TOSOH

Molecular weight determination of peptides by SEC-MALS

Your Challenge

- You need to know the MW of your peptide.
- You need to use native conditions.

Our Solution

LenS3 MALS detector

- Accurate MW detection for molecules < 10 kDa.

What was done?

- Peptide of 4 kDa was separated by SEC and MW determined by RI and MALS.

What was the result?

- Molecular weight determination down to 4 µg sample load.

The LenS3 MALS detector is suited for non-destructive peptide analysis, accurately determining MW (e.g., 3.7 kDa) even at low sample injections (4 µg), making it an optimal alternative or complement to LC-MS.

Your Benefit

High sensitivity MALS measurements for low MW samples and low sample amounts

Analysis of Peptides

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https://www.separations.eu.tosohbioscience.com/products/chromatography-instruments/stand-alone-detectors/lens3-mals-detector

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Application Note

Peptide Characterization: Native SEC and MALS for Accurate Molecular Weight Determination

Introduction

Since the first therapeutic use of bovine insulin in 1922, peptides have become an important therapeutic class with more than 110 approved molecules. This popularity is explained by their high target specificity, efficacy and low side effects as well as continuous improvement strategies. These include PEGylation, cyclization or lipid-coupling to overcome limitations such as renal clearance and poor membrane penetration.

The characterization of the peptide structure is of crucial importance for therapeutic safety and efficacy, and liquid-chromatography – mass spectrometry (LC-MS) is predominantly used for this purpose. While the method provides detailed information on structure and modifications, it can cause undesired modifications that bias the results. These include the destruction of secondary structures, the formation of adducts of the peptide with components of the mobile phase, or the fragmentation of the peptide during ionization. Therefore, alternative methods are required to determine the molecular weight (MW) of the peptide and possible impurities under native conditions.

The combination of size exclusion chromatography (SEC) with multi-angle light scattering (MALS) is known as a non-destructive MW determination but has long been limited to larger molecules (> 10 kDa). The innovative flow cell design of the LenS3 MALS detector leads to increased sensitivity, unlocking the MALS characterization of molecules with low MW such as peptides. The LenS3 MALS detector was tested for MW determination of a 3.5-4 kDa peptide and succeeded down to injection masses of 4 µg of the peptide.

Experimental Conditions

Column:	Commercial SEC column (8 nm (80 Å) pore size; 4.6 mm ID x 5 cm L)
Mobile phase:	20 mmol/L phosphate buffer pH 7.0
Flow rate:	0.2 mL/min
Detection:	Multi-angle light scattering (LenS3) and refractive index
Temp.:	25 °C
Injection vol.:	1-10 µL
Sample:	Peptide 1 mg/mL (MW between 3.5-4 kDa according to manufacturer)
Instrument:	UHPLC System

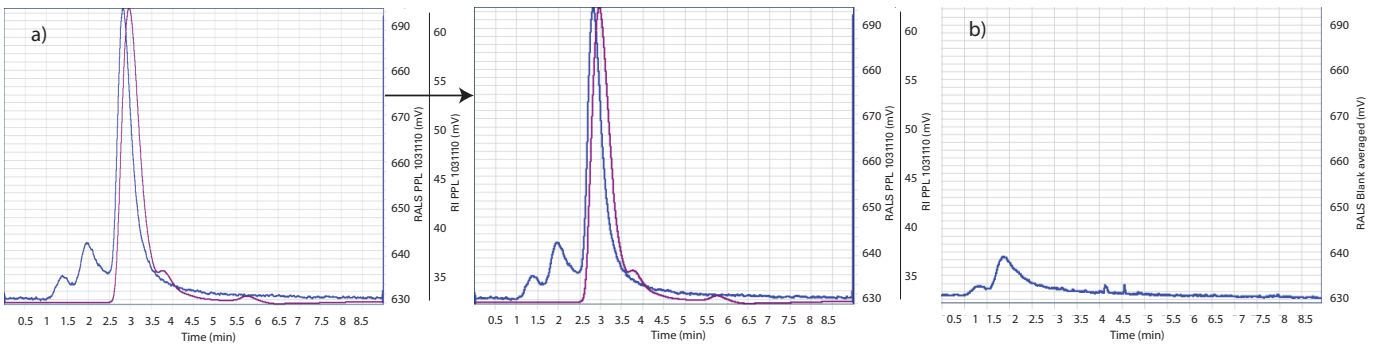
Results and Discussion

Sensitivity and robustness of peptide MW-determination

Molecular weight can be determined by coupling size exclusion chromatography with a concentration and a light scattering detector. The peptide sample was separated on an SEC column and detected by refractive index (RI) in parallel with MALS. *Figure 1* shows the chromatograms for the RI signal (purple) and the right-angle light scattering signal (RALS, blue) used for the MW calculations. The shift in retention time of the RI signal (left) due to differences in flow path and detectors' cells volume was corrected using the system calibration step in the SECview software (right).

The RALS signal shows additional peaks at 1.5 and 2 minutes before the main peak at 3 minutes. As these are not visible in the RI but are present in the RALS signal of a blank injection, the signal probably originates from particles released by the pressure spike during injection (e.g. column shedding).

Figure 1. Size exclusion peptide separation.



