APPLICATION NOTE

AN8004: Coupling MALS with preparative ionic-exchange (pIEX) for structural biology applications

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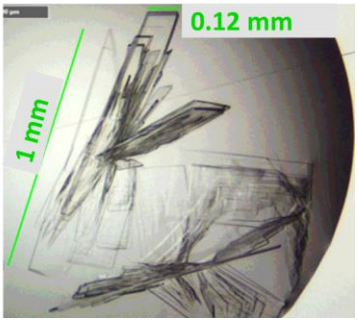
Summary

Ion-exchange chromatography is a common intermediate step in a protein purification protocol. Adding multi-angle light scattering in-line to preparative ion-exchange chromatography (pIEX-MALS) allows absolute molar mass determination of the eluting fractions in the course of the run, providing immediate identification of pure fractions, for optimal pooling with minimal time and effort.

Introduction

Common techniques in structural biology, such as x-ray crystallography and NMR spectroscopy, require producing and purifying proteins at the milligram level with purity on the order of 95% and above. In a typical workflow, crude solution is first run on an affinity chromatography column to separate the primary protein of interest from cellular lysates or supernatants. The second purification step consists of ion-exchange chromatography (IEX) to separate the desired oligomeric form (usually the monomer) from others (usually aggregates) and any remaining host cell proteins (HCPs). The final polishing step for removing oligomers such as dimers and trimers consists of preparative size-exclusion chromatography (SEC).

In the course of an IEX purification run, many eluting fractions are collected. Some of those fractions contain only the desired protein form (e.g. pure monomer), while some may contain aggregates and other impurities, or mixtures of the primary protein with those impurities in various proportions.



Fab protein obtained with pIEX-MALS produced abundant protein crystals.

The fractions containing purely monomers of the proteins of interest must be identified and pooled in order to obtain the maximum amount of the right protein, in the right form, with sufficient purity. Analysis of the fractions usually includes SDS-PAGE, to verify protein identity, and SEC-MALS, to verify monomeric/oligomeric state and purity. This workflow, outlined in Figure 1, is time-, material- and labor-intensive. We have found that adding a multi-angle light scattering (MALS) detector in-line with the IEX polishing step saves significant time and effort.

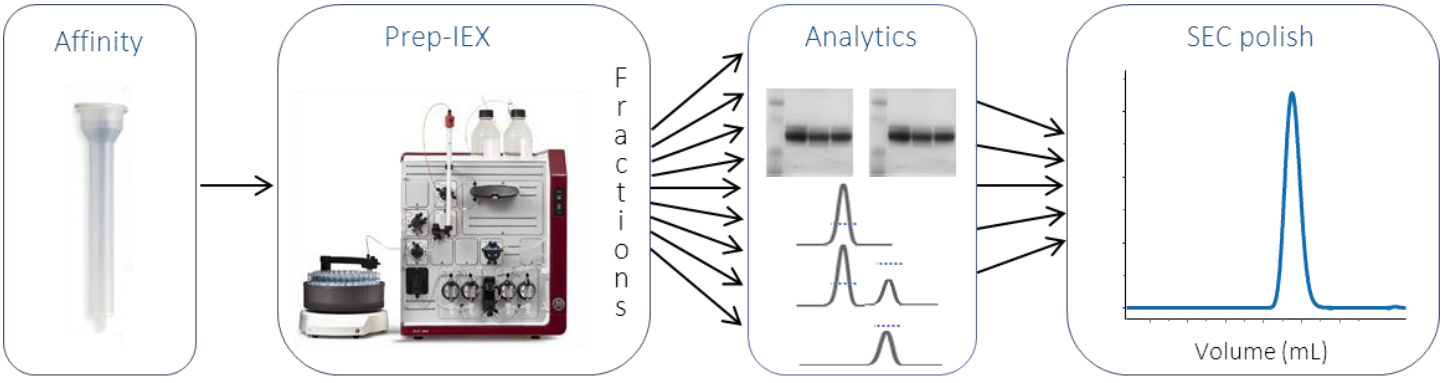


Figure 1. Standard workflow for protein purification with affinity, ion-exchange (IEX) chromatography and polishing by SEC. IEX fractions are tested on gel and SEC-MALS prior to performing preparative SEC.

