Application Note

Biopharmaceuticals

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Aggregation Analysis of Innovator and Biosimilar Therapeutic Proteins Using FTIR

Measurement of rituximab aggregation in concentrated samples using the Agilent Cary 630 FTIR spectrometer

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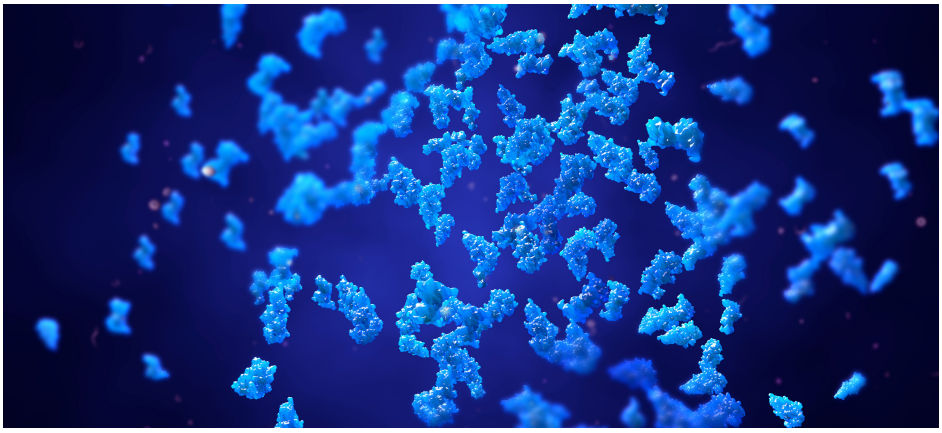

Abstract

Protein aggregation can occur during the manufacturing or storage of biologic-based therapeutics such as monoclonal antibodies (mAbs), potentially impacting their efficacy, potency, and safety. It is therefore important to monitor protein aggregation throughout the manufacturing process of mAbs. Fourier transform infrared (FTIR) spectroscopy is a nondestructive technique that can quickly monitor mAb aggregation, even in high concentration samples that are challenging for other techniques. In this application note, the Agilent Cary 630 FTIR spectrometer was used to measure thermal-induced protein aggregation. The study provides insights into the stability of high concentration mAb samples in a formulation buffer. It also shows how the Cary 630 FTIR can be used for quality control and manufacturing process optimization of innovator or biosimilar products.

Introduction

Aggregation is considered a major problem in mAb development and manufacturing, as aggregates can contribute to life-threatening immune responses. mAbs can aggregate when exposed to stressful conditions such as low pH, high temperature, and high concentration. Since therapeutic mAbs are typically administered at high concentrations, it is important to monitor mAb aggregates as a critical quality control attribute.[1]

Biosimilars are copies of innovator (original) therapeutic products that have near-identical quality, safety, and efficacy metrics as the reference product. For approval of biosimilars by regulatory agencies, the physiochemical similarities between the innovator and biosimilar product need to be established.[2]

Size exclusion chromatography (SEC) is the gold standard technique for studying protein aggregates. However, SEC may be unsuitable for monitoring high-concentration mAbs samples since the dilution, which occurs during analysis, may change the composition of aggregates in the sample. In contrast, FTIR, which is a well established and widely used technique in pharma labs or within manufacturing operations, is suitable for aggregation studies in high-concentration protein samples.

In this study, the Agilent Cary 630 FTIR spectrometer was used to monitor protein aggregation using rituximab (an mAb medication). As a model system, innovator and biosimilar rituximab were subjected to thermal stress, and aggregation was monitored by FTIR via amide band shifts.

Figure 1. The benchtop Agilent Cary 630 FTIR spectrometer is versatile, innovative, and intuitive, providing quantitative and qualitative information for the analysis of solids, liquids, and gases.

