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Hydrogels Derived from Central Nervous System Extracellular Matrix

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Abstract

Biologic scaffolds composed of extracellular matrix (ECM) are commonly used repair devices in preclinical and clinical settings; however the use of these scaffolds for peripheral and central nervous system (CNS) repair has been limited. Biologic scaffolds developed from brain and spinal cord tissue have recently been described, yet the conformation of the harvested ECM limits therapeutic utility. An injectable CNS-ECM derived hydrogel capable of in vivo polymerization and conformation to irregular lesion geometries may aid in tissue reconstruction efforts following complex neurologic trauma. The objectives of the present study were to develop hydrogel forms of brain and spinal cord ECM and compare the resulting biochemical composition, mechanical properties, and neurotrophic potential of a brain derived cell line to a non-CNS-ECM hydrogel, urinary bladder matrix. Results showed distinct differences between compositions of brain ECM, spinal cord ECM, and urinary bladder matrix. The rheologic modulus of spinal cord ECM hydrogel was greater than that of brain ECM and urinary bladder matrix. All ECMs increased the number of cells expressing neurites, but only brain ECM increased neurite length, suggesting a possible tissue-specific effect. All hydrogels promoted three-dimensional uni- or bi-polar neurite outgrowth following 7 days in culture. These results suggest that CNS-ECM hydrogels may provide supportive scaffolding to promote in vivo axonal repair.

1.0 Introduction

Biologic scaffolds composed of extracellular matrix (ECM) can facilitate the constructive remodeling of numerous tissues including esophagus [1, 2], lower urinary tract [3, 4], muscle and tendon [5, 6], and myocardium [7, 8], among others. Although the mechanisms by which ECM scaffolds promote a constructive and functional remodeling response are

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only partially understood, recruitment of endogenous multipotent progenitor cells [9, 10], modulation of the innate immune response [11, 12], scaffold degradation with the generation of bioactive molecular cues [13–15], and innervation [16] have all been shown to be important events in this process. The contribution of the innate three-dimensional ultrastructure, unique surface ligand distribution, or molecular composition to constructive, functional remodeling is largely unknown. However, hydrogel formulations of matrix scaffolds lack the native three dimensional ultrastructure of the source tissue but still possess *in vitro* and *in vivo* biologic activity [17–22], suggesting that the molecular composition of these materials is an active factor in remodeling events. There have also been reports that suggest tissue-specific biologic scaffold materials have properties that enhance greater site-appropriate phenotypic cell differentiation compared to ECM scaffolds derived from non-homologous tissue sources [23–26].

The use of biologic scaffold materials within either the central or peripheral nervous system has not been extensively investigated [27]. However, it has been shown that innervation of remodeled scaffold materials is an early event when such materials are placed in several different anatomic locations and represents a predictor of constructive and functional outcomes [16, 28, 29]. It has also been shown that innervation is a critical event in robust regenerative responses that occur in species such as the newt and axolotl [30–32]. Methods for the isolation of central nervous system (CNS) ECM have recently been described. The objectives of the present study were to develop a method to create hydrogel forms of brain and spinal cord ECM, examine the biomolecular composition and mechanical properties of the resulting hydrogels, and evaluate the *in vitro* neural cytocompatibility and neurotrophic potential of CNS-ECM hydrogels versus a hydrogel prepared from a non-CNS-ECM; specifically, porcine urinary bladder matrix.

2.0 Materials and Methods

2.1 Overview of Experimental Design

Following decellularization of porcine brain and spinal cord, the resulting brain and spinal cord ECM (B-ECM and SC-ECM, respectively) were solubilized. The ECM materials were analyzed for collagen and sulfated glycosaminoglycan content, ultrastructure, and hydrogel mechanical properties. A commonly used neural cell line for examining neurite extension, N1E-115 [33, 34], was used to identify the neurotrophic potential of ECM hydrogels in two- and three-dimensional culture. The results were compared to an ECM hydrogel manufactured from a non-CNS source, porcine urinary bladder matrix (UBM-ECM) [20].

