Published in final edited form as:
Methods Mol Biol. 2011 ; 690: 285–300. doi:10.1007/978-1-60761-962-8_19.

Chapter 19 Analysis of Glycosaminoglycans in Stem Cell Glycomics

Boyangzi Li, **Haiying Liu**, **Zhenqing Zhang**, **Hope E. Stansfield**, **Jonathan S. Dordick**, and **Robert J. Linhardt**

Abstract

Glycosaminoglycans (GAGs) play a critical role in the binding and activation of growth factors in cell signal transduction required for biological development. A glycomics approach can be used to examine GAG content, composition, and structure in stem cells in order to characterize their general differentiation. Specifically, this method may be used to evaluate chondrogenic differentiations by profiling for the GAG content of the differentiated cells. Here, embryonic-like teratocarcinoma cells, NCCIT, a developmentally pluripotent cell line, were used as a model for establishing GAG glycomic methods, but will be easily transferrable to embryonic stem cell cultures.

Keywords

Glycosaminoglycans; NCCIT cells; Chondroitin sulfate; Dermatan sulfate; Heparin; Heparan sulfate; Purification; Enzymatic digestion; Disaccharide analysis; LC-MS

1. Introduction

1.1. Glycomics

Glycomics is the study of the structure and function of glycans, glycoconjugates including glycosphingolipids, glycoproteins, such as proteoglycans (PGs), and glycan-binding proteins. An understanding of the cellular glycome should explain some mysteries associated with these frequent and important posttranslational modifications (1). The structural and functional glycomics of the glycosaminoglycan (GAG) chains (GAGome) of PGs from different tissues and cells are under intensive study in our laboratory (2–5). GAGs are linear, sulfated, heterogeneous polysaccharides consisting of various repeating disaccharide and are mainly located on both the external membrane of eukaryotic cells and within the extracellular matrix (6, 7). There are four distinct families of GAGs: chondroi-tin/dermatan sulfate (CS/DS), heparin/heparan sulfate (HS), ker-atan sulfate (KS), and hyaluronan (HA). GAGs are involved in numerous biological activities and are important as molecular coreceptors, in cell–cell interactions, cell adhesion, cell migration, cell signaling, cell growth, and cell differentiation (8–10).

1.2. GAGs in Embryonic Stem Cells

Embryonic stem cells (ESCs) have enormous potential as a source of cells for cell replacement therapy and have been used as in vitro models to study specific aspects of early embryonic development (11, 12). GAGs, particularly HS and CS, within stem cells play key roles in maintaining cell proliferation and differentiation (5). Understanding the glycomics of ESCs should shed light on development, including the differentiation of chondrocytes

from mes-enchymal cells (13–15). In our lab, we are using teratocarcinoma cells (NCCIT), a developmentally pluripotent cells, to offer a convenient model for ESCs. Methods for the elucidation of the GAGome within NCCIT cells, generally applicable for ESCs and their derivatives, such as chondrocytes, are described here for use in better understanding cell pluripotency and differentiation.

1.3. GAGs in the Extracellular Matrix of Chondrocytes

ESCs are able to differentiate into the mesenchymal cells that ultimately give rise to chondrocytes and endochondral ossification (13–15). Cartilage is a specialized connective tissue that provides support for other tissues or prevents friction of the joints. The cartilage is comprised of chondrocytes that sparsely distribute in extracellular matrix filled with collagen fibrils and PGs. The fibrous structure of collagen provides support and maintains tissue shape, while PGs form gels and act as filler to facilitate compressibility and prevent friction as well as perform other critical signaling functions. The PGs in cartilage, such as decorin, bigly-can, and aggrecan, are glycosylated with one or multiple GAG chains. In cartilage, the GAG components are mainly CS and KS and HA as well as smaller amounts of DS and HS. CS, the most abundant GAG in the cartilage, is composed of 4-*O*-sulfo, 6-*O*-sulfo, and 4,6-di-*O*-sulfo sequences (16, 17). Research has shown that both the relative amounts of these sequences within CS and the length of CS chains change in aging and in diseases such as osteoarthritis (18–21). The GAG profile of differentiating ESCs may therefore help to elucidate whether or not chondro-cytes have been formed, to what extent and may help to characterize the quality of the generated ESC-derived chondrocytes.

2. Materials

2.1. Cell Culture

1. 70% (v/v) ethanol.
2. NCCIT cells (ATCC: CRL-2073), frozen and preserved in 95% culture medium and 5% DMSO, were from American Type Culture Collection. Cells are stored in liquid nitrogen until immediately prior to use (see Note 1).
3. NCCIT medium: 500 mL RPMI-1640 medium with L-glutamine, 50 mL fetal bovine serum (FBS, Invitrogen) and 5 mL 10,000 U/mL penicillin/streptomycin stock solution. FBS and penicillin/streptomycin stock solution are stored at -20°C before use and after mixing the culture medium is stored at 4°C before use.
4. Cell detachment process solution: 0.25% trypsin (Invitrogen) and 1 mM ethylenediamine tetraacetic acid (EDTA). Store at −20°C.
5. Trypan blue stain from Invitrogen for cell viability measurements.
6. Falcon® sterile polystyrene disposable aspirating pipettes (1, 5, 10, and 25 mL), sterile centrifuge tubes (15 and 50 mL) and sterile tissue culture flasks with vented cap (canted-neck; growth area: 25 cm^2; total volume of the flask: 50 mL), for example from BD Biosciences.
7. Hemocytometer set (e.g. Hausser Scientific).
8. Microscope (e.g. CKX41, Olympus).