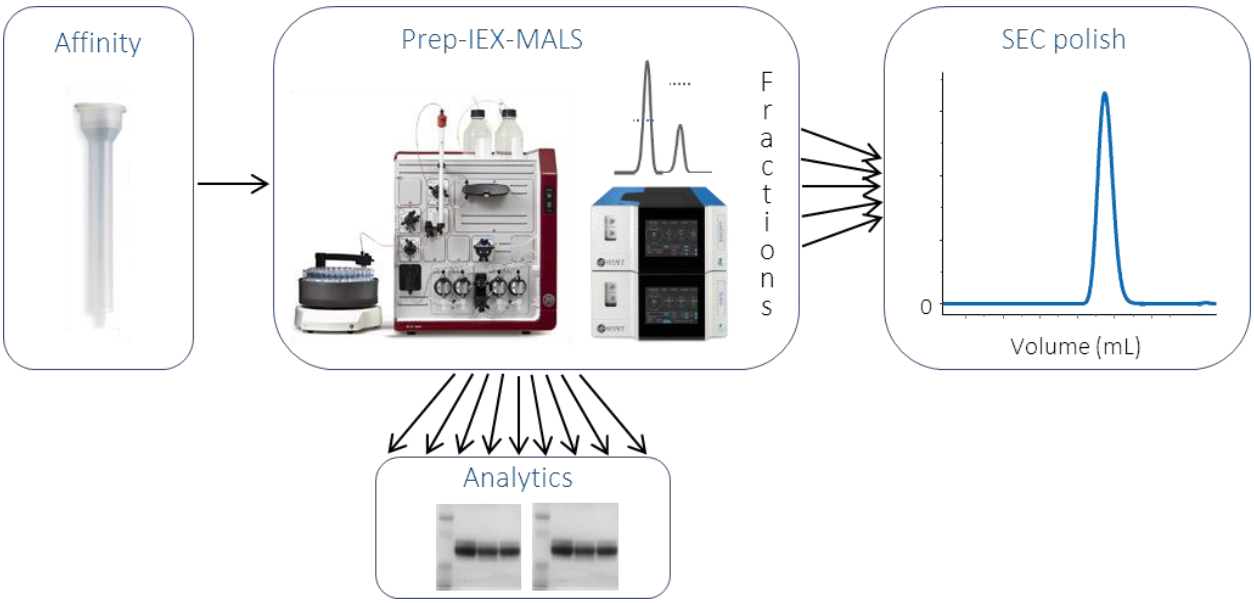


Figure 2. Optimized workflow with inline MALS. Testing fractions post-IEX on SEC-MALS is no longer needed.

The improved workflow is outlined in Figure 2. While protein identity verification by SDS-PAGE is still important, combining MALS in-line with preparative IEX provides instant identification of the correct fractions to be pooled. Multiple SEC-MALS runs, with typical time scales of 30-60 minutes each, are no longer necessary. The optimized pool can then be passed on in minimal time to the final SEC polishing step.

Elsewhere IEX-MALS has been shown to be effective as a stand-alone analytical method[1]. Here, the combination of MALS with preparative IEX, or 'pIEX-MALS', facilitates the rapid production of sufficient quantities of pure protein for crystallization. The utility of pIEX-MALS for purification is demonstrated on the Fab arm of a monoclonal antibody, producing many milligrams of pure material for crystallization and x-ray structural determination.

Materials and Methods

The sample for crystallization was the ~ 50 kDa Fab arm of a monoclonal antibody produced in CHO cells. Two variants were purified, Fab1 and Fab2. Initial purification consisted of Protein L affinity chromatography. The polishing step was performed on an ÄKTA pure FPLC system connected to a miniDAWN® MALS detector. The FPLC's UV 280 nm absorbance signal was used for concentration detection. An Optilab® differential refractive index (dRI) detector measured dRI data for additional concentration analysis.

The IEX method utilized a Mono-S cation exchange column from GE Healthcare, with 30 mM acetate buffer at pH 5.5. Loading and wash were performed at 0 mM NaCl while the elution gradient ranged from 0 to 250 mM NaCl over 20 column volumes. SEC was performed on a Superdex 200 16/60 in 10 mM Hepes 7.4, 50 mM NaCl.

