**TITLE:**

**Production of Extracellular Matrix Fibers via Sacrificial Hollow Fiber Membrane Cell Culture**

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**SUMMARY:**

The goal of this protocol is production of whole extracellular matrix fibers targeted for wound repair which are suitable for preclinical evaluation as part of a regenerative scaffold implant. These fibers are produced by the culture of fibroblasts on hollow fiber membranes and extracted by dissolution of the membranes.

**LONG ABSTRACT:**

Engineered scaffolds derived from extracellular matrix (ECM) have driven significant interest in medicine for their potential in expediting wound closure and healing. Extraction of extracellular matrix from fibrogenic cell cultures *in vitro* has potential for generation of ECM from human- and potentially patient-specific cell lines, minimizing the presence of xenogeneic epitopes which has hindered the clinical success of some existing ECM products. A significant challenge in *in vitro* production of ECM suitable for implantation is that ECM production by cell culture is typically of relatively low yield. In this work, protocols are described for the production of ECM by cells cultured within sacrificial hollow fiber membrane scaffolds. Hollow fiber membranes are cultured with fibroblast cell lines in a conventional cell medium and dissolved after cell culture to yield continuous threads of ECM. The resulting ECM fibers produced by this method can be decellularized and lyophilized, rendering it suitable for storage and implantation.

**INTRODUCTION:**

Implantable surgical scaffolds are a fixture ofwound repair,with over one million synthetic polymer meshes implanted worldwide each year for abdominal wall repair alone[1](#_ENREF_1). However, following implantation the synthetic materials polymers traditionally used in the fabrication of these scaffolds tend to provoke a foreign body response, resulting in inflammation deleterious to the function of the implant and scarring of tissue[2](#_ENREF_2). Further, as the predominant synthetic mesh materials (*i.e.,* polypropylene) are not appreciably remodeled by the body, they are generally applicable to tissues where scarring can be tolerated, limiting their clinical usefulness toward the treatment of tissues with higher-order function such as muscle. While there are many surgical mesh products which have been applied with clinical success, recent manufacturer recalls of synthetic surgical meshes and complications from interspecies tissue implants highlight the importance of maximizing implant biocompatibility, prompting the FDA to tighten regulations on surgical mesh manufacturers[3](#_ENREF_3),[4](#_ENREF_4). Implantation of scaffolds derived from patients’ own tissues reduces this immune response, but can result in significant donor-site morbidity[5](#_ENREF_5). Extracellular matrix (ECM) scaffolds produced *in vitro* are a possible alternative, as decellularized ECM scaffolds exhibit excellent biocompatibility, particularly in the case of autologous ECM implants[6](#_ENREF_6).

Because of the limited availability of patient tissue to harvest for autologous implantation and the risk of impeding function at the donor site, the ability to produce ECM scaffolds *in vitro* from the culture of human cell lines or, if possible, a patient’s own cells is an attractive alternative. The primary challenges in the manufacture of substantial amounts of ECM *in vitro* is the sequestration of these difficult-to-capture molecules. In previous work, we have demonstrated that ECM can be produced by culturing ECM-secreting fibroblasts in sacrificial polymeric foams that are dissolved after the culture period to yield ECM which can be decellularized for implantation7-10. As ECM produced in foams tend to adopt the internal architecture of the foams, hollow fiber membranes (HFMs) were explored as a sacrificial scaffold for production of threads of ECM. Described herein are methods tasked for lab scale manufacturing of cell culture quality hollow fiber membranes and the extraction of bulk extracellular matrix fibers from the same following a period of fibroblast culture. This static culture approach is readily adoptable by laboratories containing standard mammalian cell culture equipment. ECM produced by this approach could be applied toward a variety of clinical applications.

**PROTOCOL:**

1. **Production of Extracellular Matrix Using Sacrificial Hollow Fiber Membranes**

CAUTION: N-methyl-2-pyrrolidone is an irritating solvent and reproductive toxicant. Exposure to NMP may cause irritation to the skin, eyes, nose, and throat. Solvent-resistant personal protective equipment should be used when handling NMP. Use of NMP should be performed within a fume hood.

