HOW SMALL CAN WE GO? ANALYSIS OF PEPTIDES USING THE OMNISEC™ MULTI-DETECTION SYSTEM

Introduction

Peptides have an important role in biopharmaceuticals, with an increasing interest in their use for drug development, currently more than 80 peptide drugs have been approved for clinical use worldwide. As these products are used to treat diseases, they require extensive regulation to demonstrate that they are not only effective but safe. Therefore, accurately characterising these peptides is necessary for biopharmaceutical development. Characterising biopharmaceuticals and checking their stability is vital for drug efficacy, safety, and efficiency to ensure desired product quality.

Bradykinin it is a nine amino acid peptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) which causes vasodilation, it is therefore used to lower blood pressure. Bradykinin has a Mw of 1061 Da and a hydrodynamic radius (Rh) of less than 0.8 nm. Its small size can make analysis by SEC difficult, however this is not a hinderance for OMNISEC multi-detector characterisation.

The high sensitivity of the OMNISEC multi-detector system enables the enhanced characterisation of small molecules previously overlooked during SEC analysis. In this application note you will see how you can use the OMNISEC system to analyse peptides, specifically Bradykinin, with high accuracy. The OMNISEC multi-detection analysis can be applied to many other peptides/small polymers than just the specific example discussed in this app note. If you are interested to know if this is applicable to your sample, then contact us here.

Multi-detection characterisation for improved sample analysis

The OMNISEC system from Malvern Panalytical, includes a separation module, OMNISEC RESOLVE, and a multi-detector module, OMNISEC REVEAL, which contains: a refractive index (RI) detector, a UV/Vis PDA detector, a right angle and low angle light scattering (RALS/LALS) detector and a differential viscometer. By using multi-detection, the multiple measurements we make on our samples in one go allows a wealth of information to be generated, enhancing characterisation of our samples.

More information about Multi-detection and how the detectors work can be found in the white paper Triple Detection GPC/SEC: Principles and methodology | Malvern Panalytical

An important parameter that needs to be considered when analysing samples using multi-detection is the samples dn/dc value. The dn/dc is the refractive index increment and is needed to calculate the concentration as well as the absolute Mw of a sample. The dn/dc of different samples can often be found in literature, however what complicates the analysis of peptides is that it is typically difficult to define the dn/dc of a peptide, opposed to a protein whose dn/dc is known to be 0.185 mL/g in aqueous conditions. However, using the OMNISEC RI detector, Bradykinin's dn/dc was calculated to be 0.21 mL/g in aqueous conditions, this value was used to calculate the Mw of the samples.

More information about the refractive index increment and why it is important can be found on our blog: What is a dn/dc value and why is it important for GPC/SEC? - Materials Talks (materials-talks.com)

How much is enough to characterise?

In the world of biopharmaceuticals, samples are often very precious with little to spare for characterisation, especially the in-depth characterisation required for pharmaceuticals. Being able to analyse anything as small as 1000 Da is not very useful if it requires milligrams of sample to get a response from the detectors for full characterisation. Thankfully with the enhanced sensitivity of the OMNISEC detectors, small sample volumes are needed to accurately characterise the sample.

A series of injections were performed on the OMNISEC system, where 5 µg, 25 µg and 50 µg of Bradykinin was injected, the detector responses of the three injection amounts are shown in Figure 1, and the quantitative results for the samples are displayed in Table 1. When injecting as little as 5 µg of sample the RALS and RI detectors signal was strong enough to accurately characterise the molecular weight of the peptide. While an injection of just 25 µg was enough to give sufficient responses in all the detectors to allow full characterisation of the sample, including the calculation of the Mw, IV, and size in terms of Rh. An increase in sample injection to 50 µg was not needed to yield further or more accurate results but did give nice clear sample response peaks in all detector chromatograms.

