

Native mass spectrometry

Direct Mass Technology mode capturing heterogeneity of membrane proteins expressed in mammalian cells prior to cryo-EM analysis

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Overview

Membrane proteins are critical drug targets, yet their structural characterization by cryo-electron microscopy (cryo-EM) faces challenges due to the lack of standardized reference materials. Unlike soluble proteins, membrane proteins require stabilization in lipid or detergent environments, making their preparation and analysis complex.^{1,2} This issue is particularly pronounced for membrane proteins expressed in mammalian systems, as they often exhibit low expression yields, structural heterogeneity, and require specific post-translational modifications (PTMs) for functionality. Currently, no universal membrane protein standard exists for cryo-EM, hindering consistent benchmarking and quality control in structural studies. Establishing a commercially available, homogeneous membrane protein standard derived from mammalian systems would significantly advance cryo-EM applications, providing a robust tool for method validation and reproducibility.

Experimental

Protein expression and purification

Membrane proteins ATP Binding Cassette Subfamily C Member 4 (ABCC4), ATP Binding Cassette Subfamily G Member 2 (ABCG2), and Gamma-Aminobutyric Acid Type A Receptor Subunit Beta3 (GABRB3) were expressed in mammalian cells. Samples were buffer exchanged into 0.02% n-dodecyl β -D-maltoside (DDM) in 200 mM ammonium

acetate (AmAc) using a Thermo Scientific™ Zeba™ Spin Desalting Column. Native mass spectrometry (MS) and Thermo Scientific™ Direct Mass Technology™ mode analyses were performed on the Thermo Scientific™ Q Exactive™ UHMR mass spectrometer. The sample preparation for cryo-EM involves using Quantifoil™ R1.2/1.3 300 mesh grids coated with holey carbon, followed by glow discharge with the GloQube system by Quorum Technologies. The sample is then applied to the grid and rapidly vitrified using the Thermo Scientific™ Vitrobot Mark IV System, before being imaged with the Thermo Scientific™ Krios™ (300 kV) cryo-EM system for high-resolution analysis.

Results and discussion

The candidates, ABCC4, ABCG2, and GABRB3, were evaluated using native MS ensemble measurements, Direct Mass Technology mode, and cryo-EM to assess sample integrity and structural quality. ABCC4 is a dimer with 2 N-glycosylation and 15 phosphorylation sites; ABCG2 is a dimer with 1 N-glycosylation and 2 phosphorylation sites; and GABRB3 is a pentamer containing 3 N-glycosylation sites. The presence of these complex PTMs poses challenges for MS-based characterization. To address this, both ensemble and Direct Mass Technology mode measurements were employed to resolve molecular heterogeneity and improve analytical precision.

Protein expression and purification

We received 3 lots of ABCC4. Both SDS-PAGE and analytical SEC (Figure 1) confirmed the abundance and purity of the target protein. The broadening of the monomer band on the gel likely resulted from the multitude of PTMs. Ensemble measurements (Figure 2) resolved only a homogeneous protein species below 5,000, which deconvoluted to approximately 70 kDa. It is consistent with the faint band showing on SDS-PAGE. In contrast, unresolved signals from Lot-1 spanned m/z 4,000–8,000 and 8,000–21,000, indicating sample heterogeneity. When analyzed using Direct Mass Technology mode, Lot-1 exhibited a broad peak ranging from 200 to 450 kDa with an apex at 310 kDa, corresponding to dimeric ABCC4. The peak contained both high ($z \approx 55$ –70) and low ($z \approx 25$ –35) charge-state distributions, suggesting the presence of 2 conformational populations, open and closed conformations. Conversely, Lot-2 displayed a hump at lower m/z values (3,000–12,000), indicating predominance of higher charge states, which was later confirmed by Direct Mass Technology mode analysis showing an increased population of high-charge-state single ions. In Lot-3, ensemble measurement shows a 70 kDa signal below m/z 5,000, similar to the Lot-1 sample. Direct Mass Technology mode measurement reveals a wide peak apex at 343 kDa, corresponding to dimerization of ABCC4. The charge state distribution (z –25–33) indicates folded native conformation. Target peak has fronting peaks, possibly with some degradation or truncated forms.

