERIAL

Well plate with printed droplets

Bioprinter (BIO X or BIO X6) with LED modules of choice for photocuring

Cell culture medium

DESCRIPTION

- Photocure the droplets using the LED module. See Figure 1 for the comparative stiffness of PhotoHA constructs at different concentrations.

Note: It is recommended to use the 405 nm photocuring module for 30 seconds instead of 365 nm, if possible, when photocuring PhotoHA with LAP photoinitiator. Overexposure at the 405 nm and 365 nm wavelength might damage the cells.

Note: Time and distance will directly affect the stiffness of the material and the viability of cells. Use a distance between 3 and 5 cm from the plate to the LED module, and a maximum of 30 seconds of light exposure.

- Submerge the constructs in the cell culture medium and place them in the incubator (37°C, 5% CO_2 and 95% relative humidity) for 3D cell culturing according to your application.

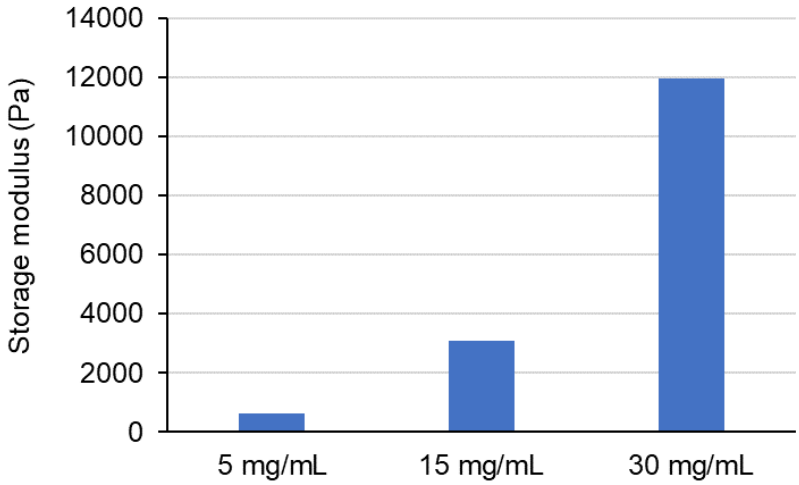

Figure 1. *PhotoHA Stiff stiffness at different concentrations. Rheology tests were performed on a Discovery Hybrid Rheometer, HR 20, TA instruments, with a UV exposure at 405 nm (LAP as a PI), for 30 seconds and at 5 cm distance from the light source.*