2.2 ECM Biologic Scaffold Production

Porcine brain, spinal cord, and urinary bladder were obtained from market weight animals (Tissue Source, Lafayette, IN). Tissues were frozen immediately after harvesting at -80°C , thawed before use, and processed by tissue specific methods described previously (Table 1A) [23]. In brief, CNS tissue was agitated (spinal cord tissue at 200 rpm; brain tissue at 120 rpm unless otherwise stated) in the following decellularization baths: deionized water (16 h at 4°C ; 60 rpm), 0.02% trypsin/0.05% EDTA (60 min at 37°C ; 60RPM; Invitrogen Corp., Carlsbad, CA, USA), 3.0% Triton X-100 (60 min; Sigma-Aldrich Corp., St. Louis, MO, USA), 1.0 M sucrose (15 min; Fisher Scientific, Pittsburgh, PA, USA), water (15 min), 4.0% deoxycholate (60 min; Sigma), 0.1% peracetic acid (Rochester Midland Corp., Rochester, NY, USA) in 4.0% ethanol (v/v; 120 min), PBS (15 min; Fisher), deionized water (twice for 15 min each rinse), and PBS (15 min). Each bath was followed by rinsing the remaining tissue through a strainer with deionized water. Decellularized B-ECM and SC-ECM were lyophilized and stored dry until use.

UBM-ECM was prepared as previously described [35]. In brief, connective tissue was removed from the serosal surface of the bladder. The tunica serosa, tunica submucosa, and majority of the tunica muscularis mucosa were mechanically delaminated, which left the basement membrane and tunica propria intact. Luminal urothelial cells were dissociated from the basement membrane by soaking the UBM-ECM in deionized water. The UBM-ECM was then agitated in 0.1% peracetic acid in 4.0% ethanol (v/v; 120 min; 300 rpm) followed by a series of PBS and deionized water rinses and lyophilization.

2.3 ECM Digestion and Solubilization

Lyophilized and comminuted B-ECM (20 mesh), SC-ECM (20 mesh or hand cut), and UBM-ECM (20 mesh or hand cut; 400–1000 μ m largest particle dimension as measured by mesh diameter or ruler) were separately placed into a 0.01 N HCl solution containing 1mg/mL pepsin (Sigma) at a concentration of 10 mg ECM/mL and stirred at room temperature for 48h as previously described (Table 1B) [20]. After 48h, B-ECM, SC-ECM, and UBM-ECM were completely digested and formed a pre-gel solution (pH~2). The pre-gel ECM solution was brought to pH 7.4 using 0.01N NaOH and diluted to the desired volume/salt concentration using 10x and 1 x PBS. Pepsin is irreversibly inactivated at pH above 7.5 [36].

2.4 Collagen and sGAG Quantification

Collagen concentration of the pre-gel ECM solution was determined for samples from each production batch with the Sircol Assay Kit (Biocolor Ltd., UK) following the manufacturer's recommended protocol (n=4 in duplicate or triplicate). Sulfated glycosaminoglycan (sGAG) concentrations were determined using digested ECM at a concentration of 50 mg ECM/ml with 0.1 mg/ml proteinase K (Sigma) in buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA for 48–72 hours at 50°C) using the Blyscan Sulfated Glycosaminoglycan Assay Kit (Biocolor Ltd., UK) and following the manufacturer's recommended protocol (n=3 in duplicate or triplicate).

