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NEUTRALIZATION AND BIOPRINTING PROTOCOL

Coll 1 Solution

This is a suggested procedure, please adjust 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 for neutralization and subsequent bioprinting of the neutralized Coll 1 Solution. This document covers the bioprinting of Coll 1 droplets with embedded cells and cell post-seeding on printed Coll 1 constructs. The biomaterial thermally gels at 37°C.

Materials needed

- Coll 1 Solution (10 mL at 10 mg/mL)*
- Collagen Buffer (5 mL)*
- Ice bath
- 1x PBS
- 1M NaOH
- Container for mixing (15 mL Falcon tube or 5 mL Eppendorf tube)
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptors*
- Cells* + culture medium*
- Cartridges, 3 cc*
- Sterile conical bioprinting nozzles*
- Temperature-controlled printhead* (optional)
- BIO X*, BIO X6* or INKREDIBLE-series* 3D bioprinter
- Well plate or mold

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

Protocol for neutralization

During the whole procedure keep all materials on ice. Cool down the Coll 1 solution after every component is added.

1. Preparing Coll 1

MATERIAL

Coll 1 Solution

Collagen Buffer

Ice bath

DESCRIPTION

- Place the vial of Coll 1 Solution and the Collagen Buffer on ice to keep them cool.
- C_S is the concentration of the original Coll 1 Solution (10 mg/mL).
- Record the desired final volume of the bioink (V_{INK}, mL).
- Record the desired final Coll 1 concentration after neutralization (C_F).

Note: C_F and C_S cannot be the same, otherwise the solution would not be neutralized (refer to Table 1).

Table 1. Preparation of Coll 1 biomaterial with different concentration.

V_{INK}, mL	C_F, mg/mL	C_S, mg/mL	$V_{Coll\,1}$, µL	V_{CB}, µL	V_{NaOH}, µL	V_{PBS}, µL
1	8	10	800	123	20	57
	6		600	92	15	293
	4		400	62	10	528

2. Calculations for neutralization

DESCRIPTION

- Volume of needed Coll 1 Solution: $V_{Coll\,1}\,(mL) = \frac{C_F \times V_{INK}}{C_S}$
- Volume of Collagen Buffer: $V_{CB}\,(mL) = V_{Coll\,1} \times 0.154$
- Volume of 1M NaOH: $V_{NaOH}\,(mL) = V_{Coll\,1} \times 0.025$
- Volume of 1x PBS to reach C_F: $V_{PBS}\,(mL) = V_{INK} - V_{Coll\,1} - V_{CB} - V_{NaOH}$

3. Neutralization

MATERIAL

1x PBS

1M NaOH

Container for mixing

Ice bath

DESCRIPTION

- Mix $V_{Coll\,1}$ and V_{CB} in a sterile container with a suitable volume capacity by vortexing or pipetting up and down. Be extra careful with keeping the solution cool once the V_{CB} is added as Coll 1 may self-assemble if heated.
- Add V_{NaOH} to the mixing container.

Note: The natural material oxidation after the vial opening may affect the material pH. Therefore, if using previously opened vial, it is recommended to proceed by adding V_{NaOH} by smaller volume steps, until the color of the solution corresponds to a pH between 6.9-7.3 (refer to Figure 1). Note down the volume used. Cool on ice.

- Add V_{PBS} to the mixing container and homogenize by pipetting up and down or by vortexing.

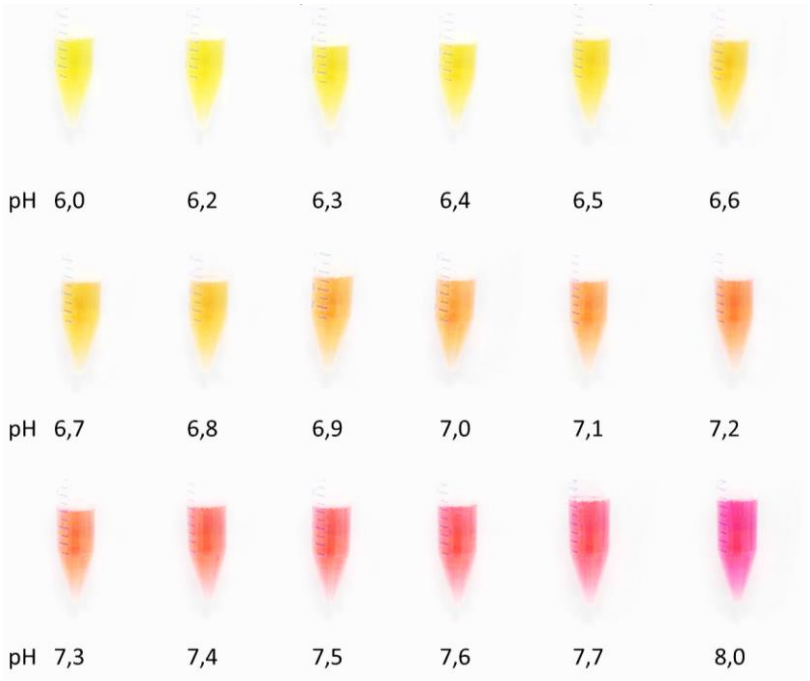

Figure 1. *Illustration of solution color correspondence to pH [1].*

[1]https://bit.ly/3lmRoCv

Protocol for bioprinting of neutralized Coll 1 solution

Make sure to follow the neutralization protocol above prior to following the bioprinting protocol. This bioprinting protocol works best using the BIO X/BIO X6 equipped with the Temperature-controlled printhead. If using the INKREDIBLE system, the bioprinting procedure should be performed fast to prevent the solution from warming and gelling in the cartridge during the experiment.

