CHAPTER

5

Glycosaminoglycan disaccharide compositional analysis of cell-derived extracellular matrices using liquid chromatography-tandem mass spectrometry

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Chapter outline

Methods in Cell Biology, Volume 156, ISSN 0091-679X, https://doi.org/10.1016/bs.mcb.2019.11.017

Abstract

Cell-derived extracellular matrices have emerged as promising scaffolds for tissue engineering (TE) strategies due to their ability to create a biomimetic microenvironment providing biochemical and physical cues to cells, without the limitations of availability and potential pathogen transmission associated with tissue-derived extracellular matrix (ECM) scaffolds. Glycosaminoglycans (GAGs) are important components of ECM with a crucial role in the maintenance of the mechanical properties of the tissue and as signaling regulators of several cellular processes, such as cell adhesion, growth and differentiation. However, despite their relevance to the field of TE, little information is available on the GAG composition of cell-derived ECM, mainly due to the lack of appropriate quantitative tools to determine different GAG and disaccharide subtypes in complex biological samples. In this chapter, we describe a highly sensitive and selective liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to characterize decellularized cell-derived ECM generated *in vitro* in terms of their GAG and disaccharide composition.

1 Introduction

Extracellular matrix (ECM), the noncellular component present in all tissues and organs, is composed of a complex and highly organized three-dimensional network of structural and functional biomolecules such as fibrillary proteins (e.g., collagens, fibronectin, laminin), proteoglycans and glycosaminoglycans (GAGs) (Badylak, Freytes, & Gilbert, 2009; Benders et al., 2013). GAGs are linear, negatively charged carbohydrates with a repeating disaccharide unit. GAGs can be grouped into four families: heparin/heparan sulfate (HS), chondroitin sulfate (CS), keratan sulfate (KS) and hyaluronic acid (HA) based on the structure of the repeating disaccharide unit (Gasimli, Linhardt, & Dordick, 2012). GAGs are crucial for the maintenance of the biomechanical environment of the tissues and for their resistance to compressive

forces through regulation of hydration and swelling pressure. GAG chains, within the ECM or mainly located on the cell membrane, interact with cell surface receptors, growth factors, cytokines, enzymes and proteins, playing a critical role in the regulation of several biological processes, such as development, microbial pathogenesis, cell growth and differentiation (Kjellén & Lindahl, 2018; Linhardt & Toida, 2004; Wang et al., 2017). The role of different GAGs in stem cell proliferation and differentiation through modulation of growth factor activity and important signaling pathways (e.g., fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling) has been previously reported (Kraushaar et al., 2012; Manton, Leong, Cool, & Nurcombe, 2007; Papy-Garcia & Albanese, 2017; Uygun, Stojsih, & Matthew, 2009). Thus, GAGs have been widely explored in a broad range of tissue engineering (TE) applications as key ECM components and as a result of their known role in determining stem cell fate (Celikkin et al., 2017; Köwitsch, Zhou, & Groth, 2018; Weyers & Linhardt, 2013).

Decellularized cell-derived ECM has been used in several TE approaches as an alternative to tissue-derived ECM. Cell-derived ECM offers several advantages over tissue-derived ECM, since it overcomes the limitations of autologous organ/tissue scarcity and possible pathogen transfer risks associated with the use of allogeneic/xenogeneic sources (Zhang et al., 2016). Additionally, cell-derived ECM can be used to mimic the composition of native ECM that is hard to isolate from tissues (e.g., stem cell niches) or combined with synthetic/natural biomaterials to generate scaffolds with enhanced bioactivity and proper mechanical support (Decaris, Binder, Soicher, Bhat, & Leach, 2012; Fitzpatrick & McDevitt, 2015; Kim et al., 2015; Lu, Hoshiba, Kawazoe, & Chen, 2011; Pati et al., 2015; Xing, Lee, Luo, & Kyriakides, 2019). In a recent study, our group demonstrated that decellularized ECM derived from a co-culture of human mesenchymal stem/stromal cells (MSC) and human umbilical vein endothelial cells (HUVEC) (MSC:HUVEC-ECM) significantly enhanced the osteogenic and angiogenic capacity of human bone marrow MSC compared to the ECM derived from the respective monocultures (MSC-ECM and HUVEC-ECM) (Carvalho, Silva, Cabral, da Silva, & Vashishth, 2019). We also combined lyophilized cell-derived ECM with the electrospinning technique to fabricate co-culture MSC:HUVEC-ECM loaded microfiber scaffolds able to enhance MSC osteogenic differentiation and provide structural support for bone TE applications (Carvalho, Silva, Udangawa, et al., 2019). Our findings suggested that a closer mimicry of the composition and structure of the native bone marrow niche is relevant for improved bone tissue regeneration. However, despite all the recent studies highlighting the potential of decellularized cell-derived ECM for improved TE settings, little information is available on the molecular composition of such matrices, specifically the content and types of proteins/GAGs retained after the decellularization process and how such composition varies among different cell sources. A method for accurately determining GAG composition of decellularized cell-derived matrices is of particular interest due to the critical importance of GAGs in regulating many cellular processes.

The goal of this chapter is to provide an outline of the steps involved in the *in vitro* preparation, morphological characterization and the GAG disaccharide compositional analysis of cell-derived matrices.

