Video Article

Tunable Hydrogels from Pulmonary Extracellular Matrix for 3D Cell Culture

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Abstract

Here we present a method for establishing multiple component cell culture hydrogels for *in vitro* lung cell culture. Beginning with healthy *en bloc* lung tissue from porcine, rat, or mouse, the tissue is perfused and submerged in subsequent chemical detergents to remove the cellular debris. Histological comparison of the tissue before and after processing confirms removal of over 95% of double stranded DNA and alpha galactosidase staining suggests the majority of cellular debris is removed. After decellularization, the tissue is lyophilized and then cryomilled into a powder. The matrix powder is digested for 48 hr in an acidic pepsin digestion solution and then neutralized to form the pregel solution. Gelation of the pregel solution can be induced by incubation at 37 °C and can be used immediately following neutralization or stored at 4 °C for up to two weeks. Coatings can be formed using the pregel solution on a non-treated plate for cell attachment. Cells can be suspended in the pregel prior to self-assembly to achieve a 3D culture, plated on the surface of a formed gel from which the cells can migrate through the scaffold, or plated on the coatings. Alterations to the strategy presented can impact gelation temperature, strength, or protein fragment sizes. Beyond hydrogel formation, the hydrogel stiffness may be increased using genipin.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55094/>

Introduction

Translating *in vitro* results to the clinic is one of the most challenging issues facing biomedical researchers. *In vitro* research on tissue culture plastic is easier, more convenient, and maintains high cell viability.¹ This approach is a reasonable starting point, but the results have limited clinical translation. Increasingly, laboratories are incorporating three-dimensional constructs to replace the traditional two-dimensional methods. Reviews are available for many three-dimensional environments, from biological scaffolds to polymeric scaffolds.^{2,3}

Biological frameworks can mimic characteristics of *in vivo* environments as they contain many of the protein and glycosaminoglycan components of the native matrix and provide familiar binding sites for cells to attach to and recognize. Extracellular matrix (ECM) derived materials have been shown to be capable scaffolds for cell attachment and proliferation.⁴ One challenge that limits the application of ECM hydrogel platforms stems from their inherently weak mechanical properties following gelation. Native tissue often has mechanical properties that are magnitudes higher than hydrogels. Non-toxic crosslinking agents can increase the mechanical properties of hydrogels to better mimic the native tissue environment. Genipin is a non-toxic, natural crosslinker derived from Gardenia plants with the ability to closely tailor mechanical properties of ECM with changes in genipin concentration.^{5,6}

Nearly all cells in the body exist in, and organize on, ECM that they either produce or maintain. New focus on the universal importance of ECM in the organization, condition, and function in every organ or system has sparked the production of matrix based platforms for *in vitro* investigation. Porcine small intestine submucosa is the most extensively studied naturally-derived scaffold, and it has been used to regenerate tendons, ligaments, skeletal muscle⁴, and even bone⁷. Matrices from other organs and donor species have also demonstrated good tissue regeneration potential. The use of foreign ECM components causes minimal issues with immunomodulation. After elimination of host cellular matter, the remaining ECM will be similar in amino acid content and organization to all other mammalian species⁸. There is a growing line of thinking that the best way to examine cell-ECM interactions *in vitro* is to utilize organ-specific ECM scaffolds. Each organ provides a unique composition of proteins and proteoglycans to create cellular niches. Niches provide structural, functional and even the enzymatic breakdown of the extracellular matrix contributing to biophysical signaling. To attain an *in vitro* microenvironment most similar to the *in vivo* microenvironment, use of tissue specific ECM would optimize the cellular niches for research.

The goal of this protocol is to provide a method for establishing a hydrogel scaffold unique to the lung ECM. This method provides a platform for *in vitro* research on lung cell-ECM interactions.

