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Kidney Decellularized Extracellular Matrix Hydrogels: Rheological Characterization and Human Glomerular Endothelial Cell Response to Encapsulation

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Abstract

Hydrogels, highly-hydrated crosslinked polymer networks, closely mimic the microenvironment of native extracellular matrix (ECM) and thus present as ideal platforms for three-dimensional cell culture. Hydrogels derived from tissue- and organ-specific decellularized ECM (dECM) may retain bioactive signaling cues from the native tissue or organ that could in turn modulate cell-material interactions and response. In this study, we demonstrate that porcine kidney dECM can be processed to form hydrogels suitable for cell culture and encapsulation studies. Scanning electron micrographs of hydrogels demonstrated a fibrous ultrastructure with interconnected pores, and rheological analysis revealed rapid gelation times with shear moduli dependent upon the protein concentration of the hydrogels. Conditionally-immortalized human glomerular endothelial cells (GEnCs) cultured on top of or encapsulated within hydrogels exhibited high cell viability and proliferation over a one-week culture period. However, gene expression analysis of GEnCs encapsulated within kidney dECM hydrogels revealed significantly lower expression of several relevant genes of interest compared to those encapsulated within hydrogels composed of only purified collagen I.

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Supplementary Material

Supplementary materials are available online at: [LINK].

Disclosures

The authors declare that they have no conflicts of interest.

Keywords

Kidney; Extracellular Matrix (ECM); Decellularized; Hydrogel; Tissue Engineering

I. Introduction

Approximately 15% of the adult population in the United States is affected by chronic kidney disease¹, and this percentage is expected to increase both in the United States and worldwide, especially in countries with aging populations and increasing prevalence of hypertension and diabetes mellitus². Current approaches to modeling kidney development and disease progression include cell culture, animal models, and stem cell-derived organoids³. Traditional cell culture using two-dimensional (2D), monolayer systems are still widely utilized though they lack critical structural signaling cues found in the native three-dimensional (3D) microenvironment^{4,5}. In comparison, animal models, specifically transgenic mouse models, enable superior correlation between genotype and phenotype with the added capacity to directly measure renal function^{6–8}; however, animal models lack utility in efficient, high-throughput drug screening and have inherent limitations in recapitulating human disease⁹. More recently, advances in stem cell research have led to the development of organoids for a variety of organ systems^{10–12}, including the kidney^{13–15}. However, organoid research is still evolving and additional investigation is required before organoids reach translational potential. Therefore, there is a compelling need for bioengineered kidney tissues as models of development, disease progression, or drug discovery to better understand and prevent the onset of chronic kidney disease and the progression to end-stage renal disease.

Traditional tissue engineering strategies rely on biocompatible materials processed into porous scaffolds for cell seeding and attachment^{16–18}; however, many of the early materials utilized such as poly-lactic-*co*-glycolic acid or poly- ϵ -caprolactone only provide mechanical support and structure for cell-seeded constructs¹⁹. Within the *in vivo* physiological environment, cells are surrounded by cell-secreted products collectively referred to as the extracellular matrix (ECM). The ECM is well-known for its role as a support structure and imparting mechanical integrity to tissues and organs; however, the ECM also presents bioactive signals to cells that regulate development and maintenance^{20–22}. By design, the ECM acts as a natural, inductive scaffold that in many ways is analogous to a composite and stimulus-responsive hydrogel^{23,24}.

Hydrogels are crosslinked networks of polymers that have demonstrated considerable potential as a 3D cell culture platform^{23–27}. The polymeric network and highly hydrated environment of hydrogels mimic the microstructure and mechanical properties of ECM and enable diffusion of oxygen, nutrients, and waste through the network^{23,24}. Furthermore, many hydrogels can be formed under mild, cytocompatible conditions amenable to cell encapsulation^{25,26}. Hydrogels derived from isolated components of the ECM such as collagen or gelatin are utilized in drug delivery, cell transplantation, and tissue engineering applications^{28,29}. These single- or even multi-component hydrogels, however, lack the full biochemical complexity of the entire ECM milieu. As research regarding tissue- and organ-

derived decellularized ECM (dECM) has gained popularity, investigators have developed methods to process a variety of dECM materials into hydrogels for cell culture applications and minimally-invasive injectable therapies³⁰. While dECM hydrogels derived from some tissue and organ systems, such as the heart^{31–34}, liver^{35–37}, and skeletal muscle^{33,38–40} have been the focus of several publications, other tissues and organs, for example the kidney, have received little investigation.

The objective of this study was to develop kidney dECM hydrogels as a cell culture platform for bioengineered kidney tissue models and compare cell response to traditional biomaterials such as collagen I hydrogels. Here, we present a method to process kidney dECM into physically crosslinked hydrogels as a substrate for cell culture or a 3D matrix for cell encapsulation. Routine histology (hematoxylin and eosin staining) and DNA quantification demonstrated the efficacy of the kidney decellularization process while immunofluorescence staining revealed retention of key ECM proteins. Rheological characterization of the hydrogels illustrated the time to gelation and measured the plateau shear moduli.

To evaluate the utility of these kidney dECM hydrogels for studies employing kidney-specific cell populations, we investigated the cell response of conditionally-immortalized glomerular endothelial cells (GEnCs) cultured on top of and encapsulated within hydrogels. The conditionally-immortalized GEnCs are a cell line generated from endothelial cells specifically isolated from kidney glomeruli⁴¹. These endothelial cells form the capillary loops through which blood flows and is filtered, forming the first layer of the glomerular filtration barrier responsible for the filtering function of the kidney⁴². Primary GEnCs are difficult to isolate and expand in culture due to the loss of important phenotypic features with increasing passage number⁴¹. However, the conditionally-immortalized cell line utilized in this study combines the advantage of continuous cell expansion and passaging when cultured at the permissive temperature while retaining the ability to acquire a mature phenotype when thermoswitched to the non-permissive temperature.

