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

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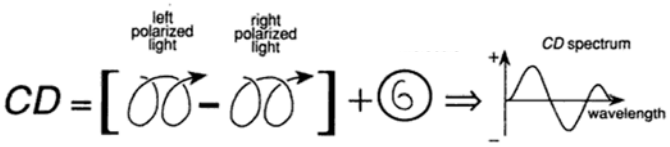
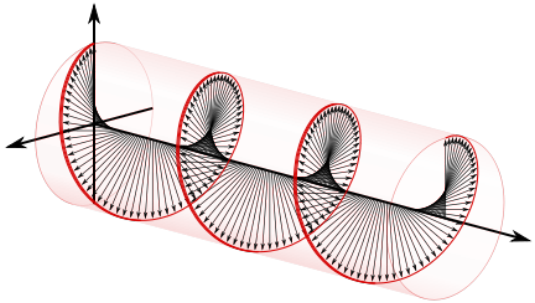
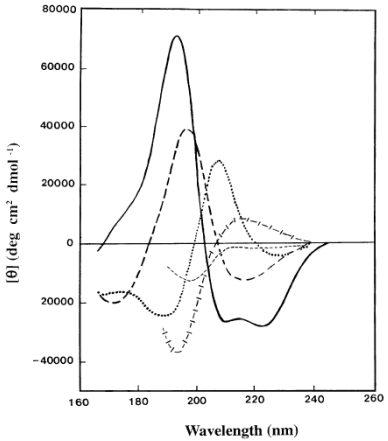
Introduction to Circular Dichroism

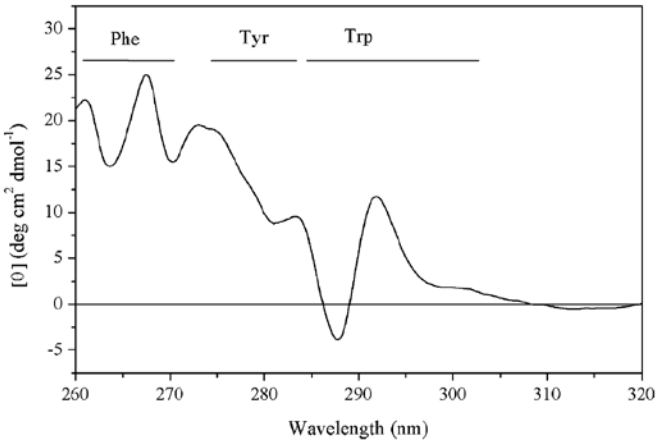
CD is the difference in absorption of left and right circularly polarised light





- 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?

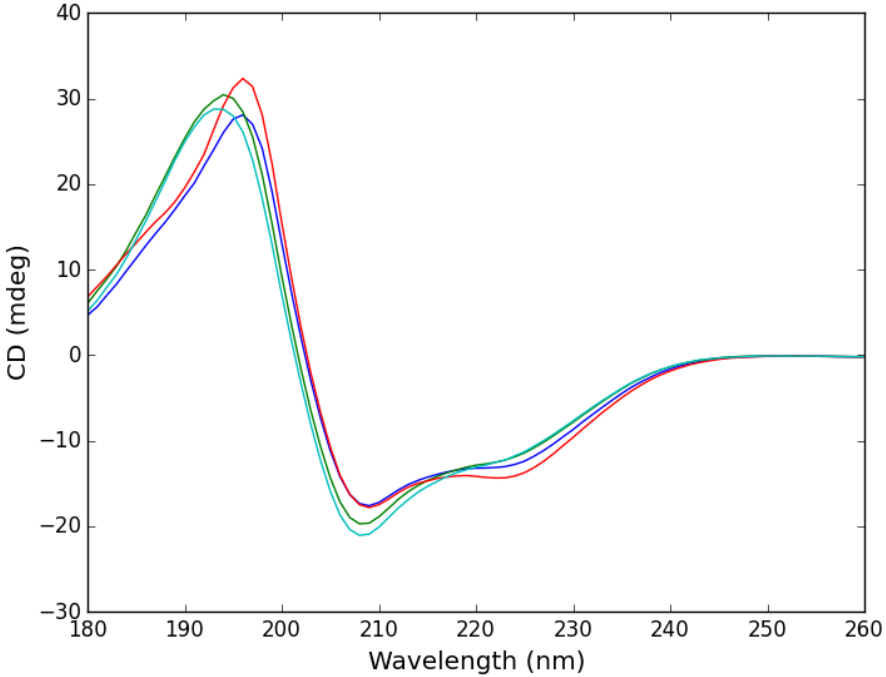
To measure differences in structure between samples