[1]NCCIT cells were established by Shinichi Teshima (National Cancer Institute, Tokyo, Japan) in 1985 from a mediastinal mixed germ cell tumor (27). This pluripotent stem cell line is capable of somatic and extraembryonic differentiation. The undifferentiated cells are equivalent to a stage intermediate between seminoma and embryonal carcinoma. They will differentiate in response to retinoic acid (28).

2.2. Recovery and Purification of GAGs

1. Defatting solution prepared from HPLC purity chloroform and HPLC purity methanol.
2. Proteolysis enzyme solution, actinase E (5 mg/mL in water) (see Note 2). Store at -20°C.
3. Protein and peptide denaturing solution: 8 M urea with 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesul-fonate (CHAPS) adjusted to pH 8.3 using 1 M HCl.
4. Prewash solution: 200 mM sodium chloride (NaCl).
5. GAG collection solution: 16% (w/w) NaCl.
6. Methanol used as GAG precipitation solvent.
7. Millex™ 0.22-μm syringe driven filter unit from Millipore to remove particulates.
8. Vivapure MINI QH columns (Viva science) for GAG recovery.
9. Microcon® Centrifugal Filter Units-Microcon Ultracel YM-3 (3,000 MWCO), i.e. from Millipore for desalting.
10. 0.1 M sodium hydroxide (NaOH).
11. ColorpHast® pH strips (universal, pH ranging from 0 to 14), EMD Chemicals or similar.

2.3. Molecular Weight Analysis of GAGs by PAGE

1. Resolving gel buffer and lower chamber buffer: 100 mM boric acid, 100 mM Tris, and 1 mM disodium ethylenedi-aminetetraacetic acid (EDTA) at pH of 8.3. Store at room temperature.
2. Upper buffer: 1.24 M glycine and 200 mM Tris as written. Store at room temperature.
3. Front gel unpolymerized solution: 20.02% (w/v) acrylamide, 2% (w/v) *N,N*-methylenebisacrylamide, and 15% (w/v) sucrose in resolving gel buffer. Store at 4°C.
4. Stacking gel unpolymerized solution: 4.75% (w/v) acrylamide, 0.25% (w/v) *N,N*-methylenebisacrylamide in resolving gel buffer at pH 6.3 using 1 M HCl. Store at 4°C.
5. Polymerization reagents: *N,N,N,N′*-tetramethyl-ethylenediamine (TEMED), and 10% (w/v) aqueous ammonium persulfate (APS). Store separately at 4°C.
6. 50% (w/v) sucrose in water for density increase in GAGs. Store at 4°C.
7. Heparin (e.g. from Celsus Laboratory). Heparin oligosaccha-rides mixture, as the standard heparin ladder for molecular weight calculation can be obtained from mixing several oligo-saccharides (e.g. tetrasaccharide, octasaccharide, decasaccha-ride, and dodecasaccharide) available from Iduron. Alternatively, heparin can be partially digested by heparinase I (Seikagaku) and used as substitute set of standards. In this protocol, we used partially digested heparin and a pure heparin-

[2]It is best to freshly prepare actinase E proteolysis enzyme solution from dry protein immediately before use.

derived octasaccharide standard prepared in our laboratory (22). Phenol red solution can be added to aid in real-time visualization during electrophoresis.

8. Gel staining reagent: 0.5% (w/v) Alcian blue in 2% (v/v) aqueous acetic acid.
9. Mini-gel electrophoresis system PowerPac 1000 from Bio-Rad.
10. Gel-loading pipette tips (200 μL).
11. UN-SCAN-IT™ digitizing software, i.e. Silk Scientific or similar.

2.4. Enzymatic Lyase Depolymerization of GAGs and Recovery of GAG Disaccharides

1. 20 mM Tris(hydroxymethyl)-aminomethane (Tris)–HCl buffer, pH 7.2.
2. Chondroitin sulfate depolymerization enzymes: 10 mU of chondroitinase ABC and 5 mU of chondroitinase ACII (Seikagaku) prepared in 20 mM, pH 7.2 Tris–HCl buffer containing 0.1% BSA. Store at -20°C.
3. Heparin/heparan sulfate depolymerization enzymes: Heparinase I, II, and III (Seikagaku) prepared as a mixture 5 mU each in 20 mM pH 7.2 PBS buffer. Store at -20°C.
4. Desalting columns: Microcon® Centrifugal Filter Units-Microcon Ultracel YM-3 (3,000 MWCO) from Millipore.

2.5. Disaccharide Analysis by LC-MS

1. Unsaturated CS/DS disaccharides standards: ΔDi-0S, ΔUA-GalNAc; Δdi-4S, ΔUA-GalNAc4S; Δdi-6S, ΔUA-GalNAc6S; Δdi-UA2S, ΔUA2S-GalNAc; Δ di-diS_B, ΔUA2S-GalNAc4S; Δ di-diS_D, ΔUA2S-GalNAc6S; ΔUA-GalNAc4S6S; Δdi-diS_E, and Δdi-triS, ΔUA2S-GalNAc4S6S (Seikagaku Corporation).
2. Unsaturated heparin/HS disaccharides standards: Δdi-0S, ΔUA-GlcNAc; Δdi-NS, ΔUA-GlcNS; Δdi-6S, ΔUA-GlcNAc6S; Δdi-UA2S, ΔUA2S-GlcNAc; Δdi-UA2SNS, ΔUA2S-GlcNS; Δdi-NS6S, ΔUA-GlcNS6S; Δdi-UA2S6S, ΔUA2S-GlcNAc6S; and Δdi-triS, ΔUA2S-GlcNS6S (Iduron).
3. Disaccharide detection system: LC-MS system (Agilent, LC/ MSD trap MS).
4. HPLC solution A for CS/DS disaccharide analysis: 0% (v/v) HPLC grade acetonitrile in HPLC grade water, 15 mM hexylamine (HXA) and 100 mM 1,1,1,3,3,3,-hexafluoro-2-propanol (HFIP).
5. HPLC solution B for CS/DS disaccharide analysis: 75% (v/v) HPLC grade acetonitrile in HPLC grade water, 15 mM HXA, and 100 mM HFIP.
6. HPLC solution C for heparin/HS disaccharide analysis: 15% (v/v) HPLC grade acetonitrile in HPLC grade water, 37.5 mM NH_4CH_3COO, and 11.25 mM tributylamine (TBA), pH 6.5 adjusted with glacial acetic acid.
7. HPLC solution D for heparin/HS disaccharide analysis: 65% (v/v) HPLC grade acetonitrile in HPLC grade water, 37.5 mM NH_4CH_3COO, and 11.25 mM TBA, pH 6.5 adjusted with glacial acetic acid.
8. ACQUITY UPLC™ BEH C18 column (Waters, 2.1 × 150 mm, 1.7 μm) for CS/DS disaccharide analysis and Zorbax SB-C18 column (Agilent, 0.5 × 250 mm, 5 μm) for heparin/HS disaccharide analysis.

