A multi-dimensional HPLC fractionation method enables the rapid analysis of diverse heparin/heparan sulfate oligosaccharide protein interactions by microarrays

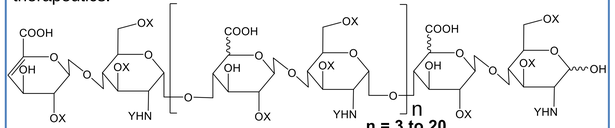
Hao Liu[1], Apoorva Joshi[2,3], Pradeep Chopra[3], Lin Liu[3], Geert-Jan Boons[2,3,4], and Joshua S. Sharp[1], *

[1]Department of BioMolecular Sciences, University of Mississippi, Oxford, MS 38677, US.; [2]Department of Chemistry, University of Georgia, Athens, GA 30602, US.; [3]Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, US.; [4]Department of Chemical Biology and Drug Discovery, Utrecht Institute for Pharmaceutical Sciences, and Biivoet Center for Biomolecular Research, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands.

Overview

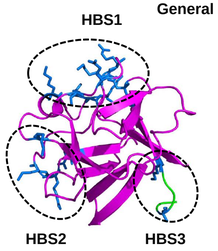
Heparin and heparan sulfate (Hp/HS) are linear complex glycosaminoglycans consisting of a reapeating disaccharide unit of GlcA-GlcNAc, modified to have widely varying and dynamic compositions. The GlcA can be epimerized to IdoA and 2-*O*-sulfated, while the GlcNAc can be *O*-sulfated at the 6- and/or 3-position, as well as deacetylated (usually followed by *N*-sulfation). This diversity mediates a wide range of protein-GAG interactions of varying specificity and affinity. However, the structural complexity brings difficulties in separation, making the study of structure-function relationships challenging.

Here we present a multi-dimensional HPLC fractionation method for Hp/HS oligosaccharide. The Hp/HS oligosaccharide is first separated by size. After size exclusion chromatography (**SEC**), the size fraction(s) of interest are highly resolved by ion-pair reversed phase chromatography (**IPRP**). After IPRP, the eluent is purified from the ion-pairing reagent and further separated by hydrophilic interaction chromatography (**HILIC**), providing both a complementary separation method and an exchange into a buffer friendlier to electrospray-mass spectrometry (ESI-MS) and microarray analysis. Our data indicates that high resolution is achieved on both IPRP and HILIC for Hp/HS isomers. In addition, the fractions co-eluted in IPRP could be further separated by HILIC, with both separation dimensions capable of resolving some isomeric oligosaccharides. We demonstrate both structural analysis by MS, as well as functional analysis by microarray printing and screening using a prototypical Hp/HS binding protein i.e. basic-fibroblast growth factor (FGF2). We demonstrate that this method has high resolving power and is directly applicable to microarray functional studies. Collectively, this method is invaluable in recognizing complex protein-carbohydrate interactions and is also essential to reveal functional Hp/HS structures as novel biomaterials or therapeutics.

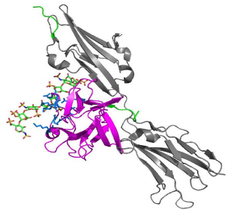


n = 3 to 20
X = H or SO_3H
Y = H or SO_3H or Ac

General heparin structure[1]

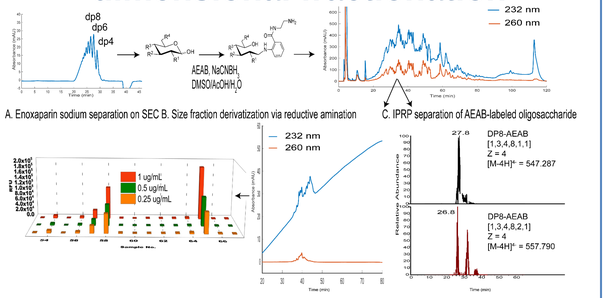


Heparin binding sites of FGF2[2]



A heparin octasaccharide forms a ternary complex with FGF2 and FGF receptor[3,4]