* 1. **Preparation of polysulfone polymer solution for hollow fiber membranes**
     1. Weigh 70 g of polysulfone pellets (molecular weight of 35 kD) in a weigh boat using an analytical balance.
     2. Transfer 314 mL (323.3 g) of N-methyl-2-pyrrolidone (NMP) to a clean borosilicate flask.
     3. Place a stir bar in the flask with NMP and place flask onto stirrer. Set the stirrer to a moderate rate of rotation.
     4. Use a dry funnel to slowly insert the prepared 70 g of polysulfone into the flask with NMP.
     5. Allow the polymer “dope” solution to stir for three days at room temperature, or until homogenized.
  2. **Setting concentricity and cleaning of the hollow fiber membrane spinneret**

Note: Spinnerets are commercially available; correct spinneret dimensions are critical to successful membrane fabrication and are listed in the **Table of Materials**. Handle the spinneret very gently, as the spinneret needle is particularly fragile.

* + 1. Use a screwdriver or drill with an appropriate screwdriver bit to carefully disassemble the spinneret.
    2. Gently flow acetone through the inlet and outlet of the spinneret, collecting any acetone and residual polymer in a glass beaker for disposal.
    3. Secure the upper body of the spinneret with the needle facing upward in a table vise.
    4. Place the outlet (bottom) body of the spinneret onto the upper body.
    5. Move the outlet body of the spinneret laterally while holding the upper body fixed on the vise until the spinneret needle is directly in the center of the outlet body.
    6. Carefully and slowly reinsert the screws connecting the two bodies of the spinneret while ensuring that the needle visible at the spinneret outlet remains centered.
  1. **Fabrication of polysulfone hollow fiber membranes**
     1. Prepare a “bore solution” containing 45 mL of N-methyl-2-pyrrolidone and 255 mL of deionized water, forming a 15% mixture of NMP with deionized water.
     2. Mount a concentric hollow fiber membrane spinneret 8 cm above the surface of a room-temperature tap water bath.
     3. Connect the polymer inlet of the spinneret and the bore inlet of the spinneret to two separate steel pressure vessels having an inner volume of at least 350 mL. Both vessels must have check valves at their outlets.
     4. Connect each of the pressure vessels to the regulators of separate N2 gas cylinders
     5. Use a funnel to insert the prepared dope solution (~315 mL) into the pressure vessel connected to the polymer inlet of the spinneret. Ensure that the top of the vessel is tightly sealed.
     6. Use a funnel to insert the prepared bore solution (300 mL) into the pressure vessel connected to the bore inlet of the spinneret. Ensure that the top of the vessel is tightly sealed.
     7. Open the check valve for the bore vessel first, then the polymer vessel second. If the spinneret is sufficiently clean, the bore solution will begin to stream out of the spinneret outlet.
     8. Pressurize both vessels to 1 PSI. Visually confirm that both polymer and bore solutions are exiting the spinneret, with a continuous white filament precipitating as the combined streams contact the water bath.
     9. Use long forceps to guide the nascent fiber under the rollers in the center of the bath, and then wind the nascent fiber around a rotating wheel connected to a motor.
     10. Set the take-up wheel and motor to rotate at a rate of approximately two meters per minute.
     11. Make minor adjustments to the regulators or take-up wheel speed as needed until a steady-state flow is achieved. The nascent fiber should form a 90° angle with the surface of the water bath.
     12. Close the N2 cylinder regulators and pressure vessel check valves and disengage the take-up wheel motor after all polymer and bore solution has been extruded from the vessels.
     13. Remove the prepared hollow fiber membranes and place them into a deionized water bath for three days, exchanging deionized water once per day to remove residual solvent.
     14. Sterilize the hollow fiber membranes by autoclaving them at 121 °C for 30 minutes or by immersing them for one day in 70% ethanol.
  2. **Cell seeding of hollow fiber membranes**

Note: All cell culture procedures should be performed within a biosafety cabinet.