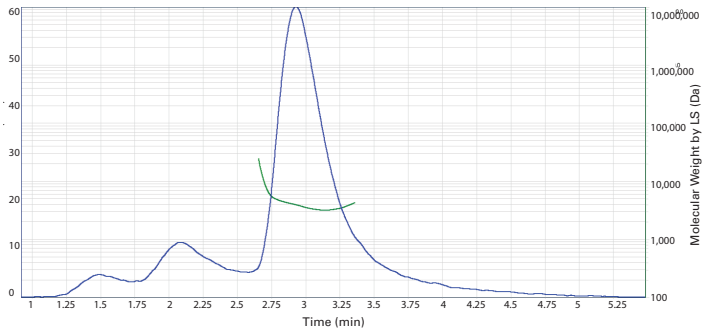
(a)Refractive index (purple) and right-angle light-scattering signals (blue) of a 10 µg injection before (left) and after calibration (right).
(b) Blank injection showing system peak.

Molecular weight determination

The molecular weight of the main peak was calculated by the SECview software based on the concentration (using the RI signal and a dn/dc of 0.185) and the RALS signal. In this example of a 10 µl injection, the average MW was calculated to be 3,947 Da. However, the MW curve shows a steep rise at the peak front due to the system peak, which can lead to an overestimation of the MW. The fact that a MW of 3,705 Da is calculated for the peak maximum (MWp) supports this assumption. Both values are within the MW range given by the manufacturer of 3.5-4 kDa.

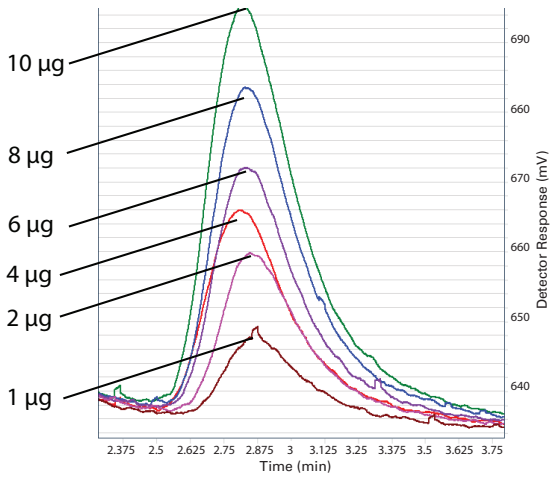
With a molecular weight of around 3.5-4 kDa, the analyzed peptide has a relatively low light scattering response compared to typical MALS analytes such as proteins or polymers. Since the light scattering detector's signal depends on MW and concentration, analyzing small sample amounts is even more challenging and only possible with a sensitive MALS detector (for detailed information read Light scattering for determination of molecular weight and radius of gyration). The LenS$_3$ MALS detector's sensitivity was demonstrated with sample quantities from 1 µg to 10 µg.

Figure 2. MALS analysis of a peptide.



Right-angle light scattering signal and molecular weight trace of a 10 µg peptide injection. At peak maximum the MW is calculated 3,705 kDa, the peak average MW is 3,947 kDa.

Figure 3. Overlay of RALS signals (injection amounts 1-10 µg).



For all injected sample amounts, the RALS signal resulted in a visible peak that is clearly above the noise level (*Figure 3, Table 1*). According to the manufacturer, the MW of the peptide is between 3.5 and 4.0 kDa, which is consistent with the MW of ~3.7 determined by the MALS detector. However, a reliable MW calculation is only possible down to ~4 µg, as the system peak interferes with the peptide peak (see also *Figure 1 b*). By using columns with a larger pore size to achieve a later elution of the peptide (e.g. TSKgel® UP-SW2000), or by using non-shedding columns (TSKgel UP-SW3000-LS), the limit of reliable MW determination is potentially shifted to lower injection masses.

The repeatability of peptide MW determination by MALS was tested with triplicate injections. The standard deviation was below 1 % for all triplicate injections (*Table 1*) hinting to a robust method.

Table 1. MW determination of a peptide by SEC-MALS.

Injected Mass (µg)	Calculated MWp (kDa)	Std. Dev. (kDa)	Std Dev (%)	Signal/ Noise
10	3.717	0.009	0.254	203
8	3.703	0.017	0.459	167
6	3.751	0.007	0.176	130
4	3.867	0.025	0.679	93
2	4.202	0.070	1.673	47
1	4.79	0.070	1.465	26

Increasing impact of system peak – no reliable MW calculation.
MW determination of a peptide by SEC-MALS across different injection amounts. Standard deviation was calculated based on triplicate injections. The signal to noise ratio is based on the average of three injections.

Conclusion

Our analytical approach demonstrates the capability to detect low scatterers such as peptides effectively using both refractive index and, more significantly, multi-angle light scattering. Even at the injection of as low as 1 µg, the limit of detection as defined by IUPAC (3x noise + baseline) was not reached and demonstrates high detector sensitivity.

The MW determination was successful and yielded results of 3.7 kDa while a value between 3.5-4 kDa was expected. Sample injections down to 4 µg of peptide resulted in reliable and repeatable MW determination. When requiring a reliable and non-destructive mass analysis as an alternative or complement to LC-MS, the LenS$_3$ MALS detector proves to be the optimal solution.

Featured product.

P/N	Description
0040000	LenS$_3$ Multi-Angle Light Scattering Detector

Data generated in collaboration with PolyPeptide Laboratories France S.A.S.