2 GAG compositional and structural analysis using LC-MS/MS

The functional relevance of GAGs in many developmental and disease mechanisms support a need for novel methods to precisely determine the GAG composition, structure and sulfation patterns in complex biological samples. Over the years, different qualitative and quantitative methods (e.g., alcian blue staining; 1,9-dimethylmethylene blue (DMMB) assay; enzyme-linked immunosorbent assay (ELISA); capillary electrophoresis (CE); high-performance liquid chromatography (HPLC) and liquid-chromatography-tandem mass spectrometry (LC-MS/MS)) have been used to study the significance of GAGs in different types of cells, tissues and biological fluids (Kubaski et al., 2017). Dye-spectrometric methods, including alcian blue and DMMB assays, have insufficient sensitivity and selectivity and are limited to determining total sulfated GAG content. ELISA methods are simple, rapid and reproducible, but expensive, since no currently available assay allows the detection of several GAGs simultaneously, requiring the use of multiple assays. CE has been used for GAG analysis due to its simplicity, high separation efficiency and advantage of allowing simultaneous assays for several GAGs in a rapid manner with low sample consumption. However, this method when used alone is only semi-quantitative and the determination of the amounts of GAGs separated by CE requires a combination with other techniques (Ucakturk et al., 2014; Zamfir, 2016). HPLC is a reproducible and sensitive method to analyze specific GAG types accurately. Nevertheless, the high complexity and time required for HPLC has limited its application in high-throughput screening. By combining the physical separation abilities of LC with tandem MS analysis, LC-MS/MS method offers a higher sensitivity and specificity for detecting all subtypes of GAGs.

LC-MS/MS analysis of GAGs has been performed following both bottom-up and top-down approaches. In the bottom-up analysis, GAG polysaccharides are depolymerized by either enzymatic digestion or chemical treatment into smaller disaccharides/oligosaccharides, which are then separated by LC and analyzed by MS. In contrast, a top-down approach comprises the analysis of intact GAG chains without need for sample preparation steps (Solakyildirim, 2019). Nevertheless, top-down analysis has been limited to small GAG chains such as those of low molecular weight heparins (LMWHs) (Li, Zhang, Zaia, & Linhardt, 2012).

A bottom-up approach comprising a highly sensitive LC-MS/MS method using multiple reaction monitoring (MRM) was previously reported by our group, in which the limit of detection for each GAG disaccharide was reduced to few picograms as compared with 1–5 nanograms usually associated with standard LC-MS analysis. MRM also enables a considerable reduction of sample preparation time (Li et al., 2015).

Such method was successfully applied to determine the GAG and disaccharide composition of a wide variety of human biological samples, including different cells and stem cell types, urine and blood samples, different regions within the intervertebral disk and cell-derived extracellular matrices (Li et al., 2015; Liu et al., 2018; Mikael et al., 2019; Silva et al., 2019; Sladden et al., 2019; Sun et al., 2015). We employed LC-MS/MS MRM, for the first time, to determine the GAG disaccharide composition of three different decellularized cell-derived matrices generated by cell sources relevant for cartilage regeneration strategies (human bone marrow-derived MSCs, synovium-derived MSCs and chondrocytes). Our results showed significant differences in GAG and disaccharide amounts, composition and sulfation patterns between the three types of cell-derived matrices, suggesting tissue-ECM specificity (Silva et al., 2019). In the following sections of this chapter, we describe the application of this method to different cell-derived matrices, previously used in bone TE strategies.

3 Overview of the protocol

A schematic overview of the sample treatment steps required for the GAG disaccharide compositional analysis using LC-MS/MS is shown in Fig. 1. GAGs were isolated and purified from the different decellularized matrices derived from different cell types (MSCs, HUVECs and co-culture of MSC:HUVEC 1:1 ratio). The GAG mixtures obtained were digested using GAG lyases to generate disaccharides, which were then fluorescently labeled and analyzed by LC-MS/MS in combination with known concentration of external disaccharide standards. By comparing the spectra of our samples with the ones of the disaccharide standards, it is possible to obtain the GAG disaccharide composition of each cell-derived ECM type.

4 Preparation of decellularized cell-derived matrices

4.1 Materials, equipment and reagents

- Cell sources: human bone marrow MSCs were isolated from bone marrow aspirates following previously established protocols (Dos Santos et al., 2010). HUVECs were purchased from Lonza.
- Low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco).
- Endothelial growth medium-2 (EGM-2, Lonza).
- Fetal bovine serum (FBS, Gibco).
- Phosphate buffered saline (PBS) no calcium, no magnesium (Gibco).
- Antibiotic-antimycotic (Anti-anti) solution (Gibco).
- Trypsin-EDTA 0.25% (Thermo Fisher Scientific).
- Trypan blue stain 0.4% (Life Technologies).
- Triton X-100 solution (Sigma-Aldrich).
- 4% Paraformaldehyde (PFA) solution in PBS (Santa Cruz Biotechnology).

