Published in final edited form as:
*Glycoconj J*. 2009 February ; 26(2): 211–218. doi:10.1007/s10719-008-9177-x.

# Structural characterization of glycosaminoglycans from zebrafish in different ages

**Fuming Zhang**, **Zhenqing Zhang**, **Robert Thistle**, **Lindsey McKeen**, **Saori Hosoyama**, **Toshihiko Toida**, **Robert J. Linhardt**, and **Patrick Page-McCaw**
*F. Zhang, R. J. Linhardt, Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA*

*Z. Zhang, R. J. Linhardt, Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA*

*R. Thistle, L. McKeen, R. J. Linhardt, P. Page-McCaw, Department of Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA*

*S. Hosoyama, T. Toida, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan*

## Abstract

The zebrafish (*Danio rerio*) is a popular model organism for the study of developmental biology, disease mechanisms, and drug discovery. Glycosaminoglycans (GAGs), located on animal cell membranes and in the extracellular matrix, are important molecules in cellular communication during development, in normal physiology and pathophysiology. Vertebrates commonly contain a variety of GAGs including chondroitin/dermatan sulfates, heparin/heparan sulfate, hyaluronan and keratan sulfate. Zebrafish might represent an excellent experimental organism to study the biological roles of GAGs. A recent study showing the absence of heparan sulfate in adult zebrafish, suggested a more detailed evaluation of the GAGs present in this important model organism needed to be undertaken. This report aimed at examining the structural alterations of different GAGs at the molecular level at different developmental stages. GAGs were isolated and purified from zebrafish in different stages in development ranging from 0.5 days to adult. The content and disaccharide composition of chondroitin sulfate and heparan sulfate were determined using chemical assays, liquid chromotography and mass spectrometry. The presence of HS in adult fish was also confirmed using $^{1}$H-NMR.

### Keywords

Zebrafish; Glycosaminoglycans; LCMS; NMR

## Introduction

The zebrafish is a popular model vertebrate organism because of its fecundity, its morphological and physiological similarity to mammals. The existence of many genomic tools and the ease with which large, phenotype-based screens can be performed also makes zebrafish an attractive model. Researchers from disparate fields, including developmental biology, neuroscience, and cardiovascular research all rely on the zebrafish model and over the past few years, the research in this area has resulted in a wealth of fundamental information about

Correspondence to: Robert J. Linhardt, linhar@rpi.edu; Patrick Page-McCaw, pagem@rpi.edu.

embryonic development and diseases [1-4]. The zebrafish genome has been sequenced and human disease gene homologs have been identified [5,6]. Zebrafish have also been used at various stages of the drug discovery process and offers a cost-effective alternative to some mammalian models [7,8].

The GAGs are long, linear, sulfated, and highly charged heterogeneous polysaccharides that are involved in numerous biological functions, including organogenesis and growth control, cell adhesion, signaling, inflammation, tumorigenesis, and interactions with pathogens [9]. A series of carbohydrate modifying enzymes and sulfotransferases are involved in the biosynthesis of GAGs. Zebrafish have been used as a vertebrate model organism for study the biological function of GAGs in different developmental stages through knockout or mutation of genes encoding the GAG biosynthetic enzymes. GAGs are believed to be essential in many zebrafish developmental processes, such as gastrulation [10,11], angiogenesis, muscle development [12], cardiac valve development [13], and axon guidance during embryonic development [14,15]. HSPG synthesis by zebrafish Ext2 and Extl3, which encode glycosyltransferases for heparan sulphate biosynthesis, is required for FGF10 signalling during limb development [16,17]. While many reports clearly indicate the presence of GAGs in zebrafish, very little is known about GAG structure, especially in the early development stages. Recently, Souza and coworkers [18] reported that the GAGs in adult fish are primarily chondroitin sulfate (CS) and keratan sulfate (KS) and that no heparin or heparan sulfate was detected. These surprising results seem to conflict with previous reports [10,11,16,19]. In light of the essential role of HS in zebrafish biology, we decided to undertake the detailed structural analysis of the GAGs at different stages of zebrafish development.

## Materials and methods

### Materials

Actinase E was from Kaken Biochemicals (Tokyo, Japan). Chondroitin sulfate, chondroitin lyases ABC and ACII and heparin lyases 1, 2 and 3 were from Seikagaku (Tokyo, Japan). Polyacrylamide, urea, CHAPS, alcian blue dye, and tetra-*n*-butylamonium hydrogen sulfate, were from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of regent grade. Vivapure MAXI QH columns were from Viva science (Edgewood, NJ, USA). Unsaturated disaccharides standards of CS/DS (ΔDi-0S: ΔUA-GalNAc, ΔDi-4S: ΔUA-GalNAc4S, ΔDi-6S: ΔUA-GalNAc6S, ΔDi-2S: ΔUA2S-GalNAc, ΔDi-di$S_B$:ΔUA2S-GalNAc4S, ΔDi-di$S_D$: ΔUA2S-GalNAc6S, ΔDi-di$S_E$: ΔUA-GalNAc4S6S, ΔDi-triS: ΔUA2S-GalNAc4S6S) and unsaturated disaccharides standards of heparin/HS (ΔDi-0S: ΔUA-GlcNAc, ΔDi-NS: ΔUA-GlcNS, ΔDi-6S: ΔUA-GlcNAc6S, ΔDi-2S: ΔUA2S-GlcNAc, ΔDi-2SNS: ΔUA2S-GlcNS, ΔDi-NS6S: ΔUA-GlcNS6S, ΔDi-2S6S: ΔUA2S-GlcNAc6S, ΔDi-triS: ΔUA2S-GlcNS6S) were obtained from Seikagaku Corporation (Japan).

