Published in final edited form as:

Methods. 2015 August ; 84: 53–59. doi:10.1016/j.ymeth.2015.03.024.

Fabrication and Characterization of Injectable Hydrogels Derived from Decellularized Skeletal and Cardiac Muscle

J. L. Ungerleider^a, T.D. Johnson^a, N. Rao^a, and K. L. Christman^{a,b}

^aSanford Consortium for Regenerative Medicine, Department of Bioengineering, University of California, San Diego La Jolla, CA, USA

Abstract

Biomaterials, which can contain appropriate biomechanical and/or biochemical cues, are increasingly being investigated as potential scaffolds for tissue regeneration and/or repair for treating myocardial infarction, heart failure, and peripheral artery disease. Specifically, injectable hydrogels are touted for their minimally invasive delivery, ability to self-assemble *in situ*, and capacity to encourage host tissue regeneration. Here we present detailed methods for fabricating and characterizing decellularized injectable cardiac and skeletal muscle extracellular matrix (ECM) hydrogels. The ECM derived hydrogels have low cellular and DNA content, retain sulfated glycosaminoglycans and other extracellular matrix proteins such as collagen, gel at physiologic temperature and pH, and assume a nanofibrous architecture. These injectable hydrogels are amenable to minimally invasive, tissue specific biomaterial therapies for treating myocardial infarction and peripheral artery disease.

Keywords

decellularization; hydrogel; extracellular matrix; myocardial infarction; peripheral artery disease

1. Introduction

Cardiovascular disease is the leading cause of death in the United States [1]. One consequence of cardiovascular disease, myocardial infarction (MI), afflicts more than 900,000 Americans annually and can lead to heart failure and death. MI is caused by acute occlusion of a coronary artery, leading to myocardial ischemia, cardiomyocyte necrosis, collagen scar formation, and subsequent diminished pump function [2]. The only treatments for heart failure post-MI are either a heart transplant or a mechanical left ventricular (LV) assist device, and no current therapy prevents negative left ventricular remodeling and heart failure. As such, the five-year mortality rate post-MI is 50 percent [1]. Another manifestation of cardiovascular disease, peripheral artery disease (PAD) is a cardiovascular

^bCorresponding author: christman@eng.ucsd.edu, 858-822-7863, Sanford Consortium for Regenerative Medicine, Department of Bioengineering, University of California, San Diego, 2880 Torrey Pines Scenic Drive, La Jolla, CA, USA, 92037.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

condition that afflicts 12 to 20 percent of Americans over age 65 [3]. Its most common cause is atherosclerosis, but it predominantly afflicts smokers, diabetics, and African Americans. The disease first presents as pain during exercise due to chronic plaque build-up and reduced blood flow in peripheral arteries, most commonly in the legs [1]. Over time, the plaque occludes more of the major arteries and leads to critical limb ischemia (CLI), the most severe form of the disease. CLI affects about a quarter of the overall PAD population [1]. Currently, the only effective treatment is surgical revascularization; however, few patients are eligible for this therapy, and it carries a high failure rate due to restenosis, which is why amputation rates due to PAD have remained relatively unchanged in the last 30 years [4, 5]. In both instances, atherosclerosis leads to acute or chronic muscle ischemia, followed by negative remodeling and fibrosis. Thus, an ideal therapy would encourage positive remodeling post-ischemia and prevent chronic ischemia and inflammation from leading to overall tissue damage and failure over time.

Direct injection of growth factors [6, 7], cells [8], and gene therapy [9, 10] are some examples of tissue engineering approaches used to stimulate angiogenesis in the ischemic limb or heart. Recently, biomaterial scaffolds have begun to be used for their potential in prolonging the release of angiogenic factors and for their inherent ability to encourage tissue-scale regeneration. Naturally-derived [6, 7, 11, 12] and synthetic [13, 14] hydrogel scaffolds have been tested for their ability to recruit endogenous progenitor cells and promote tissue remodeling post-ischemia. While promising results have been seen, with material delivery of growth factors or stem cells [5, 15–17], the expensive nature of preparing such a combination product and sub-optimal clinical trial results have halted these therapies' progression to market [18]. In contrast, material-alone therapies have significant potential for many translational reasons, including minimally invasive delivery and reduced costs compared to a cell- or growth factor-based therapy [19]. In particular, injectable hydrogels derived from decellularized muscle ECM, have shown significant potential for treating both MI and PAD. Singelyn et al. first showed the capability of a decellularized cardiac ECM hydrogel to increase vascular cell migration *in vitro* and vessel density *in vivo* [20]. The material has since shown efficacy to increase cardiac muscle, reduce fibrosis, and improve cardiac function post-MI in small and large preclinical animal models [21, 22] and is now planned for testing in a Phase I clinical trial (clinicaltrials.gov: NCT02305602). DeQuach et al. showed skeletal muscle progenitor recruitment and neovascularization due to injection of a skeletal muscle ECM hydrogel alone in a preclinical model of hindlimb ischemia, thus indicating the potential for ECM hydrogels to be used alone to treat PAD and regenerate ischemic damaged skeletal muscle [12].

