Far-UV CD Analysis During Biotherapeutic Development: A Method Capability Study

- Qualification of far-UV CD spectroscopy as a technique for characterizing a mAb biotherapeutic
- High sensitivity enabled low level detection of an IgG_1 mAb in a solution of a highly similar second mAb
- Usage of short-pathlength cells to minimize interference by formulation buffer excipients
- Establishment of Limit of Detection (LoD) and Limit of Quantification (LoQ)
- Contribution to a successful biotherapeutic submission

This study was conducted during early development of a monoclonal antibody (mAb) biotherapeutic derived from IgG_1. The aim was to determine if this mAb could be detected in a highly similar IgG_1 sample by far-UV CD. Results would enable an informed decision as to whether future higher order structure (HOS) comparisons still include far-UV CD to complement information on minor differences in tertiary structure gained from near-UV CD.

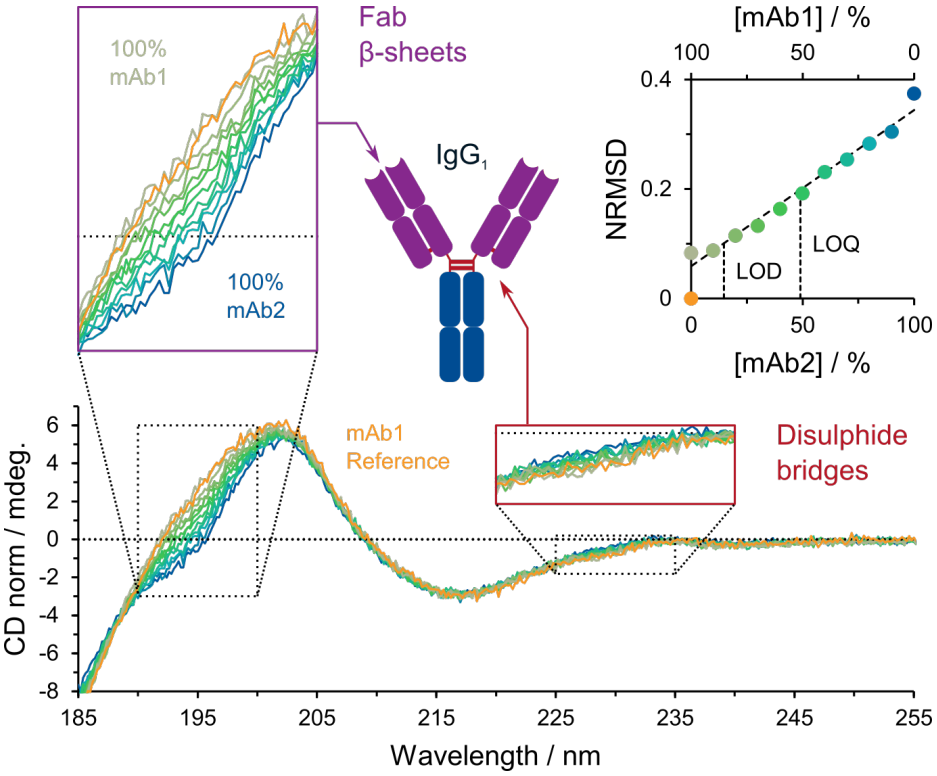


Figure 1: Spiking experiment for two mAbs, mAb1 and mAb2. CD signals in the far-UV are sensitive to changes in the Fab's variable region and dihedral angles of disulphide bonds. Inset: Spectral changes were assessed by normalized root-mean-square deviation (NRMSD) calculated for comparisons between mAb1/mAb2 mixtures and a mAb1 reference. LoD and LoQ were determined by linear regression. Data obtained with a Chirascan V100™.

Data courtesy of Dr. Alexander Bepperling, Laboratory Head Biophysical Characterization II, Global Drug Development Novartis, Germany.

Case Study

A method capability study was developed that included a spiking experiment for two mAbs: a preparation of antibody 1 (mAb1) was mixed in different ratios with a structurally similar antibody 2 (mAb2). The structural difference between these two mAbs was known from X-ray crystallography to be 1.5%. The objective of the spiking experiment was to determine the lowest fraction of mAb2 that could be detected in the spiked mAb1 sample by far-UV CD spectroscopy.

A series of eleven dilutions of mAb1 into mAb2, ranging from 0% to 100%, was prepared. To establish the limit of detection (LoD) and limit of quantification (LoQ), each sample in the series was compared to the 100% mAb1 sample.