### Zebrafish

Zebrafish embryos and adults (3–4 months of age) were raised and maintained at 28.5°C under a 14-/10-h circadian cycle under standard laboratory conditions [20]. Mixed populations of TL and wik strain animals were used for all samples. Animals were 12 and 24 h post fertilization and 5 days post fertilization or retired breeding stock adults.

### Isolation and purification of GAGs from zebrafish [21]

Zebrafish samples were crushed with dry ice into very fine homogenized powder using a mortar and pestle. The homogenized samples (in 5 ml water) were individually subjected to proteolysis at 55°C with 10% of actinase E (20 mg/ml) for 18 h. After proteolysis, dry urea and dry CHAPS were added to each sample (2 wt.% in CHAPS and 8 M in urea). Particulates were removed from the resulting solutions by passing each through a syringe filter containing a 0.22-μm

membrane. A Vivapure MAXI Q H spin column was prepared by equilibrating with 3 ml of 8 M urea containing 2% CHAPS (pH 8.3). The clarified filtered samples were loaded onto and run through the Vivapure MAXI QH spin columns under centrifugal force (500×*g*). The columns were first washed with 3 ml of 8 M urea containing 2% CHAPS at pH 8.3. The columns were then washed five-times with 5 ml of 200 mM NaCl. GAGs were released from the spin column by washing three-times with 1 ml of 16% NaCl. Methanol (12 ml) was added to the GAG solution in sodium chloride to afford an 80 vol% solution and the mixture was equilibrated at 4°C for 18 h. The resulting precipitate was recovered by centrifugation (2,500×*g*) for 15 min. The precipitate was recovered by dissolving in 0.5 ml of water and the recovered GAGs were stored frozen for further analysis.

### Quantification of GAGs by carbazole assay

The isolated GAGs were subjected to carbozole assay [22] to quantify the amount of GAG in each sample using heparan sulfate as standard. A standard curve of the heparan sulfate gave the equation $y = 17.521x + 0.0023$, $r^2=0.979$

### Polyacrylamide gel electrophoresis (PAGE) analysis

Polyacrylamide gel electrophoresis (PAGE) was applied to analyze the heparan sulfate and chondroitin sulfate in the GAG sample from adult fish. To each lane ~5 μg of intact GAG mixture or GAG mixture treated with heparinase or chondroitinase GAGs was subjected to electrophoresis against a standard composed of heparin oligosaccharides prepared enzymatically from bovine lung heparin [23]. The gel was visualized with alcian blue. The gel was then digitized with UN-Scan-it software (Silk Scientific, Utah, USA) and the average MW of the GAGs was calculated based on the heparin oligosaccharide standard [23].

### Disaccharide composition analysis using LCMS

Enzymatic depolymerization of GAGs: GAG samples (20 μg/5 μl) were incubated with the chondroitinase ABC (10 m-units) and chondroitinase ACII (5 m-units) at 37°C for 10 h. The enzymatic products were recovered by the centrifugal filtration (YM-3, 3000 MWCO, Millipore, Bedford, MA). CS/DS disaccharides, passed through the filter, were freeze-dried and ready for LC-MS analysis. Next, the heparinase I, II and III (5 mU each) were added into the remainder and incubated at 37°C for 10 h. The products were again recovered by centrifugal filtration and the heparin/HS disaccharides were similarly collected and freeze-dried and ready for LC–MS analysis. The LC–MS analysis was performed on a LC–MS system (Agilent, LC/MSD trap MS) [24]. Solution A and B for HPLC were 15% and 70% acetonitrile ($CH_3CN$), respectively, containing the same concentration of 37.5 mM $NH_4HCO_3$ and 11.25 mM tributylamine. The pH values of them were adjusted to 6.5 with acetic acid. The flow rate was 10 μl/min. The separation was performed on a C-18 column (Agilent) using solution A for 20 min, followed by a linear gradient from 20 to 45 min of 0% to 50% solution B. The column effluent entered the source of the ESI–MS for continuous detection by MS. The electrospray interface was set in negative ionization mode with the skimmer potential −40.0 V, capillary exit −120.5 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 per second). Nitrogen was used as a drying (5 l/min) and nebulizing gas (20 p.s.i.).

### Disaccharide composition analysis using high performance liquid chromatography with post-column fluorescence detection

Unsaturated disaccharides produced from heparin lyase or chondroitin lyase treatment of GAGs were also determined by high performance liquid chromatography with post-column fluorescence detection [25]. A gradient was applied at a flow rate of 1.1 ml/min on a Docosil column (4.6×150 mm) at 55°C. The eluents used were as follows: A, $H_2O$; B, 0.2 M sodium