In this article, we present detailed methods for fabricating injectable hydrogels derived from either decellularized cardiac or skeletal muscle extracellular matrix (ECM). We also present methods, which we recommend should be performed on each batch of material prior to *in vitro* or *in vivo* use, to ensure limited batch-to-batch variability and more consistent results.

2. Materials and Methods

2.1 Fabrication of injectable hydrogels

2.1.1 - Day 0 – Initial Tissue Processing—Tissue specific injectable hydrogels were derived from either porcine myocardium or skeletal muscle. In order to fabricate a sterile material, all steps in the protocol were conducted with sterile solutions and autoclaved beakers or in a biosafety cabinet where possible. Decellularization was accomplished with a 1% wt/vol sodium dodecyl sulfate (SDS) solution, made by adding appropriate volumes of 20x PBS, 10x SDS, and ultrapure water. The psoas muscle or heart was harvested from Yorkshire farm pigs weighing 30–45 kg. Note that larger animals or other sources of skeletal muscle are more likely to have greater interstitial adipose tissue within the muscle, which interferes with tissue processing. The skeletal muscle was obtained and isolated from skin, superficial fat, and fascia, leaving only the homogenous skeletal muscle tissue behind. For cardiac ECM fabrication, the left ventricle (LV) free wall and septum were isolated from the right ventricular free wall, atria, and valves by blunt dissection and cleared of any fat or fascia. Papillary muscles and chordae tendinae in the LV lumen were also removed, leaving only myocardium remaining. Muscle was cut into regularly sized cubes approximately 3–5 mm (skeletal muscle, Figure 1A) or 2 mm (cardiac muscle) per side at the smallest, as tissue is prone to degradation and collapse during decellularization. A larger piece of muscle was set aside for histological analysis as a “before decellularization” sample. Tissue was weighed and divided into 1L autoclaved beakers with 20–35 g of tissue in each beaker, and ultrapure water was added to a total volume of 800mL and spun with a stir bar at 125 rpm for 30–45 minutes. Tissue was strained in an autoclaved fine mesh strainer, rinsed under ultrapure water, and returned to the beaker. Previously mixed 1% SDS solution was added to the beaker so that the total volume of tissue and SDS was 800 mL and was stirred at 125 rpm for 2 hours as an initial rinse. Again, after 2 hours the tissue was rinsed in the fine mesh strainer with ultrapure water and returned to the beaker, also rinsed with ultrapure water. Fresh 1% SDS was added to the beaker to a final volume of 800 mL. Four mL of 10,000 U Penicillin/Streptomycin (PenStrep) was then added to each beaker, giving a final working concentration of 50 U PenStrep in 1% SDS. The beaker was kept sealed with a square of parafilm and the tissue was spun at 125 rpm for 24 hours.

2.1.2 - Day 1–5 – SDS solution changes—Tissue was strained and the beaker/stir bar were thoroughly rinsed with ultrapure water. On the first day only, larger pieces of tissue were more finely cut into smaller pieces to ensure consistent rates of decellularization (larger pieces tended to have a deeper red or pink center after the first day of decellularization, Figure 1B). Tissue was returned to the beaker and fresh 1% SDS was added to 800 mL with 4 mL 10,000 U PenStrep. Through this process, beakers were kept covered with parafilm whenever possible to reduce the risk of contamination. Rinses and solution changes were repeated every 24 hours until the tissue was completely white, usually 3–4 days (Figure 1C). Remaining ECM was spun for an extra 24 hour period to ensure full decellularization. Additional days of solution changes were minimized once tissue was fully white to avoid degradation and loss of ECM proteins. Cardiac ECM was then processed starting with the water rinse step (2.1.4), while skeletal muscle was processed first with the IPA lipid removal step (2.1.3).

2.1.3 - IPA Lipid Removal (skeletal muscle ECM only)—The presence of lipids after decellularization could inhibit subsequent gelation of the digested material, and therefore an isopropyl alcohol (IPA) lipid removal step was implemented only for fattier skeletal muscle tissue. After final SDS solution change, ECM was rinsed in the mesh strainer with ultrapure water and the beaker and stir bar were rinsed thoroughly to remove any trace SDS. ECM was spun in ultrapure water at 800 mL total volume for 2 hours to remove residual SDS. Up to 5 beakers of rinsed ECM were placed into a single clean 1L beaker with a clean stir bar. In a fume hood, IPA was added up to 400 mL total volume. The beaker was sealed with parafilm and spun at 125 rpm for 12–24 hours.

2.1.4 - Water Rinse, Freezing, Milling—ECM was rinsed in ultrapure water and for skeletal muscle ECM, used IPA was properly disposed of as hazardous waste. Both skeletal and cardiac muscle ECM was spun in 800 mL ultrapure water at 125 rpm for 30–45 minutes to remove residual SDS and IPA from ECM. ECM was rinsed and fresh ultrapure water was added and spun at 125 rpm for an additional 24 hours. ECM was thoroughly rinsed with ultrapure water in the mesh strainer and added to new autoclaved 1L bottle with ultrapure water up to 800 mL total volume. The lid was tightly sealed and the bottle was shaken vigorously for 30 seconds. The bottle and ECM were strained and rinsed several times to remove residual SDS; this shaking step was repeated at least twice until no bubbles persisted after 30 seconds of vigorous shaking. ECM was evenly divided into 50 mL conicals, which were filled to less than 20 mL. A few pieces of ECM were prepared for histological analysis to compare to the pre-decellularization sample (see 2.2.2). The 50 mL conicals were frozen at -80°C , with the conicals laid on their side and the ECM evenly distributed along the length of the conical for more uniform freezing and subsequent lyophilization. Frozen ECM was lyophilized and milled (Wiley Mini-Mill, #40 or #60 filter) to generate a particulate for subsequent protease digestion. ECM was milled in larger batches (greater than 1 gram) to avoid significant ECM loss within the mill mechanism.