3. Methods

Disaccharide analysis is useful for assessing the structure of the GAGome in pluripotent cells such as teratocarcinoma cells and embryonic stem cells. Changes in the GAGome can then be correlated to alteration in the transcription levels for enzymes involved in GAG biosynthesis, PG core proteins, GAG-binding proteins such as growth factors, growth factor receptors, chemokines, and adhesion proteins. An improved knowledge of structural glycomics of GAGs should result in a better understanding of the relationship of the GAGome to the functional glycomics associated with stem cell differentiation.

The following protocol will explain how to characterize the GAGome of embryonic stem cells and their differentiated progeny using teratocarcinoma cells as a model. In brief, teratocarcinoma cells are grown to confluency and 10^7 cells are collected. The washed cell pellet is defatted, proteolyzed and GAGs are extracted into CHAPS/Urea. Spin column-based ion chromatography is then used to recover the CS and HS GAGs that are washed and then released in salt. After membrane-based desalting, the molecular weights of purified CS and HS GAGs are analyzed by PAGE. The CS and HS GAGs are then individually depolymerized to disaccharide mixtures using either chondroitinases or heparinases. The resulting disaccharide mixtures are analyzed by reversed-phase ion-pairing high performance liquid chromatography and detected by UV and MS.

3.1. Preparation of Cells

Before performing the following steps, media should be taken out of the refrigerator and warmed to 37°C using a water bath. Make certain that the temperature never rises above 40°C. All steps performed in Subheading 3.1 were done in a laminar flow hood in a Biosafety Level 2 laboratory. The hood was sterilized with 70% (v/v) ethanol and exposed to UV light before use. All items taken into the hood were swabbed with 70% (v/v) ethanol.

3.1.1. NCCIT Cell Culture Inoculation and Maintenance

1. Remove a vial of cells from the liquid nitrogen tank and thaw quickly by swirling in the 37°C water bath. Make sure not to submerge the vial. Transfer the contents of the vial to a 15-mL centrifuge tube. With swirling add in a dropwise fashion 9 mL of warmed (37°C) NCCIT medium.
2. Centrifuge the cells at 250 × *g* for 5 min. Discard the supernatant and resuspend the pellet in 10 mL of prewarmed (37°C) NCCIT medium. Transfer the cells to a T25-cm^2 flask and place in the 37°C incubator.
3. Change culture medium when the color indicator in the medium changes from rosy pink to yellow (approximately every 2 days, change the media at least three times per week).
4. Take the cell culture flask out of the 37°C incubator and remove the medium carefully using sterile glass pipette. Avoid touching the side where cells are growing and add 10 mL of fresh medium to the flask before returning the cell culture flask to the incubator.
5. NCCIT cells should be passaged when they reach 80% of the confluency on the wall of cell culture flask (in our experience about 4 days) (Fig. 1). For passaging, take the cell culture flask out of the 37°C incubator. Using a microscope (20-fold magnification), estimate the confluency of cells growth on the flask wall.
6. Take the cell culture flask out of the incubator and remove the medium carefully using sterile glass pipette. Avoid touching the side where cells are growing and add 1 mL of the cell detachment solution.

7. Lay the flask down to let the solution completely contact with the layer of cells. Wait for about 5 min.
8. Agitate flask to make sure the cells are completely detached. Add 9 mL of NCCIT medium. Repeatedly rinse the flask wall with the medium in the flask to detach as many cells as possible from the wall of the flask. Make sure most of the cells are at the bottom of the flask suspended in the medium. Transfer the entire suspension into a 15-mL centrifuge tube and cetrifuge at 1,000 x g for 3 min. Remove the supernatant and resuspend the pellet in 10 mL of fresh NCCIT medium. Transfer an equal volume of 2.5 mL cell suspension into four new culture flasks. Add 7.5 mL of fresh and warmed (37°C) culture medium to each of those new cell culture flasks. Return the cell culture flasks to the 37°C incubator.

