WHITE PAPER

WP8003: IEX-MALS- a method for protein separation and characterization

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Summary

Ion-exchange chromatography coupled to multi-angle light scattering (IEX-MALS) allows absolute molar mass determination of proteins, protein complexes and peptides in a heterogenous sample. IEX-MALS can overcome some of the restrictions of SEC-MALS that arise from the limited separation ability of analytical SEC column. The IEX-MALS method is valuable for quality assessment as well as characterization of native oligomers, charge variants, modified proteins and mixed-protein samples.

Introduction

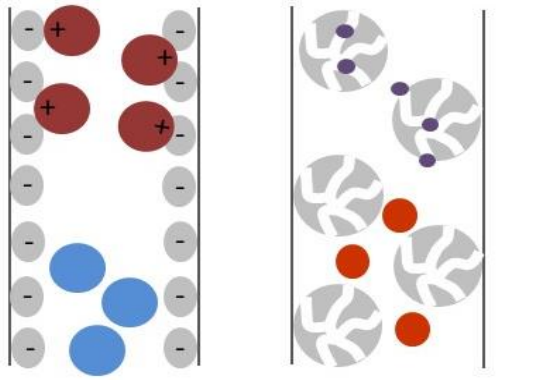
SEC-MALS is a standard, common method for protein characterization. It performs molar mass (*M*) and oligomeric determination as well as structural characterization for the analysis of aggregates, complexes and modified[1,2]. Simultaneous dynamic light scattering (DLS) measurements determine the size of each eluting species (hydrodynamic radius, R_h).

Attaining optimal results with SEC-MALS or SEC-DLS requires sufficient separation of the various protein species present in the sample prior to MALS analysis. In some cases, analytical SEC columns fail to properly separate between different proteins that are present in the sample, for example acidic and basic variants of monoclonal antibodies (mAbs), different proteins with similar hydrodynamic radius, or oligomers with close molar masses. Moreover, **when high-order oligomers or aggregates are not fully separated from the protein peak** by analytical SEC, the intense LS signal of these aggregates affect **the molecular mass determination of lower oligomers** that elutes later [3].

Combining MALS with a separation technique based on molecular properties other than hydrodynamic size can overcome these limitations of SEC and allow reliable analysis by MALS of such cases. Here we present the combination of ion exchange chromatography with MALS (IEX-MALS) as a powerful tool for separation and characterization of heterogenous protein samples.

IEX chromatography

IEX is a high-selectivity adsorption chromatography that separates proteins (and other macromolecules) according to their surface charge, based on different ionic interactions with the support matrix. Anion exchange (AIEX) and cation exchange (CIEX) matrixes bind, respectively, negatively and positively charged variants. Unlike analytical SEC, IEX does not have an associated column calibration method that can estimate the molecular weight of an eluting peak, so adding MALS is essential for online molar mass characterization.



UV versus dRI

Salt gradient elution during IEX chromatography usually leads to very large changes in the refractive index of the mobile phase, often much larger than the change due to the sample peak. For this reason, it is easiest—and often necessary—to use UV absorbance signals to determine the concentration and carry out MALS analysis of molar mass. [Of note, IEX based entirely on pH gradients rather than salt gradients should not exhibit significant refractive index changes.]

But there are several benefits to using differential refractive index (dRI) detection for concentration:

- dRI is a universal measurement that does not rely on the presence of chromophores or fluorophores
- almost all pure proteins in aqueous buffers have the same dRI response (dn/dc) to within one or two percent.
- The combination of UV and dRI can be analyzed to characterize conjugates such as glycoproteins or protein-polysaccharide vaccines.

Notably, only Wyatt's Optilab® dRI detector can measure the entire range of refractive index change produced by the gradient without loss of sensitivity for the relatively small sample signal.

The examples presented here utilized UV detection, but we have also performed analysis by refractive index. IEX-MALS analysis with dRI will be discussed in a separate application note.