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. For species that elute at salt concentrations above 250 mM NaCl, a slightly lower value of *dn/dc* may be appropriate.

Results and Discussion

Fab1 – early elution

As seen in Figure 3, elution of Fab1 began very close to the beginning of the gradient (top); in fact, the RI signal overlay (bottom) indicates that the Fab began eluting as soon as the NaCl concentration increased above zero, making it difficult to define an RI baseline for the peak. In addition, an early shoulder appears in the light scattering and UV signals between 22 – 26 mL where the RI is nominally flat. Hence this sample is best analyzed by MALS + UV.

The RI behavior also provides a good indication that the purification can be improved, by modifying buffer pH so that the Fab elutes somewhat later in the gradient for better separation from the material eluting at zero salt.

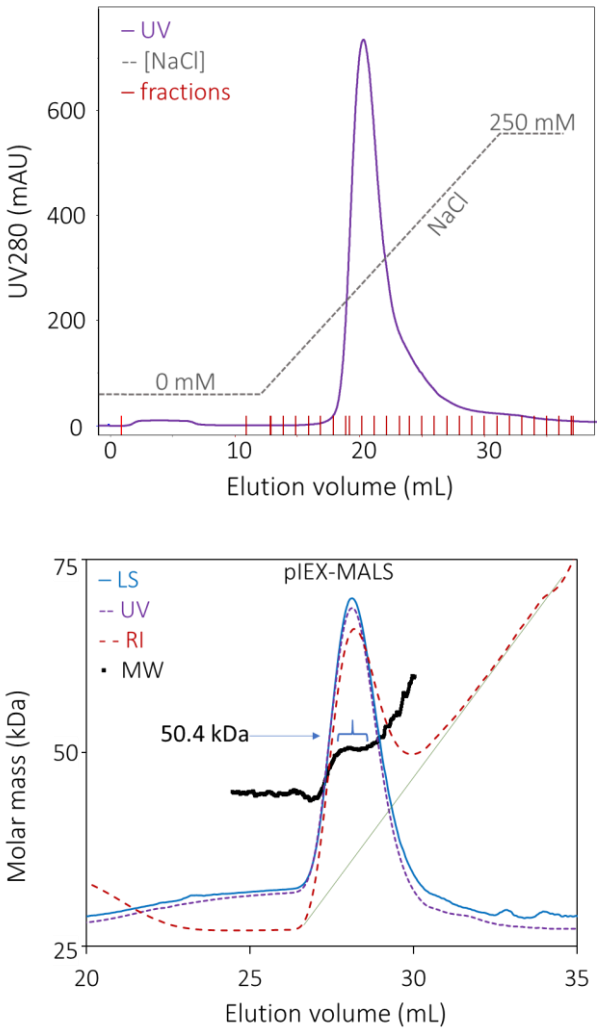


Figure 3. pIEX-MALS purification of Fab1. Top: elution position is close to the beginning of the gradient. Fractions collected are indicated in red. Bottom: Inspection of the RI signal (aligned by ASTRA to the LS and UV signals, with green line as a guide to the eye) shows that Fab1 actually begins eluting as soon as the salt concentration increases from zero.

MALS analysis (Fig. 3, bottom, black symbols) shows that the pure fractions elute between 27.5 – 29 mL. The leading edge of the peak is mixed with a low-MW species that begins eluting around 22 minutes, while the trailing edge of the peak is mixed with higher-MW species. Since protein crystallization requires monodisperse material, only the purest fractions were pooled. Abundant crystals were obtained in later crystallization trials.

Fab2 - elution within the gradient

Fab2 is seen in Figure 4 to begin eluting well after the salt gradient begins. In this case it was straightforward to draw the RI baseline in ASTRA and determine a reliable MW from RI, which is 50.2 kDa (48.5 by UV, using the extinction coefficient from sequence). The entire peak from 22 to 26 mL is found to be quite homogeneous, leading to a large quantity of pooled, pure sample that produced many crystals. In-line IEX-MALS greatly reduced the time and effort that would have been invested in testing these fractions on individual SEC-MALS analyses.