2.1.5 - Pepsin Digestion—In order to liquefy the ECM, milled ECM was partially digested in pepsin (protocol modified from [23]). All steps with open vials were performed in a biosafety cabinet. Fresh pepsin was dissolved in 0.1 M HCl at 1 mg/ml through shaking for 5–10 minutes. While pepsin was shaking, approximately 20 to 30 mg of milled ECM were added to a 20 mL scintillation vial with a small stir bar. Once dissolved the pepsin solution was sterile filtered with a $0.22\ \mu\text{m}$ filter and added to the ECM vial to reach a concentration of 10 mg ECM / 1 mL pepsin solution. The closed vial was then placed on a stir plate (~ 60 – 120 rpm) for 48 hours at room temperature. To ensure the entire material was digested in the pepsin solution, material on the sides of the vial was gently scraped down with a spatula one or two times during the 48 hour digestion. After 48 hours, the liquid ECM was titrated to pH 7.4, 1x PBS and a final material concentration of 6 mg ECM/ml. First, pH was titrated to 7.4 by adding sterile 1 M NaOH (1/10 the acidic liquid ECM volume) and by adding subsequent volumes of sterile 1 M NaOH or 0.1 M HCl after measuring pH of the well-mixed solution. Then, salt concentration was titrated to a final concentration of 1x PBS by adding 1/9 of the resulting liquid ECM volume of sterile 10x PBS. Finally, a sufficient volume of sterile 1x PBS was added to reach a final ECM concentration of 6 mg/ml, based off of the initial mass of ECM added. Material was then aliquoted, frozen and lyophilized

for long term storage for up to one year at -80°C . When ready for use, lyophilized ECM was resuspended in an equivalent volume of sterile water for use. For example, if the aliquot was a 600 μL aliquot of the original batch of digested and titrated ECM, 600 μL of sterile water was added and mixed until homogeneous by pipetting up and down repeatedly. There was some material loss due to bubbles forming upon mixing, so approximately 10% additional material than required was aliquoted.

2.2 *In vitro* characterization of injectable hydrogels

2.2.1 - Gelation Test—Each batch of decellularized ECM was characterized to ensure minimal batch-to-batch variability. First, gelation was tested by adding 500 μL of resuspended digested ECM to a 4 mL scintillation vial and incubating at 37°C for 1 hour. If material did not flow upon tilting the vial, a gel was formed.

2.2.2 – Decellularization Verification

Histology: Sufficient decellularization was first verified through hematoxylin and eosin staining and Hoechst staining. Briefly, fresh and final samples were either fresh frozen by freezing in Tissue-Tek OCT (Fisher-Scientific, Waltham, MA) or fixed in 10% formalin for 24 hours and paraffin-embedded. Samples were sectioned in 10 μm thick slices and whole mounted on slides. Hematoxylin and eosin staining was performed on both fresh and final samples (fresh frozen or paraffin-embedded) in order to assess morphology of the decellularized ECM as well as nuclear hematoxylin-labeled content of the fresh and final tissue. Fluorescent nuclear staining was performed by acetone-fixing both fresh and final slides (fresh frozen) for 1.5 minutes, followed by three 5 minute 1x PBS washes and 10 minutes of incubation in Hoechst 33342 (diluted by adding 1 μL of Hoechst 33342 in 10 mL DI water, Life Technologies, Grand Island, NY). After incubation, samples were kept with minimal light exposure, washed again 3 times for 5 minutes in 1x PBS, and cover slips were mounted using Fluormount. H&E stained slides were imaged on a Leica Aperio ScanScope CS² at 20x magnification (Leica Biosystems, Buffalo Grove, IL), and Hoechst-labeled slides were imaged on a Carl Zeiss Observer D1. Images were acquired at 10x with an equivalent exposure time for both fresh and final samples for comparison (usually about 40 ms).

dsDNA quantification: Double stranded DNA (dsDNA) was quantified to further determine the extent of decellularization. DNA was isolated from digested and lyophilized 1 mg aliquots of ECM using a standard kit (we suggest NucleoSpin, Macherey-Nagel, Bethlehem, PA). Lyophilized ECM aliquots were resuspended in lysis buffer T1 and proteinase-K by pipetting up and down multiple times, and DNA was isolated according to kit instructions. Proteinase-K digestion of the material was confirmed by a visually clear solution before running ECM through the remainder of the NucleoSpin kit. NanoDrop (Thermo Scientific, Waltham, MA) readings were conducted on eluted DNA to confirm a DNA concentration of zero, since DNA remaining in the ECM should be below the detectable threshold of NanoDrop spectrophotometers. PicoGreen fluorescent reporter was used (Life Technologies, Grand Island, NY) to more precisely quantify dsDNA content according to kit instructions with 100 μL of eluted sample DNA.

