Application Note

Wyatt Technology Corporation

Discriminating Heparin from Chondroitin Sulfate by Charge:Mass Ratio

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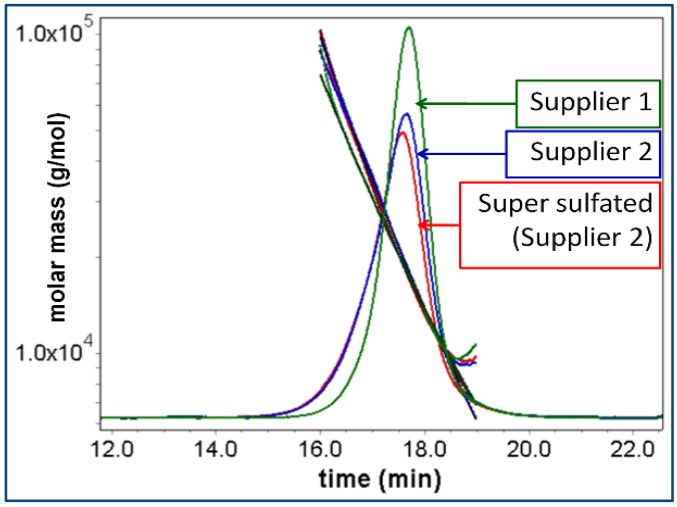
Summary

Characterizing key parameters of different heparin lots requires multiple techniques. Weight-average molar mass measured by multi-angle static light scattering (MALS) and hydrodynamic radius measured by dynamic light scattering (DLS) can be used to characterize the size and polydispersity of the molecule. These quantities can also be used for lot-to-lot comparison and to qualify heparin from different suppliers. Massively-parallel phase analysis light scattering (MP-PALS) and DLS are used to calculate the net charge and characterize the purity of the sample. In this study, we compared heparin from two suppliers, including one lot modified with super-sulfated material.

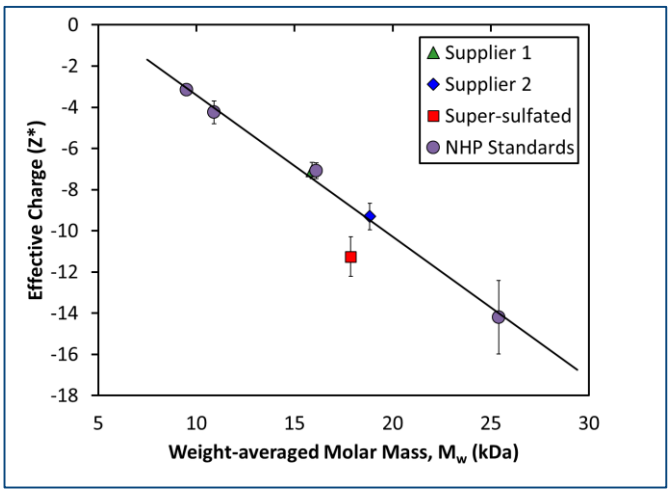
Weight-average molar mass (M_w) for the different heparin samples and for heparin mass standards was determined by size exclusion chromatography (SEC) coupled with MALS. The difference in M_w and hydrodynamic radius (r_h) between the two suppliers was <10%. Very little difference in M_w and r_h (<5%) was observed between unmodified heparin and heparin modified with super-sulfated material. Thus, size alone could not be used to qualify contaminated heparin samples.

Using the Wyatt Möbiuζ, z-averaged electrophoretic mobility and r_h were measured simultaneously to yield net charge. The net charge for a set of heparin standards obeyed a linear relationship between as a function of M_w, indicating a constant charge:mass ratio. Unmodified heparin from both suppliers obeyed the same linear relationship, but the super-sulfated material exhibited a 30% increase in negative charge. The increase in negative charge per unit mass, is consistent with an increase in sulfate groups in the modified heparin sample.

Thus, the combination of M_w by SEC-MALS and net charge by MP-PALS provides clear differentiation between unmodified and contaminated heparin. This multi-technique approach for determining the charge:mass ratio enables rapid, nondestructive characterization of different lots of heparin.



Overlay of SEC-MALS data for heparin from two different suppliers, including a one sample that has been contaminated with "super-sulfated" material.



Pure heparin exhibits a constant charge:mass ratio whereas heparin contaminated with super-sulfated material exhibits an increased negative charge compared to its molar mass.