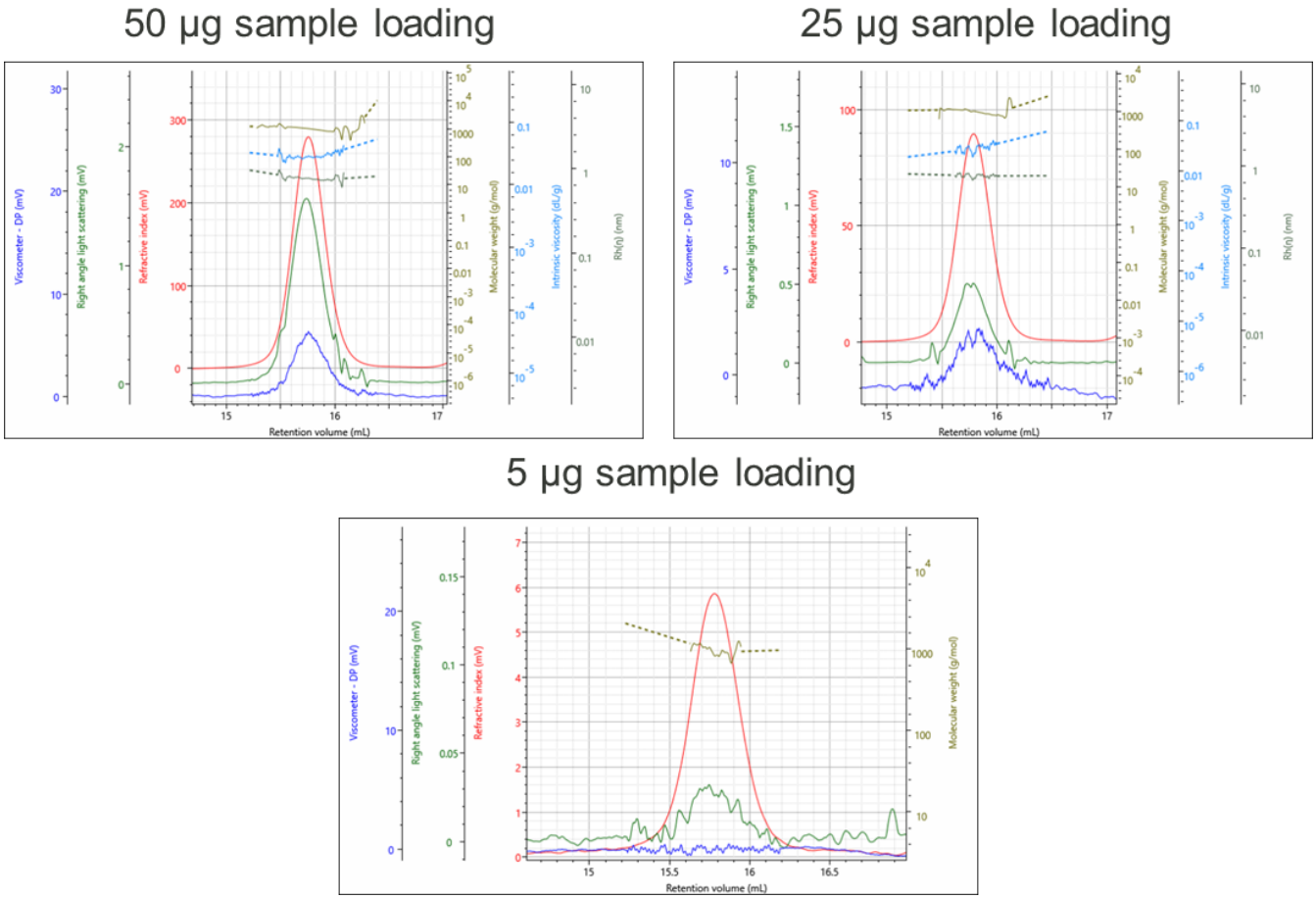

Figure 1: OMNISEC multi-detector chromatograms of Bradykinin resulting from 5 µg, 25 µg, and 50 µg of sample loading. Shown in each plot are the RI (red), RALS (green) and Viscometer (blue) sample chromatograms, overlayed with the derived data for Mw (brown), intrinsic viscosity (Blue) and Rh (grey).

Table 1: Quantitative characterisation of three sample loadings of Bradykinin, 50 µg, 25 µg, and 5 µg.

Results by sample and peak			
Parameter	**50 µg**	**25 µg**	**5 µg**
	Peak 1	**Peak 1**	**Peak 1**
RV (mL)	15.76	15.79	15.78
Mw (g/mol)	1,061	1,059	1,051
Mw/Mn	1.045	1.03	1.036
IVw (dL/g)	0.029	0.029	N/C
Rh(n)w (nm)	0.7798	0.7733	N/C

Aggregation of peptides

Aggregation of peptides or proteins can trigger an increased immune response, therefore the stability and aggregate levels of biologics are of great concern during final product development as they can affect the efficacy and immunogenicity. Analysis of the aggregation of biotherapeutics is necessary to help to indicate the stability of drug products.

Bradykinin is prone to self-associate in PBS at pH 7 over time. To test the stability of Bradykinin, several sample aliquots were left for 5 weeks at room temperature in PBS, before characterisation using the OMNISEC. The results were compared to new Bradykinin samples to assess the effect of time and stress on the samples molecular characteristics.