2.2.3 – Material Composition

sGAG quantification: Sulfated glycosaminoglycans (sGAGs) were quantified in both cardiac and skeletal muscle ECM using the 1,9-dimethylmethylene blue dye (DMMB) assay (modified from [24]). Chondroitin sulfate dilutions from 0 to 50 μg per 100 μL in a 1.5 mL Eppendorf tube were used to create a standard curve. Resuspended digested ECM at 6 mg/mL was run in triplicate samples of 100 μL per Eppendorf tube. Working solution was made fresh by combining 5 mL of formate solution (2.5g sodium formate in 240 mL 1 M guanidine hydrochloride (GuHCl) and 2.795 mL of 85% formic acid), 1.25 mL of 0.64 mg DMMB/mL ethanol, and ultrapure water to a total volume of 25 mL. Decomplexation solution was made by combining 2.05 g sodium acetate, 200 mL ultrapure water, 50 mL 1-propanol, and 250 mL of 8 M GuHCl. Working solution was added to each sample or standard concentration tube (1 mL/tube), and tubes were vortexed at level 3.5 for 30 minutes. Tubes were then centrifuged for 10 minutes at 12,000 rpm in a microcentrifuge to collect a pellet of precipitated DMMB-sGAG complex. If no pellet formed in the standard tubes, the entire protocol was restarted with fresh standard solutions. Supernatant was carefully aspirated so as not to disturb the pellet. Then, the pellet was gently broken up by slowly pipetting with 1 mL of decomplexation solution. Tubes were again vortexed at level 3.5 for 30 minutes. Solutions were confirmed to be homogenous with the pellet fully dissolved. Absorbance was then read at 656 nm on a plate reader by pipetting 100 μL in triplicate into a 96 well plate. Chondroitin sulfate concentrations were plotted against absorbance to calculate a standard curve for determination of sGAG concentration in resuspended ECM samples.

Protein content: Resuspended ECM was run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel to assess protein fragment content. Rat tail collagen I at 2.5 mg/mL (Life Technologies, Grand Island, NY) was run on adjacent wells to compare ECM protein content, as collagen is the main protein component of decellularized ECM hydrogels [25]. Gels were run using the general protocol from the Invitrogen NuPage® kit (Life Technologies, Grand Island, NY). Digested ECM was resuspended and 7.5 μL were added to each running sample. To each sample was also added 3.75 μL of NuPAGE LDS 4x Sample Buffer, 1.5 μL NuPAGE 10x Reducing Agent, and 2.25 μL DI water, to a final volume of 15 μL . Samples were heated at 70°C in a water bath or heating block for 10 minutes to denature proteins. Running buffer was made by mixing 50 mL of 20x NuPAGE SDS Running Buffer with 950 mL DI water. For the upper buffer chamber, 200 mL of the Running Buffer was set aside and 500 μL NuPAGE Antioxidants was added within 30 minutes of running the gel. The remaining running buffer (~600 mL) was added to the outer chamber of an assembled mini cell, and the 200 mL of running buffer with antioxidants was loaded into the inner chamber. In each well of a 12% Tris-Acetate gel, 10 μL of each sample was added. We recommend running resuspended 6 mg/mL ECM samples mixed in the above ratios with 2.5 mg/mL collagen I in the same ratios (7.5 μL of sample added to the mixture) for comparative analysis. The gel was run at a constant voltage (200 V) for approximately 50 minutes, or until dye reached the bottom of the gel. Gel was removed from the cartridge and stained with Imperial Protein Stain overnight on a shaker plate in a fume hood. The stained gel was then rinsed with ultrapure water for 2–3 hours, or until bands became

distinct. Placing a Kim-Wipe in the container with the gel and the water helped absorb more of the Imperial Protein Stain.

2.2.4 – Mechanical Properties—Resuspended ECM was tested via rheometry in liquid form for complex viscosity and gel form for storage and loss moduli. Samples were run on a parallel plate rheometer (ARG2 Rheometer, TA Instruments, New Castle, DE). For complex viscosity measurements, 200 μ L resuspended liquid ECM was pipetted into the center of the parallel plate geometry. Plate temperature was fixed at 25°C and gap height was set to 500 μ m to ensure the liquid filled the entire gap between plates. Material was run in a flow procedure programmed with frequencies ranging from 0.1 – 100 Hz. For storage and loss moduli, lyophilized ECM was resuspended in DI water and 500 μ L was pipetted carefully into a 4 mL scintillation vial to avoid bubble formation. ECM was gelled for 24 hours at 37°C. Samples were run with the plate pre-set to 37°C. Samples were carefully transferred from the 4 mL scintillation vial to the parallel plate; if gels were broken, the gel was discarded and another gel was used, as any impurities or tears in the gel can affect the measured mechanical properties. Excess water used to help transfer the gel from the vial to the plate was also carefully removed from the gel by using a Kim Wipe. Gap height was set to 1200 μ m in order to ensure the gel filled the entire gap between the parallel plates and an oscillatory frequency sweep was run from 0.1 to 100 rad/s to measure the storage and loss moduli. The maximum suggested gap height for running gels of this size is 1500 μ m.

