



APPLICATION NOTE

mAb AGGREGATE DETECTION - ANALYTICAL HIC AS AN ORTHOGONAL CHROMATOGRAPHIC APPROACH

ABSTRACT

During the last decades, mAbs have proved to be a very valuable medication for severe illnesses like autoimmune diseases and cancer. However, to ensure a successful therapy and the least possible side effects, a thorough investigation of potential aggregates is crucial. The quality of aggregates can be diverse in terms of physico-chemical and physiological properties. Besides a declined therapeutic effect, mAb aggregates may also be immunogenic. A detailed characterization of the different aggregate species requires resolution of the different species by an online analytical method, as aggregation is a dynamic process. Due to the rather hydrophobic nature of mAb aggregates, analytical HIC using 2.5 μm particles offers outstanding resolution of the different aggregates. Therefore, a targeted analysis of every single contained species is possible. Fluorescence detection and an applied light scattering device ensure maximum analysis sensitivity. Furthermore, we could show that the highly efficient non-porous resin allows a quantitative analysis, providing an actual back-up method for the verification of SEC results.

MATERIAL & METHODS

mAb AGGREGATION

mAb aggregation was performed by acidic incubation. The stock solution contained 5 mg mAb/ml, buffered in 0.1 M citrate, pH 6.1. To induce aggregation, the mAb was titrated to pH 2.7 using 1 M HCl. The solution was incubated for 1 h at room temperature.

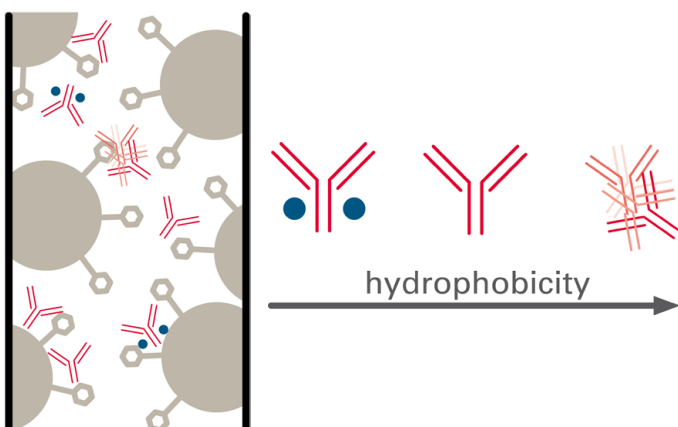


Figure 1

HIC column loaded with the mAb sample. The miscellaneous species behave differently in terms of hydrophobicity. Glycosylation decreases hydrophobicity, whereas aggregates are more hydrophobic than monomers.

Afterwards, the pH of the solution was increased to pH 6.5 by the addition 0.5 M disodium hydrogen phosphate. The aggregated mAb samples were stored at 4°C. Aggregation was accomplished on a daily base.

ANALYTICAL HIC

HIC was performed using two different column hardware formats: TSKgel Butyl-NPR 4.6 mm ID x 3.5 cm L and a 4.6 mm ID x 10 cm L. The shorter column was used in combination with fluorescence detection. The appropriate experiments were performed on a Dionex Ultimate 3000 RS system. A flow rate of 1 ml/min was applied. To induce hydrophobic interaction between the stationary phase and the mAb species, the loading buffer contained 3 M sodium chloride and 10 mM sodium phosphate, pH 7.0. 10 mM sodium phosphate, pH 7.0 comprised the elution buffer. 2 μg protein were injected. A linear gradient from 0% to 100% within 25 minutes was applied.

For HIC-MALS, the extended column hardware version was used. The column was connected to a Shimadzu Prominence HPLC system, including a Wyatt MiniDawn Treos light scattering device and a Wyatt Optilab TrEX refractometer. Flow rate was reduced to 0.7 ml/min. 10 μg mAb were injected. The sample was bound to the resin by applying either 3 M sodium chloride in 10 mM sodium phosphate buffer or 0.75 M ammonium sulfate, 0.5 M sodium sulfate and 10 mM sodium phosphate, both pH 7.0. 10 mM sodium phosphate, pH 7.0 or 2 M sodium chloride containing 30% methanol and 10 mM sodium phosphate, pH 7.0, were applied for the elution. The methanol containing elution buffer allowed a reproducible RI signal, which is necessary for molecular weight determination with static light scattering. This method is based on a two detector concept. On the one hand, the light scattering device provides the Rayleigh ratio, on the other hand a second device providing a concentration signal must be implemented into the system. Typical detectors measure the refractive index or the UV absorption. Both approaches have pros and cons; while the RI detector is a very general approach that is commonly used for isocratic chromatographic separations, using the UV signal might be more straightforward for non-isocratic chromatographic separations.