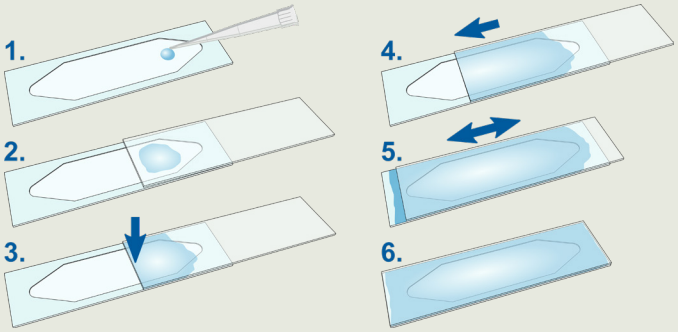
Results

Results of this study are shown in Figure 1. It is known that the secondary structure of mAbs is dominated by β-sheets. Thus, as expected, all spectra have a characteristic peak at about 200 nm and a trough at about 215 nm.

However, the spectrum of 100% mAb2 (blue) was characterized by a shoulder in the deep far-UV (wavelengths below 200 nm). The mAb1 spectrum did not exhibit this shoulder leading to the conclusion that there was a difference between the two mAbs in the wavelength region 190-205 nm. The difference was most pronounced at 195 nm.

The high sensitivity of the Chirascan V100 spectrometer revealed a second, less obvious, difference at around 230 nm. This difference could be attributed to a contribution from disulphide bridges (Hider et al. 1988, *Biophys. Chem.* 31: 45–51). Spectral differences were quantified by comparing each spectrum with a mAb1 reference spectrum (orange). There are various options for performing pairwise comparisons. In this study,

Small Pathlength Cells for Minimal Baseline, Low Volumes and High Concentrations



Demountable cells offer the **shortest pathlengths possible** for CD measurements. Their use can be considered if:

- **Sample volume is limited**, but high concentrations are feasible
- **Dilution of highly concentrated samples is prohibited** by the specific application
- **Challenging buffers** with highly absorbing or CD-active components result in a strong baseline, even with short-pathlength cuvettes, and exchange against a different buffer is not an option even though high sample concentrations are feasible

Demountable cells consist of two quartz glass plates mounted together and which are **easily cleaned** between measurements. One plate has a groove that holds the sample and defines the pathlength. Different pathlengths cells are positioned within a Chirascan V100 via an adapter.

Assembly of 0.01 mm pathlength cells can be mastered reproducibly using the following procedure:

1. Place the base plate on a clean and flat surface, with the groove facing up. Pipette the sample at one end equidistant from the groove edges. Use soft pipette tips to avoid scratching the quartz glass! To prevent the introduction of bubbles, overfill the cell by loading more microliters than the nominal cell volume of 3 µL.
2. Place one end of the cover plate onto the droplet, with its edges equidistant to the droplet, so that it rests on the base plate at an angle.
3. Gently push down the end of the cover plate so that the sample adheres to both plates and sticks them together.
4. Slowly slide the cover plate against the base plate.
5. To displace air that might still be present at the ends of the groove, gently slide the cover plate back and forth by a few millimeters.
6. Ensure that there are no bubbles in the region of the light path. Ensure the outside of the cell is clean and dry on both sides and on the edges.

Insert the assembled cell into the adapter and inspect the window to confirm that no bubbles are present. As the groove must be filled with sample by its full width, the frosted rim of the base plate is usually wetted. The thin liquid film between the frosted rim and the cover plate holds the two plates together by capillary forces.

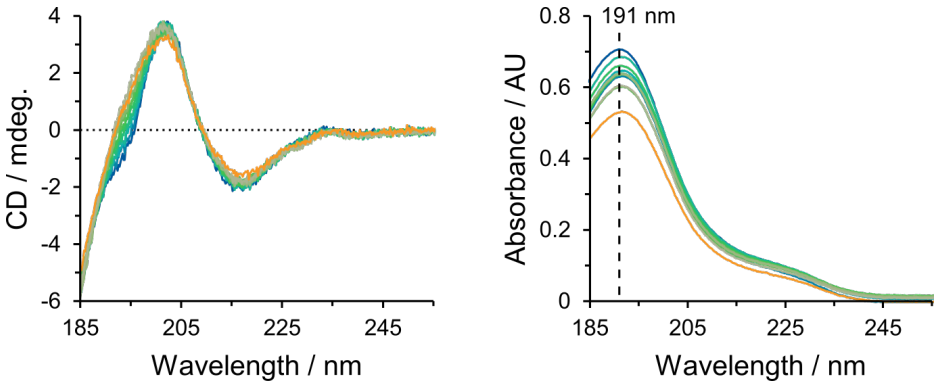


Figure 2: Data of the spiking experiment before normalization. Averaged and baseline-corrected far-UV CD (left) and absorbance (right). CD data in Figure 1 was obtained by normalization to an absorbance of 1 AU at 191 nm.

normalized root-mean-square deviation (NRMSD) was used as a measure for similarity according to the following equation

$$NRMSD = \sqrt{\frac{\sum_\lambda (x_1 - x_2)^2}{\sum_\lambda |x_1 \times x_2|}}$$

where x_1 and x_2 are the wavelength-dependent CD signals of sample 1 and 2, respectively.

