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Influence of residual composition on the structure and properties of extracellular matrix derived hydrogels

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ABSTRACT

In this work, hydrolysates of extracellular matrix (hECM) were obtained from rat tail tendon (TR), bovine Achilles tendon (TAB), porcine small intestinal submucosa (SIS) and bovine pericardium (PB), and they were polymerized to generate ECM hydrogels. The composition of hECM was evaluated by quantifying the content of sulphated glycosaminoglycans (sGAG), fibronectin and laminin. The polymerization process, structure, physicochemical properties, *in vitro* degradation and biocompatibility were studied and related to their composition. The results indicated that the hECM derived from SIS and PB were significantly richer in sGAG, fibronectin and laminin, than those derived from TAB and TR. These differences in hECM composition influenced the polymerization and the structural characteristics of the fibrillar gel network. Consequently, the swelling, mechanics and degradation of the hydrogels showed a direct relationship with the remaining composition. Moreover, the cytocompatibility and the secretion of transforming growth factor beta-1 (TGF-β1) by macrophages were enhanced in hydrogels with the highest residual content of ECM biomolecules. The results of this work evidenced the role of the ECM molecules remaining after both decellularization and hydrolysis steps to produce tissue derived hydrogels with structure and properties tailored to enhance their performance in tissue engineering and regenerative medicine applications.

1. Introduction

The extracellular matrix (ECM) is the non-cellular component present within all tissues and organs that provides not only essential physical scaffolding for the cellular constituents but also initiates crucial biochemical and biomechanical cues, which are required for tissue morphogenesis, differentiation and homeostasis [1–2]. This matrix is composed of a variety of proteins and polysaccharides that are locally secreted and assembled into an organized network in close association with the surface of the cell that produced them [3]. Several studies have reported the preparation of hECM based on porcine dermis [4], bovine pericardium [5], porcine urinary bladder [6] and rat tail tendon [7] to mimic the structure and function of the ECM *ex vivo*. The polymerization of the hECM has allowed to develop biomedical hydrogels, which have been commonly used to create three-dimensional (3D) culture systems, to investigate the cell-ECM interactions and recently to build scaffolds for tissue engineering (TE) [5,8–9]. In this respect, several advances have been made in the hydrogel design to deliver cells and biomolecules. This has allowed to get a more effectively harness of the cell–material interactions in regulation of cell fate and functions, and to modulate the environment of both normal and injured/diseased tissues towards regeneration [10].

Ideally, the hECM and their derivatives would retain the bioactivity associated with the ECM, and thus, the appropriate preparation and characterization of hECM will avoid purification steps that could remove the biomolecules present in the native ECM. Moreover, the choice of raw material tissue and the standardization of the hECM preparation could expand the biomedical utility of the ECM hydrogels. It is widely accepted that biomimetic hydrogels should include cell induction ligands such as growth factors and other biomolecules that can be delivered from injectable hydrogel systems [10]. The *in situ* gelation of hECM could be explored as a method that deliver definite and precise signals in an appropriate spatial and temporal manner [10–11].

To study the influence of the ECM composition on the properties of biomedical hydrogels, we started from the hypothesis that the tissue source and hECM composition could determine the polymerization kinetics (hydrogel formation), the structure and properties of the hydrogels obtained using hECM. With this in mind, four animal tissues

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with differences in native composition in their (decellularized) lyophilized state were chosen, i.e., rat tail tendon (TR), bovine Achilles tendon (TAB), porcine small intestine submucosa (SIS) and bovine pericardium (PB). PB and SIS can be considered as ECM biomolecules-rich tissues [12–14], such as sulphated glycosaminoglycans (sGAG), fibronectin and laminin. Albeit it is recognized that TAB and TR have as a main component the type I collagen [15]. The hECM were obtained from the decellularized tissues using acid hydrolysis assisted by pepsin. The hECM compositions, the polymerization kinetics, viscoelastic properties, degradation, swelling, microstructure and *in vitro* biocompatibility were also evaluated. Ultimately, these properties were related to the hECM source and their composition. This work represents an essential stage in the design and standardization of hECM formulations that could enhance the biomedical applications of ECM hydrogels.

2. Experimental section

2.1. Materials

The PB, SIS and TAB tissues were acquired in local slaughterhouses. The TR were obtained from the University of Guanajuato. Pepsin, type I collagenase, ethylenediaminetetraacetic acid (EDTA), 3-(4,5-dimethyl thiazol yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2-dihydroxy-1,3-indanedione (ninhydrin), t-octylphenoxypolyethoxyethanol (Triton X-100) and other salts were purchased from *Sigma-Aldrich*. The LIVE/DEAD® kit to assess viability/cytotoxicity and tissue extraction reagent I were purchased from *Invitrogen*. Human laminin and fibronectin, and human/mouse TGF beta-1 ELISA kits were purchase from eBioscience.