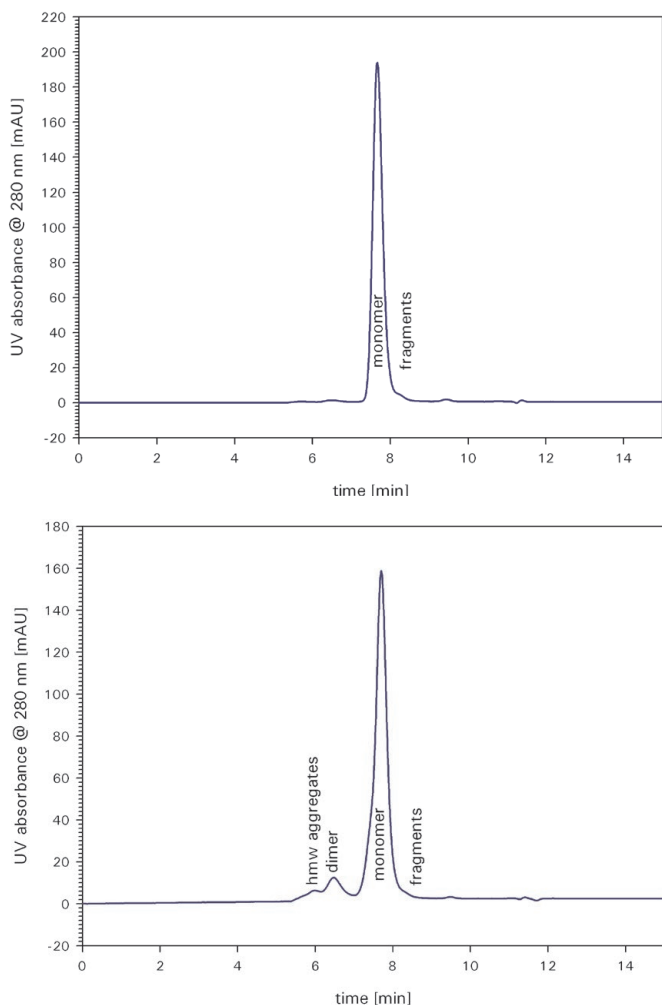
Both methods were employed to investigate the molecular weight. For the RI based approach, a linear gradient from 100% A to 60% A in 33 minutes was used, for the UV based approach a linear gradient from 100% A to 0% A in 40 minutes was used.

SEC

SEC was performed using a TSKgel G3000 SWXL column. 20 µg mAb or aggregated mAb, respectively, in a total volume of 20 µl were injected. A 0.1 M sodium phosphate buffer containing 0.1 M sodium sulfate and 0.05% sodium azide, pH 6.7 was used for the liquid phase. Applying 1 ml/min led to a analysis time of 15.5 minutes for one column volume.

DEGLYCOSYLATION

Deglycosylation was achieved by PNGase F from Elizabethkingia miricola. pH of the mAb stock solution was increased to 7.5 by addition of 0.1 M Tris/HCl, pH 8.0. 1 U was employed for 20 µg mAb. The reaction was incubated at 37 °C and 300 rpm. After every 24 hours aliquots of the reaction mix were analyzed using HIC with the fluorescence approach. For enzyme stability issues, the reaction was stopped after 72 hours. A blank mAb sample without enzyme was treated, respectively. Deglycosylation was also monitored by SDS-PAGE (data not shown).



► **Figure 2 & 3** SEC chromatograms of the mAb and the aggregated mAb sample. Fragments, monomer and the various aggregate species can be seen.

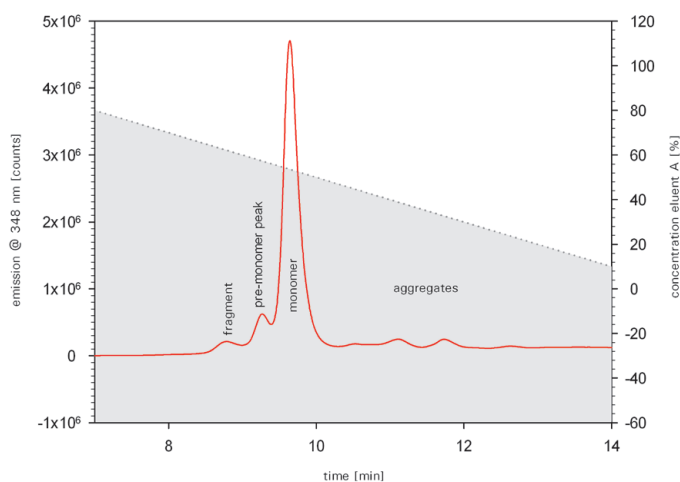
RESULTS

SEC

SEC chromatograms of the mAb and the aggregated mAb sample are shown in Figure 2 and 3. The high molecular weight (hmw) aggregates elute first, followed by the dimer and the monomer.