3.1.2. NCCIT Cell Harvest

1. Take the cell culture flask out of the incubator and remove the medium carefully using sterile glass pipette. Avoid touching the side where cells are growing and add 1 mL of cell detachment solution.
2. Lay the flask down to let the solution completely contact with the layer of cells and wait for about 5 min. Add 9 mL of NCCIT medium.
3. Repeatedly rinse the wall cells grow on for several times with the medium in the flask, detaching as many cells as possible from the wall of the flask. Ensure that most of the cells are at the bottom of the flask suspended in the medium.
4. Transfer the entire suspension into a 15-mL centrifuge tube.
5. Centrifuge the cultured cells at 1,000 × *g* for 3 min and remove the supernatant.
6. Resuspend the pellet in 5 mL of fresh NCCIT medium.
7. Always count cell before harvesting and only harvest cells when they reach 10^6 cells/mL.
8. Before cell counting, make sure the medium and the cells are mixed well. Combine 10 μL cell suspension and 10 μL trypan blue stain in one well of a 96-well plate and mix well.
9. Inject 10 μL mixture into the cleft of the hemocytometer and count the number of both living cells (transparent) and dead cells (blue).
10. Determine the percentile of cell viability and calculate the approximate total cell number in the medium. If viability is >50%, replate at higher density.
11. Repeat step 5. To the pellet, add 10 mL of PBS buffer.
12. Gently mix the cells and buffer using a pipette. Centrifuge at 1,000 × *g* for 3 min and rinse with PBS another two times, then collect the cell pellet after the last centrifugation minimum amount of PBS buffer (see Note 3).

3.2. Recovery and Purification of GAGs

Start with approximately 10^7 NCCIT cells prepared and counted using a hemocytometer as described in Subheading 3.1. Total GAG extraction as described previously (23) requires a multistep procedure (see Note 4).

[3]We tested series of different number of cells for GAGs extraction, recovery and subsequent disaccharide analysis, we found that 1 × 10^6 is currently the minimum number of cells required for disaccharide quantification.

1. Lyophilize cells by freezing the cell pellet from Subheading 3.1.2.12 at −60°C for 30 min. Place the frozen cell pellet into a tube in a freeze dryer bottle (Fisher Scientific) and attach to a lyophilizer. The sample is freeze-dried overnight under pressure of $<1.3 \times 10^{-8}$ bar at a collector temperature of −40°C.
2. Defatting involves the three-step washing of the cell pellet with 3 mL each of 2:1, 1:1, 1:2 (v/v) chloroform/methanol. Samples are placed on a shaker at a speed of 200 rpm at room temperature. Each step takes about 8–10 h.
3. Between the steps, leave the mixture to sediment. Remove the supernatant portion with a glass pipette before adding the new wash.
4. Perform a proteolysis step by incubating defatted cell pellets with actinase E proteolysis solution at 55°C overnight.
5. For GAGs extraction, add dry urea and dry CHAPS to the proteolyzed aqueous sample to obtain a final concentration of 2% (wt%) in CHAPS and 8 M in urea. Remove particles from the resulting solutions by either centrifuging at 4,000 × *g* or by passing the samples through a 0.22-μm syringe filter.
6. To recover and purify GAG use a Vivapure Mini Q H spin column (see Note 5). Wash and preequilibrate spin columns with 200 μL denaturing solution by centrifuging at 2,000 × *g*. Load sample (approximately 0.5 mL) onto the wet spin column and run through the spin columns under 2,000 × *g*. Wash the spin column once with 200 μL denaturing solution at 2,000 × *g*.
7. Next, wash the spin column five times at 2,000 × *g* with 200 μL prewash solution to remove nonspecific binding materials.
8. Elute HS and CS GAGs from column by washing three times at 2,000 × *g* with 50 μL of collection solution.
9. Desalt GAGs with a Microcon® Centrifugal Filter Units YM-3 (3,000 MWCO) spin column (see Note 6). To do so, load 100 μL of NaOH to prewash the spin column and centrifuge at 12,000 × *g*.
10. Rinse the column five times with 400 μL of water to remove all the NaOH, centrifuging after each wash at 12,000 × *g*. Make sure the eluate is at pH 7 before proceeding further using pH strips.
11. Load GAG samples and centrifuge at 12,000 × *g*.
12. Wash the membrane five times with 400 μL of water to completely remove salts and other small molecules, centrifuging at 12,000 × *g* after each wash.
13. The GAGs are recovered from the top layer of the filtration membrane by inverting the membrane and centrifuging at 1,000 × *g*.
14. Then rinse the surface of membrane three times, each time with 100 μL of water centrifuging at 1,000 × *g* to obtain residual GAGs on membrane. Store the GAG-containing wash (approximately 350 μL) at 4°C or lyophilize for future use.

[4]This method for total GAGs extraction and recovery was developed in our lab, involving in the use of a simple recovery and purification that relies on protease digestion and strong anion-exchange chromatography on a spin column followed by salt. Urea, a nonionic denaturant, is known to solubilize most proteins, and Chaps, a zwitterionic surfactant, is commonly used to solubilize hydrophobic molecules such as triglycerides. Approximately 90% of GAGs can be recovered using this method.

[5]When Vivapure spin filters are used, make sure the centrifugal force is not >2,000 × *g*. For each centrifugation, adjust time carefully so that there is always residual liquid above on the top of the membrane to avoid dryness and membrane cracking.

[6]When Microcon® Centrifugal Filter Units YM-3 (3,000 MWCO) spin columns are used, make sure the centrifugal force is not >14,000 × *g*. For each centrifugation, adjust time carefully so that there is always residual liquid above on the top of the membrane to avoid dryness and membrane cracking.