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WYATT TECHNOLOGY

I. Introduction

Although heparin has been used as a clinical anticoagulant for over seventy years, recent contamination of certain pharmaceutical lots by chondroitin sulfate has spurred new interest in precise biomolecular analysis of this molecule. Molecular weight and molecular weight distribution may not be sufficient to determine the specific chemical makeup of a lot of heparin, and advanced techniques are required for complete characterization. Measurement of electrophoretic mobility enables calculation of the average molecular charge. Together with the molecular weight, the net charge can be used to identify heparin lots contaminated with super-sulfated molecules.

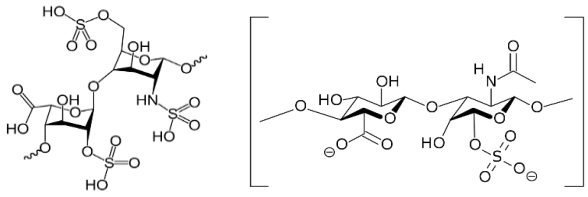

Figure 1: Molecular structure for heparin (left) and chondroitin sulfate (right). The sulfate groups are expected to impart an increased negative charge on heparin samples contaminated with chondroitin sulfate.

II. Materials and Methods

Unfractionated heparin samples and fractionated heparin standards (Heparin Derived Polysaccharides, Neoparin, Inc.) were provided by Baxter. Samples were dissolved in 0.1 M ammonium acetate to a final concentration of ~5 mg/mL and allowed to equilibrate overnight at room temperature prior to beginning analyses.

MP-PALS analysis was performed using the Wyatt Möbiuζ. Samples were filtered to 0.02 µm using a syringe tip filter (Anotop, Whatman) as they were injected directly into the Möbiuζ flow cell. Electrophoretic mobility by massively-parallel phase analysis light scattering (MP-PALS) was measured simultaneously with hydrodynamic radius by dynamic light scattering (DLS).

Weight-average molar mass (M_w) for each sample was measured using multi-angle static light scattering (MALS). Molar mass and polydispersity of fractionated heparin samples was measured by size exclusion chromatography (SEC) coupled with MALS. Unfiltered samples were injected onto a chromatography column, and the molar mass of the eluting sample was measured using a DAWN HELEOS MALS detector and Optilab rEX refractive index detector. SEC-MALS data for unfractionated heparin was provided by Baxter.

III. Results and Discussion

Although slight differences were measured in the molar mass of the different unfractionated heparin standards, this change was not enough to be used as a conclusive metric for determining if a given lot of heparin was contaminated with super-sulfated material. As shown in Table 1, the difference in molar mass (5%) between the pure heparin and heparin contaminated with super-sulfated material from Supplier 2 was less than the difference in molar mass of the pure heparin coming from two different suppliers (18%). Furthermore the hydrodynamic radius of the pure heparin is indistinguishable from the sample contaminated with super-sulfated material.

	M_w (kDa)	r_h (nm)
Unfractionated Heparin, Supplier 1	15.9 ± 0.1	2.30 ± 0.02
Unfractionated Heparin, Supplier 2	18.8 ± 0.3	2.48 ± 0.01
Super-sulfated Heparin, Supplier 2	17.9 ± 0.0	2.49 ± 0.02

Table 1: Weight-average molar mass and z-average hydrodynamic radius of unfractionated heparin samples

Since super-sulfated material was expected to increase the negative charge on the sample, electrophoretic mobility measurements were made to determine if this metric could be used to distinguish between pure heparin and contaminated samples. Figure 2 shows a typical "V-graph" for the measurement of the electrophoretic mobility. The data represent the average of 300 electric field oscillations, multiplexed across 30 detectors. The negative electrophoretic mobility (µ) indicates the heparin sample has a negative net charge. The effective molecular charge and zeta potential are then calculated from the electrophoretic mobility and hydrodynamic radius. These parameters are summarized for the unfractionated heparin samples and fractionated samples (NHP) in Table 2.

As expected, the super-sulfated material exhibited the largest net charge among the three unfractionated heparin samples. However, the difference in charge between the super-sulfated and pure samples was of the same magnitude as the difference in charge for the two suppliers. The difference might only reflect the variance in molar mass or polydispersity. This may imply that net charge alone is not an appropriate metric for qualifying different lots of heparin.