Culture of GEnCs on top of hydrogel substrates confirmed favorable cell viability and proliferation over a twelve-day culture period. Encapsulation of GEnCs within these hydrogel matrices, which has not been previously investigated by others, resulted in favorable cell viability and proliferation during a week in culture, but evaluation of gene expression demonstrated lower fold-change expression for cells encapsulated in kidney dECM hydrogels compared to collagen I hydrogels.

II. Materials & Methods

2.1. Kidney Decellularization

Female Yorkshire pig (3–4 months in age) kidneys were obtained fresh from Northwestern Simulation (Northwestern University Feinberg School of Medicine), following approval by the Northwestern Institutional Animal Care and Use Committee (IACUC), and stored at -80°C until decellularization. Prior to decellularization, kidneys were thawed for several hours in warm water and then minced into pieces approximately $0.5\text{ cm} \times 0.5\text{ cm} \times 0.25\text{ cm}$ in size with a clean razor blade. Kidney pieces were then rinsed with deionized H_2O under constant stirring for one day at room temperature with intermittent H_2O changes. After

rinsing, kidney pieces were decellularized with 0.1% (m/v) sodium dodecyl sulfate (Sigma-Aldrich, #L3771) under constant stirring for two days. After decellularization, kidney dECM was rinsed with deionized H₂O under constant stirring for one day with intermittent H₂O changes to ensure removal of residual detergent in the tissue. Kidney dECM was frozen at −80 °C until further processing.

2.2. Evaluation and Characterization of Decellularized Kidney Tissues

2.2.1. Histological Analysis—Fresh native and decellularized kidney tissues were fixed in 10% neutral-buffered formalin (Thermo Scientific, #5701) for at least 48 hours before paraffin embedding, sectioning, and routine hematoxylin and eosin staining. After staining, coverslips were mounted with Cytoseal XYL (Thermo Scientific, #8312-4). Slides were imaged on a Zeiss Axioskop upright microscope with a Zeiss AxioCam MRc5 camera.

2.2.2. DNA Quantification—Native and decellularized kidney tissue pieces were lyophilized for at least two days, and the dry weight was recorded. Tissue samples were digested with Proteinase K (Sigma-Aldrich, #P2308) in digestion buffer composed of 0.05 M Tris base, 1 mM CaCl₂, pH 8 at 60 °C overnight. DNA was quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, #P7589) following the manufacturer's protocol and a dsDNA standard included in the kit to generate a standard curve. Fluorescence was measured on a BioTek Cytation 3 Multi-Mode Reader with an excitation wavelength of 480 nm and emission wavelength of 520 nm. Samples were run in triplicate ($n = 3$) with technical replicates in triplicate. DNA content was normalized to the dry weight of the sample, and data is presented as a percentage of native kidney tissue DNA content.

2.2.3. Immunofluorescence Staining—Paraffin-embedded sections were deparaffinized and rehydrated before antigen retrieval in a Decloaking Chamber™ NxGen (Biocare Medical, model no. DC2012) at 95 °C for 40 min. with antigen retrieval buffer (Abcam, #ab94674). Sections were rinsed with 1× Phosphate-Buffered Saline (PBS, Mediatech, #21-040), permeabilized with 0.05% TWEEN-20 (Sigma-Aldrich, #P9416) for 10 min., and then blocked with Sea Block (Fisher Scientific, #37527) for 30 min. before incubation with primary antibodies. Primary antibodies were diluted in Sea Block as follows: rabbit anti-collagen I at 1:100 (Abcam, #ab34710), rabbit anti-collagen IV at 1:500 (Abcam, #ab6586), and rabbit anti-laminin at 1:200 (Abcam, #ab11575). Sections were incubated with primary antibodies for 1 hr at room temperature, rinsed, and then incubated with secondary antibodies for 1 hr at 37 °C (goat anti-rabbit Alexa Fluor 488, Thermo Fisher, #A11034, diluted 1:300 in Sea Block). After secondary antibody incubation, sections were rinsed and mounted with Mowiol mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) to stain for cell nuclei. Slides were imaged on a Nikon A1 Confocal Laser Microscope System.

2.2.4. Sulfated Glycosaminoglycan Quantification—Sulfated glycosaminoglycan (sGAG) content was quantified using the 1,9-dimethyl-methylene blue (DMMB) dye assay. Tissue samples were digested with Proteinase K as described previously in section 2.2.2. DMMB dye solution was prepared by dissolving 8.0 mg DMMB dye (Sigma-Aldrich, #341088), 1.52 g glycine (Sigma-Aldrich, #G-8898), and 1.19 g NaCl (Fisher Scientific,

#S642) in 500 mL ultrapure water and adjusting the pH to 3.0. Samples were diluted as necessary in 1× Tris-EDTA buffer (Invitrogen, #T1493), and 20 µL of sample was mixed with 200 µL of DMMB dye solution in clear, 96-well plates. Absorbance was measured on a BioTek Cytation 3 Multi-Mode Reader at 530 nm (A_{530}). Samples were run in triplicate ($n = 3$) with technical replicates in duplicates, compared to a standard curve of chondroitin sulfate (Biocolor, #B1010), and normalized to dry weight of samples.

2.3. Preparation of Hydrogels from Decellularized Extracellular Matrix

Kidney dECM hydrogels were prepared similarly to previously described protocols for other decellularized tissues⁴³. Briefly, frozen kidney dECM was lyophilized for two days, snap frozen, and milled with a Thomas Wiley Mini-Mill Cutting Mill. Milled dECM was enzymatically digested at 10 mg/mL kidney dECM, 1 mg/mL pepsin (Sigma-Aldrich, #P7000), and 0.01 M HCl (Sigma-Aldrich, #H9892) for 48 hours. The resulting pepsin digest was aliquoted and frozen at -80°C . Aliquots were thawed overnight at 4°C as needed for experiments. Pre-gel solutions were prepared by neutralizing the pepsin digest with one-tenth the volume of 1.0 M NaOH (Sigma-Aldrich, #S2770), adding one-tenth of the total volume desired of 10× phosphate-buffered saline (PBS, Mediatech, #46-013-CM) to achieve a final concentration of 1×, and diluting the mixture with sterile MilliQ H₂O. The final hydrogel was formed after incubating the pre-gel solution at 37°C for at least one hour. Collagen I hydrogels were prepared similarly from purified porcine collagen I (Advanced BioMatrix, #5169-100ML) as control groups for experimental studies.