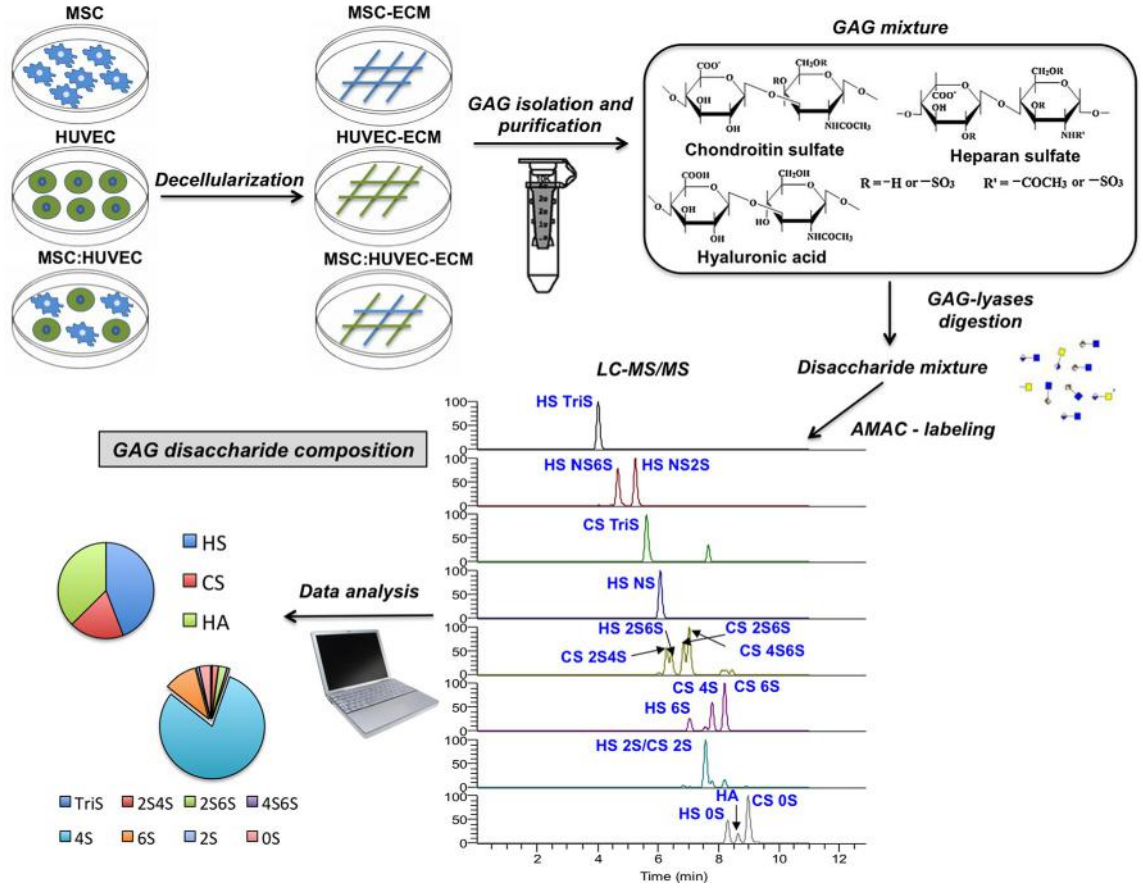

FIG. 1

Flowchart of the sample treatment steps followed to perform the GAG disaccharide compositional analysis of cell-derived matrices.

- Ammonium hydroxide (NH_4OH) solution (Sigma-Aldrich).
- 4,6-Diamino-2-phenylindole (DAPI, Sigma-Aldrich).
- Phalloidin-TRITC (Sigma-Aldrich).
- Tissue culture treated plastic ware (15 mL Falcon tubes/T-75 cm^2 flasks/ 35×10 mm Petri-dish/six-well plates, e.g., BD Biosciences).
- Hemocytometer (e.g., Hausser Scientific).
- Laminar flow hood.
- Temperature adjustable water bath set to 37 °C.
- Temperature and humidity-controlled tissue culture incubator (37 °C, 5% CO_2).
- Cell culture centrifuge.
- Fluorescence microscope (e.g., Olympus IX51 Inverted Microscope).

4.2 Step-by-step methods

4.2.1 Cell culture

1. Thaw MSCs and HUVECs and plate them on different T-75 cm^2 flasks within a cell density range of 3–6 $\times 10^3$ cells/cm^2. MSCs should be cultured using low glucose DMEM supplemented with 10% FBS and 1% Anti-anti, while HUVECs should be cultured using EGM-2 and 1% Anti-anti. Cells should be kept in a temperature and humidity-controlled tissue culture incubator at 37 °C and 5% CO_2. Culture medium renewal should be performed every 3–4 days. Cells between passages 2 and 5 should be used.
2. When MSCs and HUVECs reach around 80% cell confluence, wash the culture twice with PBS, add the appropriate volume of trypsin and incubate the cell cultures for 7 min at 37 °C. Note: Trypsin volumes vary with the surface area of the culture platform (e.g., 4 mL for T-75 cm^2 flasks).
3. Add DMEM + 10%FBS (the same volume as trypsin) to the cell cultures in order to neutralize the enzymatic reaction. Recover the cell suspensions to Falcon tubes. Centrifuge the cell suspension at 1250 rpm for 7 min.
4. After centrifugation, discard the supernatant and resuspend the cell pellet in a known volume of culture medium (DMEM + 10%FBS for MSCs or EGM-2 for HUVECs).
5. Count the cells using the trypan blue exclusion method. Viable (unstained cells) and dead cells (blue-stained cells) are identified using a hemacytometer under the optical microscope.
6. Plate MSCs, HUVECs and a co-culture of MSCs:HUVECs (1:1 ratio) at a seeding density of 5×10^3 cells/cm^2 in tissue culture polystyrene six-well plates or Petri-dish. MSCs should be maintained in DMEM + 10%FBS + 1%Anti-anti, HUVECs cultured with EGM-2 + 1%Anti-anti and the co-culture of MSCs: HUVECs (1:1) should be kept in a mixture between DMEM + 10%FBS and EGM-2 (1:1) culture mediums supplemented with 1%Anti-anti. Medium should be fully renewed every 3–4 days.

4.2.2 Decellularization of cell-derived matrices

1. When cell cultures reach confluency (between days 7 and 10), discard the culture medium and wash the cells with PBS.
2. Add a solution (enough volume to immerse the confluent cell layers) of 0.5% Triton X-100 containing 20 mM NH_4OH in PBS for 5 min at room temperature, based on previous protocols (Carvalho, Silva, Cabral, da Silva, & Vashishth, 2019; Carvalho, Silva, Udangawa, et al., 2019; Kang, Kim, Bishop, Khademhosseini, & Yang, 2012; Yang et al., 2018) to decellularize the cell cultures and isolate cell-derived ECM.

   Note: Different decellularization methods (e.g., other alkaline/acidic reagents or detergents and physical methods such as lyophilization and freeze-thaw cycling) can be used to obtain the decellularized cell-derived ECM.
3. Wash the decellularized cell-derived ECM gently three times using PBS and allow it to air dry inside the laminar flow hood. The cell-derived matrices produced can be frozen for further use/analysis or can be immediately used as a substrate to culture cells.
4. DAPI/Phalloidin staining should be performed before and after decellularization to confirm the efficiency of the decellularization process (Fig. 2). Wash the cells or the decellularized ECM twice with PBS and fix the samples with 4% PFA for 20 min at room temperature. The samples are permeabilized with 0.1% Triton X-100 for 10 min and incubated with Phalloidin (1:250 dilution in PBS, 2 μg/mL) for 45 min in the dark. The samples are then washed twice with PBS and counterstained with DAPI (1.5 μg/mL) for 5 min. After washing the samples with PBS, fluorescent staining can be imaged using a fluorescence microscope. Note: Other possible criteria to confirm successful decellularization is the DNA quantification (<200 bp DNA fragment length), which can be performed using commercially available kits (e.g., Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific) or QuantiFluor® dsDNA system (Promega)).