* + 1. Treat the hollow fiber membranes with a 20 µg/mL solution of bovine plasma fibronectin in PBS (pH = 7.4) to promote cell attachment.
       1. Weigh 1 mg of powdered bovine plasma fibronectin in a weigh boat and dissolve it in 1 mL of sterile PBS (pH = 7.4) in a sterile 1.5 mL microcentrifuge tube.
       2. Incubate the 1 mg/mL bovine plasma fibronectin solution in the incubator for 30 minutes. Add the solution to 49 mL of sterile PBS in a sterile 50 mL conical centrifuge tube.
       3. Incubate the fibers in the prepared 50 mL of bovine plasma fibronectin in an incubator at 37 °C with 5% CO2 for 1 hour. Recollect the fibronectin solution for later use and store in a sterile 50 mL conical centrifuge tube at 4 °C.
    2. Use sterile microscissors to cut the fibers to desired length (<6 cm).
    3. Seed fibroblast cells directly into the lumina of the hollow fiber membranes at a seeding density of 100,000 cells per fiber using a 21-gauge needle with 1 mL syringe.

Note: A practicable target for preparing the cell-suspension for loading in the syringe is a suspension density of 100,000 cells for every 30 microliters of medium.

* + 1. Place six seeded fibers into a 6 cm diameter Petri dish. Allow the seeded fibers to incubate at 37 °C with 5% CO2 for five minutes.
    2. Remove the seeded fibers from incubation and culture them for up to 3 weeks in DMEM/F-12 containing final concentrations of 50 µg/mL L-ascorbic acid and 150 µg/mL L-ascorbic acid 2-phosphate (freshly prepared and sterile filtered), 5 ng/mL TGF-β1 (from sterile filtered aliquots stored at -20 °C), and 1%penicillin-streptomycin in a 6 cm Petri dish within a 5% CO2 incubator at 37 °C. Exchange cell medium every two days.
  1. **Extraction of extracellular matrix from cultured hollow fiber membranes**
     1. Place cultured fibers into individual scintillation vial using forceps.
     2. Place up to 5 mL of NMP into each vial using a glass pipette.
     3. Immerse the hollow fibers in NMP, performing a total of three exchanges of NMP. Slowly aspirate the old NMP using a 1 mL pipette to prevent tearing of extracted ECM.

Note: When adding fresh NMP, it is helpful to tilt the vial and allow the NMP run down the sides, otherwise the remaining ECM scaffold is subjected to shear which may cause tearing.

* + 1. Remove NMP and rinsed the resulting ECM thread 3 times in deionized water.
    2. Cut a 1/32-inch thick sheet of silicone rubber to a length of 3 inches, width of 1 inch and cut an 8 mm by 4 mm rectangular mold in the center of the length of rubber.
    3. Place the prepared piece of rubber onto a standard 3-inch by 1-inch microscopy slide and autoclave at 121 °C for 30 minutes.
    4. Lay each ECM fiber side by side in the 8 mm by 4 mm silicone mold until there is no visible open space.
    5. Place the mold into a 50 mL conical centrifuge tube and freeze at -80°C until completely frozen.
    6. Lyophilize frozen ECM mesh overnight or until completely dry. Store the mesh at 4°C until ready for decellularization.

1. **Decellularization of Extracellular Matrix**
   1. Prepare a solution of 1% (w/w%) sodium dodecyl sulfate (SDS) in deionized water.
   2. Incubate extracted extracellular matrix in the mold in 1% SDS for 24 hours at room temperature on a rocker with gentle agitation.
   3. Rinse extracted extracellular matrix three times in sterile phosphate-buffered saline (PBS) with gentle agitation, using 3 mL of PBS per rinse.

Note: Rinses should be performed gently to minimize tearing of prepared scaffolds.

* 1. Prepare 1 L of DNAse/RNAse digestion buffer.

Note: DNAse/RNAse digestion buffer may be stored for several months at 4°C.

* + 1. Weigh 1.54 g of Tris HCl, 308 mg of MgCl2, and 56 mg of CaCl2 in separate weigh boats.
    2. Combine Tris HCl, MgCl2, and CaCl2 with 1 L of deionized water in a large flask with a stir bar and stir until all components are dissolved.
    3. Sterile filter the digestion buffer with a 0.22 µm filter into a sterile 1 L borosilicate bottle and store at 4°C.
  1. Prepare 5 mL of DNAse/RNAse digestion solution.

Note: Digestion solution should be used within 24 hours of preparation.