Materials and Methods

Analyses were performed with an AKTA pure FPLC system connected to a miniDAWN® MALS detector incorporating a WyattQELS™ embedded DLS detector. The FPLC's UV 280 nm absorbance signal was used for concentration detection. An Optilab® differential refractive index (dRI) detector acquired dRI data for concentration analysis. Usually dRI analysis is appropriate when the salt concentration profile is linear and elution of the peaks of interest occurs entirely within the gradient.

Data were acquired and analyzed by ASTRA® chromatography software. The dn/dc value used, 0.185 mL/g, is appropriate for the entire gradient region where protein elution occurred (50 – 200 mM NaCl).

IEX methods were designed to ensure complete elution of low-charge impurities and return to LS baseline before the onset of the salt gradient, and complete elution of high-charge species and return to LS baseline at the end of the salt gradient.

IEX-MALS of BSA

BSA was separated using a GE MonoQ HR 5/50 AIEX column. Several runs were performed to test reproducibility of the method.

The running and washing buffer consisted of 20mM Tris-HCl buffer at pH 8 with 50 mM NaCl. The BSA injection load was 0.5 mg or 1 mg. The elution gradient was 15-70% of 500 mM NaCl in the running buffer over 30 or 15 column volumes (CV). For improved separation of BSA oligomers, a stepwise program of 40% and 50% was used with the same elution buffer, loading 2 mg BSA.

SEC- and IEX-MALS of mAbs

Two different mAbs were analyzed using SEC-MALS and IEX-MALS. For SEC-MALS, a GE Superdex 200 Increase analytical SEC column was used while for IEX-MALS a Sepax SCX-NP5 analytical CIEX column was used.

MAb1

SEC: The running buffer consisted of 20 mM sodium phosphate at pH 7.4 with 100 mM NaCl. 0.2 mg of mAb1 were injected.

IEX: The running and washing buffer consisted of 20 mM sodium acetate buffer at pH 5 with 100 mM NaCl. 0.2 mg of mAb1 were injected. The elution gradient was 15-25% of 1 M NaCl in the running buffer over 13 CV.

MAb2

SEC: The running buffer was 20 mM sodium acetate at pH 5 with 150 mM NaCl. 0.23 mg of mAb2 were injected.

IEX: The running and washing buffer was 20mM sodium acetate buffer at pH 5 with 50 mM NaCl. 0.6 mg of mAb2 were loaded on the column. The elution gradient was 25-50% of 500 mM NaCl in the running buffer over 15 CV.

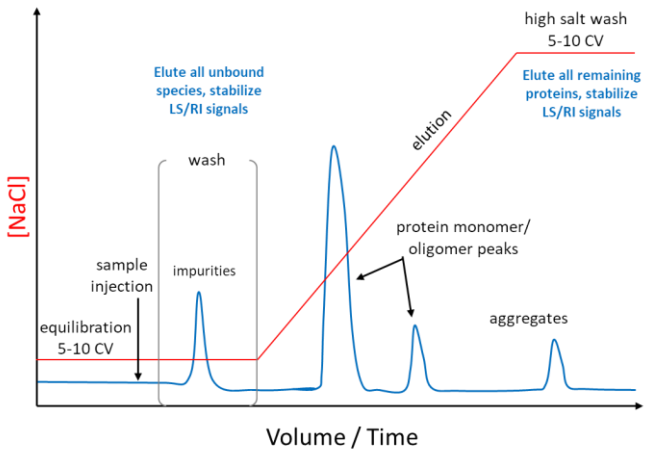


Figure 1. Schematic of ion-exchange chromatography.

Results and Discussion

BSA is commonly used to validate SEC-MALS instrumentation and therefore was analyzed to assess accuracy and repeatability as well as application of different IEX gradient profiles. Two commercially available mAbs were analyzed to demonstrate the applicability of MALS to understanding the presence of charge variants, a critical quality attribute of these biotherapeutics.