Many pharma labs use the Cary 630 FTIR for their FTIR analyses due to its robustness, flexibility, high performance, ease-of-use, and ultracompact design (Figure 1). Typical applications include the identification of drug products, incoming goods, and packaging materials, and quantitative measurements, such as the concentration of pharmaceutical ingredients. Depending on the application, the Cary 630 FTIR can quickly be reconfigured with optimized sample modules that require no user alignment.

For further ease-of-use, the Agilent MicroLab software provides step-by-step guidance with instructive pictures to intuitively navigate users through the entire analytical workflow (Figure 2). Agilent also offers MicroLab Expert, an advanced FTIR spectroscopy software that provides more analytical flexibility and spectral visualization than the standard software. MicroLab Expert allows analysts to view spectral information during data collection, which is useful for aggregation studies.

Figure 2. The intuitive Agilent MicroLab software for the Agilent Cary 630 FTIR guides the user through the analytical workflow, reducing training needs, and minimizing the risk of operator-error.

Experimental

Instrumentation

The Cary 630 FTIR spectrometer was fitted with a single reflection diamond attenuated total reflection (ATR) module. Data acquisition was carried out using the MicroLab Expert software using the parameters shown in Table 1.

Table 1. Experimental parameters for the Agilent Cary 630 FTIR.

Parameter	Value
Spectral Range	4,000 to 650 cm^{-1}
Background Scans	140
Sample Scans	140
Resolution	4 cm^{-1}
Zero Fill Factor	None
Apodization	Triangular
Phase Correct	Mertz
Sampling Technology	ATR

Materials

- Innovator and biosimilar rituximab were bought from a local distributor in Singapore. Both mAb samples were concentrated using Vivaspin 500 centrifugal concentrator spin columns (10 kDa MWCO; Sartorius).
- Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Merck Millipore).
- The formulation buffer consisted of 5.35 mg/mL sodium citrate monobasic anhydrous pH 6.5 (CAS 18996-35-5), 9 mg/mL sodium chloride (CAS 7647-14-5), and 0.7 mg/mL polysorbate 80 (CAS 9005-65-6). All reagents were bought from Sigma-Aldrich, St. Louis.

Workflow

The workflow for the study of aggregation of innovator and biosimilar rituximab using the Cary 630 FTIR is shown in Figure 3.

First, the concentration of two spin-column concentrated rituximab samples were measured by UV absorbance using the Cary 60 UV-Vis spectrophotometer. An extinction coefficient of 1.7 $mLmg^{-1}cm^{-1}$ was used to calculate the protein concentration in each sample. The concentration of innovator and biosimilar rituximab was estimated to be 50 mg/mL.

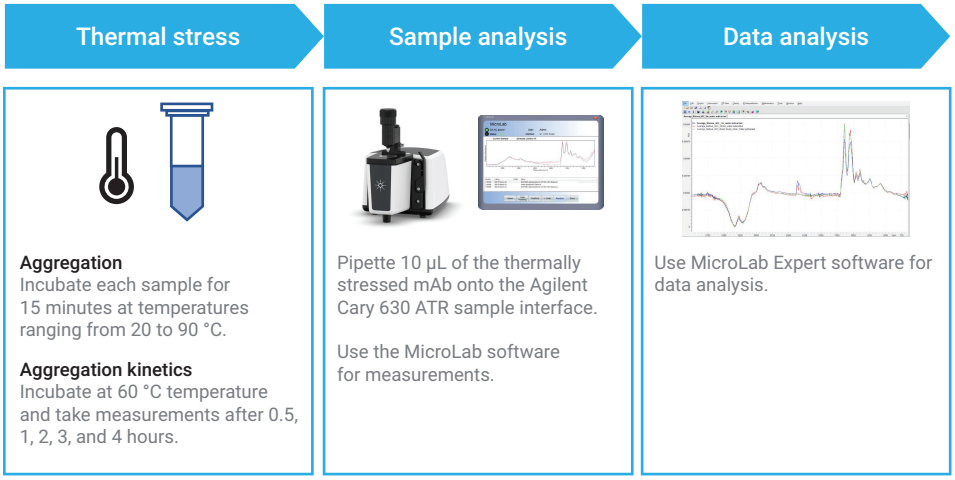

Figure 3. Aggregation workflow for the analysis of innovator and biosimilar rituximab using the Agilent Cary 630 FTIR.

