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## A NOVEL SEC-MS METHOD FOR THE STUDY OF PROTEINS AND THEIR INTERACTIONS

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### **INTRODUCTION** –

Size exclusion chromatography (SEC) is widely used for molecular weight estimations of proteins in their native state. SEC has found applications in studies on protein purity, protein-protein interactions and protein aggregation. The major limitations of SEC are that the retention time of proteins can be affected by their hydrodynamic properties and also due to tendency of some proteins to interact with the column matrix. A hyphenated size exclusion-mass spectrometric method (SEC- MS) was developed for the study of proteins and their interactions. The method utilizes a mass spectrometry compatible mobile phase consisting of ammonium formate with a post-column addition of acetonitrile and formic acid. SEC-MS combines the high mass accuracy of electrospray ionization mass spectrometry with the ability of SEC to resolve protein complexes.

We found that noncovalent protein complexes do not survive electrospray ionization and overlapping isotopic envelopes are obtained for monomeric, dimeric, and aggregates of BSA, which after deconvolution also yield identical molecular weights for the three isoforms. This property of identifying monomeric molecular weights of proteins in a multimeric complex is unique to SEC-MS. Other methods such as on-line light-scattering, absorbance, and refractive index detectors can only measure the mass of intact complex and hence, cannot identify different components in a protein complex. We have shown that this novel SEC-MS method can be very effective in the study of covalent and noncovalent protein aggregation, and in the analysis of lgG1.

### EXPERIMENTAL -

Waters Alliance® HPLC system consisting of: Either 2695 or 2795 HT Separations module Waters® 2996 PDA detector Waters<sup>®</sup> ZQ<sup>TM</sup> Mass detector -Source = ESI(+)-Capillary (kV) = 3.3 -Cone (V) = 30 -Temperature (°C) -Source = 150-Desolvation = 425 -Gas Flow (L/Hr) -Cone = 50 Desolvation = 500-Scan Mode Waters<sup>®</sup> Q-TOF™ -Source = ESI(+)-Capillary (kV) = 3.3-Cone (V) = 30 -Collision Energy = 10 **HPLC** Conditions -Isocratic -Mobile phase = 50 mM Ammonium formate (pH 6.5) -Flow Rate = 1 ml/minSEC Column-BioSuite<sup>™</sup> 250, 5µm HR SEC (7.8mmX30mm) IgG Purification Column- Protein G packed in Advanced Purification(AP<sup>®</sup>)glass column, Protein Pak<sup>™</sup> Q 8 HR



Figure 1- Schematic of SEC-MS.

SEC-MS utilizes a mass spectrometry compatible mobile phase consisting of ammonium formate. The flow is split with a post column splitter at ratio of 8:2. 80% of the sample is analyzed by a UV detector while the remaining 20% is analyzed online by the mass spectrometer to obtain accurate molecular weight of proteins.

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## Figure 2- Comparison of different mobile phases in SEC of proteins.

 I ml of protein standard mixture containing 5 mg each of Thyroglobulin, human transferrin, carbonic anhydrase, cytochrome C, lucine enkhaphalinamide was injected on to the SEC column A. The mobile phase was composed of either formate buffer (50 mM ammonium formate) or phosphate buffer (100 mM phosphate buffer, 100 mM sodium sulfate, pH 6.5) Protein were eluted isocratically from the column with a flow rate of 1 ml/min (A). B is the plot of log molecular weight versus retention time in different mobile phases.



### Figure 3- SEC-MS of protein standards.

Mass spectrum of protein peaks from figure 2 when using formate buffer. X4 in (3) indicates six fold amplification of the transferrin signal.

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Figure 4: Study of BSA aggregation using SEC-MS.

The UV chromatogram of untreated and heat treated BSA is shown in **A**. Heat treated BSA elutes in three distinct peaks comprising of monomer, dimer and aggregates of BSA. It can be seen from **B** that monomer and aggregate form of BSA have overlapping charge envelopes while dimeric form has a distinct charge envelope. After deconvulution (the monomeric form yielded a distinct peak at mass 66343 (**B1**). Dimeric form on the other hand (**B2**) shows a major peak at Mass 132855 comprising of covalently bound dimers and a minor peak at 66343 representing the noncovalently dimmers which are separated by chromatography but which do not survive ESI. The Aggregated form yields peaks at 132855 and 66343 (**B3**) indicating that the large noncovalent aggregates do not survive ESI. It also shows that the aggregates are composed both monomeric and covalently linked dimeric forms of BSA.

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Figure 5- SEC-MS analysis of reduced IgG.

(A) SEC chromatogram of reduced and non-reduced IgG. (B) Mass spectrum of IgG1 and reduced IgG1 showing two distinct charge envelopes. (C) Deconvoluted mass of the spectrum from (B)

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## **CONCLUSIONS** –

- SEC-MS is a platform independent method for the study of intact proteins.
- SEC-MS method can be used for the study protein aggregation.
- Monomeric molecular weights of a protein in aggregated or multimeric state can be obtained by SEC-MS.
- Denaturing SEC-MS can be used to resolve light chain, heavy chain and half molecule of IgG1

## **REFERENCES** –

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Figure 6- Analyses of light chain, heavy chain and half molecule from reduced IgG1 with denaturing SEC-MS.

(A) SEC-MS total ion current (TIC) chromatogram of reduced IgG with modified mobile phase consisting of 25% acetonitrile 1.5% formic acid and 25 mM Ammonium formate buffer. The flow rate was 1 ml/min, 0.4ml/ min of which was introduces into the ESI using a post column splitter. (B) Mass spectrua and deconvoluted mass of peaks from (A)

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