chloride; C, 10 mM tetra-*n*-butyl ammonium hydrogen sulfate; D, 50% acetonitrile. The gradient program was as follows: 0–10 min, 1–4% eluent B; 10–11 min, 4–15% eluent B; 11–20 min, 15–25% eluent B; 20–22 min, 25–53% eluent B; and 22–29 min, 53% eluent B. The proportions of eluent C and D were constant at 12% and 17%, respectively. To the effluent were added aqueous 0.5% (*w/v*) 2-cyanoacetamide solution and 0.25 M NaOH at the same flow rate of 0.35 ml/min by using a double plunger pump. The mixture passed through a reaction coil (diameter, 0.5 mm; length, 10 m) set in a temperature controlled bath at 125°C and a following cooling coil (diameter, 0.25 mm; length, 3 m). The effluent was monitored fluorometrically (excitation, 346 nm; emission, 410 nm). The following standard unsaturated disaccharides from heparin/HS, ΔUA-GlcNAc, ΔUA-GlcNS, ΔUA-GlcNAc6S, ΔUA2S-GlcNAc, ΔUA-GlcNS6S, ΔUA2S-GlcNS, Δ UA2S-GlcNAc6S, and ΔUA2S-GlcNS6S were used to prepare a standard curve for HS analysis. The unsaturated disaccharides from chondroitin/dermatan sulfate, ΔUA-GclNAc, ΔUA2S-GlcNAc, ΔUA-GalNAc, ΔUA-GalNAc4S, ΔUA-GalNAc6S and ΔUA-GalNAc4S6S and ΔUA2S-GalNAc4S6S were used to prepare a standard curve for chondroitin sulfate analysis.

### NMR analysis

After treating the total GAG isolate from zebrafish with chondroitin lyase (to remove the CS), the heparin/HS was analyzed by $^{1}$H-NMR spectroscopy to characterize its structure. $^{1}$H-NMR was performed on Bruker 800 spectrometer with Topsin 2.0 software. Commercial HS (from porcine intestine, Celsus Co.) and HS (μg) from fish was each dissolved in 0.5 ml $D_2O$ (99.996%, Sigma, Co.) and freeze-dried repeatedly to remove the exchangeable protons. The samples were re-dissolved in 0.3 ml $D_2O$ and transferred to an NMR tube (Sigma). The operation conditions for spectra were as follows: frequency, 800 MHz; wobble sweep width, 20 MHz; filter width, 125 KHz; pre-scan delay, 6 μs; transmitter frequency offset, 4.704 ppm; temperature, 300 K.

## Results and discussion

### Quantification of GAGs by carbozole assay

Using a simple three-step procedure involving protease digestion, strong-anion-exchange chromatography on a spin column followed by salt release and methanol precipitation that had been previously established to quantitatively isolation of heparin from human plasma [21], we isolated the total GAGs from zebrafish samples. The dry weight of the fish sample and isolated GAGs were determined and the GAG samples were subjected to carbozole assay to quantify the amount of GAG in each sample (Table 1). The content of GAG (microgram per gram dry sample) in the adult fish was an order of magnitude lower than fish of 0.5, 1 and 5 days of age.

### Polyacrylamide gel electrophoresis (PAGE) analysis

GAGs isolated from the adult fish were next analyzed by using PAGE with Alcian blue staining (Fig. 1). PAGE analysis with alcian blue staining confirmed that GAGs were present by a broad band of expected polydispersity (Fig. 1). After digitizing the gels, the average MW of GAGs were calculated based on the heparin oligosaccharide standards [23]. The average molecular weight of GAGs from adult fish was 12.95 kD. PAGE analysis also clearly demonstrated that GAGs were susceptible to both heparin lyase and chondroitin lyase digestion, demonstrating that the major GAG in zebrafish is CS/DS.

### Disaccharide composition analysis of GAGs

Composition analysis of disaccharides gives important structural information on the GAGs being analyzed and is an efficient method to measure the variation of structures of GAGs derived from different fish samples. Heparin/HS GAGs are comprised of eight repeating

disaccharide sequences. As a result, exhaustive enzymatic digestion of HS can produce up to eight different unsaturated disaccharides: ΔUA-GlcNAc, ΔUA-GlcNS, ΔUA-GlcNAc6S, ΔUA2S-GlcNAc, ΔUA-GlcNS6S, ΔUA2S-GlcNS, ΔUA2S-GlcNAc6S, and ΔUA2S-GlcNS6S (where ΔUA is Δ-deoxy-α-L-*threo*-hex-4-enopyranosyl uronic acid, GlcN is glucosamine, Ac is acetyl, S is sulfo). Similarly, CS/DS also is comprised of variable sequences from which up to 8 disaccharides can be obtained: ΔUA-GalNAc, ΔUA-GalNAc4S, ΔUA-GalNAc6S, ΔUA2S-GalNAc, ΔUA2S-GalNAc4S, ΔUA2S-GalNAc6S, ΔUA-GalNAc4S6S, ΔUA2S-GalNAc4S6S.

We developed a LC/MS method to analyze the disaccharide composition of GAGs in our lab. The ion-paring reverse phase capillary HPLC gave good resolution of eight standard heparin/HS disaccharides (Fig. 2b), their MS spectra were obtained with ESI micro spray MS [24]. In this disaccharide composition analysis, CS was digested with chondroitin lyases prior to digestion with heparin lyases. The CS disaccharide analysis results for 5-day and adult fish are present in Table 2 (the disaccharide was not detectable with the 0.5-day and 1-day fish). It is noteworthy that in adult fish, there was significantly increase in ΔDi-4S and ΔDi-diS (B or D) while ΔDi-6S decrease in comparison with the 5-day fish. HS and CS disaccharide analysis (Fig. 2 and Table 3) showed no detectable disaccharide in 0.5-day and 1-day samples. A significant decrease in NS6S, 6S and increases in 0S and tri S was observed in adult fish.

