New Approaches to HPLC Analysis of Antibody Aggregates and Fragments

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Antibody therapeutics are enjoying high growth rates in the biopharmaceuticals market, major therapeutic areas being cancer and immune/inflammation-related disorders. The characterisation of monoclonal antibodies (mAbs) is a major challenge in process monitoring and quality control. The main product characteristics to be monitored are aggregate and fragment content, glycosylation pattern and charge variants. Fast size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) on nonporous stationary phases are new approaches for mAb aggregate analysis whereas reversed phase chromatography (RPC) is a suitable tool for fragmentation analysis.

Aggregation is a common issue encountered during expression, purification and formulation of therapeutic proteins and needs to be controlled due to its potential immunogenicity. Moreover, the therapeutic effect of aggregates remains unclear, impeding a correct dosage.

Fast aggregate analysis by SEC

Protein aggregates can easily be separated from monomers by an appropriate SEC method. As SEC does not rely on any kind of interaction different mAbs can be analysed with the same method. There is no need for elaborate optimisation steps, as is the case for most separation techniques that are based on interactions. Accelerating SEC has recently turned out to be a hot topic in order to improve this chromatographic mode. Shortening the analysis time per sample is absolutely necessary to keep up with increasing production, as analytics are often considered to be a bottleneck for the whole downstream process. Especially for daily analytical routines, slightly cutting back on performance in favour of a significant reduction of analysis time might be favourable. For instance, focusing on the total mAb aggregate content, one might accept to lose resolution between higher molecular weight aggregates. Figure 1 shows an aggregated mAb sample analysed on TSKgel SuperSW mAb HTP, 4.6 x 150mm. The resolution for aggregates and the mAb monomer remains high, while analysis time is cut down to four minutes; this is one quarter of the conventional analysis time, including fragment elution. A dual strategy was used here to save analysis time: on the one hand, column dimensions were changed, and on the other, the linear flow rate was increased compared to standard SEC columns.

Due to the use of 4µm silica particles, connection to conventional HPLC systems is possible. Also pressure doesn't exceed 130 bars, thereby circumventing any pressure related problems. High pressure might cause frictional heating inside the column, although the solvent as well as the column are temperature controlled. In this event, temperature sensitive samples like proteins might suffer from aggregation or denaturation on column, effecting the proper determination of sample composition. Aggregated proteins elute in the exclusion volume, regardless of what kind of molecular species was involved. In a worst-case scenario, the sample might stick onto the column. A loss in efficiency would then further impair the achievement of correct quantitative results.

Alternative approaches to SEC

Besides aiming for faster analytics, a back-up method for the verification of SEC results is desirable. The most common quantitative methods for aggregate detection are based on the difference in size for aggregates and monomers. Analytical ultra-centrifugation and field flow fractionation are less frequently used alternatives to SEC. Instead, a separation criteria independent from size - the different hydrophobicities - can be utilised for analysis. The advantages of such an orthogonal separation criteria are obvious.

