TOSOH

Application Note

Analysis of PEGylated Antibody Fragments using SEC-MALS

The covalent modification of proteins by conjugating polyethylene glycol (PEG) is an advanced tool to improve the pharmacokinetic behavior of small biotherapeutic drugs such as antibody fragments (e.g. Fabs or scFvs) and peptides. PEGylation is mainly used to improve drug solubility, increase serum half-life, reduce sensitivity to proteolysis, and reduce renal uptake, by masking the physical and chemical characteristics of the biotherapeutics (e.g. its conformation and hydrophobicity) resulting in reduced immunogenicity and new prospects of drug delivery[1].

One major challenge is the complete characterization of PEGylated protein samples. Since the PEGylation reaction is unspecific, mono-PEGylated as well as poly-PEGylated proteins may be present. Furthermore, by evaluating the amount of PEGylated protein, free protein, and free PEG, PEGylation efficiency can be determined, which enables optimization of the PEGylation reaction conditions.

Since PEGylation changes the hydrodynamic volume of the molecule, the proteins with various degrees of PEGylation can be separated by size exclusion chromatography (SEC). In this application note, SEC was combined with multi-angle light scattering (MALS), refractive index (RI), and UV detection to calculate the molecular weight (MW) of the individual peaks in PEGylated Fab and scFv samples (*Figure 1*). This allows the determination of the degree of conjugation (DOC) as well as the analysis of reaction byproducts in the final biotherapeutic product.

Experimental Conditions

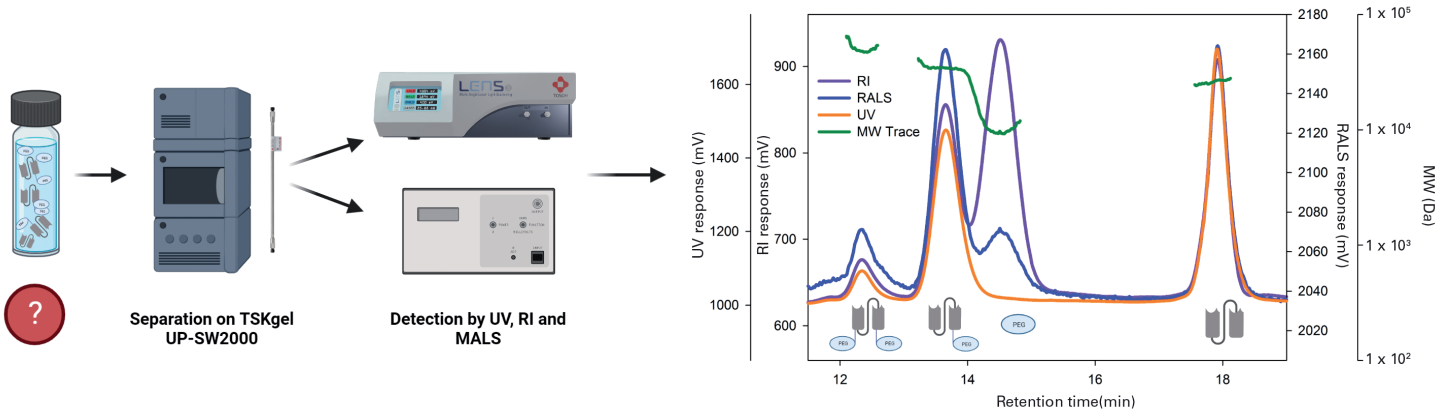
Instrumentation:	Vanquish™ UHPLC System Refractive index (RI) detector LenS™3 MALS detector
Column:	TSKgel® UP-SW2000, 2 µm, 4.6 mm ID × 30 cm
Mobile phase:	100 mmol/L sodium phosphate (pH 6.2) + 300 mmol/L arginine + 10 % isopropyl alcohol
Flow rate:	0.175 mL/min
Temperature:	25 °C
Injection vol.:	10 µL
Detection:	UV @ 280 nm; RI and MALS
Cal. standard:	bovine serum albumin (BSA)
Samples:	20 kDa PEGylated antigen binding fragment conjugate (20 kDa PEG-Fab) 10 kDa PEGylated single chain variable fragment conjugate (10 kDa PEG-scFv)
Data acq. & proc.:	SECview™ software

Results and Discussion

Analysis of PEGylated scFv

The detectors (RI, RALS, UV) were calibrated with BSA as depicted in the overlay of all signals (*Figure 2*). The calibration procedure in the SECview software corrects the offset and difference in band broadening of the detector signals caused by the dead volume between the detectors. Furthermore, the detector constants for UV, RI, and MALS are calculated and the three angles of the MALS detector are normalized.

Figure 1. Analysis of mAb fragment PEGylation by SEC-MALS.



Created with BioRender.com

Figure 2. Calibration of the detectors using a BSA standard.