To obtain the disaccharide composition in GAGs samples from 0.5-day and 1-day, disaccharide analysis was performed by using high sensitivity HPLC with post-column fluorescence detection (Fig. 3). With this method, ΔDi-6S was detected in the GAGs from 0.5-day fish in the CS disaccharide analysis. For the HS disaccharide analysis, four types of disaccharides (ΔUA-GlcNS, ΔUA-GlcNAc6S, ΔUA-GlcNS6S, and ΔUA2S-GlcNS) and one disaccharide (ΔUA-GlcNS) were detected in the GAGs from 0.5-day and 1-day samples, respectively. The results of the disaccharide composition of the GAGs from 5-day and adult are comparable between this method and LCMS. Apparently the CS and HS disaccharide composition was different in different age.

### HS 1D-$^1$H-NMR spectra

The spectra of commercial standard HS, and HS from fish are shown in Fig. 4. Comparing assigned peaks HS from fish with HS standard, the $^1$H-NMR spectra obtained for fish HS shows a pattern of HS with a higher content of residues containing 2-*O*-sulfated iduronic acid (IdoA2S) (peaks b, c, e, f), but lower content of GlcA residues (peaks d, g, j). IdoA2S was also observed in the HS/heparin disaccharide composition analysis by LC–MS.

## Discussion

Glycosaminoglycans are long, linear heterogeneous chains of charged polysaccharides that play an important role in mediating cell signaling events important for diverse normal biological processes such as embryogenesis and organogenesis as well as pathological processes such as tumorigenesis and inflammation. GAGs are synthesized in the Golgi and modified by carbohydrate modifying enzymes and sulfotransferases to create domains in the GAGs, called the fine structure, that are important in modulating GAG function. The zebrafish has proven to be a useful system in which to study vertebrate development and numerous genes GAG genes or genes required for the GAG synthesis have been studied in zebrafish development. While significant effort has been placed in describing the expression pattern of GAG modifying enzymes, relatively little work has been done to describe the carbohydrate modifications found during development of the zebrafish model. Indeed it remains controversial whether HS is present in zebrafish [18,26-28]. To redress this imbalance, we have performed disaccharide analysis of staged zebrafish embryos, larvae and adults. We find that

chondroitin sulfate and heparan sulfate modifications are dynamically modulated during development.

The first result of this study is that both CS and HS disaccharides are present in the zebrafish. This result is not surprising given that several HSPGs have been demonstrated to be essential for normal zebrafish development. For example, syndecan-4 is required for neural crest migration [29] while syndecan-2 is required for angiogenesis. Further, multiple enzymes required for formation and modification of CS and HS have been identified in zebrafish, their expression patterns in embryogenesis determined and some are known to be essential for development. The present work demonstrates that CS and HS are present in the zebrafish during development and in whole adults. Interestingly, the amount of GAG present as a proportion of total dry mass of tissue is highest during the first 5 days of development, falling to approximately an eighth the embryonic and a tenth the larval concentration in adulthood. This result is consistent with a role of GAGs in playing an essential role in signaling events that drive development and differentiation.

The second major result of this study is that GAG modification is dynamic during zebrafish development. Both CS and HS show developmentally regulated changes in disaccharide composition. CS disaccharide analysis demonstrates that the ΔDi-6S disaccharide is the only detectable disaccharide in 0.5-day animals, while 5-day animals have both ΔDi-4S and ΔDi-6S. Adults have additional detectable disaccharides ΔDi-diSB and ΔDi-diSD. Other disaccharides may be present but were below the threshold of detection. HS analysis showed a different compositional profile during development, having a diverse composition at the earliest time point tested, decreased apparent diversity at 1-day and then increasing diversity at later time points. The earliest time point disaccharide composition was similar to that observed in the adult animals which may be consistent with the presence of maternally contributed HS modification enzymes or maternally contributed HS. Loss of diversity in 1-day old animals may reflect turn-over of maternally contributed material and *de novo* synthesis of HS that is less diverse than in the more mature 5-day old and adult animals.

These changing patterns of GAG modification suggest that these modifications may play an important role in development. It will be interesting to determine whether there is spatial as well as temporal regulation of these heterogeneous GAG modifications.

## Acknowledgements

This work was supported by the National Institute of Health (Grants HL62244 and GH38060 to RL).

## References

1. Rohde LA, Heisenberg CP. Zebrafish gastrulation: Cell movements, signals, and mechanisms. Int Rev Cytol 2007;261:159–192.10.1016/S0074-7696(07)61004-3 [PubMed: 17560282]
2. Itoh N. The Fgf families in humans, mice, and zebrafish: their evolutional processes and roles in development, metabolism, and disease. Biol Pharm Bull 2007;30:1819–1825.10.1248/bpb.30.1819 [PubMed: 17917244]
3. Goessling W, North TE, Zon L. New waves of discovery: modeling cancer in zebrafish. J Clin Oncol 2007;25:2473–2479.10.1200/JCO.2006.08.9821 [PubMed: 17557959]
4. Renshaw SA, Loynes CA, Elworthy S, Ingham PW, Whyte MK. Modeling inflammation in the zebrafish: how a fish can help us understand lung disease. Exp Lung Res 2007;33:549–554.10.1080/01902140701756778 [PubMed: 18075830]
5. Dooley K, Zon L. Zebrafish: a model system for the study of human disease. Curr Opin Genet Dev 2000;10:252–256.10.1016/S0959-437X(00)00074-5 [PubMed: 10826982]