FLUORESCENCE FOR SENSITIVE AGGREGATE DETECTION

As shown in Figure 4, the short column provides separation of fragments, a pre-monomer peak, the monomer peak and various aggregates. A first attempt to identify the various peaks separated by HIC was done according to the relative peak quantities resolved by SEC. Though, the pre-peak remains unresolved by SEC.



► **Figure 4** Aggregated mAb sample on TSKgel Butyl-NPR. Fragments, a pre-monomer peak, the monomer and aggregates can be resolved. A linear gradient was chosen.

MOLECULAR WEIGHT MEASUREMENTS

In order to investigate the pre-monomer peak more thoroughly, an online multi-angle light scattering detector was used. As this detector is less sensitive than a fluorescence detector, a bigger hardware version of TSKgel Butyl-NPR was chosen. The 4.6 mm ID x 10 cm L format allows crude protein loadings up to 10 µg, while the pre-monomer can still be observed as a shoulder of the monomer peak. An appropriate salt combination such as 0.75 M ammonium sulfate and 0.5 M sodium sulfate, pH 7 (Fig. 6) must be used. The UV-signal provides the concentration source for the molecular weight determination. These conditions do not allow RI based molecular weight determination, as the RI signal shows unsteadiness due to solvent mixing effects for above described conditions.

In consequence, aiming for the more generalized, RI based MALS approach requires an adjusted protocol. Relatively stable RI baselines can be achieved for a loading with 3 M sodium chloride and a salt containing elution buffer that consists of 2 M sodium chloride containing 30% methanol, both pH 7.0 (Fig. 5). However, resolution suffers from overloading and the detector caused conditions. The molecular weights determined with the RI and the UV based MALS measurements are shown in Table 1.

DEGLYCOSYLATION MONITORING

Aliquots of the deglycosylation reaction were analyzed using TSKgel Butyl-NPR. The appropriate chromatograms are shown in Figure 7 and 8. The blank control exhibits a reproducible chromatogram for aliquots of the sample before incubation was started and after 48 h at 37°C (fig. 8). Despite this behavior, a retention shift can be detected for the deglycosylation samples. The fragment peak, the pre-monomer peak and the monomer peak are shifted towards later elution, monitoring increasing hydrophobicity for all of the three species. No significant quantitative difference for the single mAb species can be obtained from these data.

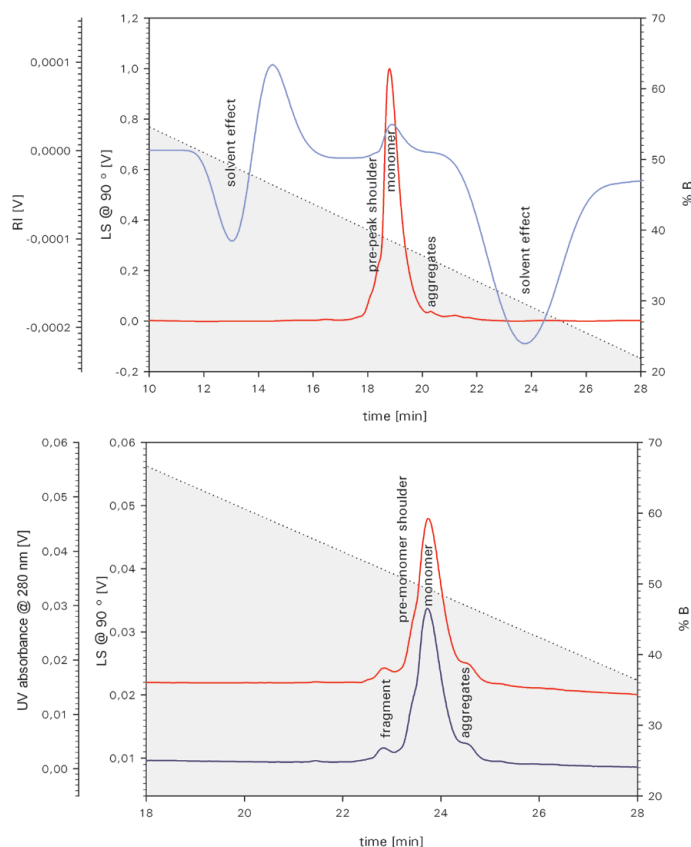


Figure 5 & 6

HIC-MALS chromatogram of the mAb. Fig. 5 shows the chromatogram for the RI based MALS, Fig. 6 shows the UV based MALS chromatogram. Different conditions were chosen due to varying detector requirements. Due to the relatively low RI response, the columns were slightly overloaded which causes the pre-monomer to affiliate to the monomer.