2.5 Scanning Electron Microscopy

Scanning electron microscopy was used to examine the surface topography of brain, spinal cord, and UBM-ECM hydrogels. Five hundred micron thick hydrogels were prepared and then fixed in cold 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) for 24 hours followed by three 30 minute washes in 1x PBS. Hydrogels were dehydrated in a graded series of alcohol (30, 50, 70, 90, 100% ethanol) for 30 minutes per wash, and then placed in 100% ethanol overnight at 4°C. Hydrogels were washed 3 additional times in 100% ethanol for 30 minutes each and critical point dried using a Leica EM CPD030 Critical Point Dryer (Leica Microsystems, Buffalo Grove, IL, USA) with carbon dioxide as the transitional medium. Hydrogels were then sputter-coated with a 4.5 nm thick gold/palladium alloy coating using a Sputter Coater 108 Auto (Cressington Scientific Instruments, UK) and imaged with a JEOL JSM6330f scanning electron microscope (JEOL, Peabody, MA, USA).

2.6 Turbidity Gelation Kinetics

Turbidimetric gelation kinetics were determined as previously described [37]. The pre-gel solution was diluted to 6mg/mL and maintained on ice at 4°C until placed into a 96 well plate (100 μ L/well). The plate was immediately transferred to a spectrophotometer (Molecular Devices) preheated to 37°C, and absorbance was measured at 405nm every 2 minutes for 50 minutes. Normalized absorbance was calculated using equation 1 and then used to calculate the following parameters: time required to reach 50% and 95% maximum absorbance was denoted as $t_{1/2}$ and t_{95} , respectively, the lag phase, t_{lag} , calculated by extrapolating the linear portion of the curve, and the turbidimetric speed, S , of gelation was

determined by calculating the growth portion slope of the curve normalized to absorbance [20]. The assay was repeated three times with independent samples in triplicate.

$$\text{Normalized Absorbance} = \frac{A - A_o}{A_{max} - A_o} \quad \text{Equation 1}$$

2.7 Rheological Measurements

The pH of the ECM digest was neutralized to 7.4 and diluted to 4, 6, or 8mg ECM/ml. The diluted pre-gel solution was then placed on a 40mm parallel plate rheometer (AR 2000, TA Instruments) at 1Pa stress and 10°C to ensure even distribution and the liquidity of the pre-gel solutions between the plates. A dynamic time sweep was run with the parameters of 5% strain (with the exception of spinal cord ECM gel at 8mg/mL, which was run with 0.5% strain), 1 rad/s (0.159 Hz) and rapidly increasing temperature from 10°C to 37°C to induce gelation as indicated by a sharp increase and plateauing of the storage modulus (G'), and the loss modulus (G'') (n=3 per gel per concentration).

2.8 N1E-115 ECM Cytocompatibility and Two Dimensional Neurite Extension

N1E-115 mouse neuroblastoma cells (ATCC No. CRL 2263), a commonly used experimental cell line to examine neurotrophic potential and differentiation [33, 34], were cultured in DMEM (Sigma) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham MA, USA)/1% pen/strep (Sigma) at a concentration of 100,000 cells per well in 12 well plate prior to the addition of ECM. B-ECM, SC-ECM, or UBM-ECM digest was added after cell attachment at a concentration of 100µg ECM/mL. Following 18–24 hrs in culture with ECM, the medium was removed and 4µM calcein-AM and 4µM ethidium homodimer-1 was added to each well to evaluate cytotoxicity. Live cells that hydrolyze membrane-permeable calcein-AM, but not ethidium homodimer-1, fluoresce in green and dead cells that bind and activate ethidium homodimer-1, but not calcein-AM, fluoresce in red.

The effects of the B-ECM, SC-ECM, and UBM-ECM pre-gel digest upon N1E-115 neurite outgrowth were independently evaluated and used as an indicator of neurotrophic potential of the remaining bioactive molecules in ECM materials after enzymatic degradation. N1E-115 cells in DMEM with 10% FBS/1%pen strep were seeded at a density of 5,000 cells/well in a 24 well plate. After incubation for 24h, the media was removed and replaced with DMEM with 2.5% FBS, which promoted low levels of neurite extension and allowed changes in response to ECM to be quantified. Neutralized ECM pre-gel solutions at concentrations of 10µg/mL and 100µg/mL were added after cell attachment and cells were incubated for 48h. N1E-115 cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature. Attached cells were stained with DAPI for nuclei and Alexa Fluor phalloidin 488 (Invitrogen) for F-actin filaments. Three images at 200x magnification were taken per well. The number of cells with neurite extensions were manually counted. The longest neurite of each cell was measured in pixels using ImageJ (NIH). The outgrowth assay was repeated six times per condition. Neurites were denoted as cell processes that extended a minimum length of twice the diameter of the cell body. Image stacks were imported into Imaris software (Bitplane, South Windsor CN, USA) for 3-D visualization