Protocol

Solution	Sterile Filter	Directions
DiH ₂ O	Yes	DiH ₂ O; Sterile filtered
0.1% Triton X-100 Solution	Yes	Under fume hood add 100 µl Triton-X 100 Solution to 100 ml DiH ₂ O and agitate until dissolved; sterile filter.
2% Deoxycholate Solution	Yes	Under fume hood add 2 g Sodium Deoxycholate solution per 100 ml DiH ₂ O and agitate until dissolved; Sterile filter
1 M NaCl	Yes	Add 58.44 g NaCl to 1 L of DiH ₂ O; agitate until dissolved; sterile filter
DNase solution	Yes	Add 12,000 units DNase to 1 L DiH ₂ O; Add 0.156 g MgSO ₄ (Anhydrous), and 0.222 g CaCl ₂ ; agitate until dissolved; sterile filter
PBS	Yes	Combine 27.2 g Na ₂ HPO ₄ · 7H ₂ O (dibasic heptahydrate), 80 g NaCl, and 2 g KCl with 10 L DiH ₂ O; agitate until dissolved; adjust pH to 7.4; sterile filter

Table 1: Solutions Required for Tissue Decellularization. Make the solutions above for the decellularization process. Store at 4 °C. Approximately 2.5 L will be needed for one porcine lung.

1. Hydrogel Formation

1. Porcine Lung Decellularization (adapted from references^{9,10})

- Obtain, an *en bloc* normal porcine lung, from a slaughterhouse, with heart and vasculature intact.
- Use tissue scissors or a scalpel to dissect away the heart, cutting vasculature as close as possible to the heart, remaining vasculature will be used in later steps for perfusion.
- Carefully dissect away the connective tissue surrounding the trachea, bronchi, and vasculature using a scalpel or scissors.
NOTE: Determine the best lung to use for the rest of the procedure by choosing a lung without large cuts or punctures through the pleura and large amounts of atelectasis or vascular occlusion that may impede the effectiveness of the decellularization process.
- Cut away the suboptimal lung leaving as much bronchus attached to the trachea as possible (heart, connective tissue and the lobes of the removed lung may be disposed of). There should be one lung remaining attached to the trachea and vasculature.
NOTE: Retain sections for histology if desired.⁹
- Close disconnected bronchi with clamps or suture to prevent excess backflow.
- Prepare the decellularization solutions and refrigerate at 4 °C until needed (**Table 1**).
- Using a hand pump cannulated to fit the pulmonary artery, perfuse the lung tissue 3 times with DI water through both pulmonary artery and trachea. Perfuse vasculature first each time. Begin with around 1 L into vasculature and 1.5 L into trachea for each perfusion, but as cellular debris is removed more liquid can be perfused through the system.
- Perfuse both vasculature and trachea with Triton X-100 solution.
- Submerge lung tissue in Triton X-100 solution for 24 hr at 4 °C.
- Perfuse vasculature and trachea 3 times with DI water to rinse.
- Perfuse both vasculature and trachea with deoxycholate solution.
- Submerge the tissue sections in deoxycholate for 24 hr at 4°C.
- Perfuse vasculature and trachea 3 times with DI water to rinse.
- Perfuse both vasculature and trachea with NaCl solution.
- Submerge tissue in filtered NaCl solution for 1 hr at 4 °C.
- Perfuse vasculature and trachea 3 times with DI water to rinse.
- Perfuse both vasculature and trachea with DNase solution.
- Submerge tissue in filtered DNase solution for 1 hr at 4 °C.
- Perfuse both vasculature and trachea 5 times with PBS.
- Dissect away noticeable cartilaginous tissue, trachea and all tubules 2 mm or larger in diameter (primarily found around the hilum and medial portions of the lungs) from conducting airways, leaving only respiratory zones (primarily the peripheral areas).
- Dissect tissue into 1 inch sections or smaller. Orientation of the tissue does not matter for this step.
- Pour off excess liquid and place tissue in 50 ml conical tubes. Freeze the tissue at -80 °C.