* + 1. Weigh 0.125 mg (50 kU) of DNAse I in a weigh boat and add to 5 mL of digestion buffer.
    2. Add 75 µL of RNAse A stock solution to the digestion buffer.
  1. Add 32 µL of the digestion solution to each prepared mold and incubate at 4°C for 6 hours.
  2. Aspirate the digestion solution and rinse three times in sterile PBS.
  3. Aspirate the PBS and incubate scaffolds overnight in 10% penicillin-streptomycin in PBS at 4°C.
  4. Aspirate the penicillin-streptomycin and rinse scaffolds three times in sterile PBS.
  5. Place the mold into a 50 mL conical centrifuge tube and freeze at -80°C.
  6. Lyophilize the decellularized ECM mesh overnight or until completely dry.
  7. Transfer the lyophilized scaffolds within a biosafety hood to a sterile container at 4°C pending use.

**REPRESENTATIVE RESULTS:**

Successful production of extracellular matrix from sacrificial scaffolds is contingent on appropriate scaffold fabrication, cell culture, and solvent rinse procedures. Fabrication of the hollow fiber membranes is performed using a dry-jet wet-spinning system assembled from commercially available components (**Figure 1**) which uses extrusion of polymer solution through the annulus of a commercially available steel spinneret (inner diameter = 0.8 mm, outer diameter = 1.6 mm) to generate a nascent tube of polymer solution which precipitates into a hollow fiber membrane upon contact with a water bath.

An example process for ECM extraction from HFMs is illustrated in **Figure 2**. **Figure 3A** shows a transverse cross-section of polysulfone HFMs fabricated under this protocol, exhibiting outer and inner layers of finger-like pores characteristic of an asymmetric membrane. In this protocol, cells are seeded specifically in the inner lumen of the membrane and cultured in 6 cm diameter Petri dishes (**Figure 3B**), with cells tending to proliferate on all surfaces of the membrane. Cultured membranes can then be subjected to batch NMP and deionized water rinsing in standard glass scintillation vials (**Figure 3C**), producing translucent threads of ECM (**Figure 3D**). ECM-producing cells remain viable inside HFMs throughout the 3-week period of culture (**Figure 4**).

HFMs cultured for three weeks with primary rat skeletal muscle fibroblasts (RSMF) were dissolved via three exchanges of N-methyl-2-pyrrolidone, after which they were rinsed three times in deionized water. The extracted matrix, normally being translucent in appearance when hydrated (**Figure 5A**), will tend to cloud upon hydration if not subjected to appropriate solvent rinsing due to the presence of residual polymer. It should also be noted that the ECM remaining after dissolution of the membrane is somewhat fragile, requiring care in handling with fine forceps. ECM fibers assembled into meshes and then lyophilized exhibit an off-white color and fibrous appearance with a gross longitudinal alignment (**Figure 5B**).

**FIGURE LEGENDS:**

**Figure 1: Illustrated process flow for casting hollow fiber membranes.** Hollow fiber membranes are manufactured using the ubiquitous non-solvent induced phase separation method (NIPS) using a system prepared from commercially available components listed in the table of specific materials. Polysulfone in NMP (17.8 w/w%) and NMP bore solutions (15 w/w% NMP in water) are extruded from separate stainless steel vessels by pressurized N2 into a spinneret, generating a nascent hollow fiber membrane at the spinneret outlet which fully precipitates upon contact with the tap water precipitation bath. Nascent hollow fiber is manually guided under and over guides and allowed to collect on a rotating motorized take-up wheel at a rate of 2.3 meters per minute.

**Figure 2:** **Illustrated production of ECM from cultured hollow fiber membranes**. Hollow fiber membranes are seeded with fibroblasts and cultured for three weeks, followed by exchange of NMP 3x and exchange of deionized water 3x.

**Figure 3: Culture and extraction of ECM**.Asymmetric mesoporous hollow fiber membranes **(A)** were cultured for three weeks with RSMF cells in DMEM/F-12 with supplemented ascorbic acid and TGF-β **(B)**. Cultured hollow fibers (**C, D** top) were dissolved via 3 exchanges in n-methyl-2-pyrrolidone then rinsed three times in deionized water, resulting in continuous threads of ECM (**D**, bottom). High magnification scanning electron microscopy micrograph of the ECM fiber surface **(E)**.

**Figure 4: Cell viability on hollow fiber membranes.** Representative hollow fiber membranes cultured with RSMF cells for three weeks were longitudinally sectioned using a fine razor to reveal the luminal surface of the HFMs and subjected to live-dead staining with Calcein AM and EthD-1. Viability staining revealed a confluent layer of viable cells with negligible EthD-1 fluorescence.