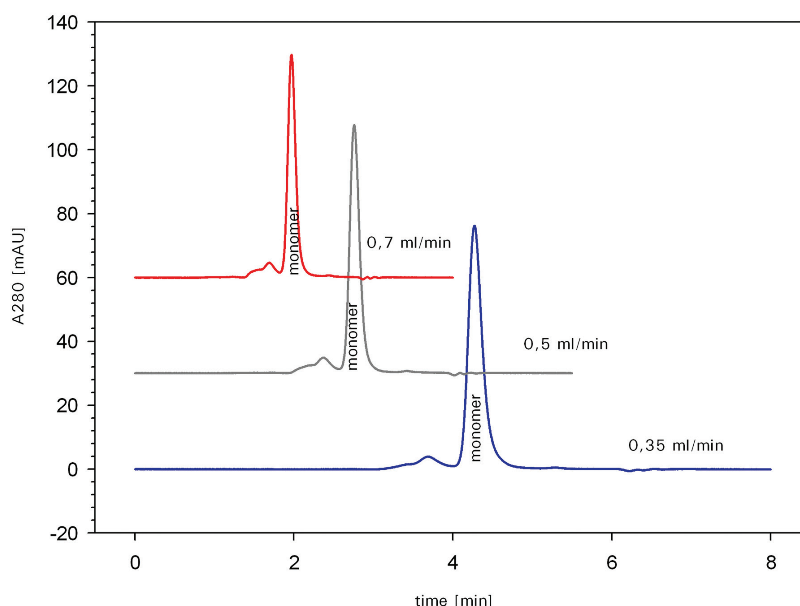

Figure 1: SEC analysis of aggregated mAb sample
TSKgel SuperSW mAb HTP column, 4µm, 4.6 x 150mm; flow rates: 0.35mL/min, 0.5mL/min & 0.7mL/min

Aggregates are more hydrophobic than the corresponding mAb monomers, leading to diverging retention in hydrophobic interaction chromatography (HIC). HIC sorts biomolecules by the degree of their surface hydrophobicity. Samples are adsorbed onto the stationary phase at relatively high salt concentrations and eluted by applying a decreasing salt gradient. The mild conditions used in the HIC separation of peptides and proteins typically maintain protein structure and biologic activity.

MAb analysis by HIC

Applying a nonporous HIC stationary phase in combination with the appropriate liquid phase provides maximum efficiency. The loading buffer contains salt, which increases the surface tension of the solution. Applying a simple linear gradient with plain water allows separate elution of the adsorbed molecules. Aggregates, monomers and fragments can easily be identified, as the relative hydrophobicity of these molecules is well known. Figure 2 shows the separation of an aggregated mAb sample on a short HIC HPLC column, TSKgel Butyl-NPR, 4.6 x 35mm, featuring 2.5µm particles. The total aggregate content of this sample is about 11%, which was also confirmed by SEC on TSKgel G3000SWXL (5 micron, 7.8 x 300mm) the current industrial standard for mAb aggregate analysis.

Besides the quantitative aspect, the qualitative separation performance of HIC is excellent. In contrast to SEC, this mode does not rely on the varying hydrodynamic radii. We know from dynamic light scattering and viscosity measurements that the hydrodynamic radius does not correlate linearly with molecular weight. There are only slight differences for the high molecular weight aggregates, whereas the monomer and the dimer differ significantly. Therefore, SEC has natural drawbacks regarding the separation of high molecular weight aggregates while HIC enables precise determination of the miscellaneous aggregate species.

However, HIC must not be considered as a replacement for SEC in standard analytics. Time- consuming method development to adjust the method to a certain sample is inevitable. Hydrophobicities of monoclonal antibodies can be quite variable, and this means that screening for the appropriate salt concentration is crucial for a successful analysis. Further, the chosen salt type or salt mixture has a great impact on resolution and efficiency. For this reason, HIC does not meet the requirements of daily routine in a high-throughput lab. On the other hand, this fact can be utilised to optimise resolution, and selectivity for the different aggregate species