3.3. Analysis of Intact GAGs

3.3.1. Preparing a Gel

1. Preparing the gel in a mini-gel electrophoresis system begins by washing all equipment and glass plates thoroughly with detergent before and rinsed extensively with distilled water before and after each use.
2. Prepare a 0.75 mm thick, 22% gel by mixing 6 mL of front gel buffer, 36 μL of 10% (w/w) aqueous ammonium persulfate solution, and 6 μL of TEMED, and mix rapidly with a needle.
3. Inject the gel into the sandwich glass plates by syringe, leaving some space for a stacking gel.
4. Cover the upper layer of the gel with water. The gel should polymerize within about 30 min depending on the room temperature. After the polymerization is set, carefully remove the water.
5. Prepare the stacking gel by mixing 2 mL of stacking gel buffer with 60 μL of 10% (w/v) aqueous ammonium persulfate solution, and 2 μL of TEMED, mixing rapidly with a needle. Using a syringe, inject the stacking gel to the top of separating gel. Insert a comb, carefully avoiding any air bubbles. The stacking gel should polymerize within about 30 min at room temperature.
6. Assemble the inner core of gel system. Once the stacking gel polymerization is done, carefully remove the comb and pour in upper buffer. Make certain there is no leakage on the assembled inner core. Add the resolving buffer to the lower chambers of the gel system.

3.3.2. Loading and Running the Gel

1. Mix 5 μL of sample and 5 μL of 50% (w/v) sucrose.
2. Load 10 μL of each sample into a well. One well should contain a mixture of heparin oligosaccharide standards, such as oligo-saccharides prepared by the partial enzymatic depolymerization of heparin, and one well should contain a purified heparin oli-gosaccharide, such as an octasaccharide. Phenol Red (0.5 μL) can be added to this well if a visible indicator if needed.
3. Complete the assembly of the gel system and connect to a power supply. Gel electrophoresis is performed at 200 V for 90 min or until the phenol red reaches the bottom of the plate.

3.3.3. Staining, Destaining, and Quantifying the Data

1. After electrophoresis is complete, carefully separate the gel from the glass plates.
2. Stain the gel with Alcian blue dye reagent for 30–60 min.
3. Completely destain the gel by shaking overnight in water.
4. Wrap the gel in clear plastic wrap and scan the gel with on a standard computer scanner (Fig. 2). The scan can then be digitized using UN-Scan-it software.
5. A standard curve, the log of the molecular weight of each heparin oligosaccharide band (disaccharide 665, tetrasaccharide 1330, hexasaccharide 1995, etc.) as a function of migration distance, is plotted.
6. The pure oligosaccharide is used to provide a counting frame by identifying the size of the oligosaccharides in the heparin oligosaccharide mixture.

7. The average molecular weights of the GAGs isolated from the cells are calculated based on this standard curve (24).

3.4. GAG Depolymerization

1. Incubate intact GAGs recovered in Subheading 3.2 with the chondroitinase ABC and ACII enzymes at 37°C for 10 h.
2. Recover the products of the chondroitinase treatment using the Microcon® Centrifugal Filter Units YM-3 (3,000 MWCO). Refer to Subheading 3.2, steps 9–14 for detailed procedures.
3. In the current case, collect both the portion, which passes through the membrane, and the portion remaining above the membrane (the retentate). The CS/DS disaccharides have a molecular weight <3,000 and passing through the membrane should be collected in three washes, combined and lyophilized and used for further LC-MS analysis (Subheading 3.5.1). Continue with the retentate with the following step.
4. GAGs in the retentate, remaining on the top of the filtration membrane, are next incubated with the heparinase I, II, and III enzyme mixture at 37°C for 10 h. The HS disaccharides have a molecular weight <3,000 and passing through the Microcon® Centrifugal Filter Units YM-3 (3,000 MWCO) membrane should be collected in three washes, combined, and lyophilized and used for further LC-MS analysis (see Subheading 3.5.2).

3.5. Disaccharide Analysis

This method has been optimized by our laboratory and has been found to work well (2, 25, 26). Two different eluent systems are required for the optimum resolution of the CS/DS and heparin/ HS disaccharides.

3.5.1. CS/DS Disaccharide Analysis

1. Inject 8 μL of disaccharide standards containing 10 ng of each disaccharide or 8 μL of CS/DS disaccharide sample from Subheading 3.4) onto an ACQUITY UPLC™ BEH C18 column.
2. Use HPLC solution A and HPLC solution B to elute CS/DS disaccharides at 100 μL/min.
3. The elution conditions at 45°C are solution A for 10 min, followed by a linear gradient from 10 to 40 min of 0–50% solution B.
4. The column effluent enters the UV detector followed by the source of the electrospray ionization mass spectrometer for continuous detection. Set the electrospray interface in positive ionization mode with the skimmer potential 40.0 V, capillary exit 40.0 V and a source of temperature of 350°C to obtain maximum abundance of the ions in a full scan spectra (350–2,000 Da, ten full scans/s).
5. Use nitrogen as a drying (8 L/min) and nebulizing gas (40 p.s.i.).
6. Use UV detection at 232 nm with simultaneous extracted ion chromatogram (EIC) detection (see Note 7). The results of this analysis are presented in Fig. 3 and Table 1.

[7]The sensitivity in this method improved to 0.2 ng/disaccha-ride of CS/DS.

3.5.2. Heparin/HS Disaccharide Analysis

1. Use a Zorbax SB-C18 column (Agilent, 0.5 × 250 mm, 5 μm) and inject 8 μL of disaccharide standards containing 10 ng of each disaccharide or 8 μL of heparin/HS disaccharide sample from Subheading 3.4.
2. Use HPLC solution C and HPLC solution D to elute heparin/HS disaccharides at 10 μL/min.
3. The elution conditions at 20°C are solution C for 20 min, followed by a linear gradient from 20 to 45 min of 0–50% solution D.
4. The column effluent should enter the UV detector followed by the source of the electrospray ionization mass spectrometer for continuous detection.
5. Add another 5 μL/min of acetonitrile just after column and before MS to make the solvent and TrBA easy spray, and easy evaporate in the ion-source.
6. Set the electrospray interface in negative ionization mode with the skimmer potential -40.0 V, capillary exit -40.0 V and a source of temperature of 325°C to obtain maximum abundance of the ions in a full scan spectra (150–1,500 Da, ten full scans/s).
7. Use nitrogen as a drying (5 L/min) and nebulizing gas (20 p.s.i.). Detect with UV at 232 nm with simultaneous extracted ion chromatogram (EIC) detection (see Note 8). The results of this analysis are presented in Fig. 3 and Table 1.