For the aggregation study, 10 µL of concentrated rituximab was diluted with an equal volume of water and incubated at an elevated temperature for 15 minutes before taking measurements with the Cary 630 FTIR. The incubation temperatures were 20, 25, 35, 45, 55, 65, 75, 85, and 90 °C.

For the aggregation kinetics experiments, 75 µL of concentrated rituximab was diluted with an equal volume of water or formulation buffer. Each solution was then incubated at 60 °C. 10 µL of each sample was collected after 0.5, 1, 2, 3, and 4 hours for FTIR measurements.

Samples obtained under each of the experimental conditions were measured three times using the Cary 630 FTIR.

Data analysis

Agilent MicroLab Expert (version 1.1.0.1) was used for the data analysis. The averaged spectra (of three measurements) were used for data processing. A blank spectrum (e.g., water or formulation buffer) was subtracted from the averaged spectra using the "Subtract Spectra" function of the Spectrum Arithmetic under the Mathematics tab. The blank subtracted spectra were merged using "Merge View" of the 2D View tab. Further processing and data analysis was done using Normalization, normalize spectrum by peak area, and Smoothening using smoothing window 9 and polynomial order 3 of the Mathematics tab. For the second order derivative spectra, a Savitzky Golay second order derivative with smoothing window 9 under the Mathematics tab was applied.

For the aggregation study, the graph follows a first-order reaction kinetics as illustrated by the following equations:

$$\text{Rate} = -d[A]/dt = k[A]$$

$$d[A]/[A] = -kdt$$

$$\ln[A] - \ln[A]_0 = -kt$$

Rearrange to solve for [A] to obtain one form of the rate law:

$$\ln[A] = \ln[A]_0 - kt$$

$$\ln[A] = -kt + \ln[A]_0$$

Where

[A] is the concentration at time t

$[A]_0$ is the concentration at time 0

k is the first-order rate constant

Therefore, the slope of the line of ln[A] versus time gives the value of –k.

The half-life of a first-order kinetic is calculated as follows:

$$t_{1/2} = 0.693/k$$

Results and discussion

Temperature induced aggregation

The temperature-dependent changes to the secondary structures of both innovator and biosimilar mAbs were examined by FTIR spectroscopy. Amide spectral bands that are commonly used for protein characterization are Amide I and Amide II[3], as shown in Figure 4. Figure 4A shows the FTIR spectra of innovator rituximab at 25 and 75 °C. At 25 °C, the Amide I band is located at 1,638 cm^{-1} which is attributed to intramolecular β-sheets secondary structure. The mAbs are β-sheet rich proteins as evident by X-ray analysis.[4]

At 75 °C, the Amide I band is shifted to 1,616 cm^{-1}, which is attributed to the formation of intermolecular β-sheet secondary structures during aggregation.[5,6] The second-derivative IR spectra of innovator rituximab at 25 and 75 °C (Figure 4B) show a clearer distinction of the shift in Amide I bands from 1,638 to 1,616 cm^{-1} during rituximab aggregation.

Figure 5 shows the change in the ratio of absorbance at 1,616 and 1,638 cm^{-1} for both the innovator and biosimilar with increasing temperature. By considering the crossover point at which absorbance at 1,616 cm^{-1} becomes greater than 1,638 cm^{-1}, the melting temperature (T_m) of the innovator and biosimilar can be estimated as 70.2 and 71.8 °C, respectively. These temperatures agree with the reported T_m of rituximab of 71.6 °C measured by differential scanning calorimetry.[7] The results show the suitability of the Cary 630 FTIR for the observation of the thermal denaturation of mAbs.