6. Postlethwait JH, Yan YL, Gates MA, Horne S, Amores A, Brownlie A, et al. Vertebrate genome evolution and the zebrafish gene map. Nat Genet 1998;18:345–349.10.1038/ng0498-345 [PubMed: 9537416]
7. Zon L, Peterson RT. *In vivo* drug discovery in the zebrafish. Nat Rev Drug Discov 2005;4:35–44.10.1038/nrd1606 [PubMed: 15688071]
8. Rubinstein AL. Zebrafish: from disease modeling to drug discovery. Curr Opin Drug Discov Dev 2003;6:218–223.
9. Handel TM, Johnson Z, Crown SE, Lau EK, Sweeney M, Proudfoot AE. Regulation of protein function by glycosaminoglyacans—as exemplified by chemokines. Annu Rev Biochem 2005;74:385–410. [PubMed: 15952892]
10. De Cat B, Muyldermans SY, Coomans C, Degeest G, Vanderschueren B, Creemers J, et al. Processing by proprotein convertases is required for glypican-3 modulation of cell survival, Wnt signaling, and gastrulation movements. J Cell Biol 2003;163:625–635.10.1083/jcb.200302152 [PubMed: 14610063]
11. Topczewski J, Sepich DS, Myers DC, Walker C, Amores A, Lele Z, Solnica-Krezel L, et al. The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension. Dev Cell 2001;1:251–264.10.1016/S1534-5807(01)00005-3 [PubMed: 11702784]
12. Bernhardt RR, Goerlinger S, Roos M, Schachner M. Anterior–posterior subdivision of the somite in embryonic zebra-fish: implications for motor axon guidance. Dev Dyn 1998;213:334–347.10.1002/(SICI)1097-0177(199811)213:3<334::AID-AJA9>3.0.CO;2-4 [PubMed: 9825868]
13. Walsh EC, Stainier DY. UDP-glucose dehydrogenase required for cardiac valve formation in zebrafish. Science 2001;293:1670–1673.10.1126/science.293.5535.1670 [PubMed: 11533493]
14. Bernhardt RR, Schachner M. Chondroitin sulfates affect the formation of the segmental motor nerves in zebrafish embryos. Dev Biol 2000;221:206–219.10.1006/dbio.2000.9673 [PubMed: 10772802]
15. Becker CG, Becker T. Repellent guidance of regenerating optic axons by chondroitin sulfate glycosaminoglycans in zebrafish. J Neurosci 2002;22:842–853. [PubMed: 11826114]
16. Bink RJ, Habuchi H, Lele Z, Dolk E, Joore J, Rauch GJ, Geisler R, Wilson SW, den Hertog J, Kimata K, Zivkovic D. Heparan sulfate 6-*O*-sulfotransferase is essential for muscle development in zebrafish. J Biol Chem 2003;278:31118–31127.10.1074/jbc.M213124200 [PubMed: 12782624]
17. Norton WH, Ledin J, Grandel H, Neumann CJ. HSPG synthesis by zebrafish Ext2 and Extl3 is required for Fgf10 signalling during limb development. Development 2005;132:4963–4973.10.1242/dev.02084 [PubMed: 16221725]
18. Souza AR, Kozlowski EO, Cerqueira VR, Castelo-Branco MT, Costa ML, Pavão MS. Chondroitin sulfate and keratan sulfate are the major glycosaminoglycans present in the adult zebrafish *Danio rerio* (Chordata–Cyprinidae). Glycoconj J 2007;24:521–530.10.1007/s10719-007-9046-z [PubMed: 17541818]
19. Chen E, Hermanson S, Ekker SC. Syndecan-2 is essential for angiogenic sprouting during zebrafish development. Blood 2004;103:1710–1719.10.1182/blood-2003-06-1783 [PubMed: 14592839]
20. Westerfield, M. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). Vol. 4. University of Oregon Press; Eugene, OR: 2000. The Zebrafish Book.
21. 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;353:284–286.10.1016/j.ab.2006.01.040 [PubMed: 16529709]
22. Bitter T, Muir HM. A modified uronic acid carbazole reaction. Anal Biochem 1962;4:330–334.10.1016/0003-2697(62)90095-7 [PubMed: 13971270]
23. 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;81:823–827.10.1002/jps.2600810821 [PubMed: 1328601]
24. Thanawiroon C, Rice KG, Toida T, Linhardt RJ. LC/MS sequencing of highly sulfated heparin-derived oligosaccharides. J Biol Chem 2004;279:2608–2615.10.1074/jbc.M304772200 [PubMed: 14610083]
25. Toyoda H, Nagashima T, Hirata R, Toida T, Imanari T. Sensitive high-performance liquid chromatographic method with fluorometric detection for the determination of heparin and heparan

sulfate in biological samples: application to human urinary heparan sulfate. J Chromatogr B Biomed Sci 1997;704:19–24.10.1016/S0378-4347(97)00478-7