2.2. Decellularization of the tissues

Approximately 4 g of tendons were excised from two adult Wistar rat tails and from one bovine leg. The TR and TAB tissue specimens were washed with fresh water to remove blood and surrounding muscular tissue. Approximately 5 g of SIS or PB were obtained from six 3 × 15 cm specimens from one intestinal tissue segment or from one 4 × 7 cm specimen from one pericardial sac. The SIS and TB tissue specimens were washed with fresh water to remove blood and surrounding muscular/fat tissue. All four tissue specimens were treated as follows: firstly, they were washed (3×, PBS). Then, they were left for 2 h in 10 mL of absolute ethanol under orbital stirring (OS), followed by a washing with 50 mL of 1% Triton ×100 (m/v) and 0.5% (m/v) EDTA (1 h for TR, and 18 h for TAB, SIS or PB; room temperature/RT, OS). After, the tissue specimens were rinsed (3×, 30 min, 50 mL of PBS). Subsequently, 50 mL of 10 mM Tris-HCl containing 0.5% (m/v) EDTA, 2.5 mg mL^{-1} RNase and 0.05 mg mL^{-1} DNase was added to the tissues (1 h for TR, 6 h for TAB, and 24 h for SIS or PB; RT, OS). Finally, the tissues were rinsed (30 min, 3×, PBS, RT, OS).

2.3. Hydrolysis of the tissues

The decellularized SIS, PB and TAB tissues were hydrolysed in 0.01 M HCl solution containing pepsin (1 mg mL^{-1}) (48 h, RT, OS). The hydrolysis of TR was carried out without pepsin. After that, all four hECM were stirred during additional 48 h at 4 °C to reach a complete tissue hydrolysis. The total protein concentration was determined by the BCA assay (bicinchoninic acid, *ThermoScientific*). The hECM solutions with 6 mg mL^{-1} of total protein were stored at 4 °C.

2.4. Biochemical composition analysis of hECM

The composition analysis was carried out in either hydrolysates or extracts from decellularized or native tissue samples, respectively. To evaluate the composition of the native tissue samples, components were extracted from trimmed samples with the Tissue Extraction Reagent I (TER; Invitrogen) according to the seller's specifications. For this, 6 mg of samples were suspended in TER (24 h, 48 °C) in the presence of protease inhibitors (cOmplete™ ULTRA Tablets, Protease Inhibitor Cocktail), then they were homogenized using a pestle (5 min), centrifuged, aliquoted, and frozen.

The DNA content in both all four hECM and native tissue extracts was evaluated using the purification kit Promega *Wizard® genomic DNA*. DNA was isolated, purified and rehydrated. The samples were evaluated in triplicate. The DNA concentration was determined by spectrophotometric measurements at 260 nm (*Thermo Scientific MultiSkan Go*) and expressed in nanograms per milliliter of hECM or native tissue extracts.

The sGAG content in all four hECM and native tissue extracts was evaluated using a dye test with 1,9-dimethyl-methylene blue [16]. The sGAG concentration was determined by spectrophotometric measurements at 540 nm using chondroitin sulfate as standard (5–50 μg mL^{-1}) and expressed in nanograms per milliliter of hECM or native tissue extracts.

The laminin and fibronectin content in all four hECM and native tissue extracts was evaluated by enzyme-linked immunosorbent assays (ELISA) kits (ab108847 and ab119599; Abcam), according to the seller's specifications. Briefly, 5 μL of standard (0.004–1.0 μg mL^{-1} for fibronectin, 0.156–5 μg mL^{-1} for laminin) or sample were plated in triplicate. The concentrations of fibronectin and laminin were derived from the standard curve and expressed in picograms per milliliter of hECM or native tissue extracts.

The technique of polyacrylamide gel electrophoresis (SDS-PAGE) was used to ensure the purity and molecular composition of type I collagen presents in the hECM. Commercial collagen standards (*Sigma-Aldrich*) and 7% polyacrylamide gels were used for separating proteins. The analysis was performed using a voltage of 50 V and run for 5 h. Finally, the gels were stained with 0.25% Coomassie blue R-250 solutions and destained with methanol/acetic acid (90:10).

2.5. Polymerization of hECM

The hECM derived from TR, TAB, SIS and PB were polymerized under identical reaction conditions to produce 3D hydrogels or matrices. For this, 1 mL of hECM was placed in the ice bath and neutralized with 150 μL of PBS 10× (0.20 M ionic strength and pH 7.4). The neutral solutions of hECM were molded inside of 24-well plates. They were sealed with parafilm and incubated at 37 °C for 24 h to obtain the respective ECM hydrogels. Fig. 1 illustrates the steps in the gel preparation.

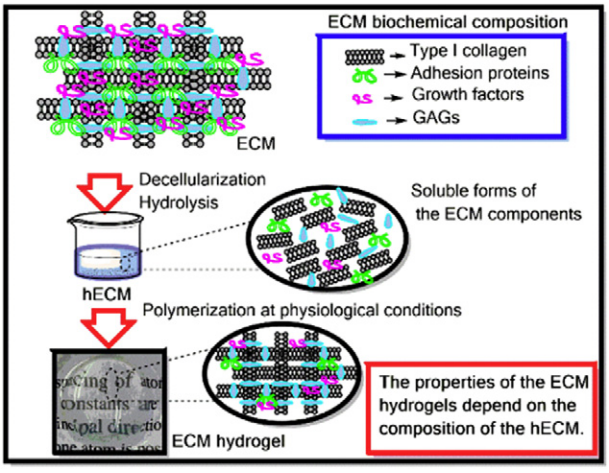

Fig. 1. Outline of the study objectives, indicating the steps in the preparation of the hECM with different composition, the subsequent polymerization at physiological conditions, and its effect on the ECM hydrogel properties.