BSA

BSA is present mostly as a monomer and contains low percent of dimers and trimers as well as some fragments. In IEX-MALS experiments, BSA monomers are separated from the higher oligomers via a simple linear salt gradient of 30CV (Figure 2A). Even though the peaks elute at slightly different times in each repetition, the molar masses identified by MALS are identical and match expected monomer values. Dimer molar mass values are lower than expected due to incomplete separation from monomers and the fragment shoulder that appears at about 19 minutes.

Since the gradient slope is low, the eluted peaks are wide and the protein is spread over a large volume, the peak concentration is relatively low. For that reason, a higher quantity of injected sample is required for these IEX-MALS experiments (0.5-1 mg for BSA) than for standard SEC-MALS.

A sharper gradient reduces the separation but produces a narrower, higher peak resulting in better signal sensitivity (Figure 2B). This can be useful for low-quantity samples where primarily analysis of the main peak is desired.

For improving separation between monomeric and dimeric peaks, a program of a 35% [NaCl] step followed by a linear gradient was designed. The value of 35% was selected according to the buffer conductivity identified for the BSA monomeric peak (figure 2C). The monomer MW of 67 kDa is uniform across the entire peak and sufficient volume was run to elute all remaining monomer. With judicious choice of salt concentration steps, this elution profile may be further refined to separate fragments, observed at the beginning of the linear portion of the gradient near 30 minutes, from the dimers and trimers that elute at slightly higher salt concentration.