2.4. Rheological Characterization

Rheological characterization of hydrogels was performed following a protocol suggested for hydrogels for tissue engineering applications⁴⁴. Testing was performed using an Anton-Paar MCR 302 rheometer with a 25-mm parallel-plate fixture under strain-controlled conditions. The lower Peltier cell was set to 4°C for sample loading and then rapidly ramped to 37°C after lowering the measuring system. Mineral oil was applied to the edges of the fixture and the system was enclosed within a solvent trap to prevent sample dehydration. All samples were mixed fresh, and the pre-gel solution was loaded onto the instrument before testing. For frequency and strain sweeps, samples were incubated on the instrument stage at 37°C for 30-45 min. to allow hydrogel formation before testing. Final time sweeps were performed for 120 min. at 37°C , 0.1% strain, and 10 rad/s. All sweeps were run in triplicate ($n = 3$).

2.5. SEM Analysis

Hydrogels were fixed in 2% (v/v) glutaraldehyde (Sigma-Aldrich, #G5882) and 3% (m/v) sucrose (Sigma-Aldrich, #S7903) in MilliQ H₂O for one hour. Samples were dehydrated in a graded ethanol series (30-100%) of 15 min. intervals, critically-point dried with a Tousimis SAMDRI-790 Critical Point Dryer, sputter coated with 5 nm of Au with a Baltec MED-020 Coating System, and imaged on a JEOL Neoscope SEM.

2.6. Cell Culture

Human conditionally-immortalized GEnCs were cultured as described previously⁴¹. These are primary cells that have been transfected with a temperature-sensitive *SV40-T* antigen and the essential catalytic subunit of human telomerase (*hTERT*) to avoid replicative senescence⁴⁵. When cultured at the permissive temperature of 33 °C at which expression of these genes is active, the cells maintain an immature state and are able to proliferate. When thermoswitched to the non-permissive temperature of 37 °C, the cells become quiescent and adopt a more mature phenotype that is comparable to freshly isolated GEnCs. Conditionally-immortalized GEnCs were cultured in Endothelial Growth Medium 2 Microvascular (EGM-2 MV BulletKit, Lonza, #CC-3202) containing 5% fetal bovine serum (FBS) and growth factors as supplied with the exception of vascular endothelial growth factor (VEGF). Media supplements included: epidermal growth factor, R3-insulin-like growth factor-1, fibroblast growth factor β , ascorbic acid, hydrocortisone, and gentamicin and amphotericin-B. Media was exchanged every other day for GEnCs, and cells were used at passage 30 or below. GEnCs were cultured for at least five days after thermoswitching to the non-permissive temperature to ensure complete inactivation of the transgenes.

2.7. Cell Studies

2.7.1. Culture on Hydrogel Substrates—Pre-gel solutions were mixed, cast into well-plates, and incubated at 37 °C for one hour. Cells were then prepared for cell seeding: the cells were rinsed once with 1× Dulbecco's Phosphate-Buffered Saline (DPBS, Mediatech, #21-030), incubated with TrypLE Express (Gibco, #12605-028) for 5 min. at 33 °C, collected and counted, centrifuged, and resuspended at a concentration of 50,000 cells/mL. Cells were then seeded on top of hydrogel substrates and cultured for one week at 33 °C plus an additional five days at 37 °C.

2.7.2. Cell Encapsulation Within Hydrogels—Well-plates were coated with poly(2-hydroxyethylmethacrylate) (poly(2-HEMA); Sigma-Aldrich, #P3932) at least one day prior following established protocols to prevent cell attachment to the well-plate surface^{46,47}. Briefly, 1.2 g of poly(2-HEMA) was added to 95% (v/v) ethanol at 40-60 °C under constant stirring until dissolved and then sterile filtered through a polyethersulfone mesh with a pore size of 0.22 μ m (EMD Millipore, #SCGP00525). Sufficient volume of the poly(2-HEMA) solution was added to cover the well surface, and the solution was allowed to evaporate overnight in a biosafety cabinet. To encapsulate cells, cells were rinsed and trypsinized as described previously, then counted. The appropriate volume of cell suspension was aliquoted to obtain the necessary number of cells for encapsulation at a concentration of 1 million cells/mL. The cell suspension was then centrifuged, and pellets were resuspended in a minimal volume of complete media. The pre-gel solutions were then prepared and mixed, and the concentrated cell suspension was added and mixed in with the pre-gel solution to achieve the desired final concentration. The pre-gel solution with encapsulated cells was then cast into well-plates and incubated at 37 °C for at least one hour before complete media was added on top of hydrogels and plates were transferred to 33 °C.

2.8. Cell Viability and Proliferation

2.8.1. Cell Viability Imaging—Cell viability was assessed with the LIVE/DEAD Assay Viability/Cytotoxicity Kit for Mammalian Cells (Molecular Probes, #L3224) following the manufacturer's protocol. Briefly, samples were rinsed once with 1× DPBS and incubated with 4.0 μM ethidium homodimer and 2.0 μM calcein in 1× DPBS for 30 min. at 33 or 37 °C. Samples were rinsed once more with 1× DPBS and immediately imaged on a Nikon A1R+ Confocal Laser Microscope System.

