Characterization of mAb aggregation using a Cary 60 UV-Vis Spectrophotometer and the Agilent 1260 Infinity LC system

Application Note
Biopharmaceuticals

Introduction

Monoclonal antibodies (mAbs) are important bioproducts, widely manufactured by pharmaceutical companies. With the increased use of mAbs in biological processes, techniques that allow them to be characterized in detail are of great interest to pharma companies. Protein aggregates are a topic of intense discussions between regulatory authorities, academics, and manufacturers due to their impact on biological activity [1]. Sub-visible protein aggregates may expose epitopes that may not be present when the protein is in a stable form. Reliably identifying the presence of aggregates is of critical importance in characterization of mAbs.
The formation of aggregates is influenced by numerous factors during manufacturing, purification, formulation, and storage. A quick QC method to identify the presence of protein aggregation is therefore advantageous. It could be used at many stages of the life cycle of antibody manufacturing (such as in clonal screening), as well as for continuous product monitoring during storage. The advantage of UV spectroscopy as an analytical method to detect protein aggregation is that it is non-destructive. It also uses low sample volumes, minimal sample preparation requirement and easy sample analysis.

A quick QC method to indicate the presence of aggregation is presented in this study. The method uses an Agilent Cary 60 UV-Vis spectrophotometer to identify aggregation of mAb solutions resulting from different stress conditions. The presence of aggregation was confirmed by size exclusion chromatography (SEC) analysis, the standard method used to characterize protein aggregation.

**Experimental**

**Reagents and samples:**
Commercially available mAb was purchased from a local pharmacy and stored according to the manufacturers’ instructions. Both monobasic and dibasic sodium hydrogen phosphate were purchased from Sigma-Aldrich. The buffers were prepared fresh and then filtered using a 0.22 µm membrane before use.

**Sample preparations:**
The mAb samples were subject to two different stress methods to induce aggregation: thermal stress; and accelerated freeze-thaw stress (see following). The untreated (unstressed) sample was used as a control for analytical measurements.

  **Thermal stress**
The mAb samples were diluted in water to 1 mg/mL concentration. 100 µL aliquots of diluted mAb sample were contained in 0.5 mL Eppendorf tubes. They were then thermally stressed by incubation at 65 °C for 5 and 10 minutes using the ThermoMixer (Eppendorf). The samples were allowed to cool down to room temperature before analytical measurements.

  **Accelerated freeze-thaw stress:**
Single 100 µL aliquots of 1 mg/mL mAb sample were placed in 0.5 mL Eppendorf tubes. These tubes were immersed in an ethanol-dry ice bath until the solution froze. The frozen samples were then immediately placed in the thermomixer for 4 minutes at 30 °C. Once the samples had thawed completely, they were flash-frozen again. This freeze-thaw cycle was repeated 15 times before analytical characterization.

**UV spectral scan:**
Spectral scans of the control and stressed mAb samples were performed using an Agilent Cary 60 UV-Vis Spectrophotometer. A 50 µL aliquot of the sample was placed in an Agilent sub-micro rectangular quartz cuvette (10 mm pathlength). The sample absorbance was recorded against a blank-corrected baseline from 240 to 380 nm. The aggregation index (A.I.) was calculated using the equation [2] shown in Figure 1. The data acquisition and analysis was carried out using the Agilent Cary WinUV Scan Application.

\[
A.I. = \left( \frac{OD_{350}}{OD_{280} - OD_{350}} \right) \times 100
\]

Figure 1. Aggregation Index calculation
Where OD 350 represents the absorbance (%Abs) at 350 nm and OD280-OD350 is difference in the absorbance at 280 and 350 nm respectively.

**Size exclusion chromatography (SEC):**
SEC analysis of the samples was carried out using an Agilent 1260 Infinity bio-inert LC system comprising:

- Agilent 1260 Infinity Bio-inert Quaternary LC pump
- Agilent 1260 Infinity High-performance Bio-inert Autosampler
- Agilent 1260 Infinity Thermostat with bio-inert heat exchangers
- Agilent Thermostatted Column Compartment
- Agilent 1260 Infinity Diode Array Detector VL Plus
- Agilent AdvanceBio SEC 300 Å, 7.8 × 300 mm, 2.7 µm LC column

LC parameters are presented in Table 1. These parameters were adopted from a different study [3] and were re-used for the separation. The peak areas of the monomer and aggregate were calculated and compared with the control samples. OpenLAB CDS ChemStation was used for data acquisition and analysis.
Table 1. LC instrument conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile Phase A</td>
<td>150 mM sodium phosphate pH 7.0</td>
</tr>
<tr>
<td>Column</td>
<td>AdvanceBio SEC 2.7 μm 300 Å 7.8 × 300 mm column</td>
</tr>
<tr>
<td>TCC Temperature</td>
<td>25 °C</td>
</tr>
<tr>
<td>Isocratic run</td>
<td>Mobile phase A</td>
</tr>
<tr>
<td>Run time</td>
<td>15 mins</td>
</tr>
<tr>
<td>Injection volume</td>
<td>5 µL</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.8 mL/min</td>
</tr>
<tr>
<td>DAD</td>
<td>220 and 280 nm</td>
</tr>
</tbody>
</table>

Results and Discussion

UV spectroscopy

UV absorbance spectroscopy offers the simplest, nondestructive way of making quantitative measurements of a protein solution. The method can be used to quantify proteins without the need for a chemical derivatization or complex dye coupling reactions.