**Figure 5: Assembly of ECM implant.** Individual extracellular matrix threads (n = 30) derived from culture of RSMF cells were placed lengthwise into a silicone mold **(A)** and decellularized by 1% SDS followed by treatment with DNAse I, RNAse A, and penicillin-streptomycin. Decellularized ECM was then lyophilized, yielding an off-white mesh with a fibrous appearance and longitudinal architecture **(B)**.

**DISCUSSION**

The processes described enable the production of bulk ECM biomaterials *in vitro* using hollow fiber membranes cast by a dry-jet wet spinning system allowing for inexpensive bulk production of membranes as well as standard cell culture equipment. While the membranes fabricated in this protocol are intended for use in cell culture, the system described can also be adapted for the production of membranes for separation purposes, with pore size distribution and hollow fiber dimensions tunable by varying spinneret dimensions, polymer used, dope and bore flow rate, take-up speed, and environmental conditions.13 Though the protocol detailed employs hollow fiber membranes, in principle any dissolvable cell culture scaffold with appropriate transport properties such as open cell foams could be used, as demonstrated in previous work[7-9](#_ENREF_8). This general approach appears useful for the production of ECM scaffolds by sacrificial scaffolds which can more faithfully mimic the internal architecture of tissues. Implants produced by this protocol in particular exhibit a gross alignment (**Figure 5B**), which may be of particular benefit toward the reconstruction of highly aligned tissues such as tendons, ligaments and skeletal muscle.

Regular exchange of medium and, in particular, supplementation of medium with ascorbic acid and TGF- β is crucial to production of ECM, as ascorbic acid is an essential enzyme in collagen biosynthesis, and TGF- β induces the synthesis of several ECM proteins[10](#_ENREF_10). Additionally, thorough rinsing of ECM with NMP must be performed, otherwise residual polymer will remain in the extracted ECM, appearing as a white film during water rinses. Care must be taken to not overly agitate ECM during membrane dissolution, as it is relatively fragile. Collected ECM scaffolds intended for implantation must be subjected to a decellularization step as described to remove xenogeneic epitopes to minimize a potential host foreign body response.

The significance of this technique lies in its production of a biocomplex scaffold of whole extracellular matrix which can be remodeled by the body’s own wound-healing processes. By using this approach to produce ECM from cells specific to a target species, it may be possible to minimize the foreign body response which hinders the clinical effectiveness of this class of biomaterials; by using cells specific to an individual, the foreign body response may be lessened further. This approach also allows for the production of ECM targeted toward particular tissues, with recent reports suggesting that tissue-specific ECM may be particularly effective in certain applications12. As this protocol allows for production of ECM across various cell lines, implants combining ECM from several cell types (*e.g.* muscle, nervous, endothelial) could be used to tailor implants to more faithfully approximate the structure and chemical complexity of target tissues. While the ECM meshes presented here were originally intended as scaffolds for wound repair, they may also have use as platforms for investigations into cell-ECM interactions, durotaxis, and biosensing. In particular, this approach lends the investigator the ability to produce ECM from specific cell types of interest which may allow for new insights into the biological significance of tissue-specific ECM structure, composition and function.

Potential improvements to the ECM production techniques presented here could include scale-up via the use of dynamic and pre-conditioning bioreactors as well as exploration of alternative sacrificial scaffold materials and architectures. In particular, transition to a solventless scaffold removal process would improve the safety of the extraction process; sacrificial scaffolds composed of materials which are degradable by enzymes, such as regenerated cellulose, may allow for solventless ECM extraction[14](#_ENREF_12). While the tensile strength of these materials is below those of synthetic materials, there exist several avenues for improvement of these scaffolds, including exploration of various culture conditions, media formulations, and sacrificial scaffold geometries. Recent genetic engineering advances could be leveraged to produce pro-fibrotic cell lines, which in combination with platforms for ECM capture could enable the production of scaffolds satisfying clinical demands. Further, existing dynamic culture systems such as continuous flow-loop hollow fiber membrane bioreactors facilitate high rates of nutrient exchange conducive to greater and more rapid ECM production, and may be of particular interest in leveraging this technique for production of larger quantities of ECM for biological research and clinical use.

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**DISCLOSURES:**

The authors declare that they have no competing financial interests.

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