5 Morphological characterization of cell-derived matrices

Immunofluorescence staining can be performed to investigate the protein composition and distribution pattern of the different decellularized cell-derived matrices. Different ECM proteins can be targeted in the analysis, including collagen I, fibronectin and laminin. In addition, scanning electron microscopy (SEM) has revealed as a helpful tool to characterize the morphology and architectural features of the different cell-derived matrices produced.

5.1 Materials, equipment and reagents

- PBS no calcium, no magnesium (Gibco).
- 4% PFA solution in PBS (Santa Cruz Biotechnology).

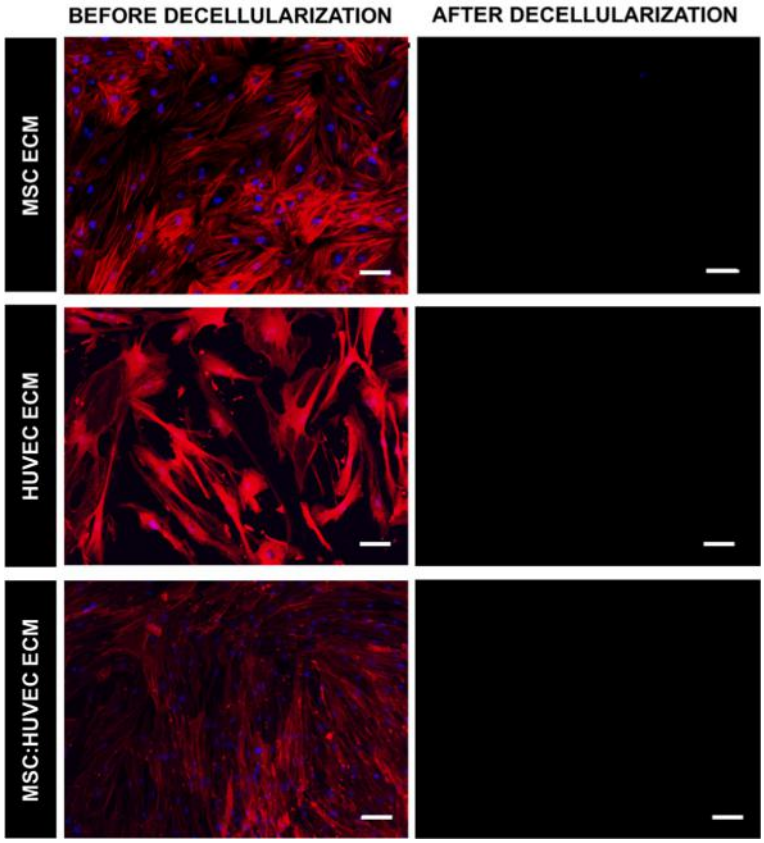

FIG. 2

DAPI/Phalloidin staining performed before and after decellularization to confirm the efficiency of the process. Before decellularization (left), confluent cell cultures show well-defined nuclei and cytoskeleton. The absence of staining in all the cell-derived matrices after the decellularization protocol (right) demonstrate the removal of cells and nuclear DNA. DAPI stains cell nuclei (blue) and Phalloidin stains actin-rich cell cytoskeleton (red). Scale bar: 100 μm.

- Bovine serum albumin (BSA, Sigma-Aldrich).
- Triton X-100 solution (Sigma-Aldrich).
- Donkey serum (Sigma-Aldrich).
- Ethanol absolute (Merck).
- Mouse anti-human collagen I (R&D systems).
- Mouse anti-human laminin (R&D systems).
- Mouse anti-human fibronectin (R&D systems).
- NorthernLights™ 557-conjugated anti-mouse IgG (R&D systems).
- DAPI (Sigma-Aldrich).

- Round glass cover slips 13 mm (VWR).
- Sputter coater setup with a gold-palladium target (e.g., Anatech Hummer IV).
- Fluorescence microscope (e.g., Olympus IX51 Inverted Microscope).
- Scanning electron microscope (e.g., FE-SEM, FEI Versa 3D Dual Beam).

5.2 Step-by-step methods

5.2.1 Immunofluorescence staining

1. After decellularization, wash the cell-derived matrices with PBS and fix the samples with 4% PFA solution for 20 min at room temperature.
2. Wash the cell-derived matrices three times with 1% BSA in PBS for 5 min.
3. Block the samples with a solution of 0.3% Triton X-100, 1% BSA and 10% donkey serum in PBS at room temperature for 45 min.
4. Remove the blocking solution and add the primary antibody solutions (mouse anti-human collagen I, laminin and fibronectin diluted at 2 μg/mL in 0.3% Triton X-100, 1% BSA and 10% donkey serum solution in PBS) into the samples. Incubate for 3 h at room temperature or overnight at 4 °C.
5. Wash the samples with 1% BSA in PBS and incubate them with the secondary antibody in the dark for 1 h at room temperature (NorthernLights™ 557-conjugated anti-mouse IgG, dilution 1:200 in 1% BSA solution in PBS).
6. Counterstain the samples with DAPI solution (1.5 μg/mL) for 5 min in the dark to assess the presence of remaining cell nuclei and confirm the decellularization procedure.
7. Wash the samples with PBS and use a fluorescence microscope (e.g., Olympus IX51 Inverted Microscope) to image the fluorescent staining of the different ECM proteins in the cell-derived matrices produced.