The effects of formulation buffer on aggregation kinetics

A detailed kinetic study was conducted as thermal denaturation studies were unable to clearly identify the effect of buffer on thermal stability. To study the effect of formulation buffer on aggregation kinetics, a constant temperature of 60 °C (lower than T_m) was maintained during the experiment. Figure 6 shows the natural logarithm of absorbance at wavenumber 1,638 cm^{-1} versus time at 60 °C for solutions of innovator and biosimilar

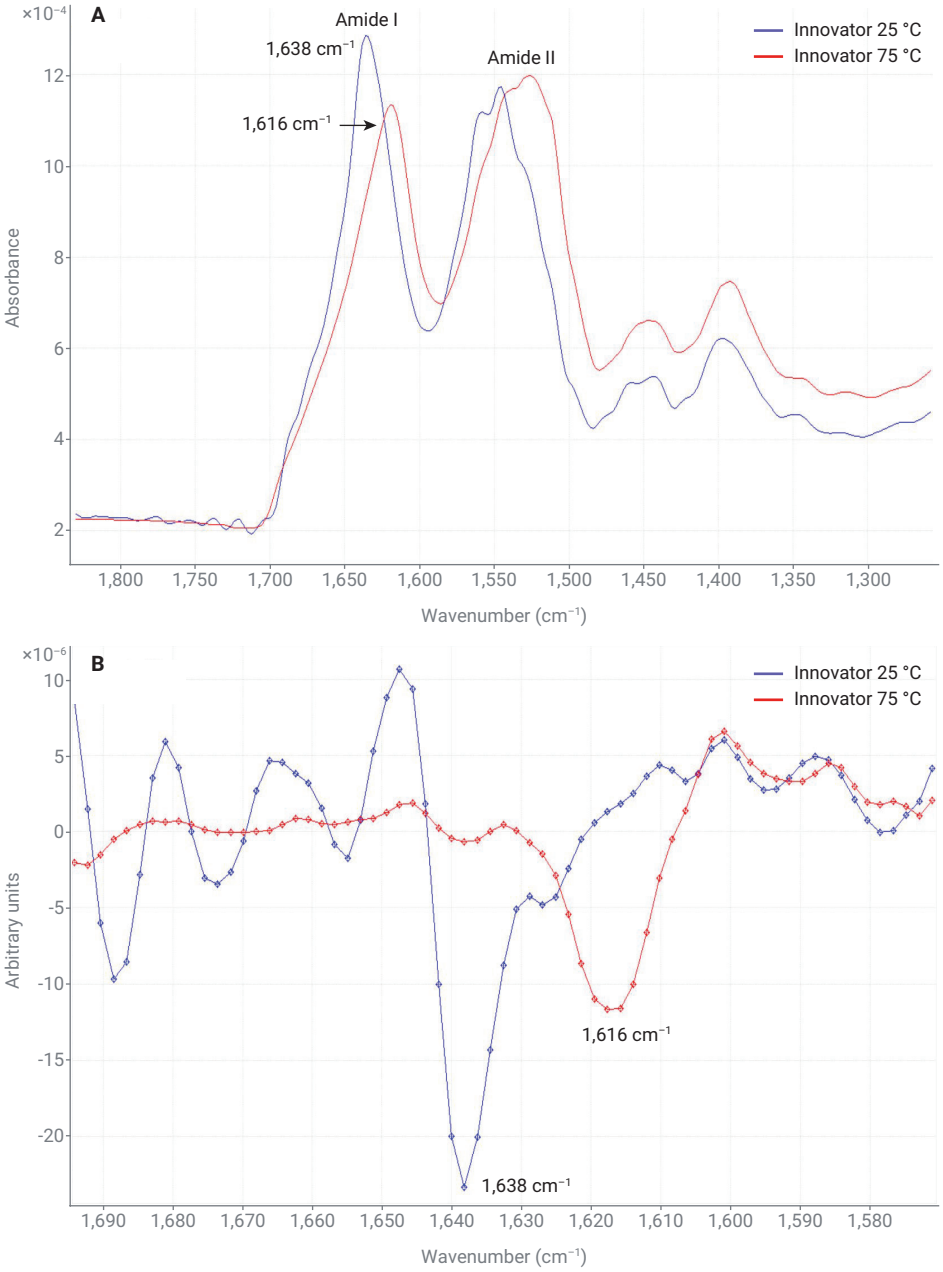

Figure 4. (A) FTIR spectra of innovator rituximab at 25 and 75 °C. (B) Second derivative IR spectra of innovator rituximab at 25 and 75 °C.

mAbs with or without formulation buffers. First-order kinetics were assumed to govern the aggregation process.