	Mobility ((µm*cm)/(s*V))	Effective charge (Z*)	Zeta Potential (mV)	r_H (nm)
Unfractionated Heparin, Supplier 1	-1.00 ± 0.05	-7.1 ± 0.40	-16.9 ± 0.9	2.30 ± 0.02
Unfractionated Heparin, Supplier 2	-1.18 ± 0.09	-9.3 ± 0.64	-19.5 ± 1.5	2.48 ± 0.01
Super-sulfated Heparin, Supplier 2	-1.46 ± 0.12	-11.2 ± 0.96	-23.8 ± 1.9	2.49 ± 0.02
NHP III	-0.42 ± 0.03	-3.2 ± 0.26	-7.9 ± 0.57	2.19 ± 0.02
NHP IV	-0.65 ± 0.10	-4.2 ± 0.56	-11.8 ± 1.7	2.09 ± 0.02
NHP VI	-0.86 ± 0.05	-7.1 ± 0.39	-15.8 ± 0.9	2.43 ± 0.02
NHP VII	-0.85 ± 0.10	-14.2 ± 1.8	-14.2 ± 1.7	3.74 ± 0.03

When both charge and mass are considered together, the difference between the super-sulfated and pure heparin samples becomes immediately apparent (Figure 3). The net molar mass of the fractionated heparin standards (NHP III, IV, VI, and VII) were used to generate a calibration curve for pure heparin. These four samples establish a linear relationship between net charge and molar mass for pure heparin, indicating a constant charge:mass ratio. Based on their measured M_w, the net charges for unfractionated heparin from Suppliers 1 and 2 fall within 2% of expected value. On the other hand, the measured net charge of the super-sulfated heparin from Supplier 2 is ~30% greater than what would be expected for a heparin of that size.

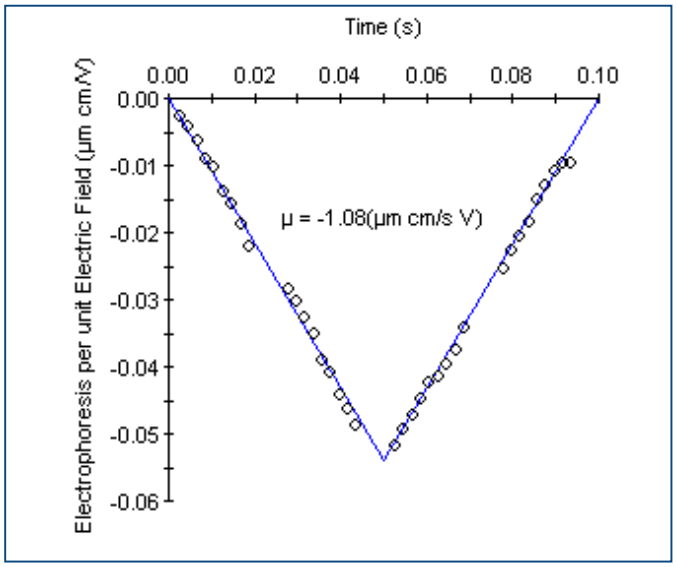

Figure 2: Electrophoretic mobility data for unfractionated heparin from Supplier 1.

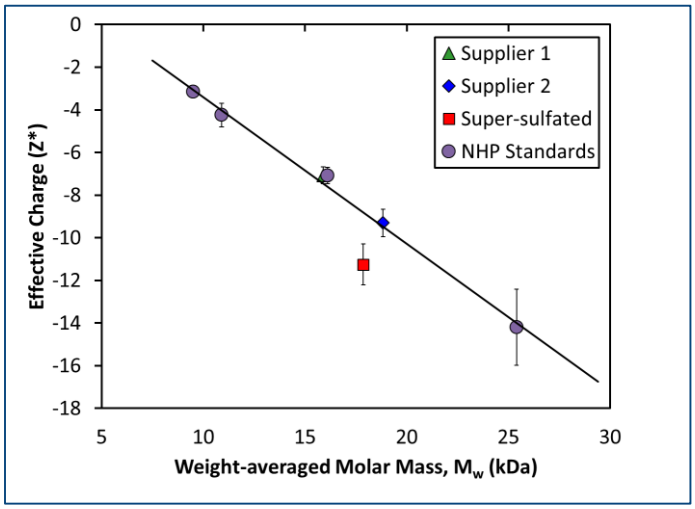

Figure 3: Measured net charge and molar mass for heparin standards exhibits a linear relationship, indicating a constant charge:mass ratio. Pure heparin samples from both suppliers obey the same linear relationship, but heparin contaminated with super-sulfated.

IV. Conclusions

Simultaneous measurements of electrophoretic mobility and hydrodynamic radius enable rapid, nondestructive characterization of effective molecular charge. The increase in negative charge, as measured by a change in electrophoretic mobility, is consistent with an increase in sulfate groups in the modified heparin sample. The increase in charge combined with the molar mass of the sample provides a unique fingerprint for pure heparin compared to heparin samples contaminated with super-sulfated material. In this study, the effective charge:mass ratio clearly distinguished between unmodified and super-sulfated heparin and can be used as a metric to qualify different samples.