2.9 Neurite Extension in Three Dimensional Culture

N1E-115 cells were maintained in 10% FBS DMEM media at 37°C. B-ECM, SC-ECM, or UBM-ECM hydrogels (1ml) were cast with a cell density of 500,000 cells/hydrogel and a concentration of 6mg ECM/ml and after gelation for 1hr at 37°C in a non-humidified

incubator. All hydrogels were then cultured for 24 hours in DMEM supplemented with 10% FBS/1% pen/strep. Serum concentration was reduced to 0% FBS and cells cultured for an additional 2 or 7 days. The hydrogels were then fixed with 4% paraformaldehyde (Fisher), stained for F-actin, and imaged using multiphoton confocal microscopy to visualize three-dimensional cell morphology inside the hydrogel scaffolds.

Hydrogels cultured with N1E-115 cells were stained with 0.1% Alexa Fluor 488 Phalloidin (Invitrogen) for 2 hours and submerged in PBS solution in a hanging drop slide and coverslipped. To visualize neurite outgrowth in three dimensions, the slide was mounted beneath an Olympus FV1000 multiphoton system. The system was equipped with a Chameleon ultra-diode-pumped laser, and a 25x XL Plan N objective with a N.A. of 1.05 and a field of view of 500 μm . The excitation wavelength was chosen at 830 nm at a 6% laser transmissivity. The sampling speed was set to 2 $\mu\text{s}/\text{pixel}$ with a 2 line kalman filter, and the scanning had an incremental z-step of 1 μm . Image stacks were then compiled into a maximum intensity z-projection in ImageJ.

2.10 Statistical Analysis

An independent Student's t-test was used to compare the effect of ECM pre-gel digests on N1E-115 cell differentiation to the effect of the no ECM control ($p < 0.05$). A one-way ANOVA was used for all other comparisons ($p < 0.05$). All statistical analysis methods used SPSS Statistical Analysis Software (SPSS, IBM, Chicago, IL, USA).

3.0 Results

3.1 Collagen and sGAG Quantification

Collagen concentration of B-ECM was $537.5 \pm 26.9 \mu\text{g}$ collagen/mg dry weight, which was less than SC-ECM and UBM-ECM at 703.2 ± 47.3 and $702.5 \pm 113.5 \mu\text{g}$ collagen/mg dry weight, respectively ($p < 0.01$) (Fig. 1A). B-ECM and UBM-ECM had a higher sGAG concentration, 5.1 ± 1.4 ($p < 0.009$) and 4.4 ± 0.4 ($p < 0.02$) μg sGAG/mg dry weight, respectively, compared to SC-ECM, which was $1.3 \pm 0.9 \mu\text{g}$ sGAG/mg dry weight (Fig. 1B).

3.2 Qualitative Assessment

B-ECM, SC-ECM, and UBM-ECM pre-gel solutions polymerized to form a hydrogel at physiologic pH (7.4) and temperature (37°C). Qualitatively, SC-ECM hydrogels were more rigid than B-ECM and UBM-ECM hydrogels (Fig. 2A–C). SEM micrographs showed dense, moderately organized collagen fibrils in B-ECM, SC-ECM, and UBM-ECM hydrogels (Fig. 2D–I). B-ECM contained the thickest fibrils (Fig. 2G), while SC-ECM hydrogels contained the most dense arrangement of fibrils (Fig. 2H). SC-ECM and UBM-ECM hydrogels contained moderately organized collagen fibers, while B-ECM contained dense clusters of randomly distributed collagen fibers.