26. Cadwallader AB, Yost HJ. Combinatorial expression patterns of heparan sulfate sulfotransferases in zebrafish: I. The 3-*O*-sulfotransferase family. Dev Dyn 2006;235:3423–3431.10.1002/dvdy.20991 [PubMed: 17075882]
27. Chen E, Stringer SE, Rusch MA, Selleck SB, Ekker SC. A unique role for 6-*O* sulfation modification in zebrafish vascular development. Dev Biol 2005;284:364–376.10.1016/j.ydbio.2005.05.032 [PubMed: 16009360]
28. Lee JS, von der Hardt S, Rusch MA, Stringer SE, Stickney HL, Talbot WS, et al. Axon sorting in the optic tract requires HSPG synthesis by ext2 (dackel) and extl3 (boxer). Neuron 2004;44:947–960.10.1016/j.neuron.2004.11.029 [PubMed: 15603738]
29. Matthews HK, Marchant L, Carmona-Fontaine C, Kuriyama S, Larrain J, Holt MR, Parsons M, Mayor R. Directional migration of neural crest cells *in vivo* is regulated by Syndecan-4/Rac1 and non-canonical Wnt signaling/RhoA. Development 2008;135:1771–1780. [PubMed: 18403410]

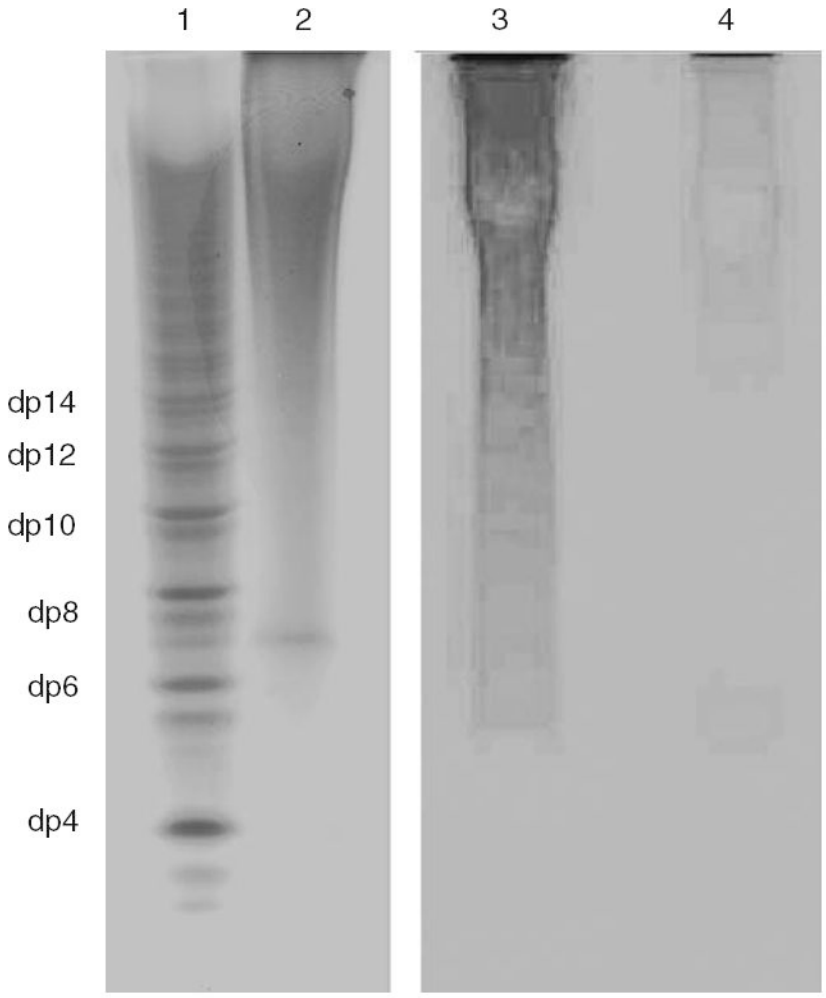


**Fig 1.**

PAGE analysis on GAGs isolated from adult zebrafish. *Lane 1* shows heparin oligosaccharide standard where the degree of polymerization (*dp*) from four (tetrasaccharide) to 14 tetradecasaccharide is labeled. *Lane 2* shows the isolated intact GAG mixture. *Lane 3* corresponds to the GAG mixture following treatment with an equi-unit mixture of heparin lyase 1, 2, and 3. *Lane 4* corresponds to the GAGs treated with an equi-unit mixture of chondroitin lyase ABC and AC II