2.8.2. Quantification of Cell Viability—Live/dead images were separated by channel and images were analyzed in MATLAB R2017a to quantify cell viability. Image pairs were loaded into the software and converted to grayscale, and objects in the images were counted by using a fast two-dimensional peak finder function available on the Mathworks File Exchange website (fastpeakfind.m) that locates local maxima in arrays after applying a threshold. Quantification of cell viability was calculated as follows:

$$\% \text{ viable cells} = \frac{\text{live cell count}}{(\text{live cell count}) + (\text{dead cell count})} * 100\%$$

Four independent samples ($n = 4$) were stained for each experimental group and time point, and three fields-of-view were imaged per sample for a total of twelve images per experimental group per time point.

2.8.3 Cell Proliferation—Samples were digested with Proteinase K digestion buffer as described previously in section 2.2.2. DNA was quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, #P7589) following the manufacturer's protocol and a dsDNA standard included in the kit to generate a standard curve. Samples for cell proliferation studies were run in quadruplicate ($n = 4$). Double-stranded DNA content was quantified from cell pellets of 50,000 cells as counted using the Trypan Blue (Gibco, #15250-061) exclusion method to determine the amount of dsDNA per cell ($n = 3$) to calculate the total cell number from dsDNA content. Proliferation is presented as the total cell number and cell number normalized to the number of cells initially seeded or encapsulated.

2.9. Histology

Samples were fixed in 10% neutral-buffered formalin (Thermo Scientific, #5701) for at least 48 hours before paraffin embedding, sectioning, and routine hematoxylin and eosin staining. After staining, coverslips were mounted with Cytoseal XYL (Thermo Scientific, #8312-4), and slides were imaged on a Zeiss Axioskop upright microscope with a CRI Nuance camera.

2.10. TEM Analysis

Samples were fixed in 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M cacodylate buffer, subsequently post-fixed with 1% osmium tetroxide for one hour then 1% uranyl acetate in maleate buffer for one hour, dehydrated in a graded ethanol and propylene oxide series, and embedded in Epon (polymerized at 60 °C for 48 hours). Ultrathin sections were visualized with a FEI Tecnai Spirit G2 TEM.

2.11. Gene Expression Analysis

RNA was isolated from samples and cell pellets using TRIzol Reagent (Invitrogen, #15596018) following the manufacturer's protocol, and RNA concentration was measured using a NanoDrop 1000 Spectrophotometer. Reverse transcription and cDNA synthesis was performed using the iScript Reverse Transcription Supermix for RT-qPCR kit (Bio-Rad, #170-8841) with an Applied Biosystems GeneAMP PCR System 9700 following the manufacturer's protocol. Quantitative PCR was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, #170-5270) and detected using an Applied Biosystems QuantStudio 7 Flex Real-Time PCR System. The thermal profile used was an initial polymerase activation step at 95 °C for 30 sec., followed by 40 amplification cycles of denaturation at 95 °C for 15 sec. and annealing and extension at 60 °C for 60 sec. The mRNA expression of each gene of interest was normalized to *cyclophilin A (PPIA)* expression, and the relative degree of gene amplification was calculated using the $2^{-\Delta\Delta Ct}$ method: $2^{[(Ct \text{ gene 2} - Ct \text{ PPIA 2}) - (Ct \text{ gene 1} - Ct \text{ PPIA 1})]}$. "Ct gene 1" represents the threshold cycle (Ct) of the target gene of the reference population, cells cultured on tissue culture polystyrene, and "Ct gene 2" the target gene of the sample of interest. Biological replicates were run in quadruplicate ($n = 4$) with technical replicates in triplicate.

2.12. Immunofluorescence Staining

Fixed and paraffin-embedded sections were processed as described previously in section 2.2.3. for immunofluorescence staining. Primary antibodies were diluted in Sea Block as follows: mouse anti-PECAM-1 at 1:100 (Abcam, #ab187377) and rabbit anti-collagen I at 1:100 (Abcam, #ab24710). Secondary antibodies were diluted in Sea Block as follows: goat anti-mouse Alexa Fluor 488 at 1:500 (Thermo Fisher, #A11029) and goat anti-rabbit Alexa Fluor 555 at 1:500 (Thermo Fisher, #A21429). Slides were mounted with Mowiol mounting medium containing DAPI to stain for cell nuclei and imaged on a Nikon A1 Confocal Laser Microscope System.

2.13. Statistical Analysis

All quantitative data is represented as the mean $\pm$ standard error of the mean. Statistical significance was determined using an unpaired two-tailed Student's *t* test assuming equal variance with Microsoft Excel (Microsoft). Significance for all statistical analyses was defined as $p < 0.05$.

III. Results

3.1. Kidney Decellularization

Minced kidney tissue began to lose color during the initial rinse step with deionized H₂O as blood was removed. After several hours in detergent, the edges of tissue pieces became white, and after two days, all kidney tissue acquired a blanched appearance (Figure 1). After decellularization, hematoxylin and eosin staining revealed removal of cell nuclei as evidenced by the lack of dark hematoxylin nucleic counterstain present, and glomerular and tubular ECM structures remained identifiable (Figure 2A). Quantification of the amount of double-stranded DNA present within native and decellularized tissues demonstrated that

98% of the DNA content (by dry weight) was removed during the decellularization process (Figure 2B). Immunofluorescence staining for key ECM proteins including collagen I, collagen IV, and laminin revealed the presence of these proteins in decellularized tissues, particularly in glomerular structures for collagen IV and laminin (Figure 2C). In addition, results from the DMMB dye assay indicated retention of approximately half of the sGAG content in decellularized tissues in comparison to native tissues when normalized to the lyophilized masses of the samples (Figure 2D).