DISCUSSION & CONCLUSION

HIC using nonporous resins offers another choice to the chromatographic toolbox for mAb characterization. Outstanding resolution of the miscellaneous mAb species potentially allows the detection of a glycosylation variant. A prototype TSKgel Butyl-NPR 4.6 mm ID x 10 cm L features higher capacity and therefore allows to apply light scattering as a detection method. Molar masses can be obtained from online measurements, providing information on the various mAb species.

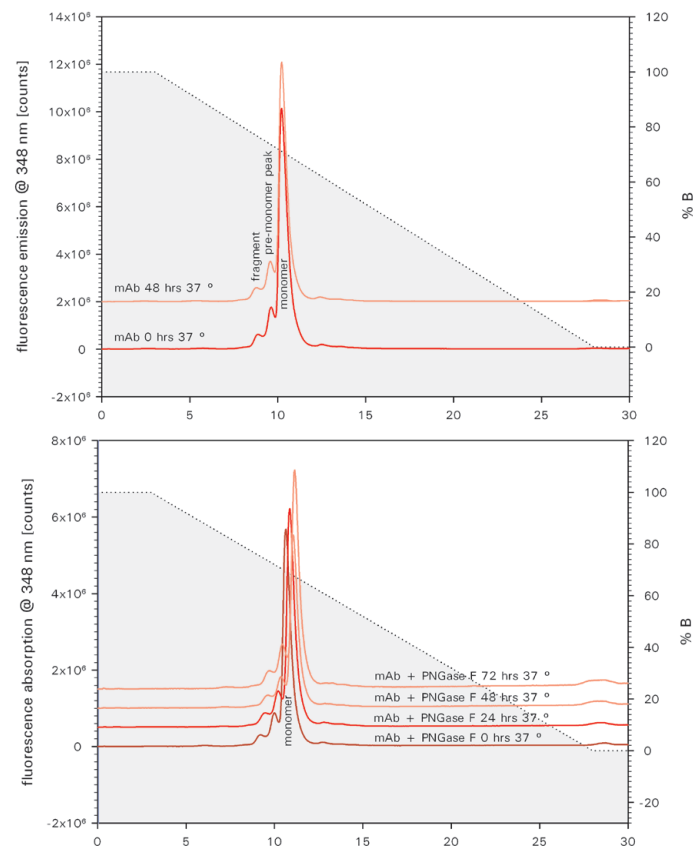


Figure 7 & 8

HIC was performed using two different column hardware formats: TSKgel Butyl-NPR 4.6 mm ID x 3.5 cm L and a 4.6 mm ID x 10 cm L. The shorter column was used in combination with fluorescence detection. The appropriate experiments were performed on a Dionex Ultimate 3000 RS system. A flow rate of 1 ml/min was applied. To induce hydrophobic interaction between the stationary phase and the mAb species, the loading buffer contained 3 M sodium chloride and 10 mM sodium phosphate, pH 7.0. 10 mM sodium phosphate, pH 7.0 comprised the elution buffer. 2 µg protein were injected. A linear gradient from 0% to 100% within 25 minutes was applied.

TABLE I

DETERMINED MOLECULAR WEIGHTS FOR THE MAB FRAGMENT, THE PRE-MONOMER AND THE MONOMER. MOLECULAR WEIGHTS WERE DETERMINED USING LIGHT SCATTERING. THE PRE-MONOMER CANNOT BE RESOLVED BY SEC.

Chromatography	Concentration Source	MW (kDa) Fragment	MW (kDa) pre-monomer	MW (kDa) Monomer
HIC				
TSKgel Butyl-NPR	UV-based detection	61	138	132
TSKgel Butyl-NPR	RI-based detection	99	151	150
SEC				
TSKgel G3000SWXL	RI-based detection	91	-	149

The necessary concentration source can be provided by a UV detector in case of a known extinction coefficient for the sample molecules. The results presented are predicated on an average IgG coefficient, which explains the deviation from the RI based results.

On the other hand, the RI based approach requires advanced fine tuning of the chromatographic separation to maintain resolution and peak shape, while doing the least possible changes to the liquid phase during sample elution. The herein presented multi-detector method leads to a resulting molecular mass of 150 kDa for the monomer peak and a molecular mass of 151 kDa for the potential glycosylation variant. A molecular weight of 99 kDa was determined for the fragment.

Retention times for all peaks are shifted towards higher hydrophobicity when PNGase F is used for glycosylation cleavage.

Concluding on this, the detected molecular weights and the qualitative SEC results of the mAb sample, the pre-monomer peak seems to be a glycosylation variant of the monomer. From these results, we deduce that the presented HIC analysis allows to separate certain glycosylation species of a mAb monomer. Further investigations on the cleaved glycosylation residues will be undertaken to confirm this.