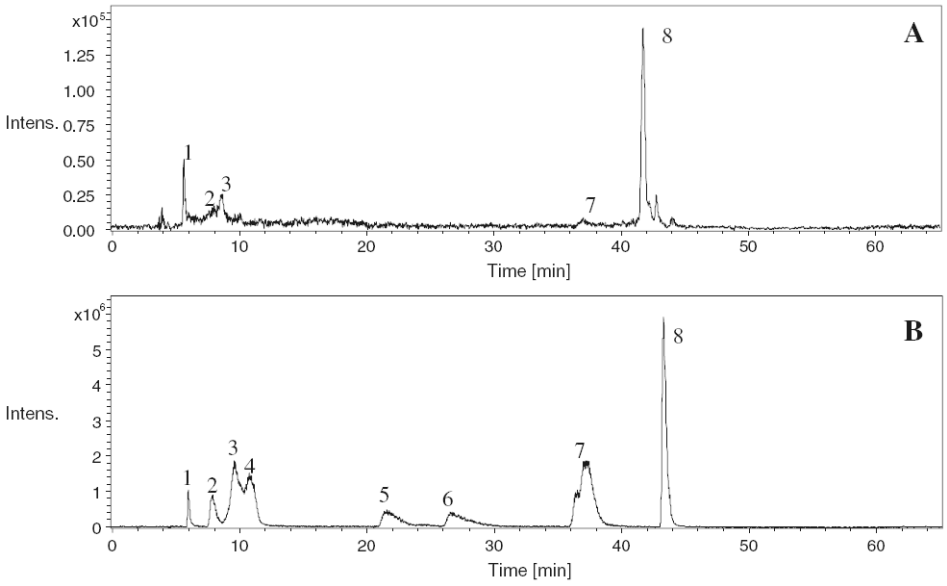


**Fig 2.**

Analysis of GAG disaccharides by LC–MS. **a** Ion-pairing chromatography for HS disaccharides from adult fish. **b** Ion-pairing chromatography for HS disaccharides standard. Peaks are assigned to *1* ΔDi-0S, *2* ΔDi-NS, *3* ΔDi-6S, *4* ΔDi-2S, *5* ΔDi-NS2S, *6* ΔDi-NS6S, *7* ΔDi-2S6S, and *8* ΔDi-triS

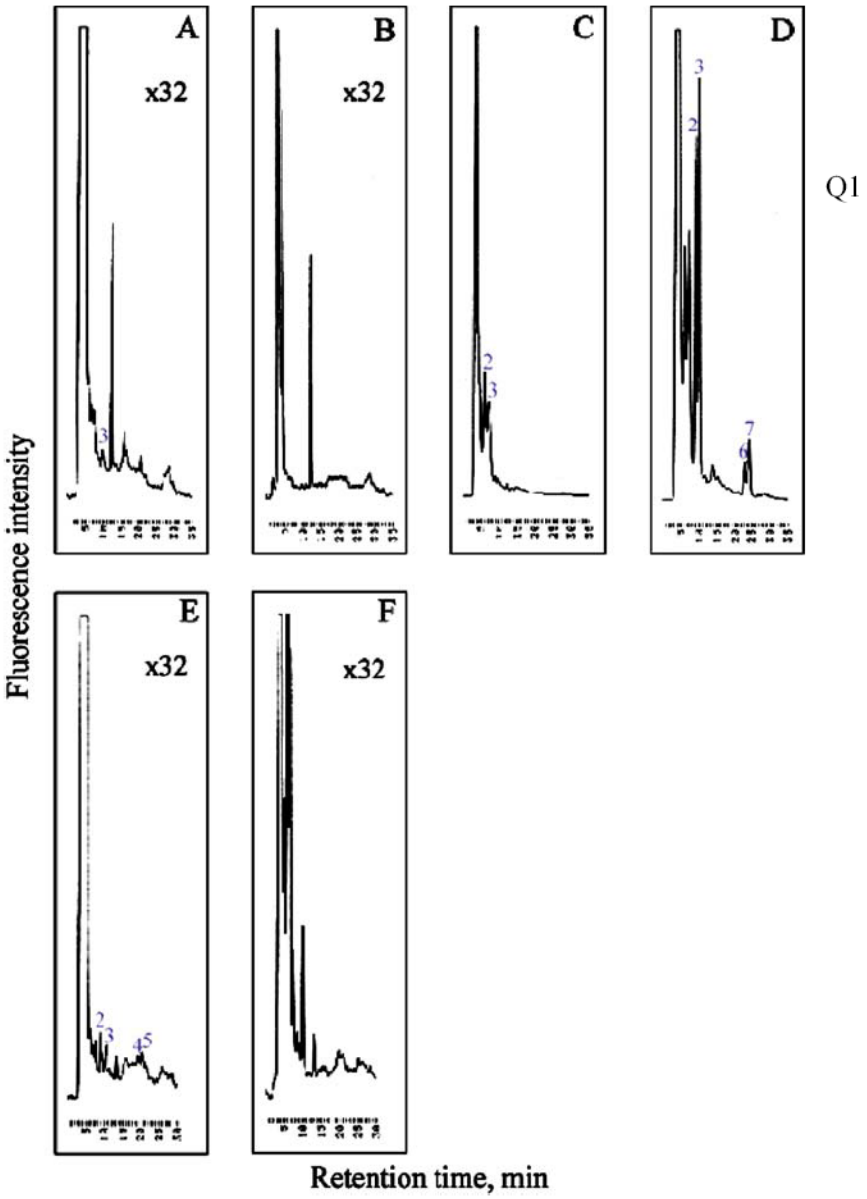


**Fig 3.**

Chromatograms of CS and HS disaccharide analysis using high performance liquid chromatography with post-column fluorescence detection. **a** CS at 0.5 day (the chromatogram magnified 32-fold); **b** CS at 1 day (the chromatogram magnified 32-fold); **c** CS at 5 days; **d** CS in adult; **e** HS at 0.5 day (the chromatogram magnified 32-fold); **f** HS at 1 day (the chromatogram magnified 32-fold)