3.2 Macroscopic and Ultrastructural Images of Hydrogels

Kidney dECM hydrogels formed at concentrations of 2.5 mg/mL and 5.0 mg/mL retained the cylindrical shape of the mold in which they were cast and were robust enough to be handled with a spatula. Kidney dECM hydrogels at 1.0 mg/mL and collagen I hydrogels 2.5 mg/mL concentrations retained the circular shape but did not meet the height of the original mold. The reduced volume of these hydrogels was likely due to the greater exclusion of water during hydrogel formation which necessitated careful handling of these fragile hydrogels (Figure 3A-D, insets). Ultrastructural imaging via scanning electron microscopy revealed fibrous morphologies of all hydrogels with randomly-oriented fibrils and interconnected pores (Figure 3A-D).

3.3 Rheological Characterization of Hydrogels

Rheological testing was performed to measure the change in shear moduli (G' , G'') over time and rate of gelation, and values are reported in Table 1. The gelation point is reported as the time at which the shear moduli are equal in value⁴⁴. The time to plateau is reported as the time at which the storage modulus (G') is approximately an order of magnitude larger than the loss modulus (G'') and both moduli have reached the plateau phase indicating that a stable hydrogel has formed. The shear moduli of all hydrogels (Figure 3E-H) were characterized by sigmoidal curves with rapid onset of gelation upon ramping the temperature up to 37 °C. Collagen I hydrogels (2.5 mg/mL, Figure 3E) presented the longest time to plateau at 25 min. with an equilibrium G' value of 15 Pa. The G' continued to increase to 60 Pa after 120 min. Kidney dECM hydrogels at 1.0 mg/mL (Figure 3F) formed stable hydrogels much more rapidly with the time to plateau being 3.5 min. and an equilibrium G' value of 2.0-7.5 Pa. Kidney dECM hydrogels at 2.5 mg/mL and 5.0 mg/mL (Figure 3G, 3H) formed stable hydrogels after just 3 min. with equilibrium G' values of 22-26 Pa and 110-130 Pa, respectively. To confirm formation of a hydrogel, samples were subject to frequency (Supplemental Figure 1A-D) and strain (Supplemental Figure 1E-H) sweeps. The G' of all samples was independent at frequencies of 25 rad/s and below, and the angular frequency value used for time sweeps was verified to be in the low-frequency plateau region for all samples. Strain sweeps demonstrated the presence of a linear viscoelastic region for all samples, confirming the formation of hydrogels, and the strain value used for time sweeps was within the linear viscoelastic region for all samples. Additionally, all samples exhibited decreasing G' values after approximately 70% strain with catastrophic failure occurring within the range of 300-500% strain.

3.4 Cell Viability and Proliferation on Hydrogel Substrates

Live/dead staining and image analysis revealed high viability (85-98%) of human conditionally-immortalized GEnCs cultured on collagen I hydrogels (2.5 mg/mL) and kidney dECM hydrogels (2.5 mg/mL) substrates at days 1 and 12 (Figure 4A,B). Quantification of DNA content of samples over time indicated proliferation of GEnCs cultured on both collagen I and kidney dECM hydrogels with cells increasing over six-fold in number during the seven-day culture period at the permissive temperature. Cell proliferation after thermoswitching and culture at the non-permissive temperature between days 7 and 12 decreased, as expected (Figure 4C).

3.5 Cellular Encapsulation Within Hydrogels

Live/dead staining and image analysis revealed high cell viability of GEnCs encapsulated within both collagen I hydrogels (2.5 mg/mL) and kidney dECM hydrogels (2.5 mg/mL) at days 1 (92-94%) and 12 (86-88%) (Figure 4D,E). Hydrogels encapsulating cells began to contract after three days in culture and resembled compacted disks after twelve days. Encapsulated GEnCs proliferated during the first seven days when cultured at the permissive temperature, approximately tripling in number for both experimental groups. The cell number was significantly greater ($p < 0.005$) for cells encapsulated in kidney dECM hydrogels than cells encapsulated in collagen I hydrogels on day 1 with 100,000 more cells present in kidney dECM hydrogels; however, there was no significant difference in cell number between the groups at time points beyond day 1 (Figure 4F). H&E staining of fixed and sectioned samples from days 7 (Supplemental Figure 2) and 12 (Figure 5) showed cell distribution throughout hydrogels and settling of cells during hydrogel formation at the bottom edge of samples. Samples from both groups demonstrated some cell spreading between days 7 and 12 but little presence of cell-cell contacts and no tube formation, which was also confirmed by TEM (Figure 5C,F).

3.6 Gene Expression & Immunofluorescence

Gene expression analysis of encapsulated GEnCs (Figure 6) revealed higher fold-change expression of cells encapsulated in collagen I hydrogels compared to cells encapsulated in kidney dECM hydrogels on days 1 and 7 for all of the genes analyzed except *ITGB1*. Genes analyzed are involved in cell-cell interactions (Figure 6A-C: *PECAM1*, *CDH5*, *ICAM2*), cell-matrix remodeling (Figure 6D: *MMP14*), signaling pathways (Figure 6E-H: *KDR*, *TIE1*, *TEK*, *PTPRB*), endothelial function (Figure 6I-L: *VWF*, *PLVAP*, *EHD3*, *EHD4*), and cell-matrix binding interactions (Figure 6M,N: *ITGA3*, *ITGB1*). Gene expression for both experimental groups was typically highest on day 12, with a few exceptions: *PLVAP*, *TIE1*, *ITGA3* and *ITGB1* for collagen I hydrogels and *ITGB1* for kidney dECM hydrogels. Immunofluorescence staining confirmed the expression of platelet endothelial cell adhesion molecule (PECAM-1 or CD31 encoded by *PECAM1*) by GEnCs encapsulated within both collagen I hydrogels and kidney dECM hydrogels on days 7 and 12 (Figure 7).

IV. Discussion

Decellularized ECM is a versatile material that has been utilized in a number of applications over the years ranging from biological wound dressings⁴⁸ to coatings for cell culture^{49,50}.

