Simplified One-stop Protocols to Extract Collagen from Various Animal Tissues for 3D Cell Cultures

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Introduction

Recent research has continued to support the premise that both the chemical composition and three-dimensional structure of cell culture substrates have measurable effects on cell activities and functions[1-4]. It is therefore necessary to maximize the likeness of the *in vitro* substrates to *in vivo* extracellular matrix (ECM) conditions. As collagen is the main structural protein of the ECM, making up to 25-35% of the total protein amount in the human body, it is necessary to ensure collagen is accurately replicated in the *in vitro* environment[5].

Collagen molecules exist in a fibril shape consisting of triple-stranded peptide helices. Each peptide chain is based on the Gly-X-Y amino acid sequence, where X and Y are often proline or hydroxyproline, and the presence of glycine at every third position allows for tight packing of collagen molecules[6]. About 30 types of collagen have been identified so far, among which, collagen type I is the most abundant, distributing in all organs and tissues[7].

Collagen is conservative between species with minimal differences between amino acids, making it feasible to use animal-sourced collagen for human cell studies[8]. Once collagen is extracted, it can be utilized to form 3D matrices for biomimetic cell environments. For instance, Bowlin's lab developed a method to directly electrospin microfibers from collagen solutions, which showed enhanced cell growth and infiltration[9]. Collagen-based hydrogels were also applied with embedded cells to mimic 3D tissue structures[10]. Because collagen is expensive to purchase, it is beneficial to extract collagen directly from animal tissues. Although various strategies have been reported to extract collagen, few detailed and instructive protocols are currently available. The most relevant one was published by Rajan et al, which however, is specific to rat tail tendons[11].

Therefore, we present here a systematic protocol to extract collagen from various animal tissues such as skin, muscle, and liver. Also, our protocol utilizes accessible materials/equipment and simplified procedures. Additionally, guidelines for applications of the extracted collagen are provided for fabricating 3D cell culture matrices. These protocols allow for a start-to-finish instructions to emulate the *in vivo* ECMs of desired tissue environments.

REAGENTS/MATERIALS

Animal tissues from local slaughterhouses

MilliQ ultrapure water or equivalent

Ice chips prepared from ultrapure water

Decellularization solution (0.5% Triton X-100 and 47.6 mM NH_4OH in deionized water)

Concentrated hydrochloric acid (37%, Sigma Aldrich, P/N 320331)

Phosphate-buffered saline (PBS Buffer, 10x)

Sodium chloride (99%, Alfa Aesar, P/N 12314)

Sodium hydroxide (98%, pellets, Sigma Aldrich, P/N S8045)

EQUIPMENT

Home-style pulse blender (Ninja professional, 800W)

Lyophilization unit (FreeZone 2.5L, Labconco, P/N 700201000)

Balance

PTFE magnetic stir bars

Stir plate

Centrifuge

Cold room/fridge and cold storage (4°C)

Beakers

Freezer (-20 °C)

50 kDa dialysis tubing (Spectrum Laboratories, P/N 132544) or chambers

Vacuum filtration flask and Büchner funnel

Screen-printing mesh (110 gauge)

Scalpels and scissors

TISSUE PREPARATION

1. Animal tissues can be obtained from local slaughterhouses. We present here collagen extraction from porcine skin, skeletal muscle, and liver tissues.
2. Fresh tissue can be used directly. Otherwise, store tissues at -20 °C and soften them at 4 °C for 24 hours prior to use.
3. Excise about 100 g of tissue by a scalpel. Remove visible fat tissues as much as possible.
4. Use clean scissors to cut the excised tissue into small pieces (~2 cm^2).
5. Place approximately 25 grams of ice chips in blender.