2.2.5 – Structural Properties—Scanning electron microscopy (SEM) was performed on gelled ECM material. Resuspended ECM aliquots of 100 μ L each were pipetted into a 96 well plate in triplicate while avoiding bubble formation. Aliquots were allowed to gel at 37°C for 24 hours and then a solution of 4% paraformaldehyde and 4% glutaraldehyde in DI water was pipetted on top of each gel for 24 hours. Fixative was added on top of each gel in the 96 well plate, or if gels were carefully transferred to a 12 or 24 well plate, the larger well size allowed for submergence on all sides for improved uniform fixation. Gels were then dehydrated in graduated rinses of ethanol. Fixed and dehydrated gels were dried and sputter coated as previously published [25]. Briefly, the critical point dryer (Leica EM CPD300, Leica, Vienna) was set to perform 40 exchange cycles of CO₂ at medium speed and 40% stirring. Before sputter coating, samples were carefully pulled apart with tweezers to expose the gels' inner nano-fiber architecture. Samples were sputter coated with 7 nm of platinum (Leica SCD, Leica, Vienna) and imaged on an FE-SEM at 0.6 kV using the in-lens SE1 detector (Sigma VP, Zeiss Ltd, Cambridge, UK).

3. Results and Discussion

To demonstrate ways to characterize decellularized porcine cardiac and skeletal muscle ECM, we present a variety of characterization assays for naturally derived ECM hydrogels. These include assessing histology, DNA content, sulfated glycosaminoglycan (sGAG) content, mechanical properties (viscosity and storage and loss moduli), protein content, and nanoscale architecture. It is important to quantify these material characteristics in order to ensure minimal batch-to-batch variability for consistent behavior in *in vitro* or *in vivo* applications.

H&E was conducted on fresh frozen or fixed and paraffin embedded 10 μ m sections of “fresh” tissue and “final” ECM for each round of decellularization to verify that the decellularization process was thorough (Figure 1). Hoechst staining was conducted on fresh frozen 10 μ m sections and imaged at constant exposure time to verify absence of nuclear content as indicated by lack of blue staining in “final” samples (Figure 2). Investigators should look for cellular content in the final product, such as hematoxylin or Hoechst positive staining, which is indicative of nuclei or residual DNA. The ECM alone should be light pink stained fibers on H&E (see Figure 2B, F). Batches with unsuccessful cellular removal should not be used for *in vivo* applications especially, as cellular content can elicit a negative immune response in host tissue [26].

DNA content was measured by isolating DNA from the ECM using a standard DNA isolation kit and a fluorescent reporter with a standard curve (PicoGreen kit), demonstrating a range from 0.1 to 5 ng dsDNA/mg ECM for both skeletal muscle and cardiac sources. Measuring double stranded DNA (dsDNA) content is important because it gives an approximation of the quantitative extent of decellularization. Removing cellular content is vital to decrease the negative immune response *in vivo*, as both allogeneic and xenogeneic biomaterials have the ability to elicit an immune response upon injection [26]. It is useful to compare measurements to a known standard, such as dsDNA content found in a variety of decellularization methods published by Reing et al [27]. Although the protocol in this study typically produces a dsDNA content an order of magnitude lower, Reing et al. suggested that 50 ng dsDNA/mg ECM should be the threshold limit for DNA content. There may be slight differences in DNA content from batch to batch or depending on the tissue source, but as long as the content is lower than the suggested threshold, the material can be considered effectively devoid of cellular content. Batch to batch or tissue source variability in dsDNA content may be due to slight differences in processing. For example, slightly different SDS exposure times or additional processing steps such as IPA rinsing may alter decellularization extent.

sGAG content was quantified with the DMMB assay, with some modifications from a previously published protocol [24], in order to account for specific ECM differences. For both muscle ECM hydrogels, sGAG content ranged from approximately 5 to 15 μ g sGAGs/mg ECM. sGAGs are growth factor binding moieties that may lend desired bioactivity to any naturally derived matrix for their ability to bind and prolong release of regenerative cytokines, either those secreted *in vivo* or exogenously delivered [28]. Although the decellularization process can wash away sGAGs [27], it is useful to quantify the remaining GAG content to compare decellularization methods or optimize a naturally derived hydrogel for a given delivery application.

PAGE shows a qualitative measure of the material protein content and extent of protease digestion. Collagen is the main content of muscle ECM-derived hydrogels, so rat tail collagen I was run in an adjacent lane to compare the characteristic bands (Figure 3). We found that 2.5 mg/mL of collagen I shows the most similar protein fragment concentrations in PAGE to 6 mg/mL of resuspended digested ECM. Figure 3 also shows one example of the batch-to-batch variability than can be possible with ECM hydrogels derived from different porcine donors. Lanes 1, 2, and 3 each show each a different sample of three

batched skeletal muscle ECM donors. Batching material from multiple donors helps ensure consistent material properties, but batched ECM can still be compared with assays such as PAGE by the presence or differing intensities of some bands. For example, batch 1 shows a higher concentration of protein fragments from 90–100 kDa than either batch 2 or 3 (Figure 3). For both muscle ECM hydrogels, the PAGE should display characteristic collagen bands as well as faint bands below 20 kDa, confirming sufficient partial digestion of the ECM by pepsin. For a more thorough analysis of the protein content, mass spectrometry can be conducted on digested material to characterize protein fractions. Furthermore, current research has identified novel methods of quantifying relative protein abundance with mass spectrometry and stable isotope labeled peptide standards [29, 30].