NOTE: Retain sections for histology to ensure removal of cells and cellular debris, if desired. We use H&E, α -galactosidase, picogreen, hydroxyproline, ninhydrin, alcian blue, SDS-PAGE, and mass spectrometry to characterize.⁹

2. Lung Processing

1. Remove lids from frozen tubes containing decellularized lung tissue.
2. Place filter paper over the tube opening and secure with rubber band.
NOTE: Tube and contents should still be frozen otherwise place in -80°C until frozen.
3. Lyophilize the tissue until all excess liquid is gone, using a freeze dryer according to manufacturer directions. Store at -80°C until ready to mill.
4. Before beginning add liquid nitrogen to freezer mill to cool insulation and internal components to working conditions.
5. Remove magnetic mill bar from mill tube and add tissue to cover bottom.
6. Replace mill bar and add loosely packed tissue. The mill bar should still move freely.
7. Close the mill tube and place in freezer mill.
8. Fill liquid nitrogen to max fill line.
9. Freezer mill all tissue into fine powder (approximately 5 min, impaction rate of $\sim 600\text{ min}^{-1}$), in a polycarbonate cylinder with a stainless steel impactor as well as stainless steel end plugs using the freezer mill according to manufacturer directions. Store at -80°C until ready for use.

3. Micro-porous gel formation (8 mg/ml) (adapted from references^{9,11})

1. Add 1% (w/v) of the decellularized powder and 0.1% (w/v) of pepsin to 0.01 M HCl, under constant agitation (should be able to see flow at the top level of liquid), at room temperature, for 48 hr.
NOTE: The powder is statically charged, so adding the HCl after the powder affords the opportunity to wash the excess off the tube walls.
2. After digestion for 48 h, place the solution and reagents on ice for 5 min.
3. Using refrigerated 10% (v/v) 0.1 M NaOH, and 11.11% (v/v) 10x PBS (to bring the entire solution to 1x concentration), bring the digested protein solution to physiologic pH of 7.4.
NOTE: The solution can now be stored at 4°C for up to one week. Perform gelation kinetics using rheometry⁹ if desired.

2. Cross-linking Hydrogels to Improve Mechanical Strength

1. Solutions of 1%, 0.1%, and 0.01% w/v genipin crosslinking solution were prepared by dissolving the necessary amount of genipin powder in 10% DMSO.
2. Vortex the solution every 15 min for 1 hr.
3. Add genipin solution to cover ECM hydrogels (100 μL for each well of a 96-well plate) that have previously been assembled in part 1.3.4.
4. Leave each ECM hydrogel to crosslink for 24 hr at 37°C .
5. Rinse the ECM hydrogel 3 times with PBS until the washing solution is no longer blue. The crosslinked hydrogels will then be ready for further characterization and cell culture studies.

3. Cell Culture with Microporous Gel

1. Two-dimensional coating

1. Using the solution from 1.3.3, add 20 μL of pregel solution to each well of a 96-well non-treated tissue culture plate.
2. Refrigerate overnight at 4°C to allow protein adsorption to plate.
3. Aspirate pregel solution and rinse with PBS.
4. Passage cells⁹.
5. Add 10,000 cells/ cm^2 to wells.
6. Increase media to 100 μL per well and incubate at 37°C .

2. Three dimensional cell culture⁹

1. Using solution from 1.3.3, resuspend pelleted cells to a concentration of 1,000,000 cells/ml of pregel solution.
2. Quickly dispense 16 μL of pregel-cell suspension into each well of a 96-well plate, to form a $\sim 500\text{ }\mu\text{m}$ thick hydrogel for 3D cell culture.
3. Incubate at 37°C for 30 min for gel to form.
4. Add 100 μL of media to the top of formed hydrogels and incubate at 37°C .

3. Three dimensional cell culture on variable stiffness gels

1. Using solution from 1.3.3, pipette 100 μL of pregel solution into each well of a 96-well plate.
2. Incubate at 37°C for 30 minutes for gel to form.
Note: Cross-linking steps from part 2 can be used here.
3. Passage cells⁹.
4. Add 10,000 cells/ cm^2 to wells.
5. Increase media to 100 μL per well and incubate at 37°C .