Although the native architecture of the dECM is lost during processing to form hydrogels, the complex tissue- and organ-specific biochemical signals of the native source are thought to be retained³⁰. As a result, hydrogels derived from dECM, as opposed to those derived from synthetic or purified natural sources, have inherent cell-interactive motifs and bioactive factors. We have demonstrated that porcine kidney dECM can be solubilized and processed to form a mechanically stable hydrogel that supports cell viability and proliferation. Kidney dECM hydrogels were evaluated by rheological characterization and *in vitro* studies were performed for comparison with purified porcine collagen I hydrogels.

The enzymatic digestion method adopted in this study allows for the solubilization of dECM material without additional purification steps that may result in the removal of desired biochemical components⁴³. The digestion occurs as pepsin, an enzyme that is active only at very acidic pH, cleaves collagen telopeptide bonds and disrupts collagen fibril aggregates⁵¹. Once the digest is neutralized, thus inactivating the enzyme, and brought to physiological temperature and salt concentration, the collagen and other matrix peptides self-assemble in an entropy-driven process^{51,52}. The resulting kidney dECM hydrogels possess a loosely organized fibrous organization with an interconnected pore structure as visualized under SEM (Figure 3A-D) that is comparable to collagen I hydrogels and various dECM hydrogels characterized by other groups³⁰.

Gelation kinetics and shear moduli (G' , G'') of kidney dECM hydrogels were analyzed by parallel-plate rheology. Time sweeps revealed that kidney dECM hydrogels at all the concentrations tested exhibited a decreased lag phase (time of nucleation) and an increased rate of self-assembly in comparison to collagen I hydrogels (Figure 3E-H). This observation agrees with previous time-lapse confocal reflection microscopy studies, which reinforces the idea that additional components present in dECM, such as proteoglycans and glycosaminoglycans, increase the polymerization kinetics of collagen self-assembly⁵³. Evaluation of steady-state G' values of kidney dECM hydrogels also revealed that increasing dECM concentration led to an increase in G' value or hydrogel stiffness. This observation agrees with rheological or mechanical characterization of various dECM hydrogels investigated by other groups³⁰, such as liver³⁶ and skeletal muscle³⁹.

Strain sweeps demonstrated that the hydrogels undergo a strain-stiffening behavior (Supplemental Figure 2E-H). This nonlinear elastic property is common among biological materials such as fibrin and is thought to prevent damage to tissues caused by large deformations⁵⁴. Kidney dECM hydrogels of all concentrations tested exhibited strain-stiffening behavior anywhere between 5-25% strain; however, strain stiffening was much more apparent in lower concentration kidney dECM hydrogels (1.0 and 2.5 mg/mL) as opposed to 5.0 mg/mL hydrogels. Collagen I hydrogels, on the other hand, exhibited strain-stiffening behavior between 25-70% strain. Strain-stiffening behavior is correlated with a material's network structure⁵⁵. For physically crosslinked networks, such as the hydrogels examined in this work, strain stiffening is dependent on the flexibility of the polymers or protein filaments. More flexible filaments result in networks that stiffen at higher strains whereas less flexible filaments result in networks that stiffen at lower strains⁵⁴. This suggests that the collagen fibrils present in the kidney dECM hydrogels are less flexible than those present in the purified collagen I hydrogels. This may be a result of the processing

procedure (*i.e.*, pepsin digestion) in combination with the presence of other ECM components that may alter collagen fibril stiffness and overall network structure.

The network structure and overall matrix stiffness act in concert with the matrix components to influence the response of cells exposed to the matrix microenvironment^{22,56–58}. Kidney dECM and collagen I hydrogels of similar concentration (2.5 mg/mL) exhibited similar G' values based on rheological characterization and were thus employed in cell studies to avoid introducing hydrogel stiffness as a confounding variable during analysis. The concentration for collagen I hydrogels was limited to a maximum of 2.5 mg/mL due to the low concentration of the material supplied by the vendor (a solution of 2.9 mg/mL).

Human conditionally-immortalized GEnCs were employed as a model cell type for investigating the cell response to interactions with kidney dECM hydrogels. This cell line is derived from endothelial cells isolated from kidney glomeruli. Unlike primary cells that are difficult to expand in culture and lose important phenotypic characteristics over time, the conditionally-immortalized GEnCs maintain the capacity to be expanded in culture when cultured at the permissive temperature, but when thermoswitched to the non-permissive temperature become quiescent and adopt a progressively mature phenotype. The *in vitro* cell studies presented here demonstrate that kidney dECM hydrogels may serve as a biocompatible substrate to support attachment, viability, and proliferation of human GEnCs with results similar to those of collagen I hydrogels (Figures 4A-C). In addition, because these hydrogels are formed under mild conditions and at physiological temperature, they are permissive for cell encapsulation. The cell encapsulation studies presented here demonstrate that human GEnCs exhibit high viability when encapsulated within kidney dECM hydrogels (2.5 mg/mL). However, as the cells began to exert contractile forces on the relatively weak mechanical properties of the collagen I and kidney dECM hydrogels in an effort to spread or migrate⁵⁹, the hydrogels contracted. Moreover, despite increasing gene expression over the twelve-day culture period of cell-cell interaction proteins *PECAM1*, *CDH5*, and *ICAM2* (Figure 6A-C) as well as *MMP14* (Figure 6D) involved in ECM breakdown⁶⁰, strong cell-cell interactions were not observed in H&E-stained sections (Figure 5).