The work flow of the multi-dimensional fractionation

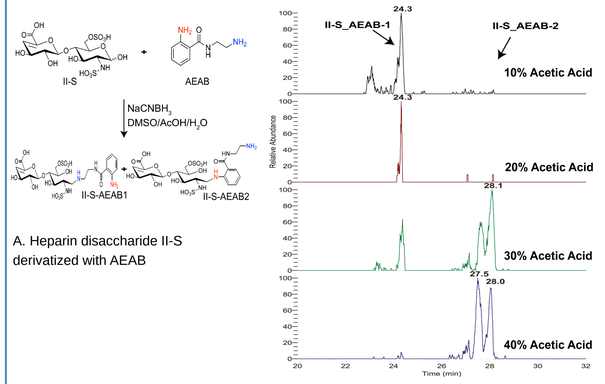


A. Enoxaparin sodium separation on SEC B. Size fraction derivatization via reductive amination C. IPRP separation of AEAB-labeled oligosaccharide

F. Protein binding affinity assay with microarry of fractions E. Offline HILIC separation of IPRP fraction D. Online HILIC LC/MS analysis of IPRP fraction

The workflow of the multi-dimensional separation. A.) SEC separation of Enoxaparin sodium; B.) Derivatization of octasaccharides with 2-Amino-*N*-(2-aminoethyl) benzamide (AEAB); C.) IPRP separation of AEAB-labeled octasaccharides; IPRP fractions could be either analyzed by D.) online HILIC LC/MS or E.) separated by offline HILIC; F.) Protein binding affinity assay with microarray of offline HILIC fractions. Oligosaccharide compositions are given as [ΔHexA, HexA, GlcN, SO_3, Ac, AEAB];

Chemo-selectivity of the reductive amination

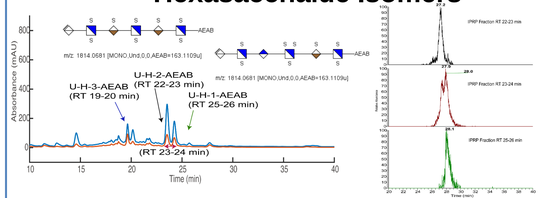


A. Heparin disaccharide II-S derivatized with AEAB

B. Heparin disaccharide II-S derivatized with AEAB in various amounts of acetic acid

By lowering the pH of the reaction, the aromatic amine is more likely to react with the reducing end of oligosaccharide, which leaves a free primary amine for future glycan microarray immobilization.

Analysis of Separation Resolution: Synthetic Hexasaccharide Isomers

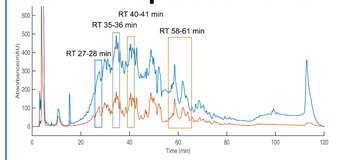


IPRP chromatogram of AEAB labeled synthetic hexasaccharides mixture with UV detection at 232 nm (—) and 260 nm(—). AEAB labeled synthetic hexasaccharides were separated on IPRP

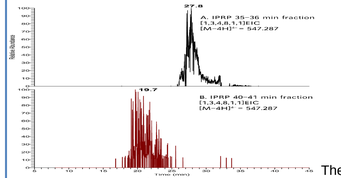
The EICs of [M-4H]$^{4-}$ ion corresponding to [1,2,3,8,0,1] from IPRP fractions. The AEAB labeled isomers were not only separated by IPRP (RT 22 to 23 min vs. 25 to 26 min), but also separated by HILIC (RT 27.2 vs. 28.1 min).

The proposed multi-dimensional separation method, **IPRP** coupled with **Amide-HILIC**, not only exchanges solvent to more compatible volatile one for MS sequencing and/or microarray study, but also provides high-resolution separation for known size Hp/HS oligosaccharides, synthetic hexasaccharides.

