

Characterization of Large Protein-Polysaccharide Conjugates Using aFFF-MALS

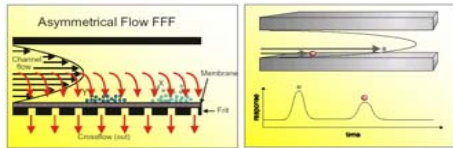
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Introduction

Size analysis of large heterogeneous samples, comprising a large range of molecular masses, presents a significant challenge. A method for protein-polysaccharide conjugate size distribution analysis was developed using asymmetrical-flow field-flow fractionation (aFFF) coupled with multi-angle light scattering (MALS), ultra violet (UV) absorbance, and differential refractometry (dRI) detection.

In this study, characterization was performed on samples of a 58 kDa protein coupled to three different large polysaccharides (conjugates A, B, and C). The polysaccharides range from an approximate weight average molar mass of 200 – 500 kDa.

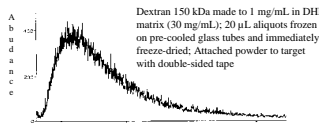
The data presented demonstrate the ability of the aFFF method to fractionate a wide range of sizes. The fractionation of a sample by aFFF, coupled with MALS, provides absolute molecular mass determination throughout large distributions of polydisperse samples. Such samples are problematic for more traditional size or mass measurement techniques (SEC, AUC, and mass spectrometry). An additional benefit of this method is that it allows for distribution analysis of the individual protein and polysaccharide components.



Drawings from Christoph Johann, Wyatt Technologies Europe

aFFF is a technique by which molecules are separated based on their diffusion against an applied cross-flow. Smaller molecules, with larger diffusion coefficients, resist the cross-flow and travel faster through the aFFF channel. Since aFFF is a non-sieving method, the size distribution analyzed is not bound by the fractionation range of a column matrix. There is also less likelihood of encountering aberrant sample-surface interactions.

150 kDa Dextran Standard Analysis by MALDI-TOF Mass Spectrometry



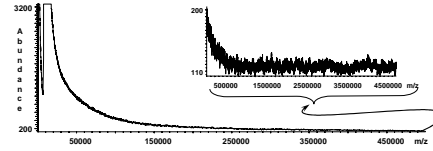
Dextran 150 kDa made to 1 mg/mL in DHB matrix (30 mg/mL); 20 μ L aliquots frozen on pre-cooled glass tubes and immediately freeze-dried; Attached powder to target with double-sided tape

Garazzo, D. et al. *Rapid Comm. Mass Spectrom.* 2002, 16, 1599

- It is widely reported that polydisperse polysaccharides fail to give reliable MALDI mass spectra and accurate average MW values.
- The MALDI mass spectrum of dextran 150 kDa standard (above) was recently reported using a “freeze-dry” preparation approach. (Garazzo, D. et al.)

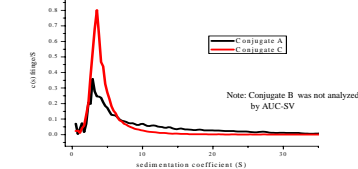
Conjugate A Analysis by SEC/MALDI-TOF Mass Spectrometry

Analyzed from a DHB matrix (freeze-dry method) by positive ion, linear mode with continuous ion extraction; Acc. V: 28.5 kV; High N_2 laser fluence; Calibrated with trypsinogen & BSA



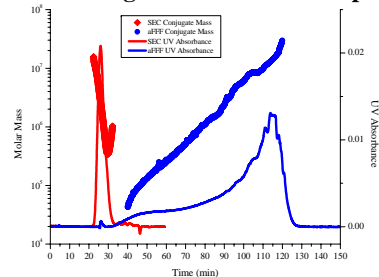
- In our hands, MALDI combined with the freeze-dry sample preparation method has produced no mass signals higher than 20 kDa for bulk dextrans or protein-polysaccharide conjugates.
- High molecular weight polysaccharides still present a challenge to current mass spectrometry techniques.