3.3 Turbidimetric Gelation Kinetics

The gelation kinetics between hydrogel forms of B-ECM, SC-ECM, and UBM-ECM were evaluated using a normalized absorbance (Fig. 3A) to define the lag phase, times to reach half and 95% of the final turbidity, and speed to reach complete gelation using turbidimetric gelation kinetics as previously described (Table 2) [20, 37]. Turbidimetric gelation kinetics showed a sigmoidal shape for SC-ECM and UBM-ECM hydrogels, whereas B-ECM showed an exponential shape (Fig. 3B). Differences observed in kinetic curve shapes translated to a longer lag phase (t_{lag} ; Fig. 3C) for UBM-ECM hydrogels (7.2 ± 1.4 min) than B-ECM (28 ± 4.2 min) or SC-ECM hydrogels (3.6 ± 5.6 min). The time required to reach half of the final turbidity ($t_{1/2}$; Fig. 3D) was also longer for UBM-ECM (11.0 ± 5.8 min.)

than B-ECM (8.9 ± 5.4 min) or SC-ECM hydrogels (8.2 ± 5.1 min.). The time required to reach 95% of the final turbidity (t_{95} ; Fig. 3E) was also longer for UBM-ECM hydrogels (21.6 ± 2.9 min) compared to B-ECM (15.1 ± 5.5 min) and SC-ECM hydrogels (12.2 ± 4.6 min). The velocity to complete gelation (S ; Fig. 3F) was faster for SC-ECM hydrogels ($0.11 \pm 0.02 \text{ min}^{-1}$) compared to B-ECM ($0.08 \pm 0.01 \text{ min}^{-1}$) and UBM-ECM ($0.07 \pm 0.02 \text{ min}^{-1}$) ($p < 0.05$). These results suggest that during hydrogel assembly, SC-ECM hydrogels reach the steady state plateau faster than B-ECM or UBM-ECM hydrogels.

3.4 Rheologic Measurements

The storage modulus (G') and the loss modulus (G'') for B-ECM, SC-ECM, and UBM-ECM hydrogels changed over time as the sample temperature increased rapidly from 10°C to 37°C . Sigmoidal storage and loss moduli curves showed increased maximum storage modulus, maximum loss modulus, and time to complete gelation as concentration increased (Fig. 4A-C). SC-ECM hydrogels had the largest storage modulus at all hydrogel concentrations (Fig. 4D). At 8mg/mL , the storage modulus for SC-ECM, 757.0 ± 74.8 Pa, was greater than both UBM-ECM and B-ECM ($p < 0.05$), which showed storage moduli of 143.8 ± 84.1 Pa and 61.8 ± 11.0 Pa, respectively. While B-ECM hydrogels have lower storage moduli than SC-ECM and UBM-ECM hydrogels, B-ECM hydrogels at a concentration of 6mg/mL reached the steady state plateau in 2.4 ± 1.3 min.; a time that was faster than UBM-ECM and SC-ECM, which had times of 8.5 ± 1.7 min. and 7.0 ± 3.6 min., respectively. Table 2 summarizes the storage modulus, loss modulus, and time to complete gelation for each ECM hydrogel at concentrations of 4, 6, and 8mg/mL .