Acknowledgments

Our laboratory acknowledges generous support from the New York State Department of Health and the Empire State Stem Cell Board in the form of grant number N08G-264.

References

1. Raman R, Raguram S, Venkataraman G, Paulson JC, Sasisekharan R. Glycomics: an integrated systems approach to structure-function relationships of glycans. Nat Methods. 2005; 2:817–824. [PubMed: 16278650]
2. Zhang F, Zhang Z, Thistle R, McKeen L, Hosoyama S, Toida T, et al. Structural characterization of glycosaminogly-cans from zebrafish in different ages. Glycoconj J. 2009; 26:211–218. [PubMed: 18777207]
3. Park Y, Yu G, Gunay NS, Linhardt RJ. Purification and characterization of heparan sulphate proteoglycan from bovine brain. Biochem J. 1999; 344(Pt 3):723–730. [PubMed: 10585858]
4. Warda M, Toida T, Zhang F, Sun P, Munoz E, Xie J, et al. Isolation and characterization of heparan sulfate from various murine tissues. Glycoconj J. 2006; 23:555–563. [PubMed: 17006646]
5. Nairn AV, Kinoshita-Toyoda A, Toyoda H, Xie J, Harris K, Dalton S, et al. Glycomics of proteoglycan biosynthesis in murine embryonic stem cell differentiation. J Proteome Res. 2007; 6:4374–4387. [PubMed: 17915907]
6. Linhardt RJ, Toida T. Role of glycosaminoglycans in cellular communication. Acc Chem Res. 2004; 37:431–438. [PubMed: 15260505]
7. Linhardt RJ. 2003 Claude S. Hudson Award address in carbohydrate chemistry. Heparin: structure and activity. J Med Chem. 2003; 46:2551–2564. [PubMed: 12801218]
8. Johnson Z, Proudfoot AE, Handel TM. Interaction of chemokines and gly-cosaminoglycans: a new twist in the regulation of chemokine function with opportunities for therapeutic intervention. Cytokine Growth Factor Rev. 2005; 16:625–636. [PubMed: 15990353]
9. Capila I, Linhardt RJ. Heparin-protein interactions. Angew Chem Int Ed Engl. 2002; 41:390–412.

[8]The sensitivity in this method improved to 2 ng/disaccharide of HS/Hp.

10. Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. Nat Rev Drug Discov. 2009; 8:235–253. [PubMed: 19247306]
11. Hoffman LM, Carpenter MK. Characterization and culture of human embryonic stem cells. Nat Biotechnol. 2005; 23:699–708. [PubMed: 15940242]
12. Giacomini M, Baylis F, Robert J. Banking on it: public policy and the ethics of stem cell research and development. Soc Sci Med. 2007; 65:1490–1500. [PubMed: 17590489]
13. Uygun BE, Stojsih S, Matthew H. Effects of immobilized glycosamino-glycans influence proliferation and differentiation of mesenchymal stem cells. Tissue Eng Part A. 2009; 15(11): 3499–3512. [PubMed: 19456238]
14. Kumarasuriyar A, Murali S, Nurcombe V, Cool SM. Glycosaminoglycan composition changes with MG-63 osteosar-coma osteogenesis in vitro and induces human mesenchymal stem cell aggregation. J Cell Physiol. 2009; 218:501–511. [PubMed: 18988189]
15. Dombrowski C, Song SJ, Chuan P, Lim X, Susanto E, Sawyer AA, et al. Heparan sulfate mediates the proliferation and differentiation of rat mesenchymal stem cells. Stem Cells Dev. 2009; 18:661–670. [PubMed: 18690792]
16. Carney SL, Muir H. The structure and function of cartilage proteoglycans. Physiol Rev. 1988; 68:858–909. [PubMed: 3293094]
17. Poole AR. Proteoglycans in health and disease: structures and functions. Biochem J. 1986; 236:1–14. [PubMed: 3539097]
18. Bayliss MT, Osborne D, Woodhouse S, Davidson C. Sulfation of chon-droitin sulfate in human articular cartilage. The effect of age, topographical position, and zone of cartilage on tissue composition. J Biol Chem. 1999; 274(22):15892–15900. [PubMed: 10336494]
19. Rizkalla G, Reigner A, Bogoch E, Poole AR. Studies of the articular cartilage proteoglycan aggrecan in health and osteoarthritis. Evidence for molecular heterogeneity and extensive molecular changes in disease. J Clin Invest. 1992; 90:2268–2277. [PubMed: 1281828]
20. Plaas AH, Wong-Palms S, Roughley PJ, Midura RJ, Hascall VC. Chemical and immunological assay of the nonreducing terminal residues of chondroitin sulfate from human aggrecan. J Biol Chem. 1997; 272:20604–20610.
21. Hitchcock AM, Yates KE, Shortkroff S, Costello CE, Zaia J. Optimized extraction of glycosaminoglycans from normal and osteoarthritic cartilage for glycomics profiling. Glycobiology. 2007; 17(1):25–35. [PubMed: 16980326]
22. Pervin A, Gallo C, Jandik KA, Han XJ, Linhardt RJ. Preparation and structural characterization of large heparin-derived oligosaccharides. Glycobiology. 1995; 5:83–95. [PubMed: 7772871]
23. Zhang F, Sun P, Munoz E, Chi L, Sakai S, Toida T, et al. Microscale isolation and analysis of heparin from plasma using an anion-exchange spin column. Anal Biochem. 2006; 353:284–286. [PubMed: 16529709]
24. Edens RE, Al-Hakim A, Weiler JM, Rethwisch DG, Fareed J, Linhardt RJ. Gradient polyacrylamide gel electrophoresis for determination of the molecular weights of heparin preparations and low-molecular-weight heparin derivatives. J Pharm Sci. 1992; 81:823–827. [PubMed: 1328601]
25. Zhang Z, Park Y, Kemp MM, Zhao W, Im AR, Shaya D, et al. Liquid chromatography-mass spectrometry to study chondroitin lyase action pattern. Anal Biochem. 2009; 385:57–64. [PubMed: 18992215]
26. Zhang Z, Xie J, Liu H, Liu J, Linhardt RJ. Quantification of hepa-ran sulfate disaccharides using ion-pairing reversed-phase microflow high-performance liquid chromatography with electrospray ion-ization trap mass spectrometry. Anal Chem. 2009; 81 :4349–4355. [PubMed: 19402671]
27. Teshima S, Shimosato Y, Hirohashi S, Tome Y, Hayashi I, Kanazawa H, Kakizoe T. Four new human germ cell tumor cell lines. Lab Invest. 1988; 59:328–336. [PubMed: 2842544]
28. Damjanov I, Horvat B, Gibas Z. Retinoic acid-induced differentiation of the developmentally pluripotent human germ cell tumor-derived cell line, NCCIT. Lab Invest. 1993; 68:220–232. [PubMed: 7680083]