Sedimentation Velocity Analysis of Protein-Polysaccharide Conjugates



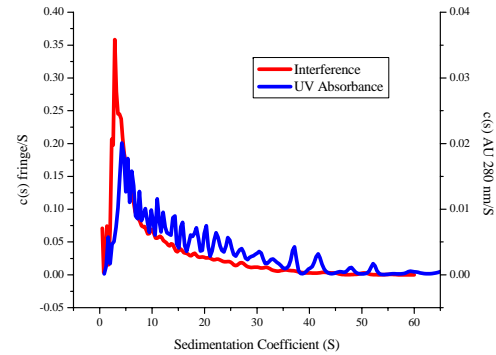
- AUC-SV failed to detect the larger species in the protein-polysaccharide conjugate distribution as seen in the aFFF-MALS method
- Hydrodynamic properties of the conjugate necessary for molecular mass determination from AUC data are difficult and impractical to measure.

Conjugate A SEC and aFFF-MALS Molecular Weight Distribution Comparison



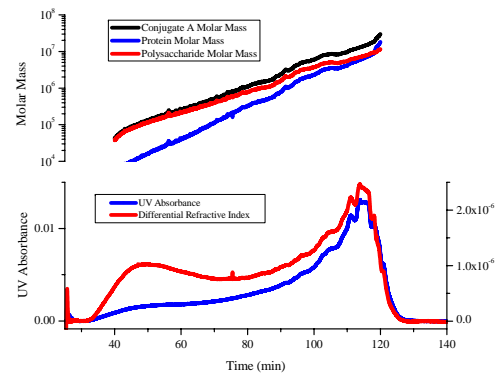
- aFFF provides a greater fractionation range compared to this SEC method.
- SEC shows evidence of large mass species interacting with the column matrix.

Conjugate A Analysis by Sedimentation Velocity Monitoring UV Absorbance and Interference



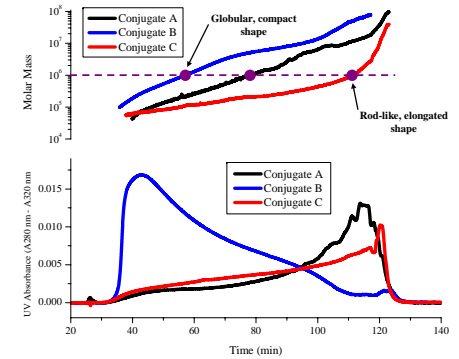
- Component size distribution information, comparing interference optics to UV absorbance, is limited due to the under representation of very large species (as observed with the aFFF-MALS method).

Conjugate A Component Analysis Using aFFF-MALS Coupled with UV and dRI



- Use of two independent concentration detectors (UV Abs and dRI) allow for quantitation of protein and polysaccharide components separately.
- The protein and polysaccharide molar mass distributions show that the relative protein content is not constant throughout the conjugate distribution.

Comparison of aFFF-MALS Fractionation of Protein-Polysaccharide Conjugates



- The aFFF method shown has the ability to reasonably resolve several orders of magnitude of molar mass.
- The conjugate molar mass distributions are overlaid to show the differences in elution time for a given mass; this difference is due to structural or shape differences of each protein-polysaccharide conjugate.

Summary

- We developed an aFFF-MALS method for the size characterization of large protein-polysaccharide conjugate distributions.
- This method has advantages over SEC, AUC and MALDI-TOF MS methods, and can easily be modified to fractionate larger or smaller sized sample distributions. These distributions may contain discretely sized species or a continuum of molecular masses, such as our conjugates.
- aFFF-MALS with UV absorbance and dRI detectors, combined with the protein conjugate module of the ASTRA V software, allowed for accurate assessment of the protein-to-polysaccharide ratio throughout each conjugate distribution.
- Using the improved aFFF separation, in conjunction with MALS, we are able to better discern conjugate shape differences resulting from the branching patterns of each component polysaccharide and the resulting conjugate conformation.

Acknowledgements

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