1. Prepare Coll 1 for mixing

MATERIAL

Neutralized Coll 1 solution

3 mL syringe with Luer lock connections

DESCRIPTION

- Cool down the neutralized Coll 1 on ice for 10 min to make sure it remains in the liquid state.
- Transfer the solution into a 3 mL syringe using the following procedure: remove the syringe plunger → cap the syringe with a tip cap → pour the Coll 1 solution in the syringe → insert the plunger → flip the syringe → release the tip cap to evacuate the air.
- *If cells will be post-seeded on printed constructs, move directly to Step 3.*

2. Mixing Coll 1 with cells

MATERIAL

Cell suspension in a syringe

Cooled Coll 1 solution

Female/female Luer lock adaptor

DESCRIPTION

- Mix ten parts of Coll 1 solution with one part of cell suspension without introducing air bubbles to the mixture. For detailed instructions see the *Mixing cells with bioink Protocol.*
    - Attach the Coll 1 solution syringe to the syringe with cell suspension using a female/female Luer lock adaptor.
    - Carefully mix the solution with the cell suspension by gently pushing them back and forth between the syringes.

Note: Suggested cell suspension density is $5x10^6$ cells/mL to $10x10^6$ cells/mL.

Note: To avoid an air gap when mixing the solution and the cell suspension, carefully pre-fill the Luer lock adaptor with Coll 1 solution before attaching the syringe with the cell suspension.

3. Preparing for print

MATERIAL

Temperature-controlled printhead (optional)

Cartridge, 3 cc

Coll 1 mixed with cells

Conical bioprinting nozzles

DESCRIPTION

- If using the BIO X/BIO X6, pre-cool the Temperature-controlled printhead to 5°C. If using the INKREDIBLE-series, cool down the cartridge on ice if needed.

Note: The bioprinting temperature can be increased to 10°C or 15°C, but this will reduce the time available for Coll 1 bioprinting process prior to its inadvertent self-assembly.

- Transfer the solution (with or without cells) to the cartridge, cap it with a bioprinting nozzle of choice and place it in the printhead.

4. Printing

MATERIAL

BIO X, BIO X6 or INKREDIBLE series bioprinter

Well plate or mold

DESCRIPTION

- Print droplets with the desired size in a mold or well plate.

5. Crosslinking

DESCRIPTION

- Coll 1 can be crosslinked via thermal gelation.
    - Warm the bioprinted construct to 37°C until gelation occurs, approx. 10-15 min. The BIO X/BIO X6 heated print bed or incubation can be alternatively used. Refer to Figure 2 for thermal gelation behavior of Coll 1 with different final concentration.
    - *If cells were mixed with Coll 1 solution prior to bioprinting, move directly to Step 7.*

Note: The crosslinking time might be adjusted based on the construct thickness.

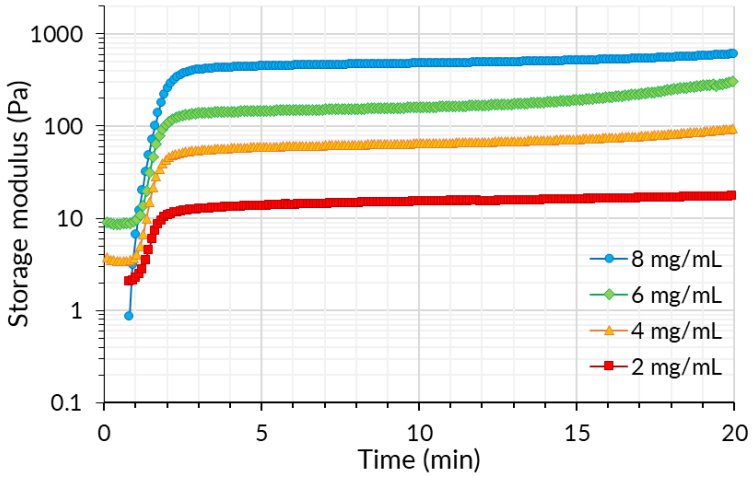

Figure 2. *Thermal gelation of neutralized Coll 1 solution with different collagen concentrations (C_F) indicated as storage moduli increase over time at 37°C.*

6. Cell post-seeding

MATERIAL

Cell suspension

DESCRIPTION

- Dispense the cell suspension in the middle of the printed hydrogel. Suggested cell suspension density: $20x10^3$ cells/cm^2 to $50x10^3$ cells/cm^2 (a highly concentrated cell suspension is suggested).

7. Incubation

MATERIAL

Cell culture medium

DESCRIPTION

- Add the desired medium to submerge the constructs and place in incubator.

Note: Ensure that the bioprinted constructs are crosslinked and do not dissolve in warm media.

- 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.