Overlaying the chromatograms of new Bradykinin and old stressed Bradykinin can give an indication of the changes in the sample. The RI chromatogram (Figure. 2A), at first glance does not give much indication of change between the new and old Bradykinin samples, however when zoomed in at retention volume (RV) 6 – 8 mL there is a clear peak (*ca.* 9 mV) in the old stressed Bradykinin sample not visible in the new sample. This peak at such low RV indicates the presence of a large sized sample population attributable to aggregates in the sample. This presence of large aggregates is made much more obvious by the RALS detector (Figure. 2B), which clearly shows an intense peak (*ca.* 40 mV) not present in the new Bradykinin sample. This highlights the value of the LS detector that can clearly show the presence of high Mw species even when they are at very low concentrations.

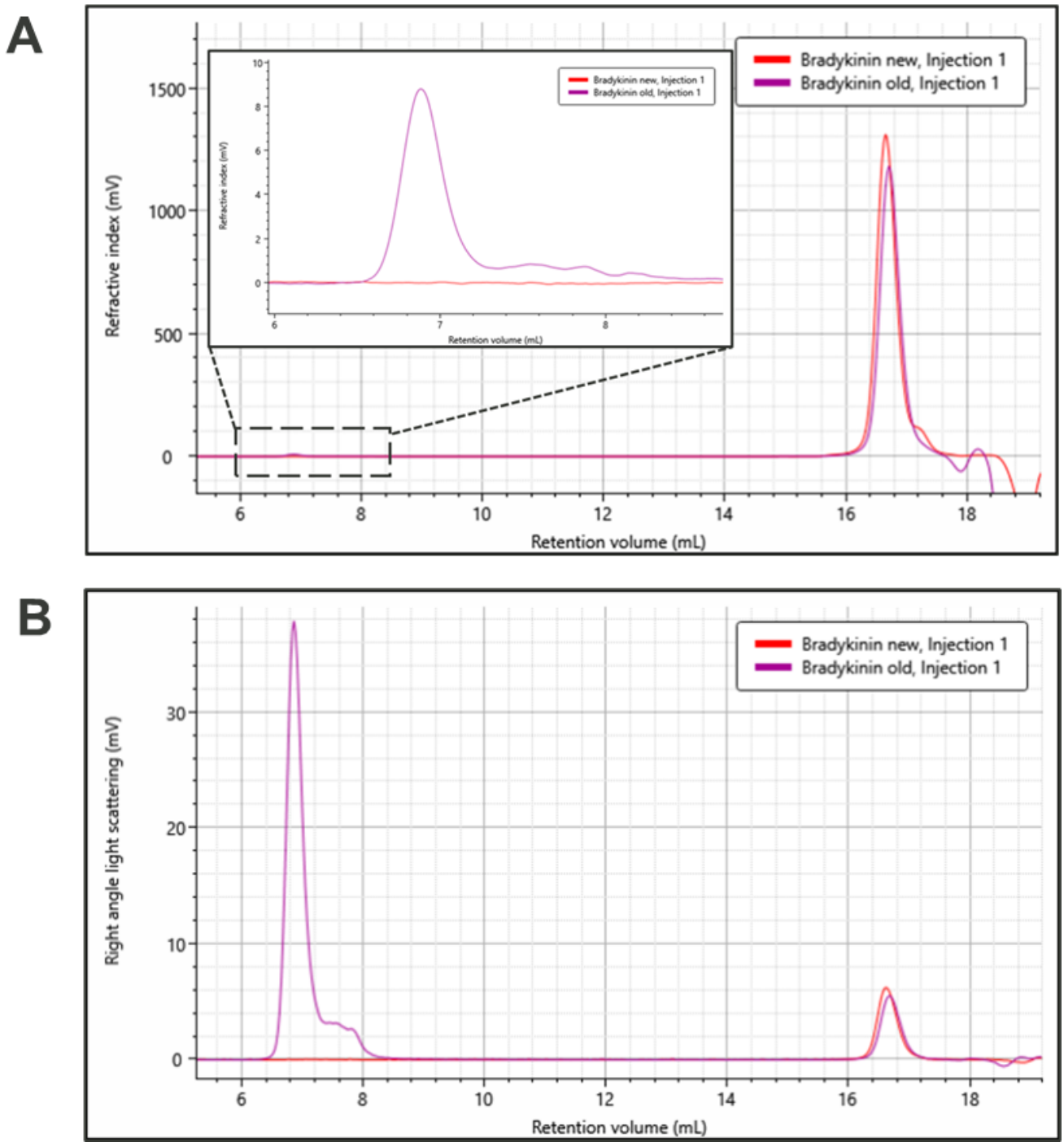

Figure 2. Overlay of RI (*a*) and RALS (*b*) chromatograms for the new Bradykinin sample (purple) and the old Bradykinin sample that had been stressed for 5 weeks at room temperature (red).