5.2.2 Scanning electron microscopy (SEM) analysis

1. For SEM analysis, culture the different types of cells in glass coverslips placed inside the culture well plates.
2. After decellularization of the samples in glass coverslips, rinse the cell-derived matrices with PBS and fix the samples with 4% PFA solution for 20 min.
3. Remove the PFA and wash the samples twice with PBS. Then, the samples should be dehydrated in a graded series of ethanol solutions (e.g., 20%, 40%, 60%, 80%, 96%, and 100% (v/v), 30 min each solution) and left to air dry inside a chemical fume hood.
4. The glass coverslips containing the cell-derived matrices samples should be removed from the plates, mounted on a holder and sputter-coated with a thin layer of 60% gold (Au)-40% palladium (Pd).
5. SEM imaging of the different cell-derived matrices can be performed at different magnifications using an accelerating voltage between 2 and 3 kV. However, it is important to note that the operating voltage and other imaging parameters should be adjusted according to the equipment, coating method used and to the specificities of the sample to be imaged.

5.3 Discussion

The presence of a biological network of ECM was confirmed in all the different cell-derived matrices produced (Fig. 3). Our analysis revealed that, although the different cell-derived matrices show the presence of common ECM proteins, such as collagen I, fibronectin and laminin, it is possible to observe clear differences in the components distribution and structure between them. Interestingly, HUVEC-ECM expresses relatively low levels of collagen I, fibronectin and laminin in comparison to the other conditions, and for the case of collagen I and laminin, the staining shows a sparse dot-like morphology. Moreover, SEM analysis demonstrates differences in the architecture and topography of the decellularized matrices derived from different cell types. While MSC-ECM presents a fibrillary architecture, HUVEC-ECM and MSC:HUVEC-ECM demonstrates a more globular structure. It is important to highlight here that the ratio of MSC:HUVEC seems to influence the ECM architecture, since it has already been shown that a MSC:HUVEC ratio of 3:1 favored the production of a ECM with a more fibrillar-like structure, similar to MSC-ECM, possibly due to the higher ratio of this cell type (Carvalho, Silva, Cabral, et al., 2019; Carvalho, Silva, Udangawa, et al., 2019).

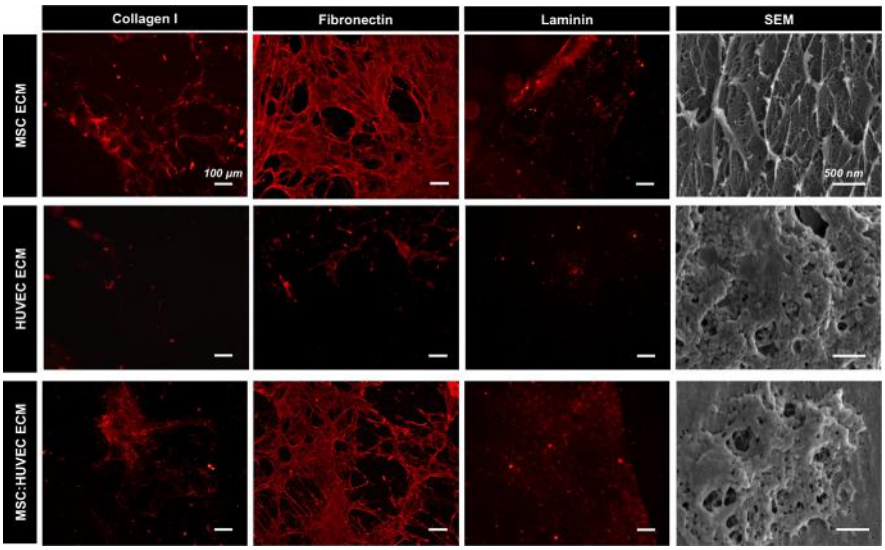

FIG. 3

Immunofluorescence staining and SEM analysis of decellularized ECM derived from different cell types, namely MSCs, HUVECs and MSC:HUVEC co-culture. Immunofluorescent staining images for collagen I, fibronectin and laminin, showing differences in the expression and distribution of these proteins between the different types of cell-derived matrices. Samples were counterstained with DAPI to confirm DNA removal. Scale bar: 100 µm. SEM micrographs revealing the architecture and structure of the different cell-derived matrices obtained after decellularization (right). Scale bar: 500 nm.

6 GAG disaccharide compositional analysis of cell-derived matrices

6.1 Materials, equipment and reagents

- Distilled water.
- Cell scrapper (e.g., VWR).
- BugBuster 10× Protein Extraction Reagent (Merck Millipore).
- 3 kDa molecular weight cut-off (MWCO) spin column (Amicon® ultra centrifugal filters, Merck Millipore).
- 1.5 mL microcentrifuge tubes (e.g., Thermo Fisher Scientific).
- Ammonium acetate HPLC grade (Fisher Scientific).
- Calcium chloride (Sigma-Aldrich).
- Recombinant *Flavobacterial* heparinum heparin lyase I, II, III and recombinant *Proteus vulgaris* chondroitin lyase ABC expressed in *E. coli* strains in our laboratory as described (Su et al., 1996; Tkalec et al., 2000).
- Unsaturated CS, HS and HA disaccharide standards (Iduron, see Table 1 for structures).

Table 1 Structures of the CS, HS and HA disaccharides analyzed by LC-MS/MS.

CS disaccharides	
$TriS_{CS}$	**ΔUA2S(1,3)GalNAc4S6S**
$2S4S_{CS}$	ΔUA2S(1,3)GalNAc4S
$2S6S_{CS}$	ΔUA2S(1,3)GalNAc6S
$4S6S_{CS}$	ΔUA(1,3)GalNAc4S6S
$2S_{CS}$	ΔUA2S(1,3)GalNAc
$4S_{CS}$	ΔUA(1,3)GalNAc4S
$6S_{CS}$	ΔUA(1,3)GalNAc6S
$0S_{CS}$	ΔUA(1,3)GalNAc
HS disaccharides	
$TriS_{HS}$	ΔUA2S(1,4)GlcNS6S
$NS6S_{HS}$	ΔUA(1,4)GlcNS6S
$NS2S_{HS}$	ΔUA2S(1,4)GlcNS
NS_{HS}	ΔUA(1,4)GlcNS
$2S6S_{HS}$	ΔUA2S(1,4)GlcNAc6S
$6S_{HS}$	ΔUA(1,4)GlcNAc6S
$2S_{HS}$	ΔUA2S(1,4)GlcNAc
$0S_{HS}$	ΔUA(1,4)GlcNAc
HA disaccharides	
$0S_{HA}$	ΔUA(1,3)GlcNAc