Quantifying mechanical properties is vital to assess the physical characteristics of naturally derived ECM hydrogels. Although they can be tissue-specific regarding biochemical composition, decellularized hydrogels are typically weaker than the tissues from which they are derived [31, 32]. This can vary due to the harshness of the decellularization method [27]. Shear storage and loss moduli can indicate the stiffness of the gel. Example traces for both muscle ECM hydrogels are shown in Figure 4A and B. Reported at 1 rad/s, values for storage modulus typically range between 5 and 10 Pa and loss modulus between 1 and 5 Pa [12, 31, 32]. Quantifying complex viscosity also shows the potential of the material for injectability and/or catheter delivery. The material should be shear thinning at frequencies from 0.1 – 100 Hz with complex viscosities of approximately 1 to 0.02 Pa•s (Figure 4C, D) [25]. Since the cardiac and skeletal muscle ECM materials are shear thinning, they are well suited to be delivered in a narrow catheter with high shear rates. Storage and loss modulus as well as complex viscosity can all be quantified on a parallel plate rheometer.

SEM can be performed to visualize the nanoscale architecture of ECM hydrogels. This three dimensional nanoscale architecture is important for cell attachment and migration through the scaffold. Although the ECM was partially digested, which allowed it to be injectable, the ECM reassembled into a three dimensional nanofibrous network upon gelation at physiologic temperature and pH. Porous nanofibrous architecture may be desirable to resemble the native ECM conditions, as the main ECM component, collagen, forms a nanofibrous architecture *in vivo* [33, 34]. Fiber diameters can also affect the mechanical properties and self assembly of the hydrogel; we have found that fibers with diameters between 30 – 250 nm and an average diameter of 100 nm form upon self-assembly of the muscle ECM hydrogels [31]. Figure 4E and F display example SEM images for the skeletal muscle and cardiac ECM hydrogels.

The final, decellularized ECM hydrogel can be used for a variety of applications. A benefit of the lyophilized digested ECM is that it has a long shelf life if stored frozen and can be quickly prepared for injection by thorough resuspension in sterile water. The shear thinning property and solution-to-gel transformation at 37°C both allow for injection and gelation *in situ* at body temperature. This allows for direct injection of the material into a tissue-specific location, such as the skeletal muscle ECM hydrogel injected intramuscularly for PAD applications, or the cardiac ECM hydrogel injected into the left ventricle via catheter for cardiac repair after MI. For example, we have shown the potential of the skeletal muscle-specific ECM hydrogel to improve neovascularization and muscle proliferation in a rat

model of hindlimb ischemia [12]. Our cardiac ECM hydrogel has been shown to increase cell migration *in vitro* [20]; it also promoted tissue repair and increased cardiac function after one and three months in small and large animal models *in vivo* [21, 22]. This general process for generating and characterizing ECM hydrogels can also be performed on many other tissue types, such as brain [35], adipose [36], bone [37], and skin [38], to develop tissue specific injectable hydrogels for a variety of *in vitro* and *in vivo* tissue engineering applications. The overall goal of the injectable ECM hydrogels is to provide biochemical cues that are specific to the tissue, which is being regenerated, as well as a physical scaffold to support cell infiltration.

4. Conclusions

We have shown that naturally derived ECM hydrogels can be fabricated through detergent based decellularization and enzymatic digestion, and subsequently characterized through a variety of mechanical and biochemical techniques. Once fabricated and properly characterized, these tissue-specific hydrogels can be used in a variety of applications for tissue regeneration and repair *in vivo* or probing tissue-specific cell interactions *in vitro*.

Acknowledgments

This research was supported in part by National Institutes of Health (NIH) through R01HL113468. Dr. Christman is co-founder, board member, and holds equity interest in Ventrix, Inc.

Abbreviations

PAD	peripheral artery disease
CLI	critical limb ischemia
MI	myocardial infarction
ECM	extracellular matrix
SDS	sodium dodecyl sulfate
PenStrep	penicillin/streptomycin
IPA	isopropyl alcohol
PBS	phosphate-buffered saline
dsDNA	double-stranded DNA
sGAG	sulfated glycosaminoglycan

References

1. Go AS, Mozaffarian D, Roger VL, et al. Heart disease and stroke statistics--2013 update: a report from the American Heart Association. *Circulation*. 2013; 127:e6–e245. [PubMed: 23239837]
2. Sutton MGSJ, Sharpe N. Left Ventricular Remodeling After Myocardial Infarction : Pathophysiology and Therapy. *Circulation*. 2000; 101:2981–2988. [PubMed: 10869273]
3. Ostchega Y, Paulose-Ram R, Dillon CF, et al. Prevalence of peripheral arterial disease and risk factors in persons aged 60 and older: data from the National Health and Nutrition Examination Survey 1999–2004. *J Am Geriatr Soc*. 2007; 55:583–589. [PubMed: 17397438]