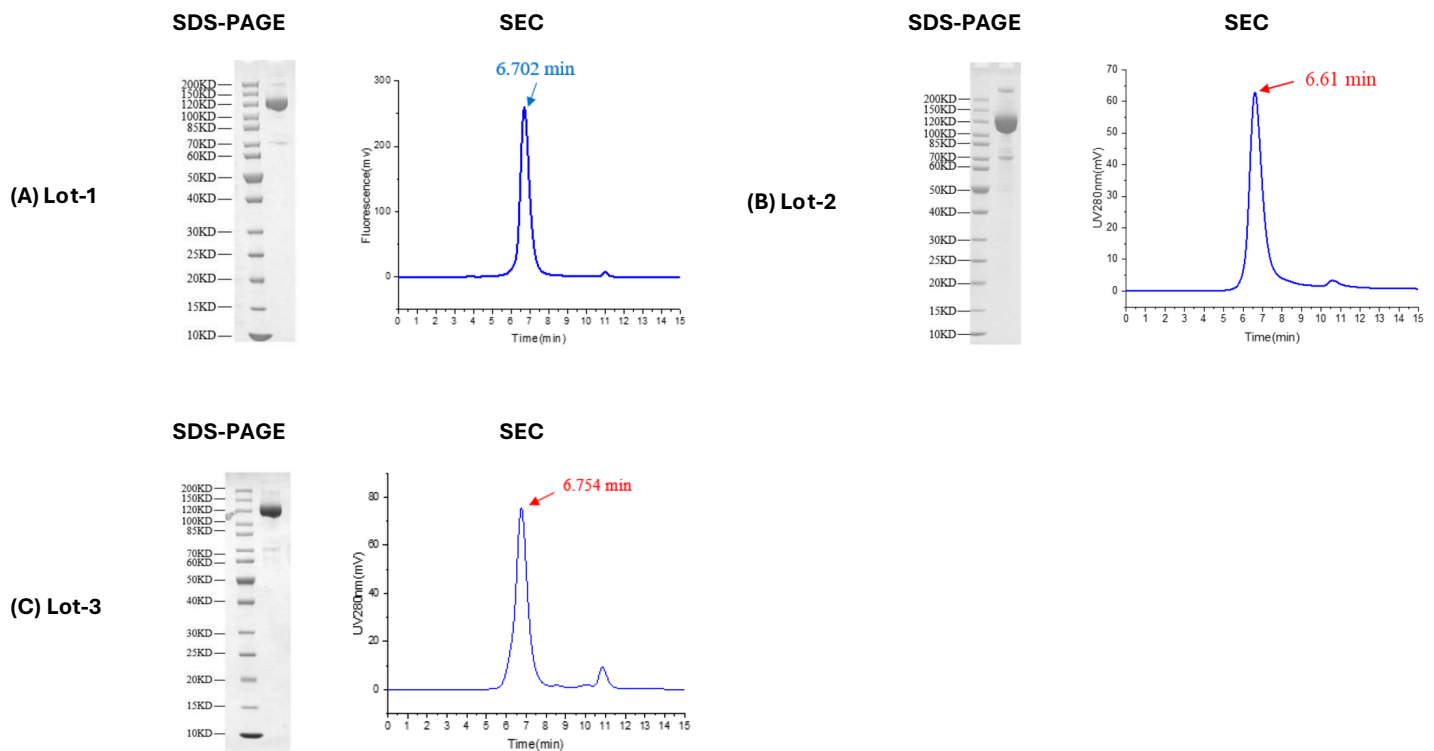


Figure 1. SDS-PAGE and SEC chromatograms of ABCC4.