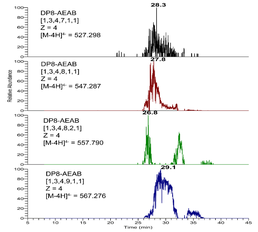
Separation of Enoxaparin dp8



IPRP chromatogram of AEAB labeled enoxaparin octasaccharides using 232 nm (—) and 260 nm(—) wavelengths.



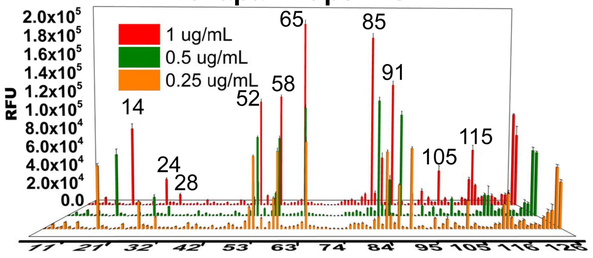
The EICs of [M -4H]$^{4-}$ ion corresponding to [1,3,4,8,1,1] from IPRP fractions. A.) In the IPRP 35 to 36 min fraction, this composition is eluted at 27.8 min. B.) In the IPRP 40-41 min fraction, this composition is eluted at 19.7 min.



The EICs of the top four possible octasaccharides from the IPRP 35-36 min fraction. The octasaccharides, which are not separated by IPRP, are resolved by HILIC, including two clearly separated [1,3,4,8,2,1] isomers, illustrating orthogonality between HILIC and IPRP chromatography.

The huge difference in retention time (**ΔRT=5 min on IPRP** and **ΔRT=8 min on amide-HILIC**) for a single oligosaccharide composition, [1,3,4,8,1,1], is achieved. In addition, the fractions co-eluted in IPRP could be separated by HILIC, for example isomers of [1,3,4,8,2,1]. This fractionation method is orthogonal with high resolving power.

Microarray Analysis of Fractionated Enoxaparin dp8: FGF2



Sample No.	RT on IPRP (min)	RT on HILIC (min)
14	28.3-29.3	40-47
24	30.3-30.7	40-41
28	30.7-31.6	40-42
52	35.8-36.8	44-51
58	36.8-38	43-44

Sample No.	RT on IPRP (min)	RT on HILIC (min)
65	38-39	43-51
85	46-46.8	44-45
91	47.8-48.5	44-51
105	51.5-52.6	45-47
115	60.9-63.2	44-51

Microarray results illustrated the various FGF2 binding Hp/HS were separated via the multi-dimensional separation method, enabling a functional analysis method complementary to MS-based structural analyses. Binding was concentration-dependent, and the pattern of binding is supported by the limited structural promiscuity of FGF2 binding to Hp/HS.

Conclusion

Our multi-dimensional separation method provides high-resolution separation of Hp/HS compatible with the generation of Hp/HS microarrays for functional study. The method is also compatible with MS, allowing structural interrogations coupled with functional analysis. Future studies will couple this separation method with more detailed Hp/HS structural interrogation methods. Overall, this method enables GAG structure-function studies from very complex initial mixtures in a microscale format, which is essential to reveal functional Hp/HS structures as novel biomaterials or therapeutics.

References

1. USP 40.
2. Ori, A., Free, P., Courty, J., Wilkinson, M. C., & Fernig, D. G. (2009). Identification of heparin-binding sites in proteins by selective labeling. Molecular & Cellular Proteomics, 8(10), 2256-2265.
3. Schlessinger, J., Plotnikov, A. N., Ibrahimi, O. A., Eliseenkova, A. V., Yeh, B. K., Yayon, A., ... & Mohammadi, M. (2000). Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. Molecular cell, 6(3), 743-750.
4. Nunes, Q. M., Li, Y., Sun, C., Kinnunen, T. K., & Fernig, D. G. (2016). Fibroblast growth factors as tissue repair and regeneration therapeutics. PeerJ, 4, e1535.

Acknowledgements

- This is supported by the National Institute of General Medical Sciences as part of the Research Resource for Integrated Glycotechnology. (P41GM103390)
- We thank Waters for donating the BEH Amide HILIC column used in this study.