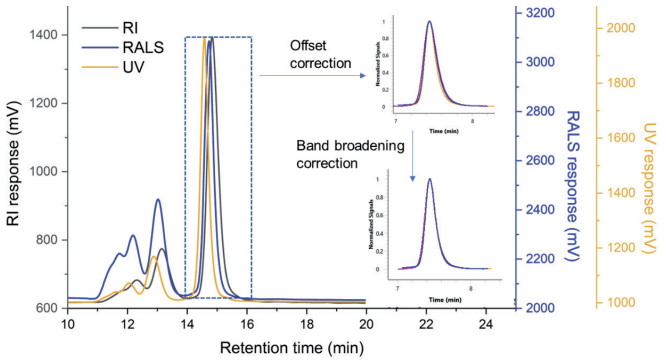
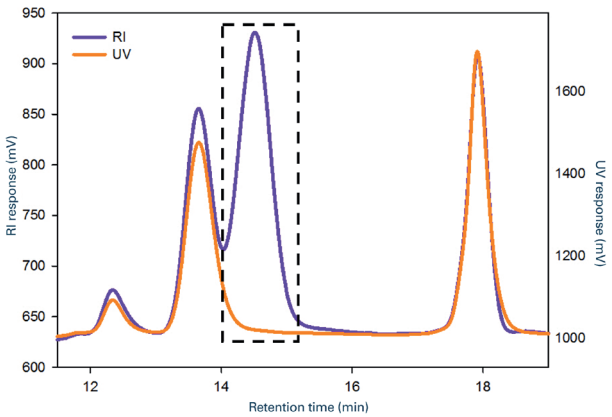


Figure 3 shows the overlaid signals from UV and RI detectors for the PEGylated 10 kDa-PEG-scFv. The highlighted portion of the chromatogram shows the signal corresponding to unreacted PEG which is UV inactive but provides a strong RI response.

Figure 3. Overlay of RI and UV detector signals of the 10-PEG-scFv sample.



To determine the molecular weight of complex protein conjugate mixtures by light scattering, the concentration and refractive index increment (dn/dc) of the individual components are required (Eq. 1).

$$MW \sim \frac{LS\ signal}{Concentration * \left(\frac{dn}{dc}\right)^2} \quad (1)$$

In the case of PEG-scFv, the PEG part is not UV-active whereas the scFv adsorbs UV, and both components generate different RI responses due to their different dn/dc values (*Figure 3*). By comparing the RI and UV signals for the different peaks, it is thus possible to determine each peak's exact composition (e.g. weight fractions of the two components) and thereby the dn/dc for each corresponding species (Eq. 2 and 3).

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

$$UV\ signal \sim dA/dc_{PEG} * Conc_{PEG} + dA/dc_{Protein} * Conc_{Protein} \quad (3)$$

Table 1 shows the literature values for dn/dc, dA/dc and expected molecular weight of the molecules used for this study.

Table 1. Literature values for dn/dc, dA/dc and expected MW of employed molecules.

Molecule	dn/dc	dA/dc	Expected MW [kDa]
scFv	0.185	1.927	26.8
Fab	0.185	1.440	47.6
10 kDa PEG	0.134	0	10
20 kDa PEG	0.134	0	20

Figure 4 depicts the results obtained from the composition analysis of 10 kDa PEG-scFv using SECview. The weight fraction (WF) of PEG and scFv for the different peaks and their corresponding dn/dc values are presented in *Table 2*.

Figure 4. Composition analysis of the 10-PEG-scFv sample. conventional calibration (CC).

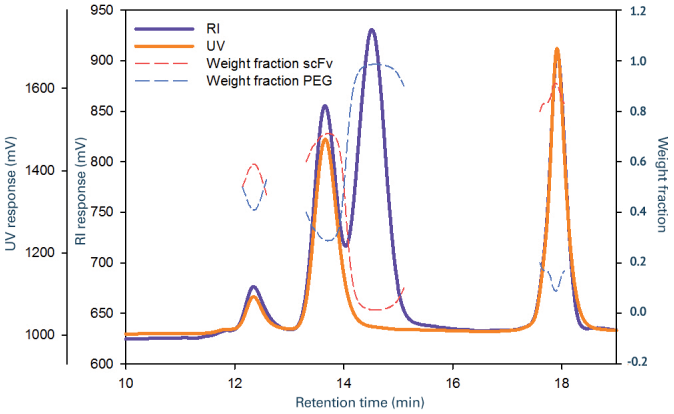
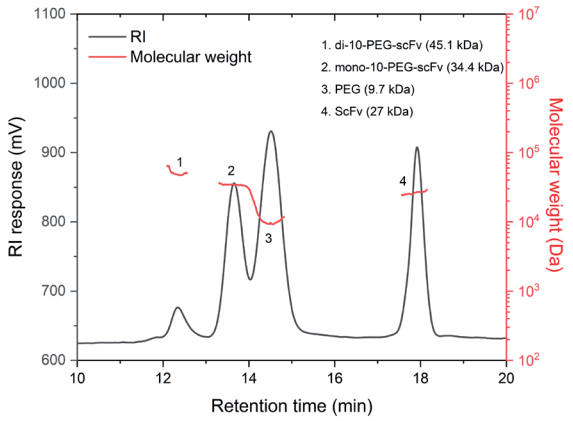


Table 2. Weight fractions and resulting dn/dc values of the peaks from Figure 4.