- 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

Teska, B. M., Li, C., Winn, B. C., Arthur, K. K., Jiang, Y. and Gabrielson, J. P. (2013) Comparison of quantitative spectral similarity analysis methods for protein higher-order structure confirmation, *Anal. Biochem.*, 434, 153-165

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

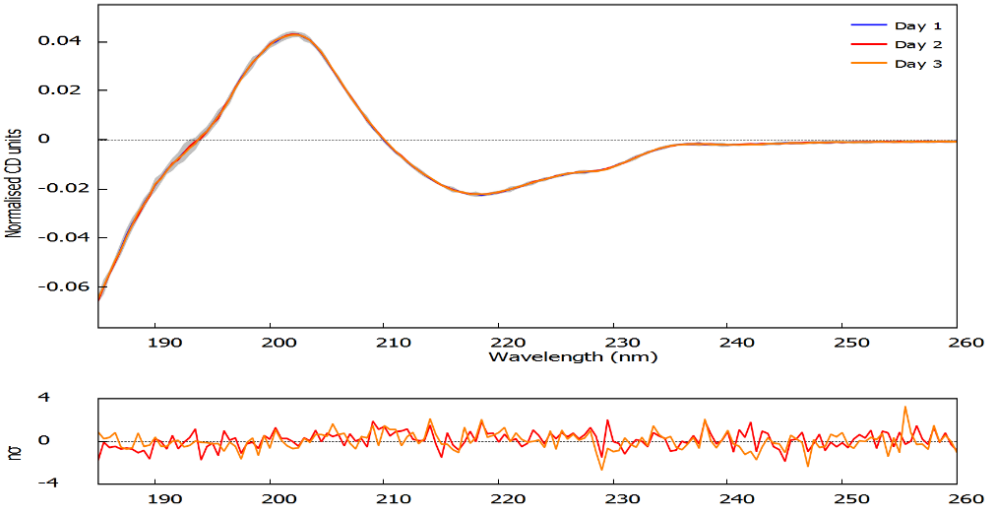
$$\mathrm{WSD} = \sqrt{\sum_{i=1}^{n}\left[\frac{1}{n}\left(\frac{|x_i|}{|x_i|_{\mathrm{ave}}}\right)(x_i - y_i)^2\right]}$$

Dinh, N. N., Winn, B. C., Arthur, K. K. and Gabrielson, J. P. (2014) Quantitative spectral comparison by weighted spectral difference for protein higher order structure confirmation. *Anal. Biochem.*, 464, 60–2

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



Sample set	Similarity score	p-value
IgG1 Day 2	0.000412	0.272
IgG1 Day 3	0.000392	0.472
IgG4 Day 2	0.000290	0.211
IgG4 Day 3	0.000257	0.145
Fab Day 2	0.000308	0.166
Fab Day 3	0.000236	0.535

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 H_2O_2
  - Deamidation by ammonium bicarbonate
- Light stress
  - 1 Mlux hours
  - 5 Mlux hours

Analysis of IgG1 degraded samples by CD

Far UV

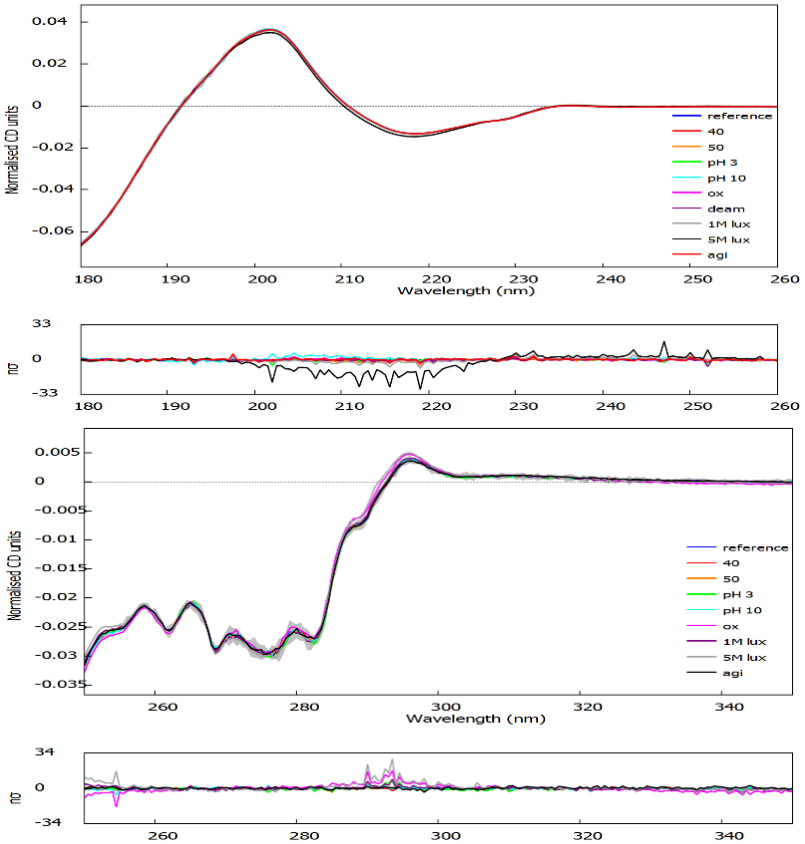
- No obvious visible differences
- Three degradation conditions statistically significant at 95% confidence