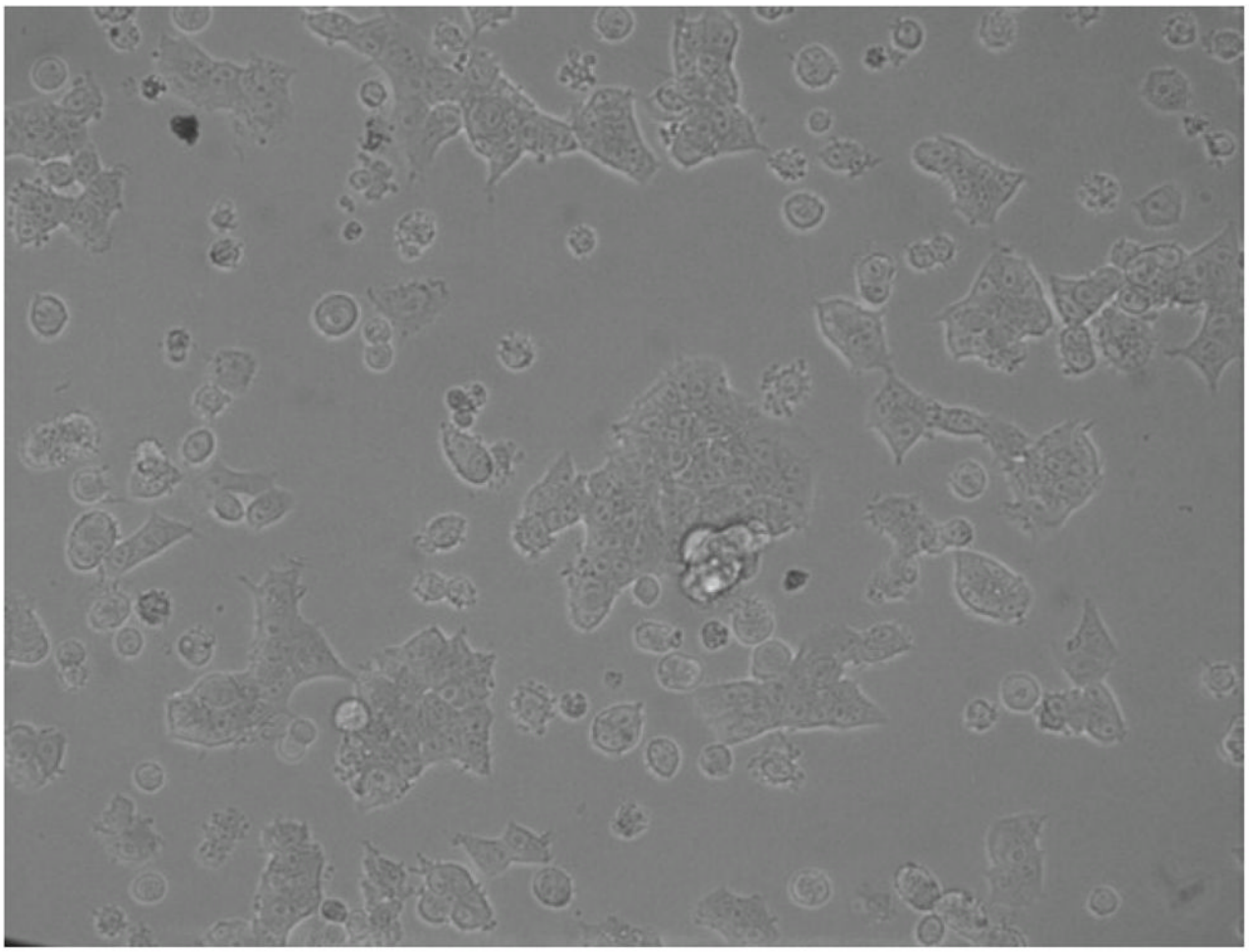

Fig. 1.
View of cultured teratocarcinoma cells using phase-contrast microscopy.