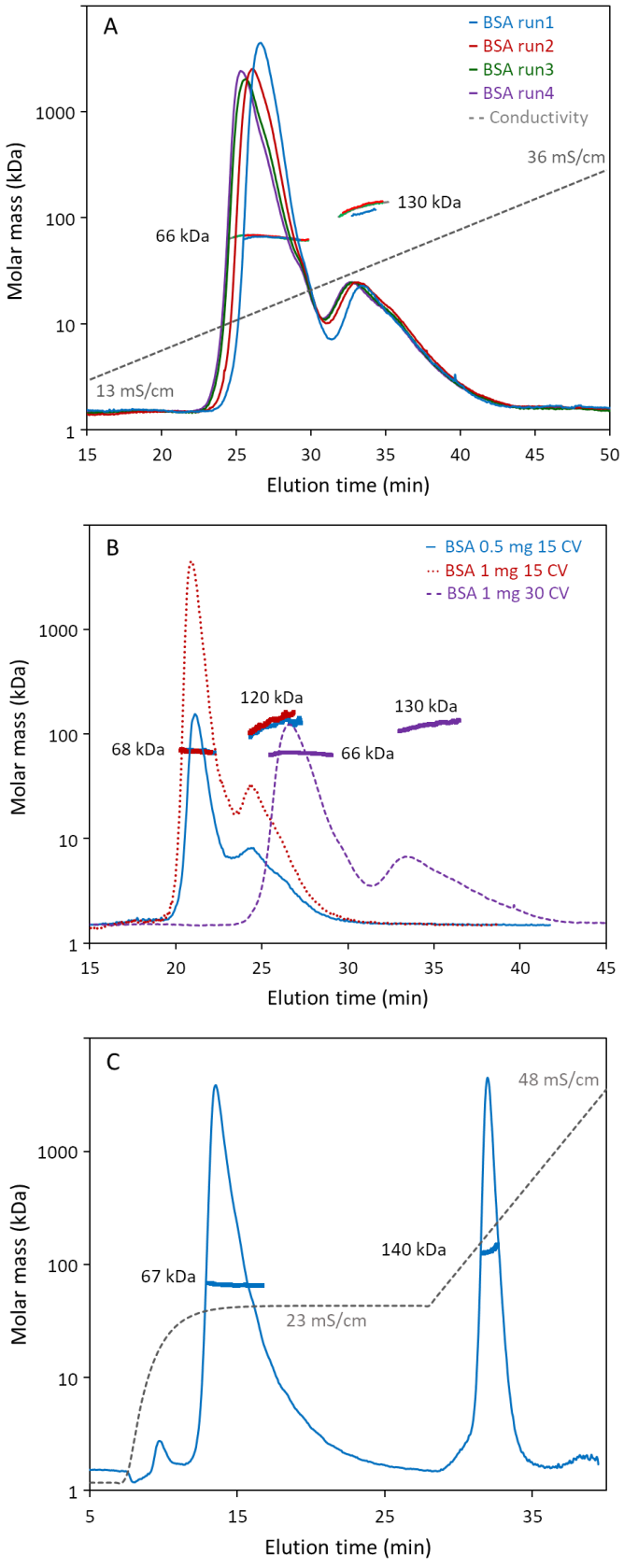


Figure 2. AIEX-MALS of BSA with different gradient programs: A) 15-70% of 500 mM NaCl over 30CV; B) 15-70% of 500 mM NaCl over 30CV and 15CV; C) 35% step and 35-100% gradient over 10CV. Chromatograms display the LS at 90° angle, UV at 280nm, and buffer conductivity with the molar mass of each peak determined by MALS.

MAbs

Antibodies usually exhibit charge variants (known as acidic and basic variants) that share very similar molar masses but vary by glycosylation pattern, amidation/deamidation, sialylation/desialylation, oxidation/reduction, C-terminal lysine cleavage, etc.[4]. Variants may also exhibit different hydrodynamic structure [5].

SEC-MALS analysis show that both mAb1 and mAb2 elute as single homogenous peaks with very high purity (Figures 3A and 3B). However, CIEX-MALS was able to separate between the mAb variants; molar mass and radius analysis by MALS and DLS were performed on each peak in the chromatograms (Figures 3C and 3D). The variants have similar hydrodynamic radii R_h and thus could not be separated by SEC.