- 2-Aminoacridone (AMAC, Sigma-Aldrich).
- Dimethylsulfoxide (DMSO, Sigma-Aldrich).
- Sodium cyanoborohydride ($NaCNBH_4$, Sigma-Aldrich).
- Acetic acid (Sigma-Aldrich).
- Methanol HPLC grade (Fisher Scientific).
- Water HPLC grade (Fisher Scientific).
- LC-MS vials, caps and inserts with bottom spring (e.g., Merck).
- Ultrasonic bath.
- Vortex.
- Freeze-dryer.
- Microcentrifuge.
- Mini spin down centrifuge.
- Incubator set at 37 °C.
- Dry block incubator able to fit microcentrifuge tubes set at 45 °C.
- Agilent 1200 LC system.
- Agilent Poroshell 120 ECC18 (2.7 μm, 3.0 × 50 mm) column.
- Triple quadrupole mass spectrometry system equipped with an ESI source (Thermo Fisher Scientific).
- Thermo Xcalibur™ software (Thermo Fisher Scientific) for data analysis.

6.2 Step-by-step methods

6.2.1 GAG isolation and digestion into disaccharides

1. Add distilled water to the wells containing the produced cell-derived matrices and detach the ECM from the wells using cell scrappers. Confirm that the ECM was successfully recovered under an optical microscope.
2. Freeze-dry the cell-derived matrices collected in distilled water overnight. Note: longer lyophilization (∼24 h) periods might be required, depending on the amount of frozen sample.
3. After freeze-drying, assess the mass of lyophilized cell-derived matrices obtained. Note: Weight tubes before collecting the samples and after completion of the freeze-drying to determine the amount (mass) of each lyophilized cell-derived ECM. The difference between the mass (tube + cell-derived ECM) and mass (tube) gives the amount of cell-derived ECM produced.
4. Add 100 μL of Bugster 10 × Protein Extraction Reagent and sonicate the samples for 1 h to initiate the isolation of GAGs from the cell-derived matrices.
5. Desalt the samples by passing them through a 3 kDa MWCO spin column with centrifugation at 13.4×10^3 rpm for 20 min.
6. Wash the samples three times with 200 μL of distilled water. For each wash, centrifuge at 13.4×10^3 rpm for 20 min.
7. Move the upper filter unit to new casing tubes and add 300 μL of digestion buffer (50 mM ammonium acetate containing 2 mM calcium chloride, adjusted to pH 7.0) to the filter unit.

8. Add recombinant heparin lyases I, II, III (10 mU each, pH 7.0–7.5) and recombinant chondroitin lyase ABC (10 mU, pH 7.4) to each sample, and mix well by pipetting.
9. Incubate the samples at 37 °C overnight (~12 h) to allow GAG enzymatic digestion.
10. Stop the enzymatic reaction by 3 kDa MWCO centrifugal ultrafiltration (13.4×10^3 rpm for 20 min). Recover the disaccharides in the lower casing tube filtrate and wash the filter unit twice with 200 μL of distilled water. For each wash, centrifuge at 13.4×10^3 rpm for 20 min.
11. Discard the columns and freeze-dry the final filtrates containing the disaccharide products overnight. Store the samples at −20 °C until labeling.

6.2.2 AMAC labeling and sample preparation for LC-MS/MS analysis

Note: The AMAC-labeling protocol should be performed on the same day of the LC-MS/MS sample run.

1. Add 10 μL of a freshly prepared 0.1 M AMAC solution in DMSO/acetic acid (17/3 ratio, v/v) to label the disaccharides of the different cell-derived ECM samples and incubate the samples at room temperature for 10 min. Note: Use a pipette to wash and dissolve all the GAG pellet spots, even in the tube walls. This is critical to accurately determine the sample GAG amounts and disaccharide composition.
2. After incubation, add 10 μL of a freshly prepared 1 M $NaCNBH_4$ aqueous solution to all the samples. Vortex the samples and place them for 1 h in a dry block incubator at 45 °C.
3. Simultaneously, perform the steps 1 and 2 to label a mixture containing all 17 disaccharide standards prepared at 0.5 ng/μL to be used for each LC-MS/MS run as external standards.
4. Upon completion of the AMAC labeling, centrifuge the samples (13.4×10^3 rpm for 20 min) and recover the supernatants.
5. Transfer 10 μL of each sample and standards to inserts with bottom spring placed in properly identified LC-MS vials. If needed, centrifuge the inserts in a mini spin down centrifuge to remove air bubbles.

6.2.3 GAG disaccharide analysis by LC-MS/MS with MRM

This method has been previously optimized by our group and was found to be efficient to determine the GAG disaccharide composition of different types of biological samples (Li et al., 2015; Liu et al., 2018; Sun et al., 2015).

1. Prepare the mobile phase solutions (mobile phase A (MPA): 50 mM ammonium acetate aqueous solution and mobile phase B (MPB): methanol).
2. Identify, organize the samples and define the parameters of the running method in the equipment software. Note: if the number of samples to analyze is high, use

several external standards placed in the initial, middle and final positions of the sample running sequence.

3. Perform the LC on an Agilent 1200 LC system at 45 °C using an Agilent Poroshell 120 ECC18 (2.7 μm, 3.0 × 50 mm) column.
4. Pass the mobile phase through the column at a flow rate of 300 μL/min. The elution of all compounds is done using the gradient as follows: 0–10 min, 5–45% MPB; 10–10.2 min, 45–100% MPB; 10.2–14 min, 100% MPB; 14–22 min, 100–5% MPB. Use an injection volume of 5 μL for all the samples.
5. The detection of the disaccharides is done using a triple quadrupole mass spectrometry system equipped with an ESI source. Perform the online MS analysis at the MRM mode with the following MS parameters: negative ionization mode with a spray voltage of 3000 V, a vaporizer temperature of 300 °C and a capillary temperature of 270 °C.
6. Perform the data analysis using the Thermo Xcalibur™ software. The GAG and disaccharides in cell-derived ECM are identified and quantified via comparison of the sample peak area to that of an external standard. The total GAG and disaccharide amounts can be normalized to the dry weight of the respective cell-derived ECM sample (e.g., Table 2). By comparing the amounts of the different GAG and disaccharide types to the total amounts in the sample, it is possible to obtain the GAG (Fig. 4) and disaccharide (Fig. 5) composition, expressed as average relative percentages.

