Using automation and statistical analysis to enhance sensitivity and reduce subjectivity of biological therapeutic comparability using circular dichroism

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Introduction to Circular Dichroism

CD is the difference in absorption of left and right circularly polarised light

\[ CD = \left[ \begin{array}{c}
\text{left polarized light} \\
\text{right polarized light}
\end{array} \right] + \odot \Rightarrow \]

- Chiral molecules absorb these two polarisations differently
- The inherent chirality of most amino acids makes CD sensitive to protein structure
  - Amide bonds absorb in the far ultraviolet
    - Secondary structure
  - Aromatics absorb in the near ultraviolet
    - Tertiary structure
Why is the comparison of spectra useful?

To measure differences in structure between samples

- Has a process/manufacturing change altered the protein product structure?
- Have samples degraded over time/after stress?
- Has a PTM or mutation affected structure?
- Do two batches have comparable structure?
- Is there contamination?
Traditional method of comparing spectra

Overlay spectra and visually inspect

- How much of a difference is a real difference?
  - Can use overlapping standard deviations to help give a measure of comparability
  - How much overlap is required?
  - How much of the spectrum must overlap?

- Trend to try to further understand structural data to inform on potential impacts on function/activity
Automation and statistical methods can improve this

Automation

- Chirascan™ Q100 features a liquid handling robotics system that can measure 96 samples in 24 hours
  - Greatly improves throughput over manual systems
  - Allows higher numbers of sample replicates to be measured which improves robustness of the comparisons

- Cells with different path lengths allow for a single sample to be prepared for multiple different measurements
  - Near UV CD
  - Far UV CD
  - Fluorescence
  - Absorbance
Automation and statistical methods can improve this

Statistics

- Previous publications have proposed several different methods for comparing spectra numerically
  - Area of overlap
  - Correlation coefficient
  - Derivative correlation
  - Spectral difference
- All have advantages and disadvantages
- No real consensus on what is or should be best practice

Automation and statistical methods can improve this

- Weighted spectral difference (WSD)
  - Independent of number of data points
  - Weighting function based on relative signal magnitude helps to exclude differences at the level of noise

- Compare spectra to a reference sample set to give a similarity score

- Similarity score and distribution of the similarity scores compared by t-test (or other methods) to determine comparability to a certain level of confidence

- This and other methods are implemented in HOS comparison software developed by Applied Photophysics

\[
WSD = \sqrt{\sum_{i=1}^{n} \frac{1}{n} \left( \frac{|x_i|}{|x_i|_{ave}} \right) (x_i - y_i)^2}
\]
Repeatability and robustness of this method

Can we determine comparability across multiple experiments?

- Molecules representative of biotherapeutics in three formats used:
  - IgG1
  - IgG4
  - Fab

- Samples were run across three days with 6 replicate buffer sample pairs and then compared using the WSD

- Can be done but for the best reliability and most accurate comparisons, samples should be run in one experiment

<table>
<thead>
<tr>
<th>Sample set</th>
<th>Similarity score</th>
<th>p-value</th>
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<tbody>
<tr>
<td>IgG1 Day 2</td>
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<td>0.272</td>
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<tr>
<td>IgG1 Day 3</td>
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<tr>
<td>IgG4 Day 2</td>
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<td>IgG4 Day 3</td>
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<td>Fab Day 3</td>
<td>0.000236</td>
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</table>
Can we detect differences as well as comparability?

How sensitive is this method to small structural modifications?

- Measure samples with known differences

To generate modifications, the three molecules were degraded using a range of stress conditions:

- Temperature
  - 40 °C
  - 50 °C

- pH
  - pH 3
  - pH 10

- Agitation

- Chemical modification
  - Oxidation by H₂O₂
  - Deamidation by ammonium bicarbonate

- Light stress
  - 1 Mlux hours
  - 5 Mlux hours
Analysis of IgG1 degraded samples by CD

Far UV

- No obvious visible differences
- Three degradation conditions statistically significant at 95% confidence

Near UV

- Small changes visible in light stressed samples
- Many more changes are statistically significant at 95% confidence
Analysis of IgG4 degraded samples by CD

Far UV

- Only one sample (pH 3) visibly different
- Three degradation conditions statistically significant at 95% confidence

Near UV

- Small changes visible in some samples
- Many more changes are statistically significant at 95% confidence
Analysis of Fab degraded samples by CD

Far UV

- No obvious visible differences
- Light stressed samples are statistically significant at 95% confidence

Near UV

- Light stressed samples very obviously different
- Additional samples are statistically significant at 95% confidence
Orthogonal testing – are the CD results meaningful?

How sensitive is CD to the types of modifications introduced?

Other techniques employed to characterise the changes in the stressed samples:

- Mass spectrometry
  - Peptide mapping
  - Intact mass
- Size exclusion chromatography
- SDS-PAGE
- Capillary isoelectric focusing
Correlation of results – IgG1

- Near UV CD seems to have better sensitivity than far UV CD
  - Tertiary structure more perturbed by small modifications

- Higher levels of modifications and/or other effects required before the effects are seen in the far UV CD
  - 100% methionine oxidation levels under oxidation and light stress
  - IgG1 heavily fragmented under oxidation

- Modifications under deamidation stress not detected
  - Very low levels of deamidation and +10% methionine and tryptophan oxidation
  - May have been detectable by near UV CD but precipitation prevented measurement

- HMWS formed under agitation not observed by CD

<table>
<thead>
<tr>
<th>IgG1 Sample</th>
<th>SDS-PAGE</th>
<th>SEC</th>
<th>Peptide mapping</th>
<th>cIEF</th>
<th>NUV CD</th>
<th>FUV CD</th>
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<tbody>
<tr>
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Correlation of results – IgG4

Near and far UV CD are complementary techniques for detecting structural modifications:
- High levels of HMWS formed under 50 °C and pH 3 stress conditions detected by far UV but not near UV CD
- Range of methionine oxidation levels detected by near UV CD
- Despite 100% methionine oxidation this is not detected by far UV CD
- No fragmentation of IgG4 under oxidation as was seen in IgG1

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Correlation of results – Fab

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Similar trend to the other molecules

- Tryptophan oxidation detected by near UV CD
  - Tryptophan is directly monitored by near UV CD so this is expected
- HMWS formed under light stress detected by far UV CD
Conclusions

- Near and far CD are complementary techniques and show good agreement with the other methods when used together.
- Far UV CD is more sensitive to formation of high molecular weight species and fragmentation.
- Near UV CD is more sensitive to PTMs such as methionine and tryptophan oxidation.
  - Deamidated samples did not show high levels of deamidation over control.
  - Higher levels of deamidation may be detectable in molecules more prone to it.
- Near and far CD can be used to screen for modifications and aggregation/fragmentation quickly to inform on where to invest in more time intensive characterisation.
- Statistical comparison is robust when using higher numbers of sample replicates allowed for by automating the analysis and removes the subjectivity of an operator visibly inspecting the data.
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