3.5 N1E-115 ECM Cytocompatibility and Two Dimensional Neurite Extension

The live/dead assay showed all ECMs to be non-cytotoxic for N1E-115 cells (Fig. S1). B-ECM, SC-ECM, and UBM-ECM pre-gel solutions at concentrations of 10 and $100\mu\text{g ECM/mL}$ increased the number of cells extending neurites (differentiated cells) compared to cells cultured without ECM digest. At $100\mu\text{g ECM/mL}$, SC-ECM digest promoted the highest percentage of differentiation, with $69.7 \pm 14.0\%$ of the cells extending neurites, whereas UBM-ECM and B-ECM promoted, $57.4 \pm 12.1\%$ and $54.3 \pm 11.7\%$ respectively. At $10\mu\text{g}$ and $100\mu\text{g ECM/mL}$, B-ECM, SC-ECM, and UBM-ECM increased the percentage of cells with neurite extensions compared to the buffered control; however B-ECM was the only scaffold that showed an increase in neurite extension length for both 10 and $100\mu\text{g ECM/mL}$ compared to cells cultured without ECM (Fig. 5).

3.6 Neurite Extension in Three Dimensional Culture

B-ECM, SC-ECM, and UBM-ECM supported the formation of three-dimensional neurite extensions at 2 and 7 days following removal of serum (Fig. 6). N1E-115 cells seeded in B-ECM hydrogels promoted a short arborizing growth pattern at two days, while SC-ECM and UBM-ECM hydrogels induce unipolar extensions. By 7-days, all ECM hydrogels promoted unipolar or bipolar extensions (Fig. 6).

4.0 Discussion

The present study shows that biologic scaffolds derived from porcine brain and spinal cord can be processed to form hydrogels that retain selected ECM-specific constituents. At comparable ECM concentrations, these hydrogel forms of CNS-ECM have distinctive composition and biomechanical properties. Furthermore, CNS-ECM hydrogels are cytocompatible, promote N1E-115 cell differentiation, and support three-dimensional neurite extension.

The mechanical properties of SC-ECM hydrogels are similar to those previously shown to support neuronal differentiation of stem cells (0.1–1kPa) [38]. It is therefore plausible that the rheologic and turbidimetric properties of CNS-ECM hydrogels could be used to influence the differentiation of endogenous or therapeutically administered stem cells following CNS injury [38]. The gelation kinetics and storage moduli of CNS-ECM hydrogels can be manipulated to a certain extent by varying ECM concentrations, such that following polymerization a hydrogel could be made with tailored *in vivo* pre-polymerization lag time, final storage modulus, and rate of polymerization [39]. Altering these parameters may enhance not only the ability of the gels to modulate stem cell behaviors, but also the cell and drug delivery properties of CNS-ECM hydrogels [40, 41].

It is logical that the distinctive collagen and sGAG concentrations play a role in ECM hydrogel gelation and rheologic properties; therefore, quantification of the collagen and sGAG concentrations provides valuable insight. Furthermore, the collagen monomers aggregate and self-assemble into thin filaments that can then crosslink into collagen fibers that interweave with themselves and other ECM components to contribute to hydrogel formation [42]. The shorter polymerization time and smaller storage modulus of B-ECM hydrogel compared to SC-ECM hydrogel may result from the higher sGAG concentration found in B-ECM hydrogels. Concentration of sGAGs has been shown to alter gelation kinetics and mechanical properties of hydrogels [43, 44]. While UBM-ECM hydrogel has a similar concentration of sGAGs compared to B-ECM hydrogel, the increased storage modulus could be due to a possible increased ratio of collagen I to collagen III in UBM-ECM hydrogel [45, 46]. The increased storage modulus of SC-ECM hydrogel compared to UBM-ECM hydrogel may result from relatively low sGAG concentration found in SC-ECM. Although collagen composition of B-ECM contains approximately 50% collagen, native brain contains very limited amounts of ECM components including collagen [47]. It should be noted that the percent collagen described herein represents a percentage of the isolated ECM, rather than a percentage of the mass of the intact brain prior to decellularization. While collagen and sGAGs affect hydrogel mechanical properties, further studies are needed to determine those additional molecules present in the ECM hydrogels that may contribute to polymerization.