6.3 Discussion

Cell source is a critical factor determining the composition of cell-derived matrices, since cells derived from different tissues typically secrete ECM that resembles the composition of its native source (Fitzpatrick & McDevitt, 2015). Thus, it is expected that different cell-derived matrices would show considerable differences in terms of GAG amounts and composition. MSC-ECM and MSC:HUVEC-ECM contained higher amounts of total GAGs than HUVEC-ECM (Table 2). The GAG composition of the different cell-derived matrices is provided in Fig. 4. While MSC-ECM had

Table 2 GAG composition of the different cell-derived ECM produced from MSC, HUVEC and MSC:HUVEC co-culture, quantified as ng of GAG/mg of dry ECM.

	Total GAG (ng/mg)		
	HS	**CS**	**HA**
MSC-ECM	90.6 ± 36.6	139.8 ± 9.2	122.6 ± 34.1
HUVEC-ECM	42.8 ± 14.3	18.1 ± 5.5	0.8 ± 0.7
MSC:HUVEC-ECM	163.7 ± 88.7	64.8 ± 21.7	136.2 ± 64.7

Results are presented as mean ± SD of three independent samples (n = 3).

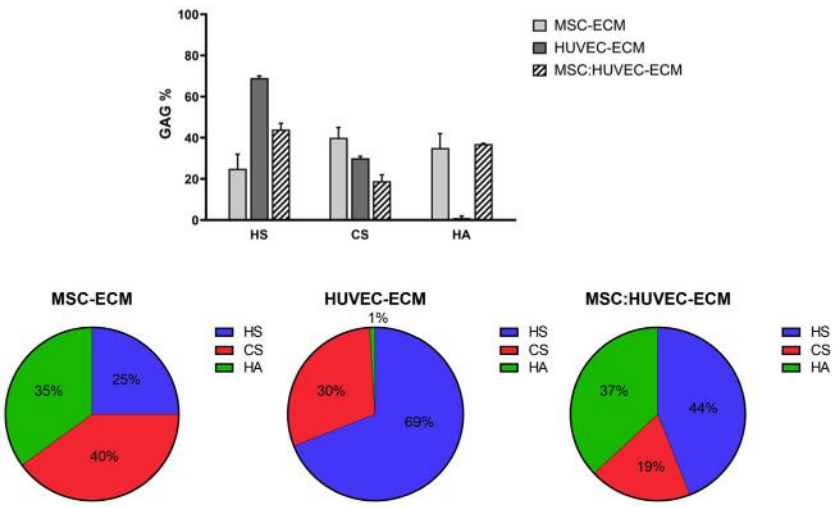

FIG. 4

Average percentage GAG composition of the different cell-derived matrices (MSC-ECM, HUVEC-ECM and MSC:HUVEC-ECM). Results are presented as mean ± SD of three (n = 3) independent samples.

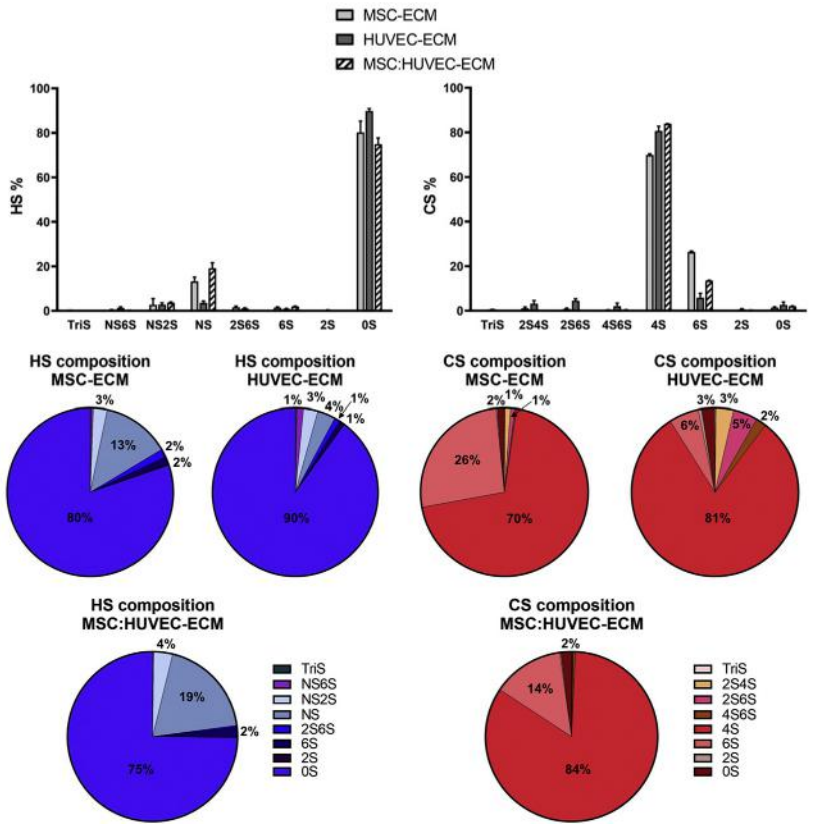

FIG. 5

Average percentage HS (left) and CS (right) disaccharide composition of the different cell-derived matrices (MSC-ECM, HUVEC-ECM and MSC:HUVEC-ECM). Results are presented as mean ± SD of three (n = 3) independent samples.

nearly similar amounts of CS, HA and HS, HUVEC-ECM was mainly composed by HS (69%), followed by CS (30%) and contained a very small amount of HA (1%). In addition, the total GAG content of the ECM derived from the co-culture of MSCs and HUVECs was composed by HS (44%) and HA (37%) with low amounts of CS (19%).