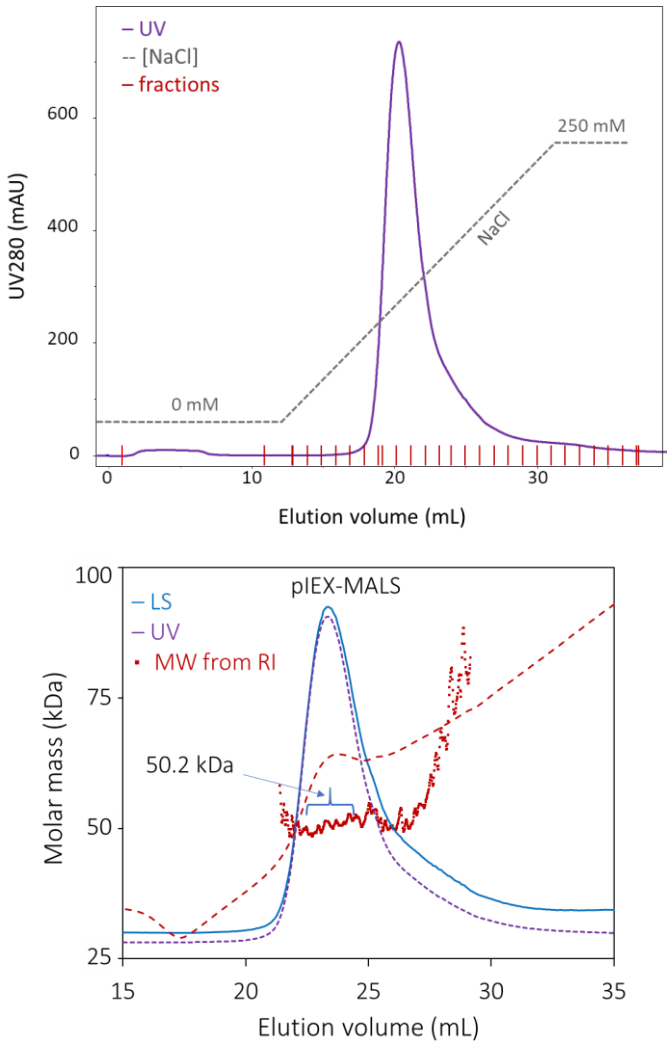


Figure 4. Top: elution position of Fab2 relative to the salt gradient. Here the sample elutes mid-gradient rather than at the beginning. Fractions collected are indicated in red. Bottom: MALS analysis (red symbols) indicating that the majority of the peak consists of purely 50.2 kDa protein, with optimal cut between 22 and 24 mL.

Pros and cons

Compared to analytical SEC-MALS, pIEX-MALS will be subjected to high levels of noise and fluctuations due to gradient mixing, particulates and impurities. However, these are overcome by the normally high protein concentrations used in preparative work. Accurate and reliable MW values are obtained despite the seemingly 'MALS-unfriendly' environment.

Unlike isocratic SEC-MALS, where either RI or UV may be used to determine concentration values that feed in to

MW calculations, the choice of concentration detector is not as flexible when coupling MALS to a gradient chromatography. In some instances it is impossible to reliably set the baseline in RI, and UV must be used, even though there may be questions raised regarding extinction coefficients, and also regarding linearity when protein concentrations exceed 10 mg/mL.

For those interested in investing a little more work—to achieve better results—ASTRA's *Baseline Subtraction* method permits acquiring a blank injection under the identical gradient and subtracting the blank signals from those measured with the sample. This method can be applied to all three signals for the most accurate analysis.

An optimal SEC-MALS-RI system for salt gradients would include the high-concentration version of the Optilab, which can cover a range equivalent to 2 M NaCl.

Conclusions

Preparation and purification of relatively large quantities of high-quality protein for crystallography on lab-scale FPLC instrumentation can be a tedious task. With the addition of MALS to IEX it has been possible to greatly reduce the necessary time and effort, obtaining well-purified protein that has led to many successful crystallization projects. Preparative IEX-MALS, or pIEX-MALS, can be readily adopted by most labs engaged in this type of activity for enhanced research productivity.

References

1. Amartely, H., Avraham, O., Friedler, A., Livnah, O. & Lebendiker, M. Coupling Multi Angle Light Scattering to Ion Exchange chromatography (IEX-MALS) for protein characterization. *Sci Rep* **8**, 6907 (2018).

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