   Critical step: Localized heat is generated by the blender during use which may be detrimental to the extracellular matrix. Ice chips help to dissipate the heat.
6. Add the tissue pieces from Step 4 into the blender and pulse blend the contents until the tissue pieces are ~2 mm or smaller.
7. Using a clean spatula, transfer the material in Step 6 into a 1 L beaker along with a magnetic stir bar.
8. Add the decellularization solution to the 1L (1000 mL) mark of the beaker.
9. Cover the beaker with a watch glass and place the beaker on a stir plate in a cold environment (4 °C).
10. After 6 hours, carefully decant the decellularization solution and refill the beaker.
11. Repeat Steps 8-10 for a minimum of 48 hours, until the tissue pieces are white, opaque, and no longer changes appearance from additional time in the decellularization solution.
12. Decant the decellularization solution and rinse the tissue pieces five times with generous cold (4 ^oC) deionized water.
13. Decant the last water rinse and use clean paper towel to pat dry the remaining tissue. Gently press the tissue to remove any residual liquid.

    Critical step: The liquid within the decellularized tissue need to be removed as much as possible. Otherwise, the residual solution will interfere with the collagen extraction later.

COLLAGEN EXTRACTION

1. Transfer the decellularized and rinsed tissue pieces ion to a clean 250 mL beaker. Add only enough 0.5 M hydrochloric acid to just submerge the tissues.

   Critical Step: If too much acid solution is used, the extracted collagen can be too dilute, which will affect collagen collection later.

2. Let the tissue and acid solution stir for 24 hours at 4 °C.
3. After 24 hours, the solution should be more viscous and slightly opaque.
4. Precut a screen-printing mesh (110 gauge) to circular filters matching a Büchner funnel. Put a filter in the Büchner funnel and vacuum filter the materials from Step3. Keep the liquid.

   Critical step: Filter papers cannot be used because it absorbs large amounts of collagen. The tissue pieces remaining in the funnel can be extracted again.

5. Add saturated NaCl solution (in PBS) to the solution collected in Step4 at a volume ratio of 1:1. The NaCl can precipitate collagen.
6. Centrifuge the material from Step 5 for 20 minutes at 8000 rpm (4 °C).
7. After the centrifugation, any floating material (likely residual fats) should be removed. Collagen appears to be globular, slightly opaque, and should be along the wall of the centrifuge tube.
8. Remove the supernatant and add approximately 5 mL of 0.5 M HCl into the centrifuge tube and shake to resuspend the collagen.

   Note: 5 mL is used to resuspend all collagen extracted from 100 g tissues. If less tissues are used, the volume of HCl should be reduced proportionally.

9. Transfer the collagen suspension from Step8 into 50 kDa dialysis tubing/chamber.
10. Dialyze the material in a large beaker (>1 L) filled with 0.5 M HCl.
11. Keep changing the 0.5 M HCl solution every day for 7 days.
12. Collect the material from dialysis tubing, flash freeze it (liquid nitrogen), and lyophilize it.
13. The dried collagen can be stored at -20 °C for at least 6 months.

    Critical data: the rough collagen extraction yield, per 100 grams of starting tissues, is 3 grams (skin), 10 mg (liver), and 10 mg (skeletal muscle).

STERILIZATION PROCEDURE

For general applications

1. Dissolve the lyophilized collagen in 0.01M HCl. A concentration of 3 mg/mL is commonly used.
2. Because HCl is a strong aseptic solvent, the collagen solution should be sterile for general applications.

   Critical data: we plated 3 mg/mL collagen solution on agar plates, together with a positive control (E. coli). As is shown in Fig. 1, the collagen plating did not show any bacterial colonies.