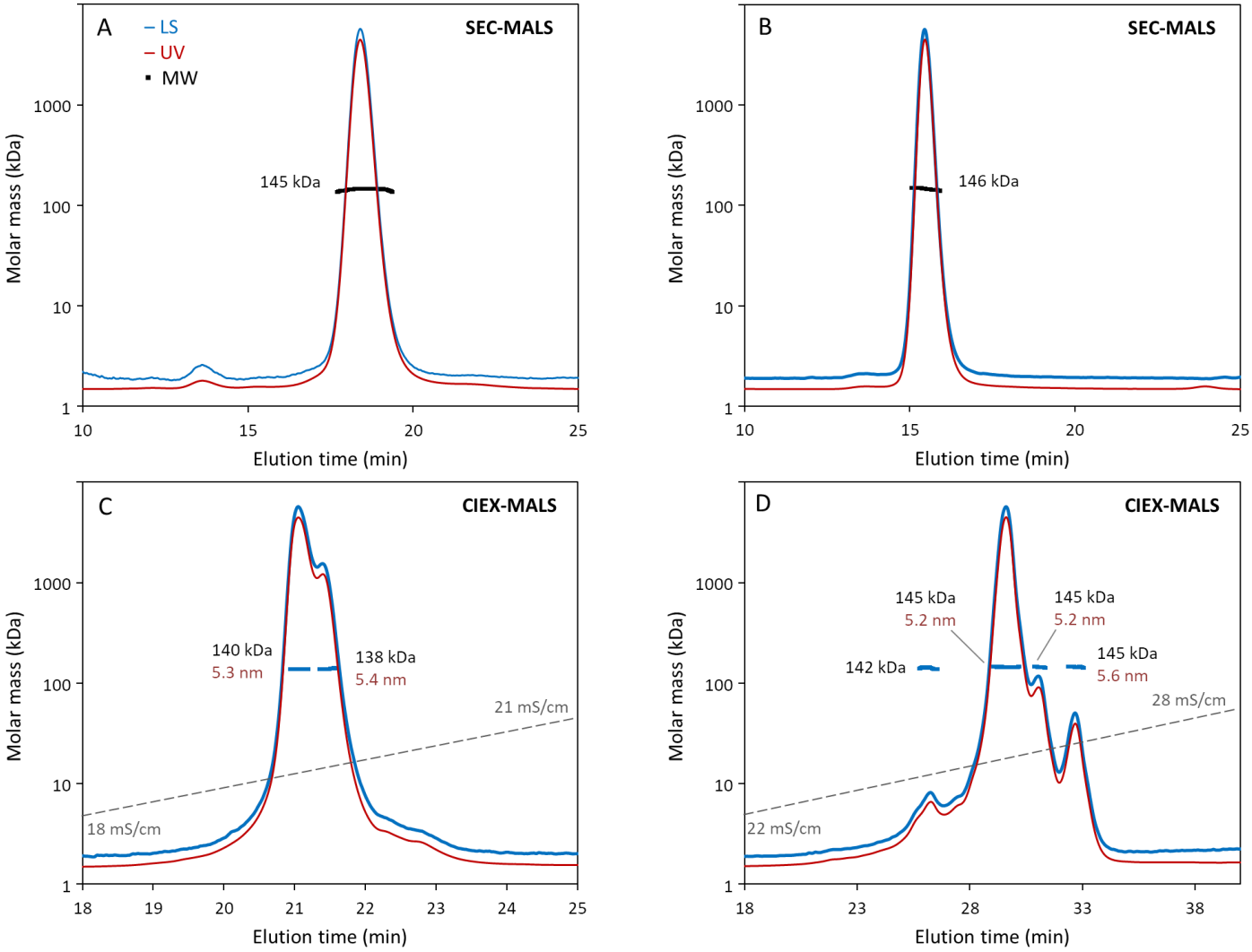


Figure 3. SEC-MALS and CIEX-MALS of monoclonal antibodies. SEC-MALS results of mAb1 (A) and mAb2 (B). CIEX-MALS results of mAb1 (C) and mAb2 (D). Chromatograms display the LS at 90° angle, UV at 280nm, and buffer conductivity with molar mass of each peak determined by MALS. LS traces are offset vertically from UV for clarity.

mAb1

Two variants are observed with a small apparent difference in molecular weight determined by MALS-UV. The difference could arise as a result of a minor sequence difference (two tryptophans replaced with a non-UV-absorbing amino acid) or different degrees of glycosylation. While a high-resolution method such as mass spectrometry is required to identify unambiguously the reason for the difference, IEX-MALS offers a first glimpse into the presence and characteristics of the variants.

mAb2

MALS shows that the variants have the same or very close molar masses values, and so truly are charge variants of the monomeric mAbs.

Outlook

The flexibility of ion-exchange chromatography to adapt to difficult separations by use of arbitrary conductivity and pH gradient profiles or sign of charge and binding strength of columns means that IEX-MALS can serve a wide range of analytes in aqueous buffers. IEX is nearly size-neutral, meaning that one column can be applied to a large range of peptides, proteins, nucleic acids, complexes, membrane proteins encapsulated in detergent micelles, nanodiscs, etc. which may not run on a single SEC column. A larger-pore column will be suitable for IgM, virus-like particles, small vesicles and many more biomacromolecules

Further development of this technique will extend the realm of absolute molar mass and size analysis by online MALS and DLS to many more analytical challenges. Progress is also being made in combining MALS with preparative IEX in order to optimize purification runs and minimize post-purification analytical work.

Conclusions

The different principle of separation used in IEX-MALS provides an additional pathway to absolute protein characterization, making it a complementary method for SEC-MALS. IEX-MALS is a valuable technique for addressing protein quality assessment and characterization; it addresses pure proteins and heterogenous samples. In particular this technique should be employed in order to characterize samples that cannot be separated by standard SEC because the species present have similar hydrodynamic size or otherwise co-elute.

To learn more about IEX-MALS capabilities, implementation, best practices and data interpretation, please contact info@wyatt.com. Additional literature may be found in Reference 3 and Wyatt application note AN8004. For an IEX-MALS protocol, see the JoVE article and video.

Acknowledgements

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