Development of Mass Spectrometry-Screening Methods for Quality Assessment of Cryo-EM Standard

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ABSTRACT

Purpose: Develop mass spectrometry methods to assess Apoferritin quality as a Cryo-EM standard

Methods: Recombinant human Apoferritin (~500 kDa, 24-mer) was expressed in E. coli. Quality, stability and heterogeneity of different batches of Apoferritin were verified by SDS-PAGE, UV HPLC, and infusion or LC-MS using a Thermo Scientific™ Vanquish™ Flex UHPLC system coupled to a Thermo Scientific™ Q Exactive™ UHMR mass spectrometer.

Results: Results clearly demonstrate the utility of native and intact LC/MS for Cryo-EM sample screening, and optimization of sample preparation for detailed Cryo-EM structural studies.

INTRODUCTION

Cryo electron microscopy (Cryo-EM) is used to determine the structure of biological macromolecules in near native state. For cryo-EM, protein particles are required to be embedded in a thin layer of vitreous ice. As the success of EM grid preparation and subsequently obtaining a high-resolution structure strongly depend on the homogeneity and stability of the sample, quality control is therefore crucial. Mass spectrometry (MS) as a widely applied technique for protein characterization provides the capability to screen biomolecules at relatively higher throughput and lower cost. Using MS to screen proteins prior to Cryo-EM imaging can thus accelerate structure elucidation and provide a much- needed quality assessment. Here, we developed Liquid Chromatography (LC)-MS-screening methods including native and intact protein assays for quality assessment of Cryo-EM standard, Apoferritin.

MATERIALS AND METHODS

Sample Preparation

Recombinant GST-Apoferritin (A51362- VitroEase Apoferritin Standard) was overexpressed in E. coli and purified by glutathione affinity chromatography. The GST tag was removed by cleavage with TEV protease and the intact ApoF complex was purified by size exclusion chromatography (SEC). Final ApoF buffer conditions and SEC protocol varied with R&D lots as conditions were optimized.

MS:

Samples analyzed for LC-MS were used as received in the formulation buffer. Samples for direct infusion were buffer exchanged into 200 mM Ammonium Acetate using 30 kDa MWCO (Millipore-Sigma).

Cryo-EM:

3.5ul of purified ApoF sample was applied to glow-discharged 200mesh 1.2/1.3 carbon grid (Quantifoil) and excess protein was blotted away before plunge-freezing in liquid ethane at 100% humidity and 4 ºC using Vitrobot Mark IV (Thermo Fisher Scientific).

Materials

Column: MAbPac™ SEC-1 column (PN 088790), 2.1 mmx 15 cm

Online buffer exchange column (Prototype), 2.1 mm x 3 cm

Mobile phase: 50-200 mM Ammonium Acetate

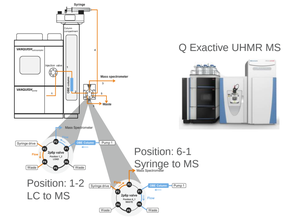
Data Analysis

Freestyle 1.8 was used to analyze UV chromatogram

Thermo Scientific™ BioPharma Finder™ 4.1 Software. was used for intact deconvolution and top-down analysis

Cryo-EM data was collected on Titan KriosG4 (Thermo Fisher Scientific) operating at 300kV equipped with bottom mounted Falcon4 direct electron detector. Data analysis was performed using Relion 3.1 software.

Figure 1. LC-MS set up for online buffer exchange or size exclusion chromatography



RESULTS

Online buffer exchange (OBE)[1] vs. Size exclusion chromatography (SEC)

Traditional native MS studies of protein complex requires either offline buffer exchange or online SEC separation prior to MS analysis. Buffer exchange is time-consuming as well as suffering from sample loss. SEC involves less sample preparation but has limitations in terms of high-throughput screening due to long runs. OBE is positioned in between by removing sample prep requirements and having short runs. This enables high-throughput screening of protein, protein complex, protein-ligand complex.

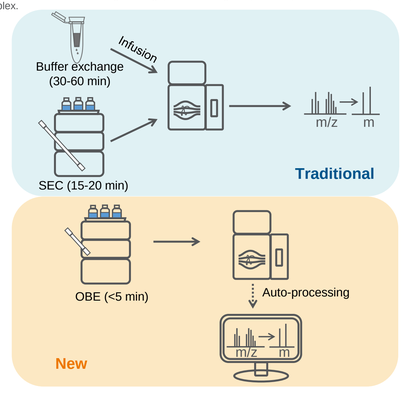


Figure 2. TIC and MS spectrum of OBE

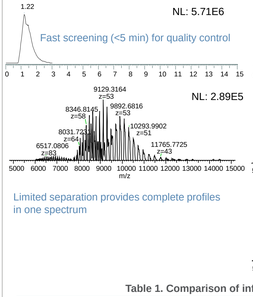


Figure 3. TIC and MS spectrum of SEC

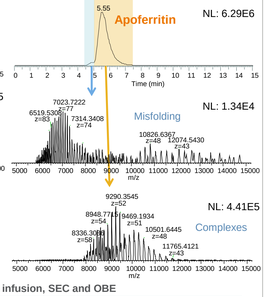


Table 1. Comparison of infusion, SEC and OBE

Methods	Sample prep time	Separation	MS time window	Throughput
Infusion	High	No	Long	Low
SEC	Low	Based on protein size	Short	Medium
OBE	Low	Proteins and salts	Short	High

Quality assessment

Understanding the quality of a Cryo-EM sample prior to microscopy is a critical step in achieving a high-resolution structure. Recombinant Apoferritin 24-mer complex is widely used as a Cryo-EM standard to test and tune a microscope. Critical attributes of such standards are subunit sequence confirmation, complex assembly and homogeneity, stability and aggregation.