[8] From the slope of the line, the first order constants, k_1 and $t_{1/2}$, were estimated. Table 2 shows the kinetic parameters for the innovator and biosimilar. Both the proteins show an increase in stability with formulation buffer, as shown by the slower rate constant and higher $t_{1/2}$ compared to no formulation buffer. This finding suggests that the formulation buffer provides protection against thermal stress. The k_1 of innovator and biosimilar mAbs were found to be similar under non-formulated conditions. With formulation buffer, the biosimilar was more stable (lower k_1 and higher $t_{1/2}$) than the innovator. Formulation and excipients can greatly influence the stability of antibodies. Excipients that maintain pH (e.g., tris, acetate, histidine, and citrate buffers) can enhance stability.[9]

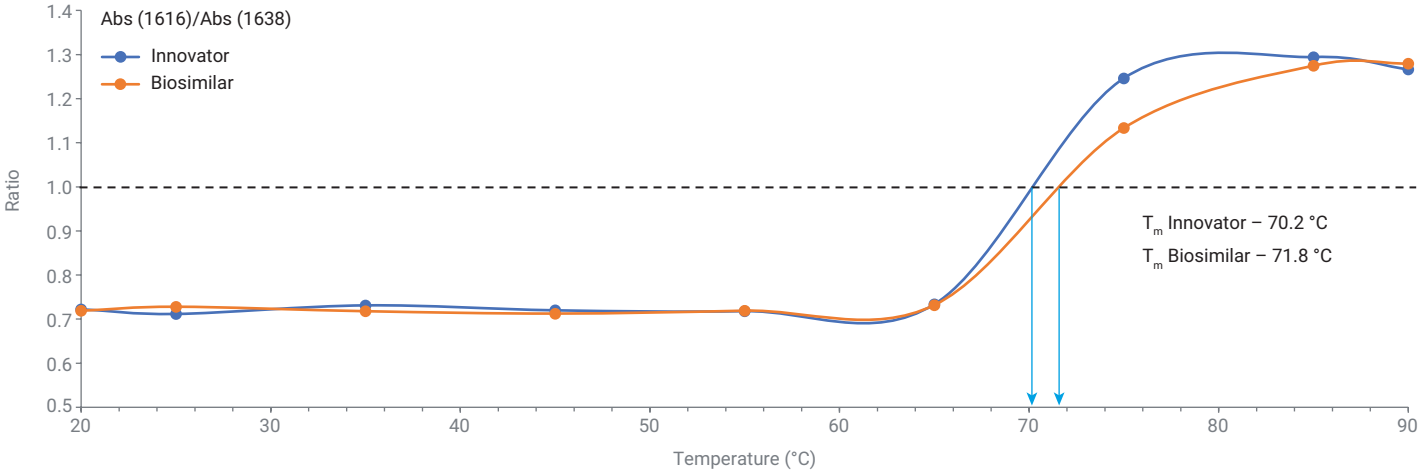

Figure 5. Change in the ratio of absorbance of wavenumbers 1,616 cm^{-1} and 1,638 cm^{-1} for both innovator and biosimilar rituximab with the increases of temperature