Both composition and material properties of the CNS derived hydrogels are distinct from a hydrogel derived from a non-CNS tissue source (UBM-ECM) [20]. The unique ECM composition of different organs [48, 49], suggests that ECM scaffolds derived from the same tissue type as that of the injury site may contain bioactive components uniquely able to induce constructive remodeling of that tissue type. An array of proteins and peptides will be generated during ECM solubilization [9, 20] that is reflective of the molecular profile of the organ from which the ECM is derived. Thus, a hydrogel derived from solubilized and digested CNS-ECM will contain a unique composition of molecular constituents resembling those found within the ECM of healthy CNS tissue. The bioactive factors retained in the ECM hydrogels in the present study are neurotrophic as evident by the formation of N1E-115 cell neurite extensions when cultured in the presence of ECM digests. In addition, the present study shows that B-ECM hydrogels increase the length of N1E-115 neurite extensions in two-dimensional culture. This effect is not seen in UBM-ECM or SC-ECM digest, a possible indication of a tissue-specific effect of B-ECM upon these brain derived cells [33, 50].

5.0 Conclusions

B-ECM and SC-ECM hydrogels, while derived by similar decellularization methods from their source tissue, each have a unique biochemical composition, mechanical properties, and neurotrophic potential. The increase in neurite length for N1E-115 cells in response to B-

ECM suggests a tissue specific effect of B-ECM hydrogels on a brain derived cell line. Each ECM elicited unique cell responses as demonstrated by neurotrophic potential in their solubilized form and support of considerable three-dimensional neurite growth and extension in their re-polymerized hydrogel forms; this finding suggests that the molecular constituents of the source ECM play an important role in the bioactivity of these scaffolds. Support of three-dimensional neurite extension by CNS-ECM hydrogels also suggests the possibility that these hydrogels provide the scaffolding necessary to promote in vivo axonal repair and warrants further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Collagen and sGAG composition in B-ECM and SC-ECM scaffolds. (A) SC-ECM contains a significantly higher percentage of collagen than B-ECM. (B) B-ECM contains a significantly higher concentration of sGAGs than SC-ECM.

Figure 2. B-ECM, SC-ECM, and UBM-ECM Hydrogels and their respective Scanning Electron Micrographs (6mg/mL). (A) B-ECM hydrogel; (B) SC-ECM hydrogel; (C) UBM-ECM hydrogel; (D) B-ECM 1000x; (E) SC-ECM 1000x; (F) UBM-ECM 1000x; (G) B-ECM 10000x; (H) SC-ECM 10000x; (I) UBM-ECM 10000x.

Figure 3. B-ECM and SC-ECM representative turbidimetric and normalized turbidimetric curves. (A) Example absorbance curve and the metrics analyzed. (B) Normalized absorbance for B-ECM (diamonds), SC-ECM (squares), and UBM-ECM (triangles). (C) Lag time comparison for B-ECM, SC-ECM, and UBM-ECM. (D) Time to 50% complete gelation for B-ECM, SC-ECM, and UBM-ECM. (E) Time to 95% complete gelation for B-ECM, SC-ECM, and UBM-ECM. (F) Velocity to complete gelation for B-ECM, SC-ECM, and UBM-ECM following the lag time.

Figure 4.

Representative rheologic gelation kinetics for B-ECM and SC-ECM hydrogels (4, 6, and 8 mg/mL). G' represents the storage modulus and G'' represents the loss modulus. SC-ECM is significantly stiffer than B-ECM at each hydrogel concentration. (A) Storage modulus time sweep for B-ECM, SC-ECM, and UBM-ECM at 8mg/mL. (C) Storage modulus time sweep for B-ECM, SC-ECM, and UBM-ECM at 6mg/mL. (C) Storage modulus time sweep for B-ECM, SC-ECM, and UBM-ECM at 4mg/mL. (D) Final storage modulus for B-ECM, SC-ECM, and UBM-ECM at each concentration. * Indicates statistical significance of $p < 0.05$.

