Size-Exclusion Chromatography, Analytical Ultracentrifugation, and the Formulation-Dependence of ADC Aggregation

Christopher Sucato and Mario DiPaola

Charles River Laboratories, 8 Henshaw Street, Woburn, MA 01801 Mario.DiPaola@crl.com 617-234-0001

ABSTRACT

Small-molecule cytotoxic compounds are often employed as functional elements in antibody-drug conjugate (ADC) constructs. The hydrophobic properties of the cytotoxic drug moiety and/or the chemical linkage between the drug and the antibody can increase the propensity for ADCs to undergo aggregation. While aggregates may be present in low concentrations, these may have a significant effect on the quality of biologics, potentially leading to activity loss, decreased solubility, and increased immunogenicity.

Size exclusion chromatography is the standard method for characterization of protein aggregation, but it may not be sufficient to fully evaluate this critical quality attribute. Here we explore analytical ultracentrifugation (AUC) as a tool to monitor ADC aggregation characteristics, as a function of formulation and ADC concentration. We demonstrate that AUC can reveal formulation and concentration dependencies that SEC does not, and we discuss the unique aspects of AUC, in comparison to SEC, that can be used to detect and more accurately quantify aggregation when developing an ADC formulation.

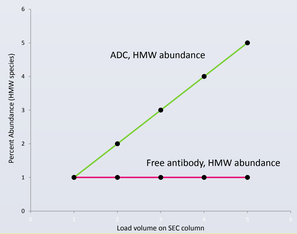


Figure 1: The trends in percent abundance of a HMW species that may be observed in SEC separations, for an ADC product and the corresponding free antibody. The increasing level of aggregate in the ADC may suggest different physico-chemical properties of the ADC compared to the antibody, but the result must be verified.

BACKGROUND:

Conjugation of a drug moiety to functional groups on monoclonal antibodies can alter the aggregation characteristics of the ADC in comparison to the unconjugated protein. One such change in the aggregation behavior is the functional dependence of high molecular weight (HMW) species abundance on concentration. We have observed that the SEC profile of an ADC may show a HMW percent abundance that increases with the loading volume onto the column, however the same trend is not observed in SEC performed on the free antibody that corresponds to the ADC. Figure 1 depicts the difference on the load-dependence of the HMW abundance, comparing an ADC and the free antibody, while Figure 2 and 3 are examples of how the appearance of the HMW peak may likewise differ as a function of the injected load.

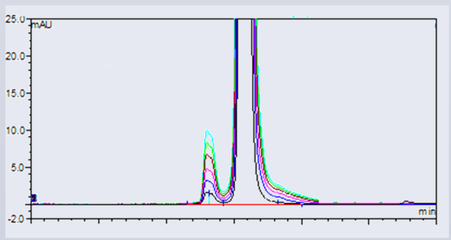


Figure 2: Illustration of the trend in the HMW peak by SEC, of a typical well-behaved antibody sample

As shown, the peak shape of the HMW species is dependent on the presence of the drug moiety, such that the HMW species of the ADC is a more broad, diffuse peak compared to that of the free antibody. The addition of hydrophobic groups to the solvent accessible surface may explain an increased propensity for aggregation of the ADC, and may necessitate a re-evaluation of the formulation conditions. In order to explore the nature of HMW species in ADC preparations, we employed analytical ultracentrifugation (AUC) assays on ADC samples that showed the type of SEC behavior as described in Figures 1 and 2. AUC assays are useful because they can be carried out directly in the formulation buffer, which may not always be the case for SEC, and unlike SEC there is no interaction of the protein or ADC with a stationary phase material or column frits, etc. Therefore AUC is an excellent compliment to SEC to determine the nature of ADC aggregation.

