TOSOH

Application Note

SEC-MALS Analysis Determines Molecular Weight and Oligomeric State of Membrane Proteins

Membrane proteins are among the most important drug targets because they regulate many cellular functions such as excitability, ion homeostasis, signaling, and solute transport. Furthermore, they are more readily accessible for small molecule drugs or biologics when they reside in the plasma membrane compared to intracellular proteins. An important parameter of membrane proteins is their oligomeric state and whether this oligomeric state is preserved in purified form. However, the molecular weight of membrane proteins is difficult to characterize by standard procedures because they can only be solubilized and purified in their native state using detergents, or detergent-like polymers. These additives inherently alter the hydrodynamic radius of purified membrane proteins, which makes molecular weight estimation by size exclusion chromatography (SEC) challenging. The limitation can be overcome by coupling SEC to multi-angle light scattering (MALS) as it determines molecular weight independent of retention time. In conjunction with a refractive index detector (RID), the UV, MALS, and RI signals suffice to measure the molecular weight of eluting particles in SEC and resolve the molecular weight of the individual components (the detergent belt and the protein component).

Experimental Conditions

This study analyzed MtTMEM175 ("Transmembrane protein of unknown function 175"), an ion channel of the bacterium *Marivirga tractuosa*, which was kindly provided by Prof. Janine Brunner and Dr. Stephan Schenck from the VIB VUB Center for Structural Biology Brussels.

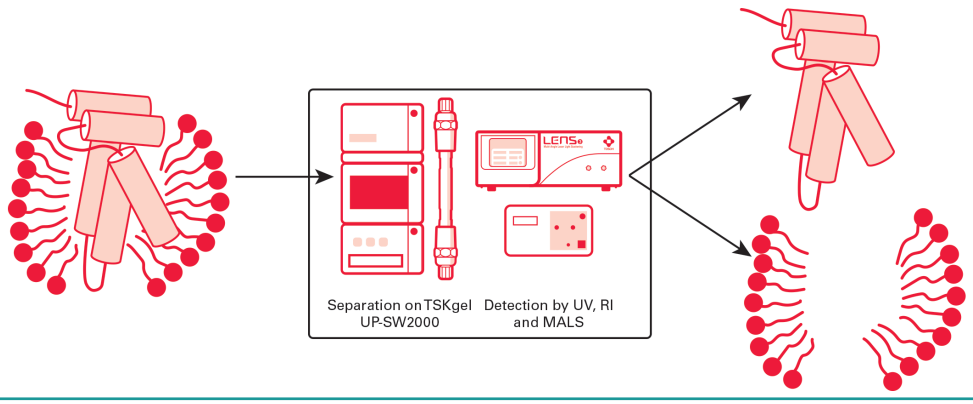
Its human isoform is a lysosomal potassium (K^+) channel which stands out from all other known potassium channels due to its different selectivity filter. It is important in the onset of Parkinson's disease and thus has become a drug target of increasing interest.

The protein was solubilized in a buffer containing a detergent, with the following composition:
135 mmol/L KCl, 9 mmol/L Hepes-Na, 10 % Glycerol, 0.03 % n-Dodecyl-beta-Maltoside (DDM), pH 7.5. Protein concentration was at 2 mg/mL.

Instrument:	Vanquish™ UHPLC
Detection:	UV @ 280 nm Refractive index detector LenS™3 MALS detector
Column:	TSKgel® UP-SW3000 LS, 2 µm, 4.6 mm ID × 30 cm L
Mobile phase:	0.1 mol/L sodium phosphate + 0.1 mol/L sodium sulfate (pH 6.7) + 0.03 % n-dodecyl-beta-maltoside (DDM)
Flow rate:	0.35 mL/min
Temperature:	21 °C
Data evaluation:	SECview™
Injection vol.:	10 µL
Calibration standard:	Bovine serum albumin (BSA)

The volume of capillaries in the system introduces delays and band broadening between each detector response, which are corrected in a single system calibration procedure with BSA.

Figure 1. Process scheme for SEC-MALS analysis of membrane proteins.

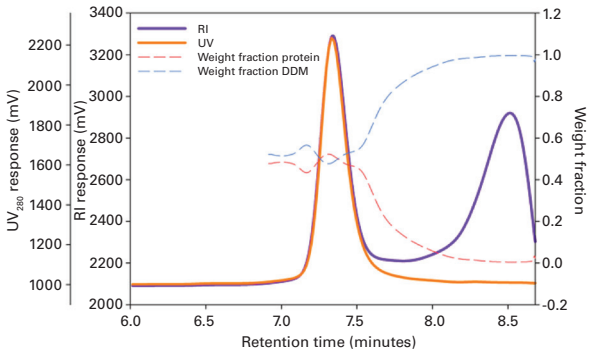


Results and Discussion

Molecular weight determination

Three detectors are employed for the analysis of the membrane protein MtTMEM175: UV provides the protein content, RI detects the presence of both DDM and protein, while MALS determines the molecular weight of the protein-DDM complex as well as that of free DDM. *Figure 2* shows the overlay of the RI and UV signals of the sample containing the MtTMEM175 membrane protein and the detergent DDM.

Figure 2. UV and RI signal of MtTMEM175.



The red and blue dashed lines indicate the calculated weight fractions of protein and DDM respectively.

A peak at 7.4 minutes is visible on both detector signals (RI and UV). The membrane protein encapsulated into a DDM-detergent belt generates a response in both UV absorbance at 280 nm and in RI, which suggests that the peak corresponds to the DDM-protein complex.

With increasing retention time, the UV signal decreases back to the baseline, while a second large peak occurs on the RI detector signal after 8.5 minutes. This peak represents free DDM from the eluent as DDM does not absorb UV at 280 nm but is visible by the RI detector. Indeed, DDM forms micelles above its critical micelle concentration (CMC).