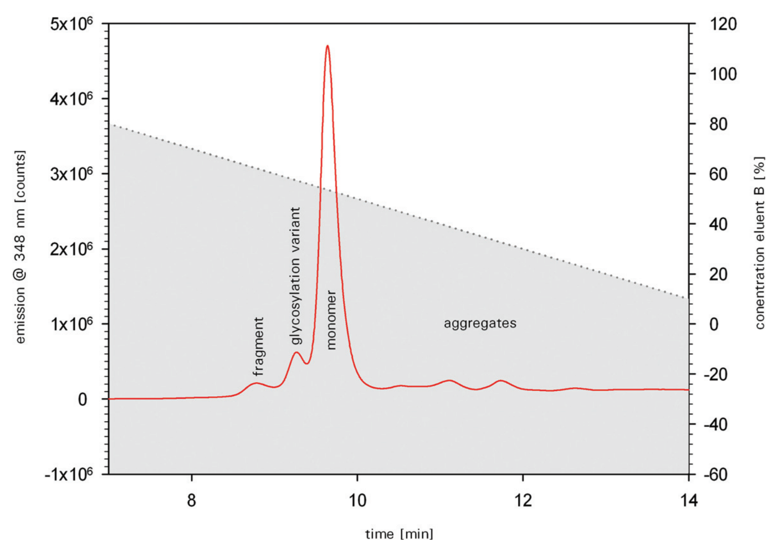

Figure 2: HIC analysis of aggregated mAb sample
TSKgel Butyl-NPR, 2.5µm, 4.6 x 35mm. Fragments elute first, followed by monomers and the aggregates.

can be modified. In view of the capacity of a nonporous resin, only small sample amounts should be injected meaning that a sensitive detector is needed. A fluorescence detector is ideal, because some salts absorb at 280nm but do not emit. Overall, HIC on nonporous particles is a very useful tool to overcome the natural limits of aggregate detection methods based on size. It opens up possibilities to get a more accurate analysis of certain mAb samples and to distinguish, for example, glycosylation variants of the monomer.

MAb fragment analysis

Fragmentation of antibodies can be detected with both modes described above, but their power to separate different fragment species is limited. Reversed phase chromatography has the necessary separation power, and can also be coupled to mass spectrometric detection for exact mass determination. A new wide pore C4 reversed phase 3µm stationary phase can be applied to separate large proteins. TSKgel Protein C4-300 is available in standard dimensions as well as in shorter column length for fast reversed phase protein separations. Figure 3 shows the separation of Fab', Fc and F(ab')2 antibody fragments on a standard column.

The antibody analysis toolbox

HPLC methods are applied in a broad range of applications used in the development, production, validation and release of antibody therapeutics. The TSKgel family of HPLC columns featuring various separation modes provides straightforward solutions for most applications that are mandatory when characterising a new biopharmaceutical. Besides the SEC, HIC and RPC solutions mentioned in this article the well-established solutions for the determination of mAb charge isomers by ion exchange chromatography (TSKgel STAT) and analysis of glycosylation pattern (TSKgel Amide-80) complete the chromatographic toolbox for antibody analysis.

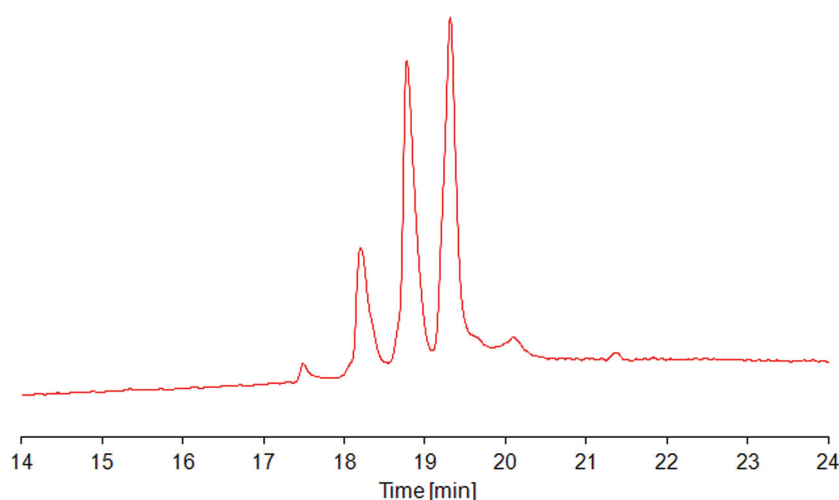

Figure 3: Reversed phase separation of mAb fragments
TSKgel Protein C4-300, 3µm 4.6 ×150mm; Linear gradient of water/acetonitrile/TFA from 90% water to 80% acetonitrile in 45 minutes.