In addition to the weak mechanical properties of the hydrogels, it is possible that the biochemical composition of the kidney dECM hydrogels developed in this investigation were not ideal for encapsulation of the GEnCs utilized. Biochemical characterization of decellularized kidney tissues presented in this work was performed, demonstrating retention of key ECM proteins such as collagen I, collagen IV, laminin, and sulfated GAGs. However, decellularized ECM hydrogels are complex and may contain any combination of proteins, cryptic peptides and bioactive motifs, sequestered growth factors, cytokines, chemokines, and even matrix-bound nanovesicles³⁰. Any of these additional bioactive components may present negative or undesired signaling cues to specific cell types. Alternatively, despite increasing gene expression of several growth factor receptors over the culture period, including *KDR*, *TEK*, *TIE1*, and *PTPRB* (Figures 6E-H), critical bioactive factors may be lost or rendered inactive during decellularization or hydrogel processing⁶¹, resulting in poor cell response. For example, VEGF was deliberately excluded from the culture media in these studies following previous investigations utilizing this conditionally-immortalized cell line^{41,62,63}; however, the lack of active and bioavailable VEGF may explain the deficiency

of cell-cell contacts formed. Future studies will aim to tune the hydrogel mechanical properties and composition by modifying the processing procedures to include covalent crosslinking methods and strategies to restore critical signaling cues thought to be lost.

The work presented here is the first to explore the use of kidney dECM hydrogels for direct encapsulation of GEnCs; however, this is not the first study to investigate the potential of kidney dECM hydrogels in general. O'Neill, *et al.* explored the regional specificity of kidney dECM in solubilized, hydrogel, and sheet forms on modulating mouse papilla-derived kidney stem cell growth and metabolism⁶⁴. The rheological or mechanical properties of the region-specific kidney dECM hydrogels were not investigated in the O'Neill, *et al.* study; however, the authors demonstrated that kidney stem cells adopted a significantly more proliferative and metabolically active phenotype when cultured in the presence of kidney dECM as opposed to heart or bladder dECM. Furthermore, they also demonstrated that kidney stem cells cultured in the presence of kidney papillae-derived dECM in any form resulted in lower cell proliferation, higher metabolic activity, and altered cell morphology in comparison to cortex- and medulla-derived dECM⁶⁴. It is thought that the renal papilla represents the native kidney stem cell niche^{65,66}, therefore, it follows that dECM derived from the papillae would maintain a stem cell phenotype as suggested by the results. Furthermore, the authors propose that cortical- and medullary-derived dECM may show potential in directing differentiation of kidney stem cells towards specific renal lineages⁶⁴.

Nagao, *et al.* examined the influence of human kidney cortex-derived dECM hydrogels on human kidney peritubular microvascular endothelial cell (HKMEC) phenotype⁶⁷. Proteomics analysis of the decellularized kidney cortex revealed that collagen IV was present in the highest abundance; however, rheological testing indicated that the complex modulus of the corresponding hydrogel at a concentration of 7.5 mg/mL only reached 15 Pa. This is much weaker than the 110-130 Pa measured for the kidney dECM hydrogels at a concentration of 5.0 mg/mL presented in this study. Nagao, *et al.* further demonstrated that HKMECs cultured on these hydrogel substrates exhibited a more quiescent and mature phenotype. The addition of collagen I to increase the hydrogel mechanical properties (up to 250 Pa) led to a more activated HKMEC state⁶⁷. The data presented by the authors coincides with *in vivo* evidence that an increased collagen I content in the kidney ECM is associated with fibrosclerosis and the development of chronic kidney disease⁶⁸⁻⁷⁰.

Most recently, Magno, *et al.* investigated the ability to utilize macromolecular crowding as a method for tailoring the fibrillar architecture of kidney dECM hydrogels⁷¹. The incorporation of increasing concentrations of a macromolecular crowder, Ficoll400, resulted in faster rates of fibrillogenesis as measured by turbidimetric analysis, generation of thicker fibrils and greater fibril alignment, and reduced hydrogel stiffness. Interestingly, this effect is the opposite of those previously observed for pure collagen I hydrogels where increasing concentrations of macromolecular crowders resulted in a decrease in fibril thickness and reduced fiber alignment⁷². In addition, the researchers found that the inclusion of Ficoll400 resulted in greater network formation by human umbilical vein endothelial cells (in the presence of VEGF) and morphogenesis of mouse kidney stem cells when cultured on kidney dECM hydrogels⁷¹.

Together, this and previous studies demonstrate that kidney-derived dECM hydrogels are able to support cell attachment, viability, and proliferation. Furthermore, region-specific kidney-derived dECM hydrogel substrates have already been shown to modulate cell response and either maintain an immature, stem cell-like phenotype⁶⁴ or a more mature, quiescent phenotype⁶⁷. Future studies may thoroughly investigate region-specific kidney-derived dECM composition or the ability to tailor such hydrogels with additives, the corresponding gelation kinetics, and the ultrastructure and rheological characteristics of the resulting hydrogel. Such investigations will continue to delineate some of the factors that influence the response of different cell types or stem cells directly encapsulated within tissue- and organ-specific 3D cell culture microenvironments.

V. Conclusion

Kidney dECM can be digested and processed to form hydrogels that retain biochemical signaling cues of the kidney dECM microenvironment. The kidney dECM hydrogels presented in this study exhibit fibrous ultrastructural networks similar to other dECM hydrogels previously presented by others but unique rheological characteristics from other tissue- and organ-derived dECM hydrogels and from purified collagen I hydrogels. These kidney dECM hydrogels may act as a substrate for cell culture or for encapsulation of cells as a 3D cell culture microenvironment. While only human glomerular endothelial cells were presented in this study, it is anticipated that the hydrogels may serve as a culture environment for any cell type, kidney-specific, stem cell, or otherwise with potential in developing a kidney tissue model for developmental studies and pharmaceutical screening applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Process to form kidney decellularized extracellular matrix hydrogels. The native kidney is minced into pieces approximately 0.5 cm × 0.5 cm × 0.25 cm in size and treated with 0.1% (w/v) sodium dodecyl sulfate to remove all of the cellular material. The remaining decellularized extracellular matrix is lyophilized and milled into a fine powder before being subjected to a pepsin digestion at acidic pH (0.01 M HCl) to disrupt collagen fibril aggregates and solubilize the material. To form a hydrogel, the pepsin digest is kept on ice, neutralized with 1.0 M NaOH, buffered to pH 7.4, and incubated at 37 °C for about an hour.