4. White CJ, Gray WA. Endovascular therapies for peripheral arterial disease: an evidence-based review. *Circulation*. 2007; 116:2203–2215. [PubMed: 17984390]
5. Tongers J, Roncalli JG, Losordo DW. Therapeutic angiogenesis for critical limb ischemia: microvascular therapies coming of age. *Circulation*. 2008; 118:9–16. [PubMed: 18591450]
6. Ruvinov E, Leor J, Cohen S. The effects of controlled HGF delivery from an affinity-binding alginate biomaterial on angiogenesis and blood perfusion in a hindlimb ischemia model. *Biomaterials*. 2010; 31:4573–4582. [PubMed: 20206988]
7. Kawamura I, Takemura G, Tsujimoto A, et al. Treatment of leg ischemia with biodegradable gelatin hydrogel microspheres incorporating granulocyte colony-stimulating factor. *J Cardiovascular Pharmacology*. 2011; 57:416–423.
8. Powell RJ, Comerota AJ, Berceli SA, et al. Interim analysis results from the RESTORE-CLI, a randomized, double-blind multicenter phase II trial comparing expanded autologous bone marrow-derived tissue repair cells and placebo in patients with critical limb ischemia. *J Vasc Surg*. 2011; 54:1032–1041. [PubMed: 21684715]
9. Powell RJ, Goodney P, Mendelsohn FO, et al. Safety and efficacy of patient specific intramuscular injection of HGF plasmid gene therapy on limb perfusion and wound healing in patients with ischemic lower extremity ulceration: results of the HGF-0205 trial. *J Vasc Surg*. 2010; 52:1525–1530. [PubMed: 21146749]
10. Shigematsu H, Yasuda K, Iwai T, et al. Randomized, double-blind, placebo-controlled clinical trial of hepatocyte growth factor plasmid for critical limb ischemia. *Gene Ther*. 2010; 17:1152–1161. [PubMed: 20393508]
11. Fan C, Gao P, Gu Y, et al. Therapeutic angiogenesis by intramuscular injection of fibrin particles into ischaemic hindlimbs. *Clinical and Experimental Pharmacology and Physiology*. 2006; 33:617–622. [PubMed: 16789929]
12. DeQuach JA, Lin JE, Cam C, et al. Injectable skeletal muscle matrix hydrogel promotes neovascularization and muscle cell infiltration in a hindlimb ischemia model. *Eur Cell Mater*. 2013; 23:400–412. [PubMed: 22665162]
13. Webber MJ, Tongers J, Newcomb CJ, et al. Supramolecular nanostructures that mimic VEGF as a strategy for ischemic tissue repair. *Proc Natl Acad Sci U S A*. 2011; 108:13438–13443. [PubMed: 21808036]
14. Lin YD, Luo CY, Hu YN, et al. Instructive nanofiber scaffolds with VEGF create a microenvironment for arteriogenesis and cardiac repair. *Sci Transl Med*. 2012; 4:146ra109.
15. Rane AA, Christman KL. Biomaterials for the treatment of myocardial infarction: a 5-year update. *J Am Coll Cardiol*. 2011; 58:2615–2629. [PubMed: 22152947]
16. Akhyari P, Kamiya H, Haverich A, et al. Myocardial tissue engineering: the extracellular matrix. *Eur J Cardiothorac Surg*. 2008; 34:229–241. [PubMed: 18502661]
17. Segers VF, Lee RT. Biomaterials to enhance stem cell function in the heart. *Circ Res*. 2011; 109:910–922. [PubMed: 21960724]
18. Ouma GO, Jonas RA, Usman MH, et al. Targets and delivery methods for therapeutic angiogenesis in peripheral artery disease. *Vasc Med*. 2012; 17:174–192. [PubMed: 22496126]
19. Ungerleider JL, Christman KL. Concise review: Injectable biomaterials for the treatment of myocardial infarction and peripheral artery disease: translational challenges and progress. *Stem Cells Trans Med*. 2014; 3:1–10.
20. Singelyn JM, DeQuach JA, Seif-Naraghi SB, et al. Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering. *Biomaterials*. 2009; 30:5409–5416. [PubMed: 19608268]
21. Seif-Naraghi SB, Singelyn JM, Salvatore M, et al. Safety and Efficacy of an Injectable Extracellular Matrix Hydrogel for Treating Myocardial Infarction. *Sci Transl Med*. 2013; 5:173ra125.
22. Singelyn JM, Sundaramurthy P, Johnson TD, et al. Catheter-deliverable hydrogel derived from decellularized ventricular extracellular matrix increases endogenous cardiomyocytes and preserves cardiac function post-myocardial infarction. *J Am Coll Cardiol*. 2012; 59:751–763. [PubMed: 22340268]