The calculated NRMSD values are plotted in Figure 1 (inset). As illustrated by the trendline, the change in NRMSD with the fraction of mAb2 was linear (R = 0.99). The calculated LoD and LoQ correspond to a fraction of 14% and 49% mAb2, respectively. Considering the small structural difference of 1.5% between the mAbs, these correspond to total structural differences of 0.2% and 0.7%. These results confirmed that far-UV analysis using a Chirascan V100 was able to reveal small differences in HOS and endorsed the method for use in further studies. Ultimately, the study supported a successful biotherapeutic submission to the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

Experimental Setup

Measurements were performed using demountable cells with a pathlength of 0.01 mm. This short pathlength minimized the contribution from excipients of the formulation buffer to the CD spectra. In consequence, a small sample volume of 3 µL was used at a total protein concentration of 5 mg/mL to reach absorbances in the linear range according to Beer-Lambert's law. In comparison, the final product was formulated at 25 or 50 mg/mL. The slight variability of pathlength that arises when mounting these cells was accounted for by data normalization.

Different formulation buffers were used to stabilize the two mAbs. To ascertain defined buffer conditions,

mixing and dilution with buffer was followed by dialysis against a buffer with the same mixing ratio. The dialysates were then used for buffer-correction of the spectra.

Far-UV spectra were obtained on a Chirascan V100 in the wavelength range 185- 260 nm, step size 0.2 nm, bandwidth 1 nm and time-per-point 0.2 s.

Each acquisition comprised ten repeat scans which were averaged. Averaged spectra were buffer-corrected and normalized by the absorbance at 191 nm to an absorbance of 1 AU. For comparison, the CD and absorbance spectra before normalization are shown in Figure 2.

For the final documentation resulting from this study, spectra were smoothed by a Savitzky-Golay filter with a window of 10. The data shown in this application note (Figures 1 and 2) represent unsmoothed spectra to illustrate the data quality obtainable with a Chirascan V100 at the given acquisition settings.

LoD and LoQ were calculated according to LoD = 3**SD/m* and LoQ = 10**SD/m*, where *SD* is the standard deviation of the data from the trendline and *m* is the slope of the trendline.

Overcoming Manual Handling Errors

In this study, a total of 140 measurements were performed on a Chirascan V100 over a duration of several months. The CD signal was confirmed to be linear with concentration and intermediate precision was established to assess method variability (data not shown).

When using a manual CD spectrometer, projects of this kind at a similar, or even larger, scale are time-consuming and the need for manual sample handling increases the risk of inconsistency during data acquisition and analysis.

Risks of inconsistency increase further if sample handling involves the use of demountable cells: excess gas must be removed from samples and cells must be over-filled to prevent the formation of air bubbles. Over-filling can result in a slight increase in pathlength by ~10% which must be accounted for by normalizing the CD data by absorbance.

Manual handling errors can be eliminated by using a fully integrated Chirascan Q100 CD spectrometer. Automating the transfer of multiple samples increases **productivity** and **reproducibility** while offering the same level of sensitivity as a manual system.

A **short pathlength flow cell** (0.1 mm) is loaded from microwell plates and cleaned with a defined protocol between measurements enabling analysis of **multiple replicates** of each sample. This, in turn, makes it possible to monitor and reconfirm the established variability of the method in subsequent experiments. For the spiking experiment described here, this could yield multiple NRMSD values for each mixing ratio of the two antibodies and ultimately lower both LoD and LoQ.

With a suitable number of replicates for each sample, **rigorous statistical analysis** becomes possible. As an alternative to NRMSD values, spectral results are converted into **Weighted Spectral Difference** scores as a measure of similarity, an approach increasingly preferred by biopharmaceutical companies and regulatory authorities. These are then subjected to a Quality Range test for Tier 2 critical quality attributes in line with FDA guidelines.

For examples of case studies in which **objective, statistically-validated data** has contributed to informed decision-making during development of a biosimilar and in a forced degradation study, visit the Applied Photophysics website to download posters:

- "A novel approach for objective, quantifiable HOS comparisons: a biosimilar case study utilizing circular dichroism"
- "Assessment of statistical significance of minor changes in HOS using circular dichroism – a new approach"