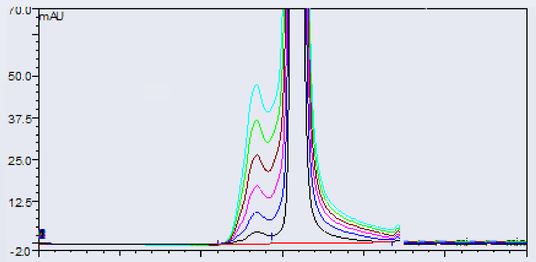


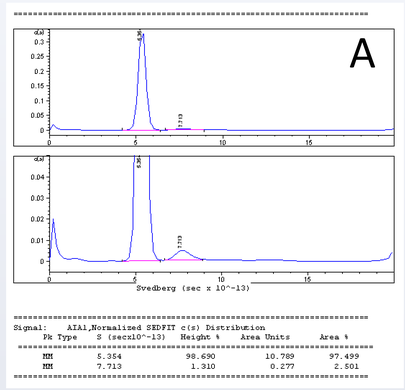
Figure 3: Illustration of the trend in the HMW peak by SEC, of an ADC product that may show an increased propensity for aggregation, *c.f.* Figure 2 (*below left*).

RESULTS AND DISCUSSION

In this study, we have investigated a single ADC whose SEC behaviour is like that depicted in Figures 2 and 3. We performed sedimentation velocity AUC assays on the ADC under two different formulation conditions, and at different ADC concentrations, to gain insight into the nature of the aggregation behaviour as seen on SEC. The AUC results are shown in Figure 4, across four panels. The AUC data are size distribution profiles, which display the distribution percentage versus sedimentation coefficient. Two different formulation conditions were studied. Panels 4A and 4B are the AUC results in 20 mM Histidine, 5.5% trehalose, 0.01% PS 20, pH 6.0, and in Panels 4C and 4D are the results in 10 mM KPO4, 200 mM NaCl, pH 6.5. In each case the top panel is the assay performed at 0.2 mg/mL and the bottom is at 0.8 mg/mL.

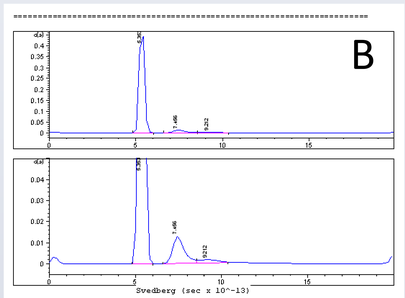
The data in Figure 4 suggest that there can indeed be a concentration effect on the levels of aggregate in the ADC preparation, but the AUC results in different matrix conditions it is dependent on the nature of the formulation. In the histidine-based buffer (Panels 4A, B) a clear concentration effect was found, as the HMW species at about 7-8 S increased in abundance roughly two-fold as a function of concentration between 0.2 mg/mL to 0.8 mg/mL. The corresponding results in KPO4 buffer however, do not follow the same pattern. In that matrix, the total abundance of HMW species greater than monomer is virtually unchanged with the protein concentration.

The SEC trends that could be observed in Figures 1-3, for an ADC and the free antibody, may be interpreted as a change in the aggregation characteristics of the product upon conjugation with the hydrophobic small-molecule. A concentration dependence on the level of aggregate that appears after ADC conjugation, may be taken to suggest a reversible propensity for aggregation driven by the properties of the drug molecule.



Signal: AIAl,Normalized SEDFIT c(s) Distribution

Pk Type	S (secx10^-13)	Height %	Area Units	Area %
MM	5.354	98.690	10.789	97.499
MM	7.713	1.310	0.277	2.501



Signal: AIAl,Normalized SEDFIT c(s) Distribution

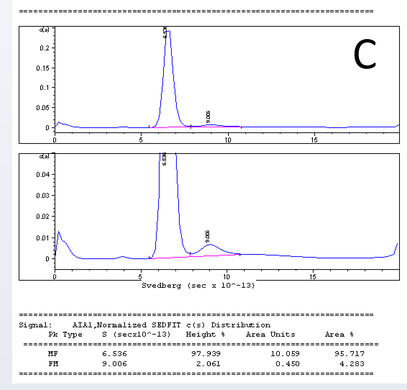
Pk Type	S (secx10^-13)	Height %	Area Units	Area %
MM	5.353	97.115	10.968	93.829
MM	7.456	2.582	0.609	5.211
FM	9.212	0.302	0.112	0.959