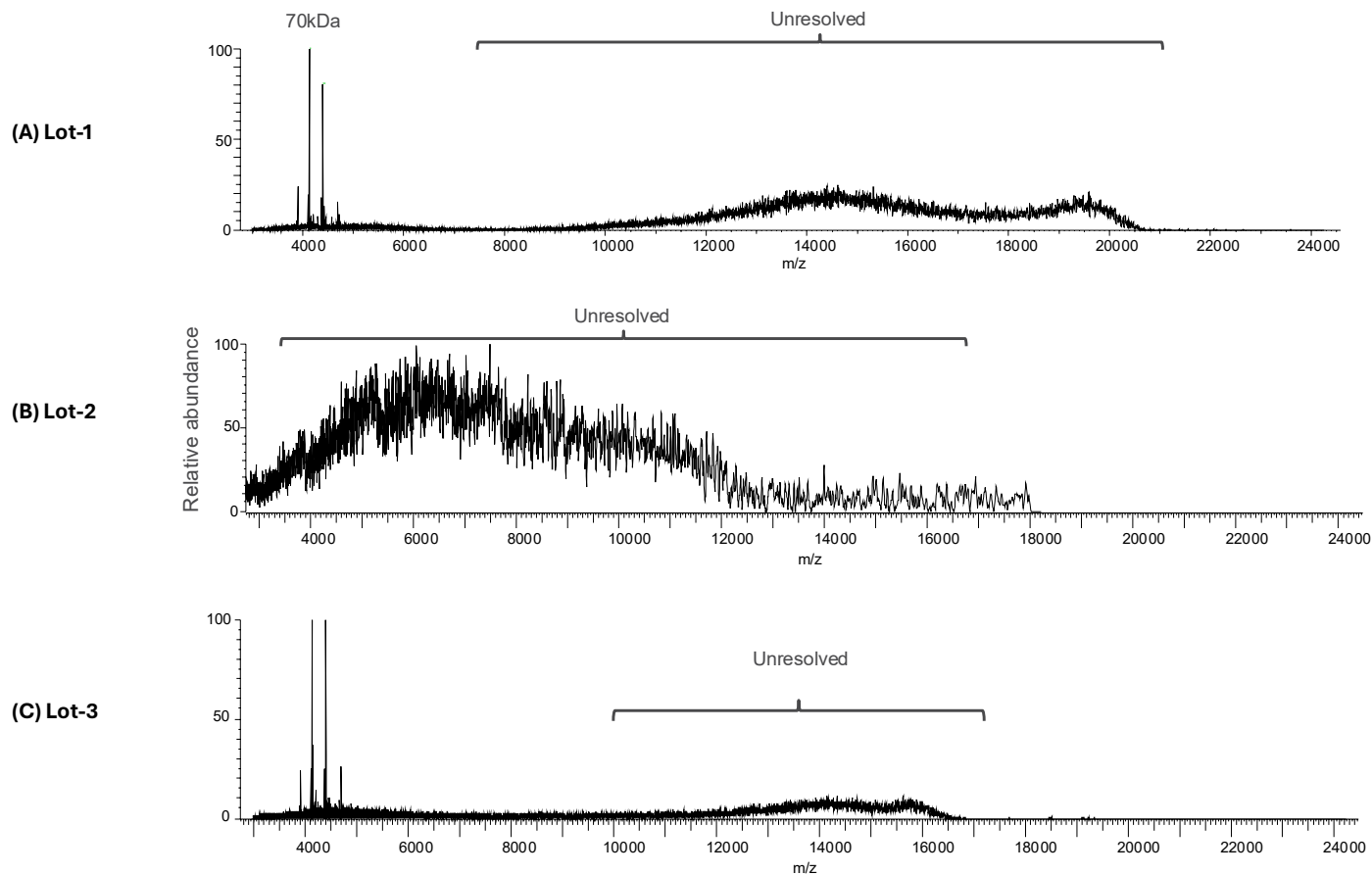


Figure 2. Native MS ensemble measurements.