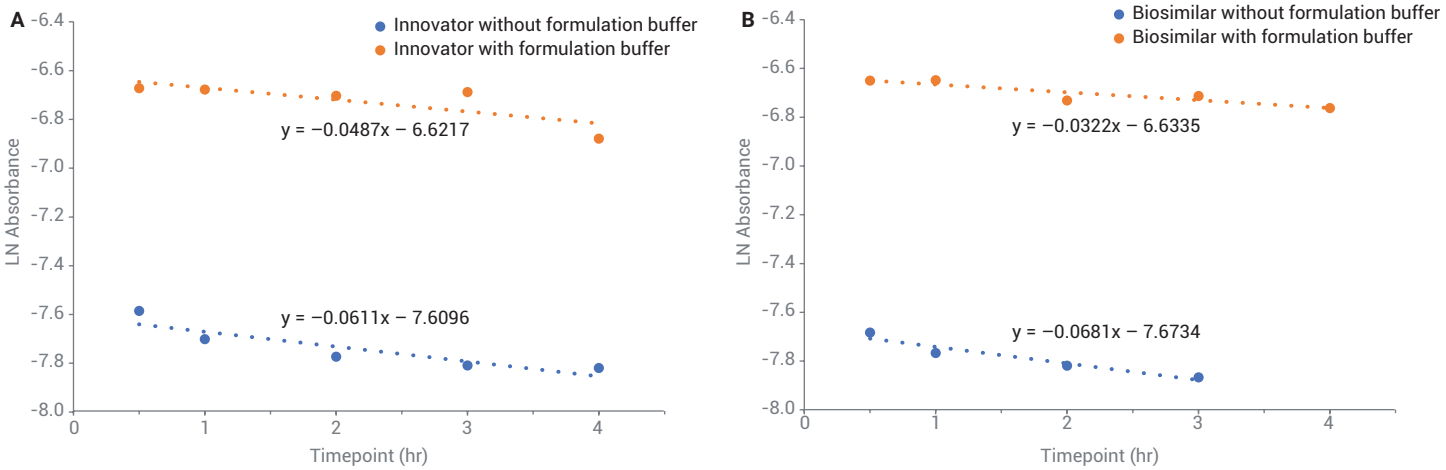

Figure 6. Natural logarithm of absorbance (1,638 cm^{-1}) versus time at 60 °C for solutions of innovator (A) and biosimilar (B) with or without formulation buffer. Due to the large variation in measurements for the biosimilar without formulation buffer at 4 hours, the data point was not included in the analysis.

Table 2. Aggregation kinetics of innovator and biosimilar with and without formulation buffer.

	Without Formulation Buffer		With Formulation Buffer	
	k_1 (h^{-1})	$t_{1/2}$ (h)	k_1 (h^{-1})	$t_{1/2}$ (h)
Innovator	0.0611	11.34	0.0487	14.34
Biosimilar	0.0681	10.17	0.0322	21.52

Conclusion

The Agilent Cary 630 FTIR spectrometer is a simple and easy-to-use instrument for the analysis of protein aggregation of biologic therapeutics, without the need for extensive sample preparation procedures. Measurement of aggregation in highly concentrated samples was performed quickly using the method, which could be used throughout the manufacturing process of mAbs. There was agreement between the FTIR-determined temperature at which rituximab thermally denatures and the temperature reported in the literature, confirming the effectiveness of the method.

The Cary 630 FTIR with Agilent MicroLab Expert software was also used to study of the stabilizing effect of formulation buffers on the aggregation kinetics of mAbs. While further work is needed to understand the mechanism of protein aggregation, this initial study has shown that the Cary 630 FTIR can provide orthogonal information on how proteins fold.

The Cary 630 FTIR is easy-to-use, requires no sample preparation, and can be widely used in the biopharmaceutical industry, including for quality control of aggregation of innovator and biosimilar protein-based therapeutics.

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