The mAb samples that had been subjected to different conditions to induce aggregate formation were measured using the Cary 60 UV-Vis spectrophotometer. The Cary 60 is suited for measuring biosamples because the xenon flash lamp source does not photodegrade light-sensitive samples. Agilent offers a wide selection of quartz cuvettes, with sample volumes ranging from 5 µL to 1 mL. A sub-micro quartz cuvette with a sample volume of 50 µL and a path length of 10 mm was used in this study. Using this cuvette ensured that sample loss was minimized and measurements were able to be taken with low sample volumes.

Any absorbance signal above 320 nm is attributed to the scattering of the light by aggregate particles. Aggregate-free protein solutions do not have absorption above 320 nm [4]. The spectral scans of the non-stressed and stressed mAb samples are presented in Figure 2. Panel A shows the absorbance spectrum of thermally stressed samples. A slight increase in the optical density at 350 nm can be observed. This spectrum contrasts with that of the freeze-thaw stressed sample in panel B, which shows a significant increase in the optical density at 350 nm.

A log-log plot of the same data is presented as insets in each of the panels on Figure 2. They show the contribution of scattering to the overall absorbance. The change in the optical density of the solution can be clearly seen in the increasing shift of the UV traces.

The aggregation index (AI) was calculated from these data using the equation in Figure 1, and the change in aggregation index is presented in Table 2. The samples that had been subject to repeated freeze-thaw cycles show a higher aggregation index than both the control samples and those subjected to thermal stress. From these data, it can be observed that freeze-thaw stress leads to a greater formation of aggregates than thermal stress. This could be due to factors such as partial unfolding at the ice-liquid interface, pH changes as buffer salt crystallizes, and solute concentration during ice crystal growth.

Table 2. Aggregation index change in non-stressed and stressed samples with different conditions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>OD 280 nm</th>
<th>OD 350 nm</th>
<th>Aggregation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Stressed IgG</td>
<td>1.57</td>
<td>0.01</td>
<td>0.5</td>
</tr>
<tr>
<td>65 °C – 5 min</td>
<td>1.69</td>
<td>0.02</td>
<td>1.1</td>
</tr>
<tr>
<td>65 °C – 10 min</td>
<td>1.72</td>
<td>0.04</td>
<td>2.5</td>
</tr>
<tr>
<td>15 x Freeze thaw (FT)</td>
<td>1.82</td>
<td>0.10</td>
<td>5.8</td>
</tr>
</tbody>
</table>

SEC analysis:

All samples were analyzed using the Agilent AdvanceBio SEC column with 2.7 µm particle size. The AdvanceBio SEC column efficiently separated the monomer peak, aggregate peaks, and also the small molecule preservatives contained in the formulation. High resolution of the monomer and the aggregate peaks were achieved within a short analysis time.

The SEC chromatogram for thermally stressed samples is presented in Figure 3 panel A and freeze-thaw samples in panel B. All samples showed a major monomer peak occupying about 99 % in total peak area. A small amount of aggregate peak was found to be present at 7 minutes in control samples. The chromatogram of the thermally stressed samples shows an appearance of the additional
peak at retention time of 6 minutes. This peak is absent in control samples. Also, the treated samples show an increase in baseline around 11 minutes indicating degraded mAb. The chromatogram shows that on thermal treatment, some of the intrinsic aggregate has changed into higher-order aggregate species. Although the overall aggregate peak area is same, the different forms of aggregate can be separated using the SEC analysis. The overlaid chromatogram of the control sample and 15X freeze-thawed samples is shown in panel B. This shows an increase in peak area of the aggregate at 7 minutes in stressed samples.

The peak area percentage of the monomer and aggregate peaks presented in Table 3 show high aggregation on repeated freeze-thaw cycles compared to thermal degradation. The UV spectra showed a similar result.

Table 3. Peak Area % of the aggregate and monomer peak from SEC chromatogram of different conditions.

<table>
<thead>
<tr>
<th>mAb sample</th>
<th>Peak Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aggregate</td>
</tr>
<tr>
<td>Non-Stressed mAb</td>
<td>0.73</td>
</tr>
<tr>
<td>65 °C – 5 min</td>
<td>0.68</td>
</tr>
<tr>
<td>65 °C – 10 min</td>
<td>0.67</td>
</tr>
<tr>
<td>15 x Freeze thaw (FT)</td>
<td>1.18</td>
</tr>
</tbody>
</table>

The UV absorbance data and the SEC data correlate well in assessing the presence of aggregate. Although UV-absorbance method cannot separate the aggregates, it can be used as a quick tool before the SEC characterization. The UV absorbance method offers advantages of low sample volume, reusable sample, and easy instrument set up and less than a minute reading.

Conclusions

The results of this study suggest that an Agilent Cary 60 UV-Vis spectrophotometer can be used to monitor the presence of aggregate in monoclonal antibody solutions. While the method cannot separate different forms of aggregate, it can be used as a quick screening method to identify the presence of aggregates, before using SEC analysis to separate them. The Agilent AdvanceBio SEC column, used with the Agilent 1260 Bio-LC system, proved capable of delivering high resolution analysis of mAb aggregates within a short run times.
References