Peak No.	WF (scFv) [%]	WF (PEG) [%]	dn/dc [mL/g]
1	59	41	0.164
2	71	29	0.17
3	0	100	0.134
4	100	0	0.185

The dn/dc distribution obtained over the full chromatogram was used to calculate the molecular weight of the multiple species in the 10-PEG-scFv sample. The obtained molecular weight trace is illustrated in *Figure 5*. The RI signal shows a small shoulder on the high molecular weight region of the chromatograms (~11.8 min) indicating a bimodal pattern, that represents the poly-PEGylated conjugates. The MW of peak 4 and peak 3 were determined at 27 kDa and 9.7 kDa, respectively, which is in agreement with the expected MW of free unreacted scFv and PEG. Peak 1 was calculated at 45.1 kDa which corresponds to the di-10-PEG-scFv whereas peak 2 shows a MW of 34.4 kDa corresponding to the mono-10-PEG-scFv.

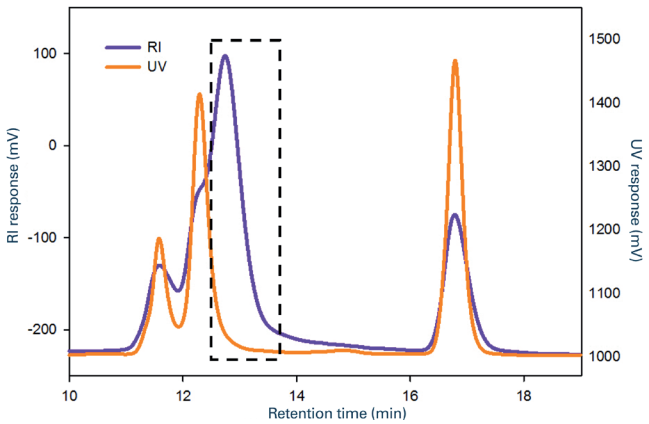
Figure 5. Molecular weight profile of the 10-PEG-scFv sample.



Analysis of PEGylated Fab

A PEG-Fab conjugate was analyzed in the same way to show the compatibility of the method for a bigger protein. The overlay of the UV and RI detector signals is shown in Figure 6 for the 20 kDa-PEG-Fab. The RI signal of UV inactive PEG is highlighted.

Figure 6. Overlay of RI and UV detector signals of the 20-PEG-Fab sample.



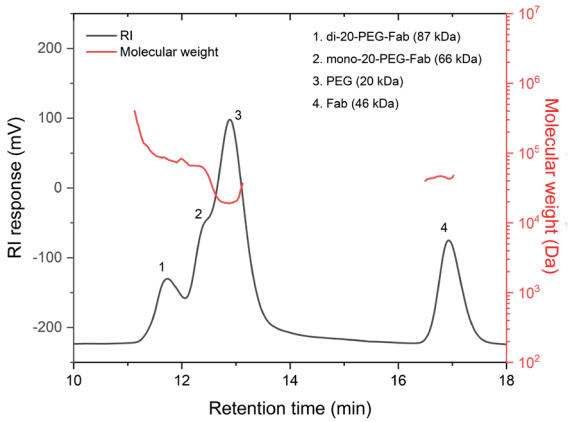
Composition analysis was performed to determine weight fractions and average dn/dc of Fab and PEG (*Table 3*).

Table 3. Weight fractions and dn/dc obtained for the 20-PEG-Fab sample obtained from the composition analysis.

Peak No.	WF (Fab) [%]	WF (PEG) [%]	dn/dc [mL/g]
1	55	45	0.162
2	78	22	0.174
3	0	100	0.134
4	100	0	0.185

The dn/dc profile obtained from the composition analysis was used to determine the molecular weight distribution of the 20-PEG-Fab sample (*Figure 7*).

Figure 7. Molecular weight profile of the 20-PEG-Fab sample.



The MW for peak 4 was determined at 46 kDa, which is the expected MW of the unreacted Fab fragment. For peak 3, a MW of 20 kDa was calculated, again nicely matching the expectation for the unreacted PEG. Peak 1 represents a di-20-PEG-Fab conjugate (87 kDa) whereas peak 2 with a molecular weight of 66 kDa represents a mono-20-PEG-Fab.

Conclusion

The complex mixtures stemming from an undirected PEGylation of antibody fragments could be characterized successfully by SEC-MALS. A versatile characterization method was established by separating the sample components on a TSKgel UP-SW2000 UHPLC column, combined with the LenS3 MALS detector as well as UV and RI detectors. By determining the MW of the individual peaks, the degree of conjugation could be identified. The SECview software streamlines the conjugation analysis so that it can be done in a matter of minutes. The results obtained in this analysis could help optimize the PEGylation reaction, leading to effective new biotherapeutics with high stability as well as determining residual impurities in final products.

References

1. Gupta et al. J Cell Commun Signal. 2019 Sep; 13(3); Turecek et al. J Pharm Sci, 2016)

Featured products.

P/N	Description
0040000	LenS3
0023514	TSKgel UP-SW2000