Representative Results

Using this method, we have produced hydrogels from normal pig, rat, and mouse lungs (**Figure 1**). Processed lungs provide an estimated 5 mg, 40 mg, and 10 g of ECM powder respectively. An overview of the process is shown in **Figure 2**. Key visualizations during the process include: white appearance of the lungs after rinsing deoxycholate; after the pregel formation, the solution should be opaque and the solution should appear homogenous for months if stored at 4 °C. We have included a basic troubleshooting table to help identify sources of error throughout the process (**Table 2**). After gelling, hydrogels can be used immediately or stored for future use. Rheological testing confirms that over 90% of the gelling occurs in the first minutes after reaching 37 °C (**Figure 3**). Formed hydrogels are stable for long periods in PBS, but cell attachment and proliferation may change (**Figure 4**).

While this protocol is written for porcine lung, space limitations limit us to use only one lung when decellularizing pig lungs. We have used the same procedure with minor modification to decellularize both lungs for other species. For rats and mice, the lungs can be left intact and the heart attached, but the connective tissue should still be removed with a scalpel or scissors. We keep the heart intact and attached for rats and mice and perfuse the solutions through the right ventricle instead of the pulmonary artery. Perfusion of the trachea is still the same; however, the cannula can be sutured in the trachea for ease of access for smaller species.

We have added multiple cell types, human mesenchymal stem cells,⁹ mouse mesenchymal stem cells, human epithelial cell lines (A549s), human primary microvascular endothelial cells (HpuVECs), and human umbilical vein epithelial cells (HUVECs) within and on the surface of the lung ECM hydrogels. We have not encountered issues regarding the xenogenic nature of the formed gels. Our previous work has shown a significant reduction in α -galactosidase in the porcine hydrogels.⁹ The pre-gel solution may also be used as a coating at 4 °C to improve cell attachment. The pregel solution improves HpuVEC attachment and proliferation when added to lung ECM coated tissue culture plates. Coated wells demonstrate significantly improved cellular attachment compared with non-treated wells (**Figure 5**).

Adding genipin increases hydrogel crosslinking. This increased crosslinking may provide a more physiologically relevant stiffness, while decreasing degradation of the ECM. The decrease in degradation, though minor *in vitro*, may occur due to increased bonding between proteins and therefore more resistance to ECM remodeling by cells. We used frequency sweeps on a rheometer to measure the mechanical properties of the crosslinked hydrogel. Hydrogel stiffness was found to be directly correlated to genipin concentration with the median and most relevant concentration being 0.01% (**Figure 6**, native tissue not shown). An MTT assay was used to quantify cell proliferation and survival on the crosslinked hydrogel. Cell proliferation is higher on the crosslinked hydrogel (**Figure 7**). This supports increased cell proliferation on increased stiffness from others.¹² Varying genipin crosslinking time or temperature may result in different crosslinking degrees.

Figure 1: Images of Lungs We Have Decellularized. Pictures from (A) Mouse lung after decellularization and heart removed, (B) Rat after Triton X-100 rinsed away, (C) Pig, before initial rinse; they yield ~5 mg, 40 mg, and 10 g of Lung ECM, respectively. (D) Mouse lung and intact heart, after decellularization, with cannula still sutured in place. [Please click here to view a larger version of this figure.](#)

Figure 2: Overview of Methods. This is an overview of the method to produce hydrogels from lungs. Beginning with en bloc pig lung decellularization occurs leaving only opaque white or translucent tissue that is comprised of ECM proteins. Then after removing any remaining water, and increasing the surface area to optimize acid digestion the tissue is solubilized. Neutralization of the acid and increasing the salt to physiological levels creates the pregel solution ready for gelation. Once the solution forms a hydrogel at 37 °C, characterization takes place with a gel rheometer and scanning electron microscopy. [Please click here to view a larger version of this figure.](#)

Figure 3: Representative Rheological Data for Gelation under Temperature Ramping. After an initial amplitude sweep to determine linear viscoelastic range, a temperature ramp was used to determine gelation kinetics. Data are presented as mean $\pm$ standard deviation. $n = 4$.