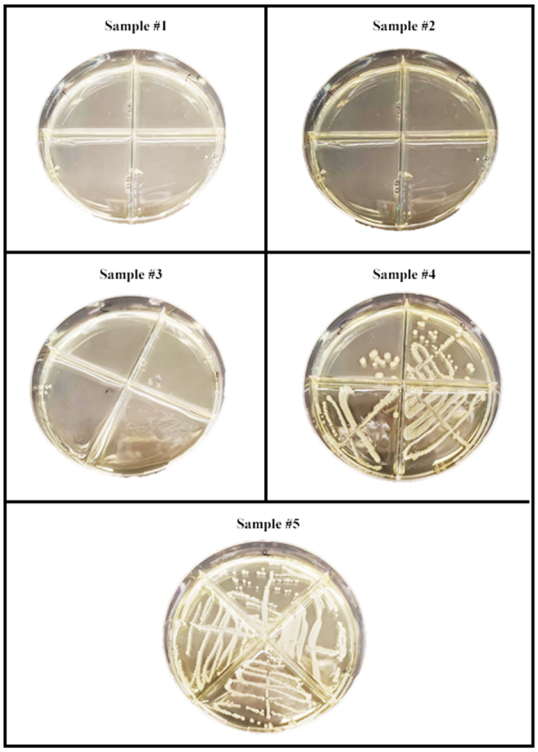

Figure 1. Testing if the collagen solution in HCl is sterile. Samples#1 and #2: collagen of 3 mg/mL made in 0.01M HCl was neutralized by sterile (filtered) NaOH; plating of the neutralized solution did not result in bacteria colonies on agar plates. Sample#3 is just an agar plate without any plating. Samples #4 and #5 are positive controls with E. coli plating.

For applications that require stringent sterilization

1. Prepare a collagen solution of desired concentration in 0.01 M HCl.
2. Add x mL of chloroform to a sterile glass vial.
3. Carefully and gently lay x mL of the collagen solution atop the chloroform. Avoid mixing the two solutions.
4. Let the solutions sit at 4 ^{o}C for 24 hours.
5. Carefully transfer the collagen solution to another sterile container.

6. Soak a dialysis membrane/chamber in 70% ethanol for 30 min, followed by being air dried in a UV biohood for 30 min.

   Note: sterilized dialysis chambers are available commercially. If such a product will be used, step 6 can be skipped.

7. Put a large beaker (> 1L, autoclaved) in a UV biohood. Fill it with sterile 0.01M HCl solution (filtered). Fill the dialysis tube with the collagen solution from Step 5. Change the HCl solution every day. Keep dialyzing for 1 week.

   Note: Step 7 should be conducted in a sterile biohood.

8. Collect the collagen solution from the dialysis apparatus and transfer it to a sterile container. This solution should be sterile and can be stored at 4 ^{o}C for at least 6 months.

9. Aliquot a small portion of the sterile collagen and any protein assay (e.g., BCA assay) to measure its concentration, which may slightly change after the dialysis. Non-sterilized lyophilized collagen can be used to make standards to generate a calibration curve.

MAKE 3D CELL CULTURE SCAFFOLDS FROM THE COLLAGEN

Hydrogel Formation

1. Dissolve the freeze-dried extracted extracellular matrix in 0.01M HCl at 6 mg/mL.
2. Neutralize the HCl in the collagen solution with the same volume of 0.01M NaOH (sterilized by 0.22 μm filters).

   Critical step: Step 2 needs to be performed on ice. Otherwise, neutralized collagen solution can gelate easily at room temperature.

3. Prepare a cell suspension in desired media (e.g. DMEM),and mix it with the neutralized collagen solution from Step 2 at a one to one volume ration.

   Critical step: The cell-collagen mixture should be added to desired containers based on research needs, such as 96-well plates.

4. Incubate the prepared cell mixture in a proper container from Step 3 at 37 °C for the collagen to gelate, which will take approximately 60 min. More dilute solutions may take longer.

   Critical step: Some cell types cannot proliferate in dense gels. Too diluted collagen solution may not gelate to form robust gels. Therefore, the concentration and gelation conditions need to be optimized based on specific studies.

Microfibrous scaffolds

Although collagen can be directly electrospun to generate microfibers, this protocol is not as easy as spinning other polymers (e.g. crosslinking will be needed). Therefore, we provide a general protocol to coat the extracted collagen on fibers electrospun from common polymers such as polycaprolactone (PCL), polystyrene (PS), and silk fibroin.

1. Electrospin a polymer solution to generate desired microfibers.
2. Make a 0.3 mg/mL collagen solution in 0.01 M HCl.
3. Soak the electrospun fiber sheet (or precut into desired shapes) in the collagen solution from Step2. Leave the setup at 4 ^{o}C for 24 hours.
4. After 24 hours, remove the fibers and rinse them thoroughly using PBS.
5. The coated fibers can be air dried at 4 ^{o}C or lyophilized. These coated fibers can be stored at -20 ^{o}C for at least 6 months.
6. Before seeding cells on the fibers, they should be sterilized by being soaked in 70% ethanol for at least 30 min, followed by air drying in UV.

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