The disaccharide composition of the GAGs present in the different cell-derived matrices is shown in Fig. 5. In all the cell-derived matrices, HS was composed primarily of HS 0S with low amounts of other disaccharides, particularly HS NS and HS NS2S, in agreement with our previous observations of cell-derived ECM generated from other cell types (Silva et al., 2019). The CS composition of the different cell-derived matrices showed that the predominant disaccharide was CS 4S, followed by lower amounts of CS 6S. Despite relevant differences, a higher similarity both in GAG and disaccharide composition was observed between MSC:HUVEC-ECM and MSC-ECM, which might result from the higher capacity for GAG production demonstrated by MSCs in comparison to HUVECs. Therefore, in the cell-derived ECM generated from the co-culture, MSCs might play a stronger role than HUVECs in determining the GAG and disaccharide composition.

The LC-MS/MS analysis employed here is a valuable tool to accurately determine the GAG and disaccharide composition of several types of cell cultures, tissues and biological fluids. Thus, this method is highly promising to identify novel biomarkers for diagnosis and clinical applications since specific changes in GAG disaccharide composition and structure have been associated with different cell differentiation states (Gasimli et al., 2014; Mikael et al., 2019) and with diseases such as osteoarthritis and cancer (Plaas, West, Wong-Palms, & Nelson, 1998; Weyers et al., 2013, 2012). In this chapter, we describe the application of the LC-MS/MS method to determine the GAG and disaccharide composition of different cell-derived matrices, which provides important information for better understanding of the role of ECM in directing cellular responses and for developing improved ECM-like biomaterials for TE applications.

7 Technical considerations and possible improvements

When preparing *in vitro* cell-derived matrices, it is important to consider several factors influencing their composition, such as cell source, donor variability, decellularization method used and the culturing system. Different decellularization protocols (e.g., chemical, enzymatic or physical methods) have been shown to impact the ECM composition differently (Gaetani et al., 2018; Lu, Hoshiba, Kawazoe, & Chen, 2012). The cell culture system and conditions selected can also influence the amounts and composition of the ECM secreted by cells. In addition to the adherent monolayer cultures employed in this chapter, cells can also be cultured as 3D aggregates, seeded on scaffolds or encapsulated within degradable carriers to promote ECM production and deposition. Moreover, the cell-derived ECM can be tuned to recapitulate specific *in vivo* microenvironments through the modulation of culture conditions such as

supplementation with proteins/growth factors or the use of co-cultures, hypoxic conditions or dynamic culture systems (e.g., bioreactors). In fact, macromolecular crowding has been applied as an effective strategy to enhance cell-mediated ECM production *in vitro* (Chen, Loe, Blocki, Peng, & Raghunath, 2011; Shendi et al., 2019). Other potential approach to modulate ECM production is the use of genetically engineered cells to augment or silence the expression of target molecules, generating specific cell-derived ECM for a particular application.

One major variable that can impact the performance of cell-derived matrices generated in tissue culture plates is their high fragility. Therefore, it is crucial to be very gentle throughout all the procedures and to add/aspirate the solutions slowly (without touching the well surface with the pipette tip) to avoid disrupting the ECM structure. Also, respecting completely the defined incubation periods and controlling the pH of the decellularization agent solution are important for process reproducibility.

Although the LC-MS/MS analysis described in this chapter provides novel and valuable knowledge about GAG and disaccharide composition of cell-derived matrices, some limitations and possible improvements still need to be addressed. This method does not allow the detection and quantification of core proteins. Thus, it would be interesting to combine this analysis with proteomic approaches to obtain better information about the composition and functionality of the *in vitro* produced cell-derived matrices. Additionally, as this method is based on disaccharide analysis using heparinases and chondroitinase ABC, it is only possible to quantify CS, HS and HA. Further methodological development will be required to allow for the detection and quantification of other GAGs (e.g., KS) and to be able to distinguish between CS and dermatan sulfate (DS).

Despite the fact that this LC-MS/MS method has resulted from previous optimization (Li et al., 2015), there is always room for improvement in order to increase the method sensitivity while reducing analysis time and cost. In fact, mass spectrometers coupled to LC systems are expensive to run and require regular maintenance, which further raises the costs. Thus, it is important to run the samples under carefully optimized conditions to ensure sensitivity and reproducibility in the analysis and to avoid resource waste. Additionally, it is important to note that laboratory environment must be well controlled to guarantee system stability and appropriate training should be provided to all LC-MS/MS users to keep the system running efficiently.

8 Conclusions

Decellularized cell-derived matrices, used alone or in combination with polymeric materials, have emerged as superior platforms for regenerative medicine due to their ability to promote cell adhesion, proliferation and differentiation through a closer mimicry of key structural and biochemical features of native *in vivo* microenvironments. Due to its growing relevance in TE, a better understanding of the molecular composition of cell-derived matrices is critical to expand our knowledge about the underlying mechanisms by which ECM regulates cell functions and foster the

development of novel and improved ECM-like biomaterials. In this chapter, we described a procedure to study the composition of cell-derived matrices in terms of GAGs, one of ECM's main functional constituents. Despite possible further improvements, we believe the highly sensitive and selective LC-MS/MS method detailed here represents a useful tool for researchers to determine the GAG and disaccharide composition of different cell-derived matrices in an accurate and rapid manner.

Acknowledgments

The authors are thankful for financial support from Center for Biotechnology and Interdisciplinary Studies-Rensselaer Polytechnic Institute, Fundação para a Ciência e a Tecnologia (FCT, Portugal) through the project UID/BIO/04565/2019 and from Programa Operacional Regional de Lisboa 2020 (Project N. 007317 and through the project PRECISE—Accelerating progress toward the new era of precision medicine—Project No. 16304). The authors also acknowledge the National Institutes of Health (NIH) grant # DK111958 and CA 231074 for supporting this work. The authors declare no conflicts of interest.

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