Using the ratio of the RI and UV signals and the specific extinction coefficient (dA/dc) and refractive index increment (dn/dc) of the protein and DDM, the weight fractions for these components can be determined following the principles indicated in equations 1 and 2. The used dA/dc and dn/dc values are listed in *Table 1*.

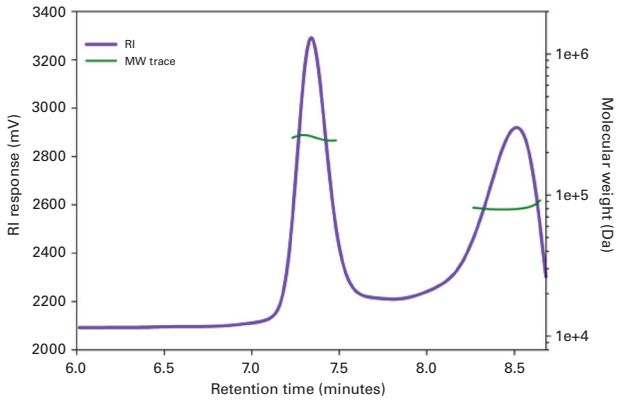
$$UV\ signal \propto dA/dc_{Protein} * Conc_{Protein} + dA/dc_{DDM} * Conc_{DDM} \quad (1)$$

$$RI\ signal \propto dn/dc_{Protein} * Conc_{Protein} + dn/dc_{DDM} * Conc_{DDM} \quad (2)$$

The calculated weight fraction traces are also shown in *Figure 3*. The protein weight fraction is on average 48.9% for the protein/detergent complex peak and at 0% for the peak representing the free DDM.

A variable dn/dc trace can be calculated across the chromatogram using the calculated weight fraction traces. This dn/dc trace is required for accurate MW determination of such bi-component samples. *Figure 3* shows the resulting MW distribution:

Figure 3. Molecular weight distribution of MtTMEM175 in buffer containing DDM.



The overall molecular weight of the membrane protein complex peak was determined at 255.1 kDa. Using the protein weight fraction average of 48.9%, a protein MW of 124.8 kDa could be calculated. The DDM belt contributes to the overall molecular weight by the remaining 130.3 kDa. This result is in good correlation with literature values[1].

By dividing the calculated molecular weight of the protein in the complex by the theoretical MW of the monomer, the oligomeric state of the protein could be determined:

$$\frac{124.8\ kDa}{29.5\ kDa} = 4.23 \quad (3)$$

This result indicates that inside the detergent belt, the proteins form tetramers, which agrees with what is reported and evident from the crystal structure.

Table 1. Literature values for dn/dc and dA/dc for employed molecules.

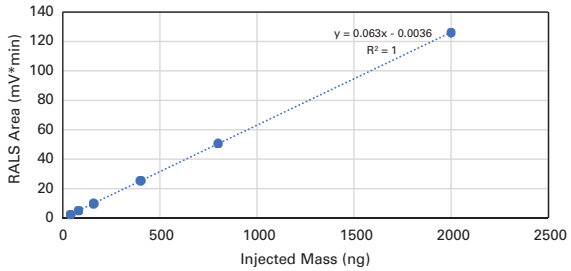
Molecule	dn/dc	dA/dc
MtTMEM185	0.185	1.404
DDM	0.144	0

Sensitivity study

In order to assess the detector's sensitivity while maintaining calculation accuracy, the sample was injected with decreasing injected mass, by varying injection volume and diluting the 2 mg/mL stock down to 80 µg/mL.

The main peak area of the RALS signal was plotted against the injected mass. The low end of this study is displayed in *Figure 4*:

Figure 4. RALS area versus injected mass.



The RALS response proved to be perfectly linear, down to an injected mass of only 40 ng. As a result, even injections of sample containing low amounts of protein can be used for MW calculations. *Table 2* shows the calculated MW for the protein/detergent complex using different injected masses. The corresponding relative standard deviation was calculated from triplicate injections.

Table 2. Calculated MW and corresponding relative standard deviations using varying injected mass.

Injected Mass [ng]	Complex Average MW [kDa]	relative Standard deviation [%]
10,000	255.1	0.05
4,000	255.0	0.09
800	252.1	0.04
80	252.8	0.26

These results regarding MW determination show very little deviation when varying the injected mass of protein. The highest difference was observed between the 10,000 ng and 800 ng injections, which differ by 1.2%. Furthermore, the triplicate injections and corresponding MW calculations for each injected mass proved to be very low. As expected, the highest relative standard deviation could be observed for the 80 ng injections, which was still as low as 0.26%. Overall, this proved that SEC-MALS using the described setup can be regarded as a very robust method for the MW determination of membrane proteins, even for low injected masses down to 80 ng.

Conclusion

Using the combination of SEC with MALS, UV, and RI detectors, the molecular weight of a membrane protein in the presence of detergent, as well as the contribution of the individual components of the complex to the overall molecular weight, could be accurately determined. Additionally, this enabled the determination of the oligomeric state in which the protein is incorporated into the detergent belt.

The TSKgel UP-SW3000-LS column enabled the efficient separation of the complex from free DDM micelles and facilitated the use of the highly sensitive LenS$_3$ MALS detector with its low noise properties.

References

[1] Brunner JD, Jakob RP, Schulze T, Neldner Y, Moroni A, Thiel G, Maier T, Schenck S. Structural basis for ion selectivity in TMEM175 K+ channels. Elife. 2020 Apr 8;9:e53683. doi: 10.7554/eLife.53683

Featured Products

Part #	Description
0040000	LenS$_3$
0023546	TSKgel UP-SW3000-LS, 2 µm, 4.6 mm ID × 30 cm