1) Subunit sequence confirmation

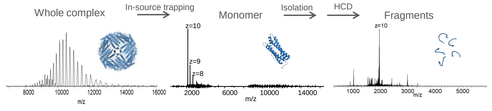


Figure 4. Deconvoluted spectrum of pseudo MS3 fragments

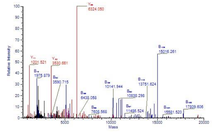
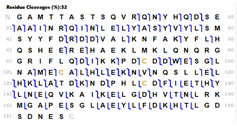


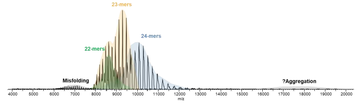
Figure 5. Fragments map of Apoferritin monomer



Native Pseudo MS3 determines the sequence of major form of monomer by matching major fragments to b- and y-ions. Sequence coverage is 52%.

2) Complex assembly and homogeneity

Figure 6. Native SEC-MS spectrum of Apoferritin from R&D lot 1



Native SEC-MS reveals multiple complexes as well as misfolding in early R&D batch (lot 1). Aggregation may be present, but sensitivity is insufficient.

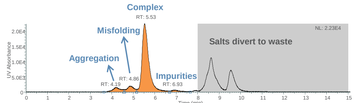
Table 2. Target and misassembled complex% from deconvoluted spectra

Lot	Target complex%	Misassembled complex%
1	71.39	28.61
2	79.35	12.05
3	91.28	8.01

Native MS followed by deconvolution in BioPharma Finder provides qualitative and quantitative assessments of each sample lot.

3) Stability and aggregation

Figure 7. UV chromatogram of Apoferritin complexes separated by SEC



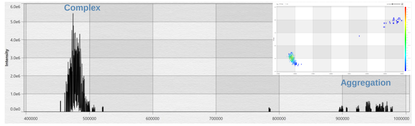
SEC-UV provides percentages of aggregation, misfolding and target complex by integrating peak areas using Avalon algorithm in Freestyle.

Table 3. Quality assessment for stability samples stored at various temperatures and days

Temperature	Days	Aggregation%	Misfolding%
-80 ºC	0	0.12	1.07
4 ºC	1	0.27	2.01
4 ºC	2	0.20	2.01
-20 ºC	3	0.33	2.14
-20 ºC	6	0.29	2.20

Both aggregation% and misfolding% increase gradually after samples are stored for several days at > -80 ºC.

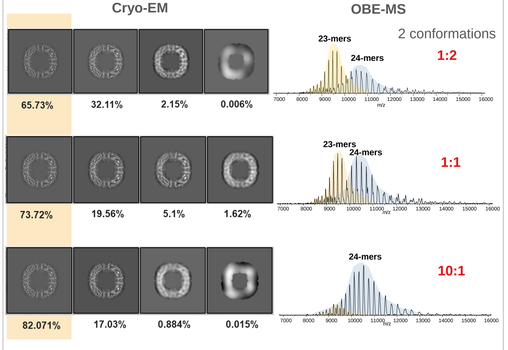
Figure 8. Apoferritin Analysis by I2MS[2].



While presence of aggregation in native SEC-MS analysis is ambiguous, individual ions mass spectrometry (I2MS) with exceptional sensitivity confirms aggregation.

Cryo-EM and MS correlation

Figure 9. Cryo-EM images and corresponded OBE-MS spectra of samples from three different purification strategies.



Three samples prepared via different purification methods show correlation between native MS and Cryo-EM results. Percentage of the most homogenous particles (shown as the leftmost images) was different for each sample from different purification procedures. The last purification yielded the greatest concentration of intact particles which is similar to result from raw MS data on the right. Native MS can help guide the biochemist to determine the best purification strategy

Workflow summary

Subunit sequence confirmation	**Denatured LC-MS** **Direct infusion pseudo MS3**
Complex assembly and homogeneity	**SEC-MS** **OBE-MS**
Stability and aggregation	**SEC-UV** **I2MS**

CONCLUSIONS

- First, we developed native MS spectrometry methods including infusion, SEC-MS and OBE-MS to characterize Apoferritin sequence, its homogeneity and stability.
- Secondly, the data demonstrates that native MS sample screening shows strong correlation to the quality of prepared Cryo-EM grids, both in terms of aggregation as well as particle distribution and stability.
- Thirdly, while going through multiple batches, the MS data helped to inform decision making on the biochemical preparations.
- These results clearly demonstrate the utility of native and intact LC/MS for Cryo-EM sample screening, and optimization of sample preparation for detailed Cryo-EM structural studies.

REFERENCES

1. Van Aernum et al Nat Protoc. 2020 Mar;15(3):1132-1157
2. Kafader JO et al.. Nat Methods. 2020 Apr;17(4):391-394

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TRADEMARKS/LICENSING

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