Figure 5. B-ECM and SC-ECM pre-gel solutions increase the number of cells with neurite extensions. All Scaffolds show a dose dependent increase for the number of cells with neurite extension, while only B-ECM shows a dose dependent increase in neurite length with increasing concentrations of ECM. * Indicates statistical significance of $p < 0.05$.

Figure 6.

Neurite Extension in B-ECM, SC-ECM, and UBM-ECM in 6mg/mL hydrogels in a 3-D cube or compressed Z-stack. (A) N1E-115 cell extension following 7 days culture in B-ECM in 3-D cube. (B) N1E-115 cell extension following 7 days culture in SC-ECM in 3-D cube. (C) N1E-115 cell extension following 7 days culture in UBM-ECM in 3-D cube. The compressed Z-stack shows B-ECM has short arborizing extensions at two days, while SC-ECM and UBM-ECM have uni- or bi-polar extensions. At 7 days all ECM hydrogels support uni- or bi-polar N1E-115 cell extensions. For 3-D cubes the major tick mark represents 50 μ m and the minor tick mark represents 10 μ m. For the Z-stacks the scale bar represents 50 μ m.

Table 1

Methods for Decellularization and Solubilizing B-ECM, SC-ECM, and UBM-ECM.

A. Decellularization Methods		
Step	B-ECM and SC-ECM (120 and 180 RPM respectively)	UBM-ECM (300 RPM)
1.	Deionized Water Soak (18–24 hours)	Mechanical Delamination
2.	0.025% Trypsin (1 hour)	
3.	3% Triton X 100 (1 hour)	
4.	1M Sucrose (30min.)	
5.	Deionized Water Soak (30min.)	
6.	4% Deoxycholic Acid (1 hour)	
7.	0.01% Peracetic Acid	

B. Methods for Solubilizing and Digesting			
	<i>B-ECM</i>	<i>SC-ECM</i>	<i>UBM-ECM</i>
<i>Particle Size</i>	<400um	400–1000um	
<i>Solubilization</i>	0.01N HCl		
<i>Digestion</i>	1mg/mL Pepsin		

Table 2

Summary of Rheologic and Turbidity Values for B-ECM, SC-ECM, and UBM-ECM.

Rheology	B-ECM (Average (STDEV))			SC-ECM (Average (STDEV))			UBM-ECM (Average (STDEV))		
	4mg	6mg	8mg	4mg	6mg	8mg	4mg	6mg	8mg
Storage Modulus (G' ; Pa)	20.3 (16.0)	49.9 (16.8)	61.8 (11.0)	138.5* (33.8)	235.5 (63.1)	757.0* (74.9)	11.43 (4.9)	72.78 (2.2)	143.8 (84.1)
Loss Modulus (G'' ; Pa)	2.6 (1.9)	9.4 (4.6)	10.2 (1.8)	16.3* (4.9)	37.5* (11.4)	93.6* (10.9)	1.4 (0.6)	10.14 (0.5)	19.3 (12.3)
Complete gelation (Time; min)	34.8 (28.9)	2.4 (1.3)	8.3 (2.8)	11.7 (5.63)	7.0 (3.6)	28.97 (4.7)	52.5 (2.2)	8.47 (1.7)	19.8 (19.1)

Turbidity	B-ECM (Average (STDEV))		SC-ECM (Average (STDEV))		UBM-ECM (Average (STDEV))	
	6mg		6mg		6mg	
Speed (S; min ⁻¹)	0.8 (0.1)		0.11 (0.02)		0.07 (0.02)	
50% gelation ($t_{1/2}$; min)	8.9 (5.4)		8.2 (5.1)		11.0 (5.8)	
95% gelation (t_{95} ; min)	15.1 (5.5)		12.2 (4.6)		21.6 (2.9)	
Lag Phase (t_{lag} ; min)	2.8 (4.2)		3.6 (5.6)		7.2 (1.4)	

* Indicates statistical significance at $p < 0.05$ when one group is statistically different from the other two.