23. Freytes DO, Martin J, Velankar SS, et al. Preparation and rheological characterization of a gel form of the porcine urinary bladder matrix. *Biomaterials*. 2008; 29:1630–1637. [PubMed: 18201760]
24. Barbosa I, Garcia S, Barbier-Chassefiere V, et al. Improved and simple micro assay for sulfated glycosaminoglycans quantification in biological extracts and its use in skin and muscle tissue studies. *Glycobiology*. 2003; 13:647–653. [PubMed: 12773478]
25. Johnson TD, DeQuach JA, Gaetani R, et al. Human versus porcine tissue sourcing for an injectable myocardial matrix hydrogel. *Biomaterials Science*. 2014:60283D.
26. Badylak SF. Decellularized allogeneic and xenogeneic tissue as a bioscaffold for regenerative medicine: factors that influence the host response. *Ann Biomed Eng*. 2014; 42:1517–1527. [PubMed: 24402648]
27. Reing J, Brown BN, Daly KA, et al. The effects of processing methods upon mechanical and biologic properties of porcine dermal extracellular matrix scaffolds. *Biomaterials*. 2010; 31:8626–8633. [PubMed: 20728934]
28. Seif-Naraghi SB, Horn D, Schup-Magoffin PJ, et al. Injectable extracellular matrix derived hydrogel provides a platform for enhanced retention and delivery of a heparin-binding growth factor. *Acta Biomater*. 2012; 8:3695–3703. [PubMed: 22750737]
29. Hill RC, Calle EA, Dzieciatkowska M, et al. Quantification of Extracellular Matrix Proteins from a Rat Lung Scaffold to Provide a Molecular Readout for Tissue Engineering. *Molecular & Cellular Proteomics*. 2015
30. Beynon RJ, Doherty MK, Pratt JM, et al. Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides. *Nat Meth*. 2005; 2:587–589.
31. Johnson TD, Lin SY, Christman KL. Tailoring material properties of a nanofibrous extracellular matrix derived hydrogel. *Nanotechnology*. 2011; 22:494015. [PubMed: 22101810]
32. Singelyn JM, Christman KL. Modulation of material properties of a decellularized myocardial matrix scaffold. *Macromol Biosci*. 2011; 11:731–738. [PubMed: 21322109]
33. Rosenblatt J, Devereux B, Wallace DG. Injectable collagen as a pH-sensitive hydrogel. *Biomaterials*. 1994; 15:985–995. [PubMed: 7841296]
34. Williams BR, Gelman RA, Poppke DC, et al. Collagen Fibril Formation: Optimal in vitro conditions and preliminary kinetic results. *J Biological Chem*. 1978; 253:6578–6585.
35. DeQuach JA, Yuan SH, Goldstein LS, et al. Decellularized porcine brain matrix for cell culture and tissue engineering scaffolds. *Tissue Eng Part A*. 2011; 17:2583–2592. [PubMed: 21883047]
36. Young DA, Bajaj V, Christman KL. Decellularized adipose matrix hydrogels stimulate in vivo neovascularization and adipose formation. *J Biomed Mater Res A*. 2014; 102:1641–1651. [PubMed: 24510423]
37. Sawkins MJ, Bowen W, Dhadda P, et al. Hydrogels derived from demineralized and decellularized bone extracellular matrix. *Acta Biomater*. 2013; 9:7865–7873. [PubMed: 23624219]
38. Wolf MT, Daly KA, Brennan-Pierce EP, et al. A hydrogel derived from decellularized dermal extracellular matrix. *Biomaterials*. 2012; 33:7028–7038. [PubMed: 22789723]

Highlights

- Injectable ECM hydrogels fabricated from decellularized porcine muscle.
- Hydrogels are characterized for biochemical, structural, and mechanical properties.
- ECM hydrogels can be used as minimally invasive scaffolds for tissue engineering.

Figure 1. Decellularization Process

(A) Images of freshly cubed porcine skeletal muscle. (B) Tissue after the first day in 1% SDS. (C) Final ECM after complete decellularization. Note larger pieces of ECM maintain an opaque, pink-tinted center after the first day of SDS rinsing (B), indicating incomplete decellularization.

Figure 2. Decellularization Verification

Hematoxylin and eosin staining shows fresh (A, E) and decellularized (B, F) cross sections of porcine skeletal and cardiac muscle, respectively. Nuclei (purple) are clearly present in fresh, but not final samples. Hoechst 33342 staining shows fresh (C, G) and decellularized (D, H) cross sections of skeletal and cardiac muscle, respectively. Nuclei (blue) are clearly present in fresh, but not final samples. Scale bars are 100 μm .

Figure 3. Material Characterization

PAGE gel showing collagen I (Col), multiple batches (1, 2, and 3) of digested skeletal muscle ECM, and a single batch of digested cardiac ECM.

Figure 4. Mechanical and Structural Properties

Sample rheometer frequency sweep for storage (G') and loss (G'') moduli for skeletal muscle ECM (A) and cardiac ECM (B) hydrogels. Sample complex viscosity traces for liquid skeletal muscle ECM (C) and cardiac ECM (D). SEM shows nanoscale architecture of skeletal muscle ECM (E) and cardiac ECM (F) hydrogels. Scale bar is 1 μm .