Figure 2.

Evaluation and characterization of decellularized kidney tissues. (A) Routine hematoxylin and eosin staining of native and decellularized kidney sections demonstrating removal of cellular nuclei (dark spots) and preservation of the ECM architecture, including glomeruli (*) and tubule structures. (B) Quantification of double-stranded DNA content in native and decellularized kidney tissue. DNA content was normalized to the dry weight of each sample, and data is presented as a percentage of native kidney tissue DNA content ($n = 3$). (C) Immunofluorescence staining of native and decellularized kidney sections demonstrating retention of collagen I, collagen IV, and laminin in decellularized tissues; glomerular structures indicated by (*). (D) Quantification of sulfated glycosaminoglycan content in

native and decellularized kidney tissue. sGAG content was normalized to the dry mass of each sample with samples run in triplicate ($n = 3$).

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Figure 3.

Scanning electron micrographs, macroscopic images (insets), and rheological characterization of collagen I and kidney dECM hydrogels. SEM and macroscopic images of (A) collagen I hydrogel at 2.5 mg/mL, (B) kidney dECM hydrogel at 1.0 mg/mL, (C) kidney dECM hydrogel at 2.5 mg/mL, and (D) kidney dECM hydrogel at 5.0 mg/mL. Rheological characterization of hydrogel formation over time with measured shear moduli plotted on a logarithmic scale of (E) collagen I hydrogels at 2.5 mg/mL, (F) kidney dECM hydrogels at

1.0 mg/mL, (G) kidney dECM hydrogels at 2.5 mg/mL, and (H) kidney dECM hydrogels at 5.0 mg/mL.

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Figure 4.

Viability and proliferation of human GEnCs cultured on collagen I hydrogels and kidney dECM hydrogel substrates (A-C) or encapsulated within hydrogels (D-F). (A) Live/dead staining and confocal imaging of GEnCs cultured on collagen I hydrogels or kidney dECM hydrogels at days 1 and 12. (B) Quantification of viability of cells cultured on top of hydrogel substrates presented as the percentage live cells from image analysis. (C) Quantification of cultured GEnC proliferation over twelve days using a Quant-iT PicoGreen dsDNA Assay Kit. Normalized values (right axis) were normalized to the number of cells seeded per sample on day 0. Samples initially seeded with 12,500 cells per sample (50,000 cells/mL). Number of independent measurements, $n = 4$. (D) Live/dead staining and confocal imaging of GEnCs encapsulated within collagen I hydrogels or kidney dECM hydrogels at days 1 and 12. (E) Quantification of viability of cells encapsulated within hydrogels presented as the percentage live cells from image analysis. (F) Quantification of encapsulated GEnC proliferation over twelve days using a Quant-iT PicoGreen dsDNA Assay Kit. Normalized values (right axis) were normalized to the number of cells encapsulated per sample on day 0. Samples initially seeded with 150,000 cells per sample (1 million cells/mL). Number of independent measurements, $n = 4$. *** $p < 0.005$.

Figure 5. H&E and TEM of GEnCs encapsulated within hydrogels after twelve days in culture. (A, B) H&E stained sections and (C) TEM micrograph of GEnCs encapsulated within collagen I hydrogels. (D, E) H&E stained sections and (F) TEM micrograph of GEnCs encapsulated within kidney dECM hydrogels.

Figure 6.

Gene expression of GEnCs encapsulated within hydrogels after 1, 7, and 12 days in culture. (A) *PECAM1* encoding for platelet endothelial cell adhesion molecule or CD31. (B) *CDH5* encoding for cadherin 5 or vascular endothelial cadherin. (C) *ICAM2* encoding for intercellular adhesion molecule 2 or CD102. (D) *MMP14* encoding for matrix metalloproteinase 14 or MT1-MMP. (E) *KDR* encoding for kinase insert domain receptor or vascular endothelial growth factor receptor 2. (F) *TEK* encoding for tyrosine kinase with immunoglobulin-like and EGF-like domains 2 or TIE2. (G) *TIE1* encoding for tyrosine

kinase with immunoglobulin-like and EGF-like domains 1 or TIE1. (H) *PTPRB* encoding for receptor-type tyrosine-protein phosphatase beta or VE-PTP. (I) *VWF* encoding for von Willebrand Factor. (J) *PLVAP* encoding for plasmalemma vesicle-associated protein. (K) *EHD3* encoding for Eps15 homology domain-containing protein 3. (L) *EHD4* encoding for EH domain-containing protein 4. (M) *ITGA3* encoding for integrin subunit alpha 3. (N) *ITGB1* encoding for integrin subunit beta 1. Values are expressed as fold-change in expression normalized to gene expression of GEnCs cultured on tissue culture polystyrene on day 0. Number of independent measurements, $n = 4$. Statistical significance denoted by: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Figure 7.

Immunofluorescence staining for PECAM-1 of GEnCs encapsulated in collagen I hydrogels or kidney dECM hydrogels after 7 or 12 days in culture. Merged images: DAPI (blue), PECAM-1 (green), and collagen type I (red).

Table 1

Rheological characterization values.

	Collagen I Hydrogels	Kidney dECM Hydrogels		
Concentration (mg/mL)	2.5	1.0	2.5	5.0
Gelation Point (min)	5.0	1.0	2.0	1.5
Time to Plateau Phase (min)	25.0	3.5	3.0	3.0
Equilibrium Storage Modulus, G' (Pa)	15.0-60.0	2.0-7.5	22.0-26.0	110.0-130.0
Average Strain Range of Strain Stiffening Behavior	25.0-70.0%	12.5-25.0%	5.0-15.0%	5.0-10.0%