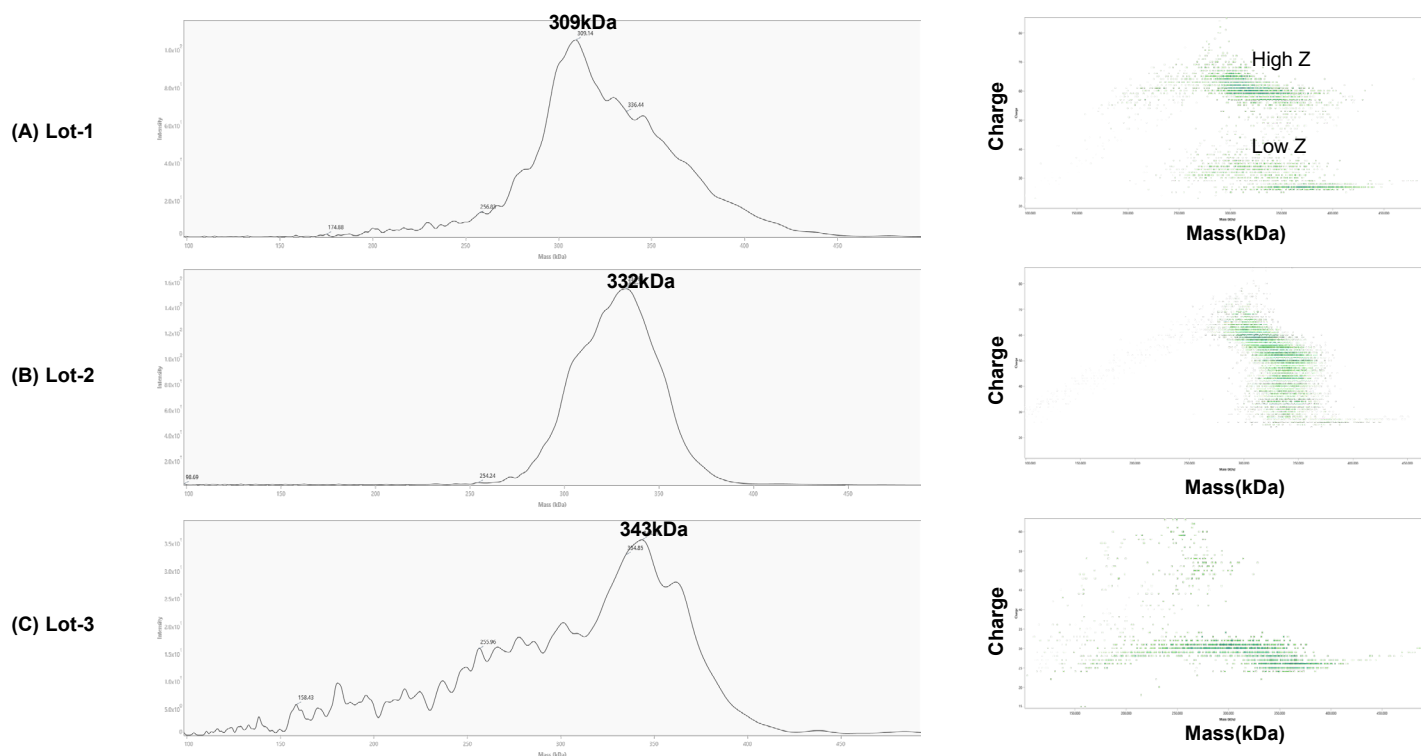


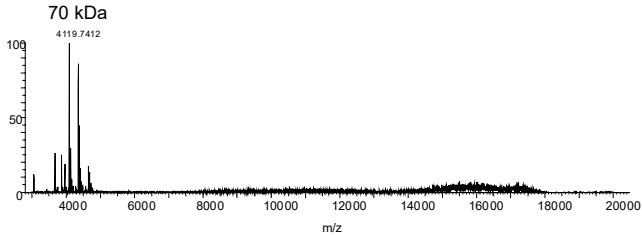
Figure 3. Direct Mass Technology mode analysis of ABCC4.

In addition to ABCC4, 2 other glycosylated membrane proteins were analyzed by native MS, as shown in Figure 4. A 70 kDa species was observed below m/z 5,000; however, top-down analysis did not match the ABCG2 sequence, indicating a common impurity present in most samples. Unresolved signals were detected across m/z 7,000–18,000. For ABCG2, dominant peaks appeared at 122 kDa and 478 kDa, with a shoulder near 143 kDa corresponding to the dimeric form.

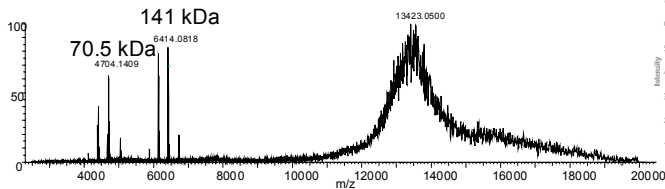
The high-abundance peak centered at 478 kDa suggests a significant level of aggregation within the sample. Although the 70 kDa impurity was also observed in GABRB3, its unresolved ensemble spectrum exhibited a uniform hump across m/z 10,000–18,000, corresponding to an apex molecular weight of 407 kDa determined by Direct Mass Technology mode analysis. The higher-than-expected molecular weight likely reflects extensive PTMs or protein aggregation.