Figure 4: Long Term Stability. An image of a pig lung ECM hydrogel stored in PBS for over 6 months at room temperature.

Figure 5: Metabolic Assay of HpuVECs. MTT proliferation data from Human primary microvascular endothelial cells (HpuVECs) grown on non-treated tissue culture plates. Pregel solution from 1.3.3 was added to plates, refrigerated overnight and rinsed to form pre-gel lung ECM coated plates. HpuVECs showed higher MTT activity on ECM coated plates compared to non-coated.*p <0.05 compared to control. Data are mean $\pm$ standard deviation n = 3 per group.

Figure 6: Rheological Data for Enhanced Crosslinking. Rheometry data for genipin crosslinking. The Stiffness increases with increased concentrations of genipin to a plateau at 1% genipin. Data is normalized to show a fold increase in crosslinking with genipin.

Figure 7; Cell Proliferation on Genipin Crosslinked Gels. Normalized MTT data for A549s on genipin crosslinked hydrogels. Increased cell proliferation seen with increased stiffness. p <0.05 compared to control. Data are mean $\pm$ standard deviation. n = 3 per group.

Problem	Issue	Resolution
Red/pink color retained after decellularization	Ineffective decellularization	Start over with new lungs
		Remove areas of incomplete decellularization
	Vascular occlusion	Start over with new lungs
		Remove areas of incomplete decellularization
Significant cellular debris retained	Ineffective decellularization	Start over with new lungs
		Remove areas of incomplete decellularization
	Vascular occlusion	Start over with new lungs
		Remove areas of incomplete decellularization
	Atelectasis	Start over with new lungs
		Remove areas of incomplete decellularization
Destruction of membranes found in histological samples	Over-pressurization of vessels during perfusion	Reduce perfusion pressure of detergents
Large particulate remains after milling	Too much lyophilized material in tube	Reduce material in grinding cylinder
	Not milled for long enough	increase milling time
Pregel solution not gelling	Solution pH too high	Make pH 7.4
		Begin pregel solution with fresh powder
	Solution pH too low	Make pH 7.4
		Begin pregel solution with fresh powder
	ECM over digested	Begin pregel solution with fresh powder
Cells do not survive	Microbes introduced after HCl digestion	Begin again with fresh powder and use sterile filtered solutions
	pH off	Begin pregel solution again
		Make pH 7.4
	Cells burst	Fix salt content in pregel solution

Table 2: Troubleshooting Guide to Identify Sources of Potential Error.

Discussion

One of the integral aspects of biology is the self-organization of molecules into hierarchal structures that perform a specific task.¹³ In the lab, self-assembly depends on numerous factors such as salt concentration, pH, and digestion duration. As shown, a self-organizing hydrogel forms when solubilized proteins return to a physiological temperature. The hydrogel formed is capable of promoting cellular attachment and proliferation *in vitro*.

Cellular response to biophysical signals from ECM cannot be researched using polymers. Tissue specific cellular response to biophysical signals cannot be researched by using other tissues. Regeneration researchers and clinicians believe using decellularized scaffolds improves tissue regeneration and wound healing. Chronic lung disease sufferers need therapeutic improvements to increase organ availability and decrease immunogenicity. Organ specific, cell-ECM interactions must be researched to further organ biology.

Using ECM as a cell scaffold for lung cells has shown consistent results.^{14,15} Using lung slices from diseased models encourages disease renewal¹⁶ stem or progenitor cells, found in distal lungs,^{17,18} and requires special niches to prevent formation of teratomas. Using tissue specific ECM as a delivery vehicle, regenerative effects of progenitor cells may be enhanced. The ECM hydrogel establishes a more applicable environment to assess cellular reactions to extra cellular matrix.