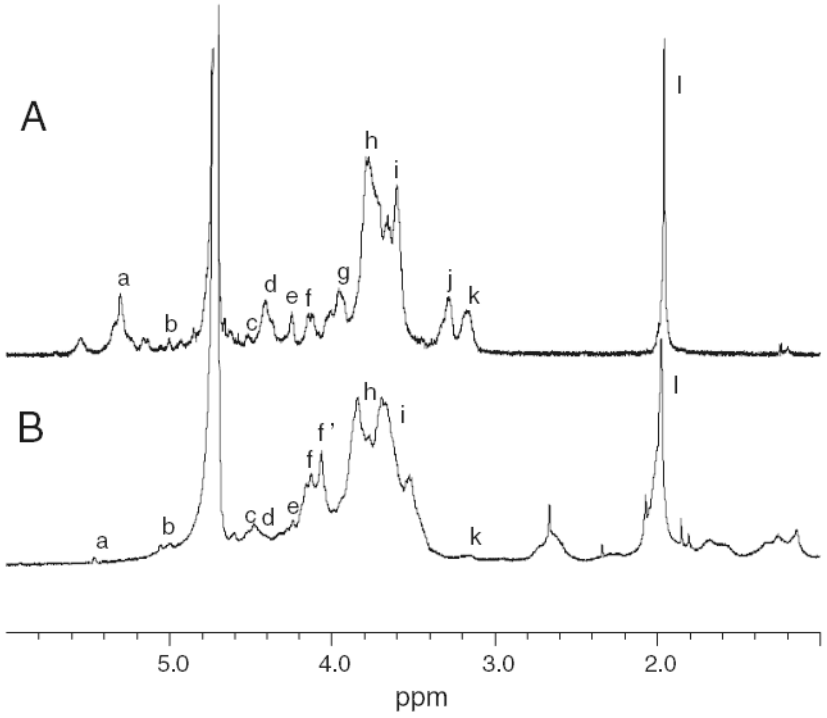


**Fig 4.**

1D-$^{1}$H-NMR spectra of HS samples. **A** $^{1}$H-NMR of standard heparan sulfate obtained from porcine intestine; **B** $^{1}$H-NMR of heparan sulfate from adult fish. *a* H-1 GlcNAc; *b* H-1 IdoA2S; *c* H-5 IdoA2S; *d* H-1 GlcA; *e* H-2 IdoA2S; *f* H-3 IdoA2S and H-6 GlcNS6S or GlcNAc6S; *f'* H-4 IdoA2S; *g* and *h* H-2 and H-3 GlcNAc, H-6 and H-5 GlcNS or GlcNAc; *i* H-3 and H-4 GlcNS, GlcNAc, GlcNS6S or GlcNAc6S, *j* H-2 GlcA; *k* H-2 GlcNS or GlcNS6S; *l* $CH_3$ of acetyl group

**Table 1**

Quantification of isolated GAGs by carbazole assay

| Sample | Dry weight (g) | Number of fish | Dry weight (mg)/fish | GAGs (μg) | GAGs (μg)/g dry tissue | GAGs (μg)/fish |
|---|---|---|---|---|---|---|
| 0.5 day | 0.0638 | 760 | 0.0839 | 30.5 | 479 | 0.0401 |
| 1 day | 0.0237 | 580 | 0.0409 | 20.4 | 860 | 0.0352 |
| 5 days | 0.0299 | 700 | 0.0427 | 20.3 | 678 | 0.0290 |
| Adult | 3.53 | 7 | 504 | 214 | 60.5 | 30.6 |

**Table 2**

CS/DS disaccharide composition analysis by high performance liquid chromatography with post-column fluorescence and ESI MS detection

| Sample | CS/DS disaccharide composition[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Unsaturated disaccharide (%) | | | | | | | |
| | ΔDi-0S | ΔDi-4S | ΔDi-6S | ΔDi-2S | ΔDi-diS$_E$ | ΔDi-diS$_B$ | ΔDi-diS$_D$ | ΔDi-triS |
| 0.5 day | n.d./– | n.d./– | 100.0/– | n.d./– | n.d./– | n.d./– | n.d./– | n.d./– |
| 1 day | n.d./– | n.d./– | n.d./– | n.d./– | n.d./– | n.d./– | n.d./– | n.d./– |
| 5 day | n.d./n.d. | 59/40 | 42/60 | n.d./n.d. | n.d./n.d. | n.d./n.d. | n.d./n.d. | n.d./n.d. |
| Adult | n.d./1.9% | 43/50 | 39/36 | n.d./n.d. | n.d./n.d. | 7.4/11[b] | 11/11[b] | n.d./n.d. |

[a] Determined by post-column fluorescence detection/determined by MS detection

[b] The 11% determined by MS detection could be either ΔDi-diSB or ΔDi-diSD

*n.d.* not detected, *en dash* not determined

**Table 3**

HS/heparin disaccharide composition analysis by high performance liquid chromatography with post-column fluorescence and ESI MS detection

| Sample | HS/heparin disaccharide composition[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Unsaturated disaccharide (%) | | | | | | | |
| | ΔDi-0S | ΔDi-NS | ΔDi-6S | ΔDi-2S | ΔDi-NS6S | ΔDi-NS2S | ΔDi-2S6S | ΔDi-triS |
| 0.5 day | n.d./– | 62/– | 13/– | n.d./– | 7.3/– | 18/– | n.d./– | n.d./– |
| 1 day | n.d./– | 100/– | n.d./– | n.d./– | n.d./– | n.d./– | n.d./– | n.d./– |
| 5 day | –/n.d. | –/18 | –/34 | –/n.d. | –/30 | –/n.d. | –/n.d. | –/18 |
| Adult | –/38 | –/12 | –/15 | –/n.d. | –/4.9 | –/n.d. | –/n.d. | –/30 |

[a] determined by post-column fluorescence detection/determined by MS detection

*n.d.* not detected, *en dash* not determined