The multi detector chromatogram in Figure 3 displays the raw data for old Bradykinin after stress and displayed two distinct populations within the sample. The second peak at RV 17 mL was characterised to be the peptide Bradykinin with a Mw of 1047 Da, while the first peak at RV 7 mL was characterised to have a Mw of 2 MDa. The quantitative results are displayed in Table 2. The data shows that despite the low concentration of aggregates it was possible to quantify the amount of aggregates and characterise them, showing that 0.7 % of the sample injected was characterised as aggregates.

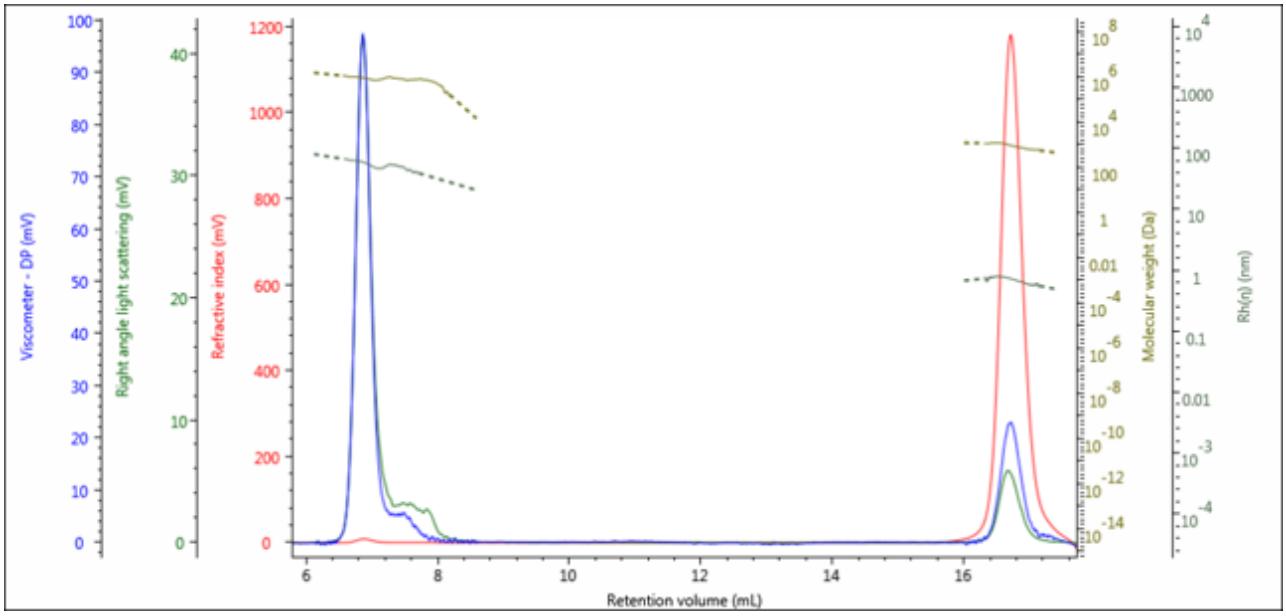

Figure 3. Multi-detector chromatogram of 5-week stressed Bradykinin sample overlayed with the derived Mw and Rh data.

Table 2: Quantitative characterisation of 5-week stressed Bradykinin

Results by sample and peak		
Parameter	**Stressed Bradykinin**	
	Peak 1	**Peak 2**
RV (mL)	6.887	16.72
Mw (g/mol)	2,147,000	1,047
Mw/Mn	1.675	1.072
IVw (dL/g)	11.36	0.02416
Rh(n)w (nm)	70.31	0.731
Rgw (nm)	79.44	N/C
Frac. Of sample (%)	0.7	99.3

Conclusions

In this study multi-detection SEC analysis has proven to be a valuable tool for the analysis of small peptides. The OMNISEC system from Malvern Panalytical was used to successfully characterize Bradykinin, a small 1000 Da peptide. As little as 5 µg of Bradykinin was used for accurate Mw characterisation, and as low as 25 µg of sample enabled full characterisation of Mw, IV and Rh, due to the high sensitivity of the OMNISEC detectors. As demonstrated the aggregation of stressed samples could also be characterised, with the LS detectors high sensitivity to larger Mw populations within a sample being of particular value.

Links

Multi-detector GPC/SEC system – the OMNISEC system
Triple Detection GPC/SEC: Principles and methodology | Malvern Panalytical
What is a dn/dc value and why is it important for GPC/SEC? - Materials Talks (materials-talks.com)
Why should I upgrade to multi-detection SEC? 10 reasons for protein characterization scientists | Malvern Panalytical

MALVERN PANALYTICAL

Grovewood Road, Malvern
Worcestershire, WR14 1XZ
United Kingdom
Tel. +44 1684 892456
Fax. +44 1684 892789

Lelyweg 1,
7602 EA Almelo,
The Netherlands
Tel. +31 546 534 444
Fax. +31 546 534 598

info@malvernpanalytical.com
www.malvernpanalytical.com