A large body of research exists looking at stiffness as it relates to cellular signaling. Our method of genipin crosslinking allow for cell-stiffness signaling as well as cell-ECM component interactions. While hydrogel stiffness can be manipulated by ECM amount and ratio of proteins, the use of genipin allows hydrogel stiffness changes somewhat independent of ECM content. Adjusting genipin concentration may allow for accurate tuning of mechanical properties to most closely resemble natural lung tissue⁶. From genipin data obtained using hydrogels from peripheral lung in the present study, a 15-fold increase (from 5 Pa to 75 Pa) was noted. Based on rheological data of biopsied normal rat lung tissue that was decellularized using the same methods, we can achieve approximate peripheral lung tissue stiffness (data not shown) by adding 1% genipin to peripheral lung hydrogels or adding 0.01% to whole lung hydrogels (estimated). Using the peripheral lung ECM consisting of lung parenchyma made a considerably softer gel compared with our previously published whole lung ECM hydrogel results⁷. Therefore in addition to genipin, selecting specific regions of lung tissue to decellularize can dramatically impact the stiffness.

There are opportunities to improve this method, which may include additional steps commonly used in perfusion decellularization of whole lung tissue. This method has been used on normal lung tissue and may require modification for decellularization of diseased lungs. We have not yet examined this process in specific disease states. We obtain our lungs shipped on ice overnight from a slaughterhouse. This method could

limit the efficacy of our process due to atelectasis or vascular occlusion; however, we rarely have to remove areas of lung that have not been thoroughly decellularized. When obtaining fresh lung tissue, we would recommend making all of the solutions required before getting the lungs to prevent any pauses in tissue processing. Previous research has found that using peracetic acid as a final sterilization step to matrix production causes a regenerative M1 macrophage phenotype in infiltrating immune cells,¹⁹ potentially improving proregenerative effects of decellularized tissue. Other research has gone into using other detergents to complete the process, but those appear to be lab specific preferences with ultimately the same impact on decellularization. The decellularization procedure and digestion steps may be optimized depending on the researcher's specific application. For example, altering the gel digestion time or neutralization methods may change the protein composition, mechanical properties, or gelation kinetics.

Alternatives to the method discussed here can vary in level of complexity depending on desired experimental outcomes. For example, if the goal was to keep the basement membrane intact as much as possible, a perfusion pump with a pressure limited control may decrease barrier damage. Additionally, if one had a laboratory setup available to keep both lungs intact for larger species, the decellularization process may improve the efficacy. Conversely, a hand grinding technique with a mortar and pestle may be applied to the lyophilized tissue for labs who do not have a cryomill. We would expect hand grinding to produce larger fragments, limiting the surface area available for the acid digestion so the digestion times may need to be altered in this case.

Alternatives are not limited to the decellularization steps and formation of hydrogels. Glutaraldehyde has been used as a crosslinker for hydrogels, providing one possible alternative to increasing mechanical strength of biological hydrogels used for model systems. Other model systems include intact decellularized lungs as an *in vitro* 3D culture system, or more commonly looking at lung slices as a model 3D *in vitro* scaffold.²⁰ We have already mentioned the limitations that come from working with polymer scaffolds as well as single component scaffolds, but those remain as possible alternatives to the procedure presented here. Additionally, there are alternative methods to determining decellularization efficacy (such as DNA isolation), gelation (can use turbidity assays), and cell attachment and proliferation (such as use of CCK8). We found that the methods described here are more than adequate to characterize hydrogels for use as model 3D scaffolds.

The lung ECM hydrogels we describe here provide an effective platform for studying cell-ECM interactions. The pregel solution has potential as a coating for polymer drug carriers, aiding in delivery of mesenchymal stem cells, or as a drug delivery platform by itself. The protocol presented herein provides the basic steps for creating a versatile hydrogel derived from lung ECM.

Disclosures

The authors have nothing to disclose.

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