Figure 4A,B: Size distribution profiles generated by SEDFIT analysis of the AUC assays, for ADC sample at 0.2 mg/mL (Panel A) or 0.8 mg/mL (Panel B) in 20 mM Histidine, 5.5% trehalose, 0.01% PS 20, pH 6.0.

However, as we show in the AUC results here, the SEC results should be interpreted with caution. Because SEC is often not done directly in the formulation conditions, results from an orthogonal method like AUC should also be evaluated, to determine if a concentration dependence is real.

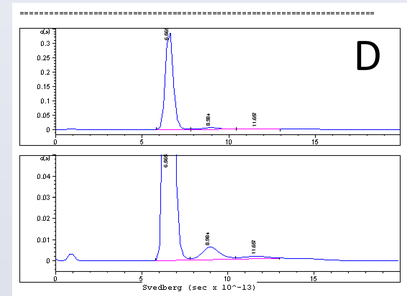
METHODS

The ADC sample was diluted to 0.2 mg/mL and 0.8 mg/mL in either histidine-based formulation buffer, or KPO4-based buffer, prior to loading into AUC cells. For the sedimentation velocity AUC, samples (0.457 mL) were loaded into the sample channel of AUC cells having quartz windows and 12-mm double-sector Epon centerpieces. The sample buffer (0.460 mL) was loaded into the corresponding reference channel of each cell. The centrifugation was carried out at 20 °C and 45,000 rpm. Radial scans of the concentration profile were collected sequentially by absorbance at 280 nm, until no further sedimentation was observed. The resulting data sets were analyzed using the program SEDFIT with a continuous c(s) distribution model, yielding best-fit distributions for the number of sedimenting species and the effective molecular weights.



Signal: AIAl,Normalized SEDFIT c(s) Distribution

Pk Type	S (secx10^-13)	Height %	Area Units	Area %
MF	6.536	97.939	10.059	95.717
FM	9.006	2.061	0.450	4.283



Signal: AIAl,Normalized SEDFIT c(s) Distribution

Pk Type	S (secx10^-13)	Height %	Area Units	Area %
MF	6.565	97.991	10.918	95.091
MF	8.984	1.678	0.444	3.864
FM	11.657	0.331	0.120	1.045

Figure 4C,D: Size distribution profiles generated by SEDFIT analysis of the AUC assays, for ADC sample at 0.2 mg/mL (Panel C) or 0.8 mg/mL (Panel D) in 10 mM KPO4, 200 mM NaCl, pH 6.5.

CONCLUSIONS

While SEC is a common analytical method used to measure aggregation in protein products, it is clear that for certain classes of molecules the use of SEC should be weighed carefully. ADCs are complex molecules that display different hydrophobic characteristics in comparison to the unconjugated antibodies; these characteristics can lead to anomalous results at least when analyzed by SEC. In this study, varying amounts of aggregate were measured by SEC as a function of protein load. However, when the same molecule in the same formulation conditions was analyzed by AUC at different concentration, the aggregate level was found to be independent of load, as one would expect.

REFERENCES

Kirk, William and Klimtchuk, Elena. (2006). Photophysics of ANS. III: Circular dichroism of ANS and anilinonaphthalene in I-FABP. *Biophysical Chemistry.* **125**(1): 24-31.

ACKNOWLEDGMENTS

We would like to acknowledge all the members of Charles River, Woburn, for their support.

The work described herein is intended for research purposes only; not intended for any human or animal therapeutic or diagnostic use, unless otherwise stated. The trademarks or copyrights mentioned herein are the property of their respective owners.