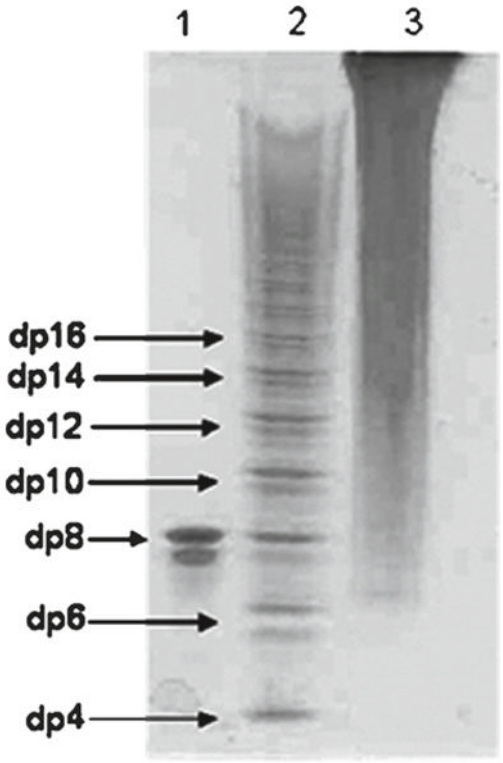

Fig. 2.

PAGE analysis on GAGs isolated from NCCIT cells. Lane 1 shows heparin oligosaccharide with degree of polymer-ization of eight (dp8). Lane 2 shows heparin oligosaccharide standard where the degree of polymerization (dp) from four (tetrasaccharide) to 14 tetradecasaccharide is labeled. Lane 3 shows the isolated intact GAG mixture. PAGE analysis with Alcian blue staining confirmed that GAGs were present by a broad band of expected polydispersity. After digitizing the gels using UN-Scan-it software, the average MW of GAGs were calculated based on the heparin oligosaccharide standards. The average molecular weight of GAGs from NCCIT cells is 15.53 kDa.

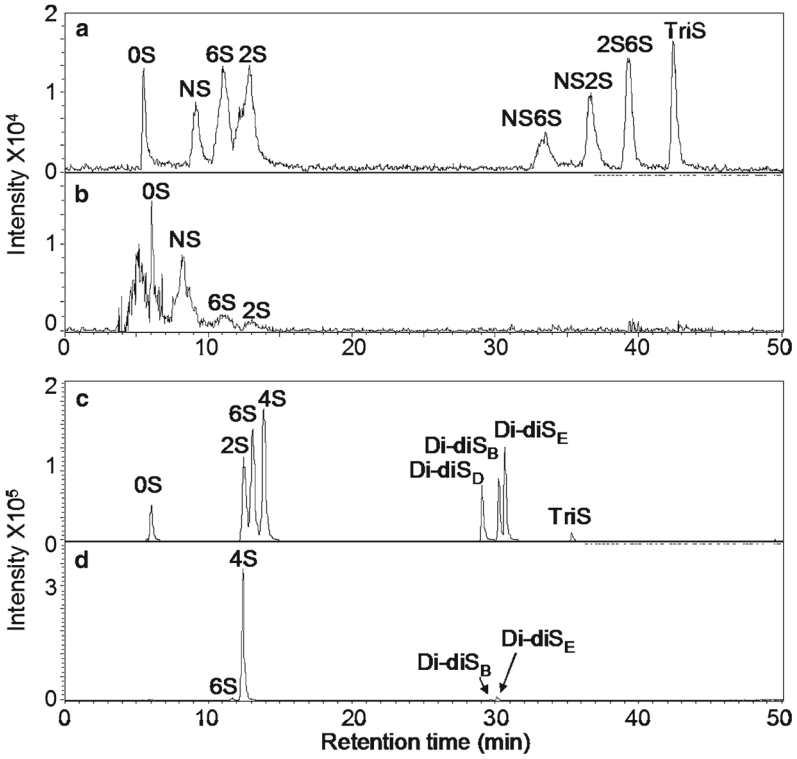

Fig. 3.

LC-MS disaccharide analysis of NCCIT cells. (**a**) EIC of HS/Hp disaccharide std. (**b**) EIC of HS/Hp disaccharide of NCCIT cells. (**c**) EIC of CS/DS disaccharide std. (**d**) EIC of CS/DS disaccharide of NCCIT cells.

Table 1

Disaccharide composition of NCCIT cells

HS/Hp	**0S**	**NS**	**6S**	**2S**	**NS6S**	**NS2S**	**2S6S**	**TriS**
	23.8%	47.6%	18.1%	10.5%	n.d.	n.d.	n.d.	n.d.
CS/DS	**0S**	**2S**	**6S**	**4S**	**Di-diS$_D$**	**Di-diS$_B$**	**Di-diS$_E$**	**TriS**
	n.d.	n.d.	1.2%	94.3%	n.d.	0.3%	4.2%	n.d.

HS/Hp: 0S, ΔUA-GlcNAc; NS, ΔUA-GlcNS; 6S, ΔUA-GlcNAc6S; 2S, ΔUA2S-GlcNAc; NS2S, ΔUA2S-GlcNS; NS6S, ΔUA-GlcNS6S; 2S6S, ΔUA2S-GlcNAc6S; TriS, ΔUA2S-GlcNS6S. CS/DS: 0S, ΔUA-GalNAc; 2S, ΔUA2S-GalNAc; 6S, ΔUA-GalNAc6S; 4S, ΔUA-GalNAc4S; Di-diS$_B$ ΔUA2S-GalNAc4S; Di-diS$_D$, ΔUA2S-GalNAc6S; Di-diS$_E$, ΔUA-GalNAc4S6S; TriS, ΔUA2S-GalNAc4S6S; n.d., not detected.