Neutralization and Printing 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 printing 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 gelates 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)
- Cartridges, 3 cc*
- BIO X* or INKREDIBLE-series* 3D Bioprinter
- Temperature-controlled Printhead (optional)
- Sterile conical bioprinting nozzles*
- Cells + culture medium
- 3 mL syringes with Luer lock connections
- Female/female Luer lock adaptors*

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

Protocol for neutralization

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

Step	Title	Material	Description
1	Coll 1 preparation	- Vial of Coll 1 Solution - Collagen Buffer - Ice bath	- 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).
2	Calculations for neutralization	- Calculator (optional)	- 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	- 1x PBS - 1M NaOH - Container for mixing - Ice bath	- 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.

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

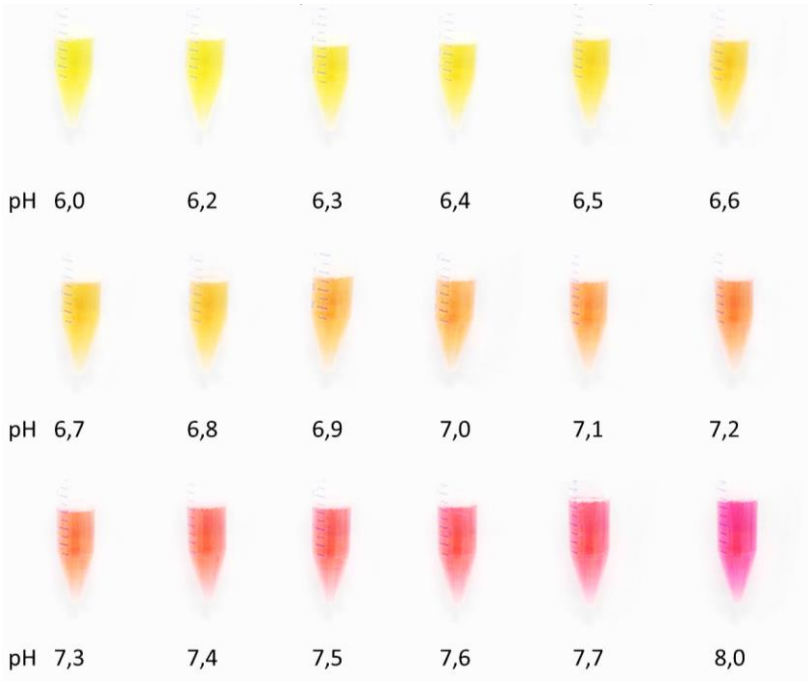

Figure 1. Illustration of solution color correspondence to pH [1].

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

Protocol for printing of neutralized Coll 1 solution

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

Step	Title	Material	Description
1	Prepare Coll 1 for mixing	- Neutralized Coll 1 solution - 3 mL syringe	- Cool down 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 a

			syringe plunger → cap the syringe → pour the Coll 1 solution in the syringe → insert the plunger → flip the syringe → release the tip cap to evacuate the air.
2	Mix Coll 1 with cells	- Cell suspension in a syringe - Cooled Coll 1 solution - Female/female Luer lock adaptor	If cells will be post-seeded on printed constructs, move directly to step 3. 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 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	Load the cartridge	- Cartridge, 3 cc - Coll 1 mixed with cells - Sterile Conical Bioprinting nozzles, 25G	- Transfer the cell-containing solution to the cartridge and cap it. - If using the BIO X, pre-cool the printhead to 5°C. If using the INKREDIBLE-series, cool down the cartridge on ice if needed. Note: The printing temperature can be increased to 10°C or 15°C, but this will reduce the time available for Coll 1 printing process prior to its inadvertent self-assembly. - Place the cartridge in the printhead and cap with a bioprinting nozzle of choice.
4	Printing	- Bioprinter (BIO X or INKREDIBLE series)	- Print droplets with the desired size in a mould or well plate.
5	Crosslinking		Coll 1 can be crosslinked via thermal gelation. - Warm the printed construct to 37°C until gelation occurs, approx. 10-15 min. The BIO X heated print bed or incubation can be alternatively used. Refer to Figure 1 for thermal gelation behavior of Coll 1 with different final concentration.

			Note: The crosslinking time might be adjusted based on the construct thickness.
6	Cell post-seeding	- Cell suspension	If cells were mixed with Coll 1 solution prior to printing, move directly to step 7. - 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 for use in a volume not exceeding 10 µl).
7	Incubation	- Cell culture medium	- 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 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.

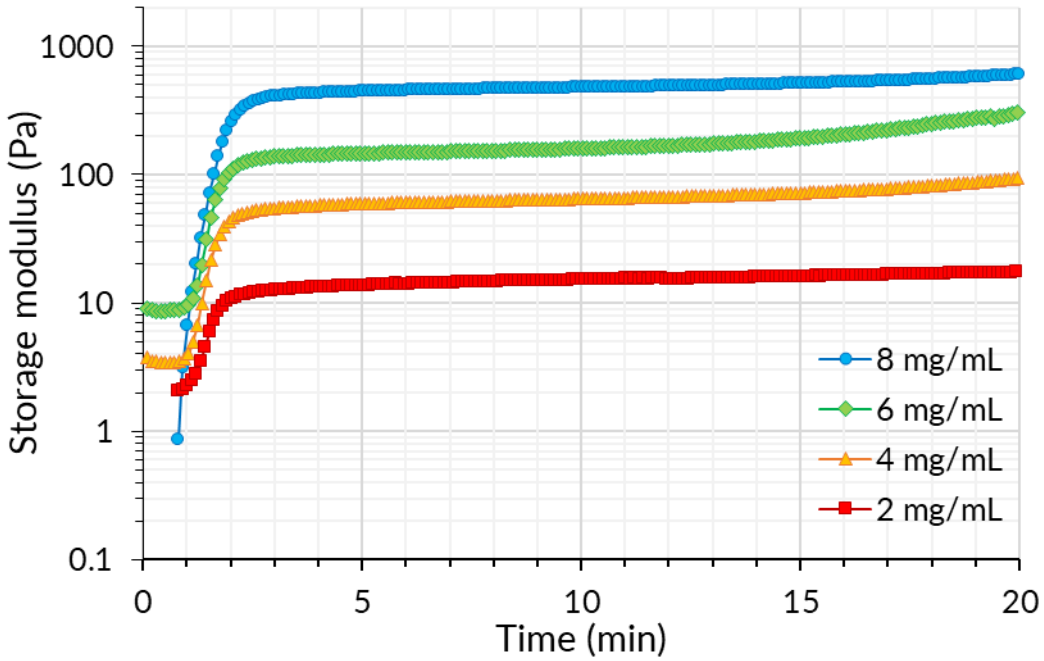

Figure 1. Thermal gelation of neutralized Coll 1 solution with different collagen concentrations (C_F) indicated as storage moduli increase over time at 37°C.