Ensemble measurements

(A) ABCG4



(B) GABRB3



Direct Mass Technology mode

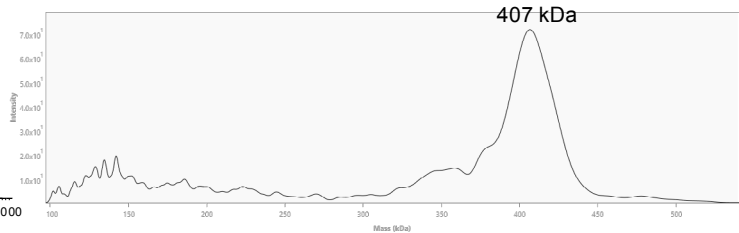
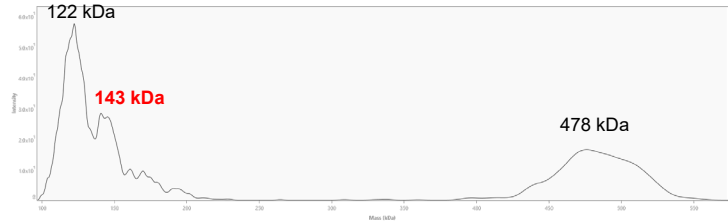


Figure 4. Native MS ensemble measurements and Direct Mass Technology mode analysis of (A) ABCG4 and (B) GABRB3.

Table 1 summarizes the characterization results for ABCC and GABRB3 obtained by both mass spectrometry and cryo-EM. Due to extensive glycosylation, ensemble mass spectrometry cannot reliably resolve the molecular weight in all cases. In contrast, Direct Mass Technology mode simultaneously measures the m/z and charge state of individual ions, enabling unambiguous characterization of these complex species. Figure 5 shows the reconstructed structure of ABCC4 and GABRB3 to atomic resolution respectively. ABCC4 was reconstructed as a dimer, while GABRB3 was reconstructed as a pentamer. Due to the aggregation on the grids, it prevented the reliable 3D reconstruction of ABCG2.

Table 1. Characterization of ABCC4, ABCG4, and GABRB3 using native MS and cryo-EM.

Samples	Ensemble MS	Direct Mass Technology mode	Cryo-EM
ABCC4 Lot-1	Unresolved	Resolved	2.96 Å
ABCC4 Lot-2	Unresolved	Resolved	2.93 Å
ABCC4 Lot-3	Unresolved	Resolved	2.70 Å
ABCG2	Unresolved	Resolved, aggregation	Aggregation
GABRB3	Unresolved	Resolved	2.82 Å

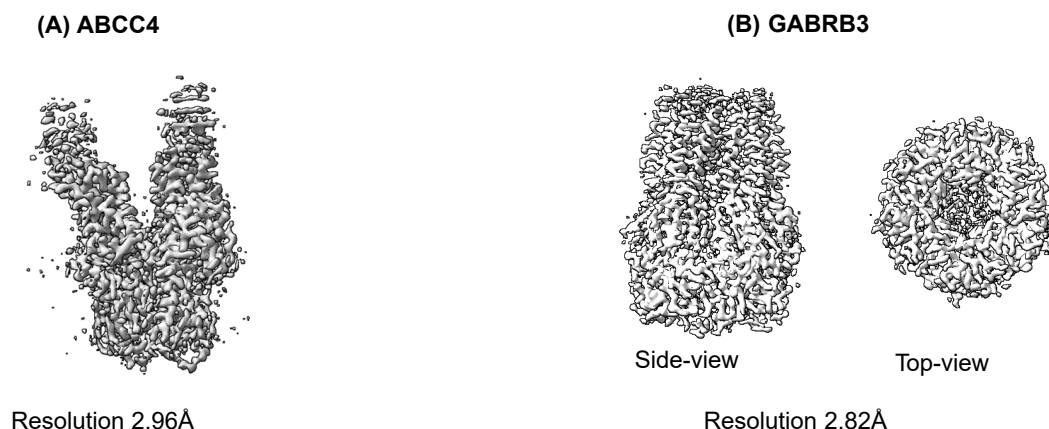


Figure 5. Cryo-EM structure of ABCC4 and GABRB3.

Conclusions

Direct Mass Technology mode enabled simultaneous charge and mass-to-charge measurements, allowing successful resolution of MW profiles of complex mammalian membrane proteins. Among the 3 candidates, ABCC4 demonstrated the most consistent performance across both MS-based and cryo-EM analyses. Its reproducible dimeric architecture, well-defined charge distribution, and compatibility with Direct Mass Technology mode screening highlight its potential as a mammalian membrane protein standard. This standard can serve as a benchmark for cryo-EM workflow validation, connecting upstream sample quality control via Direct Mass Technology mode to downstream structural outcomes.

References

1. Cheng D. et al. *Biotech Adv.* 2025;78:108483.
2. Errasti-Murugarren E. et al. *Membranes (Basel)*. 2021;11(2):155.

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