Sample	p-value
40 °C	0.490
50 °C	0.647
pH 3	0.000
pH 10	0.658
Oxidation	0.002
Deamidation	0.752
1 Mlux hour	0.589
5 Mlux hour	0.001
Agitation	0.233

Near UV

- Small changes visible in light stressed samples
- Many more changes are statistically significant at 95% confidence

Sample	p-value
40 °C	0.011
50 °C	0.020
pH 3	0.007
pH 10	0.093
Oxidation	0.000
Deamidation	Not tested
1 Mlux hour	0.001
5 Mlux hour	0.000
Agitation	0.186

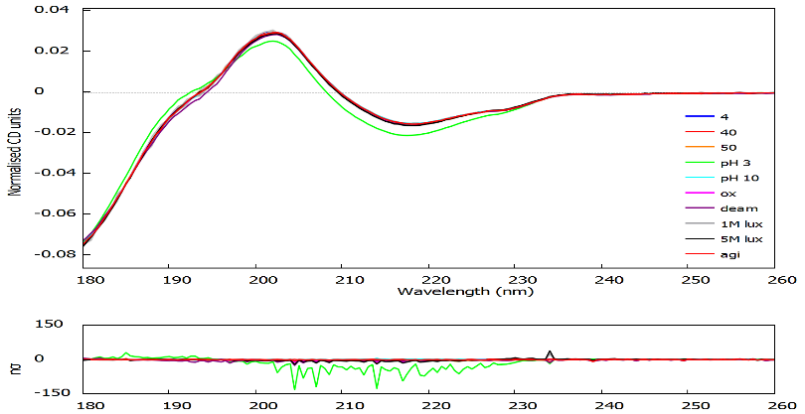


Analysis of IgG4 degraded samples by CD

Far UV

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

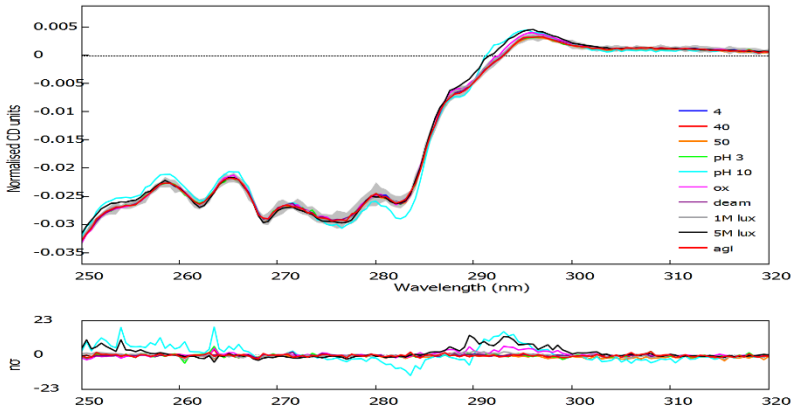
Sample	p-value
40 °C	0.572
50 °C	0.024
pH 3	0
pH 10	0.260
Oxidation	0.082
Deamidation	0.115
1 Mlux hour	0.911
5 Mlux hour	0.002
Agitation	0.850



Near UV

- Small changes visible in some samples
- Many more changes are statistically significant at 95% confidence

Sample	p-value
40 °C	0.270
50 °C	0.065
pH 3	0.810
pH 10	0.000
Oxidation	0.029
Deamidation	0.499
1 Mlux hour	0.011
5 Mlux hour	0.000
Agitation	0.568



Analysis of Fab degraded samples by CD

Far UV

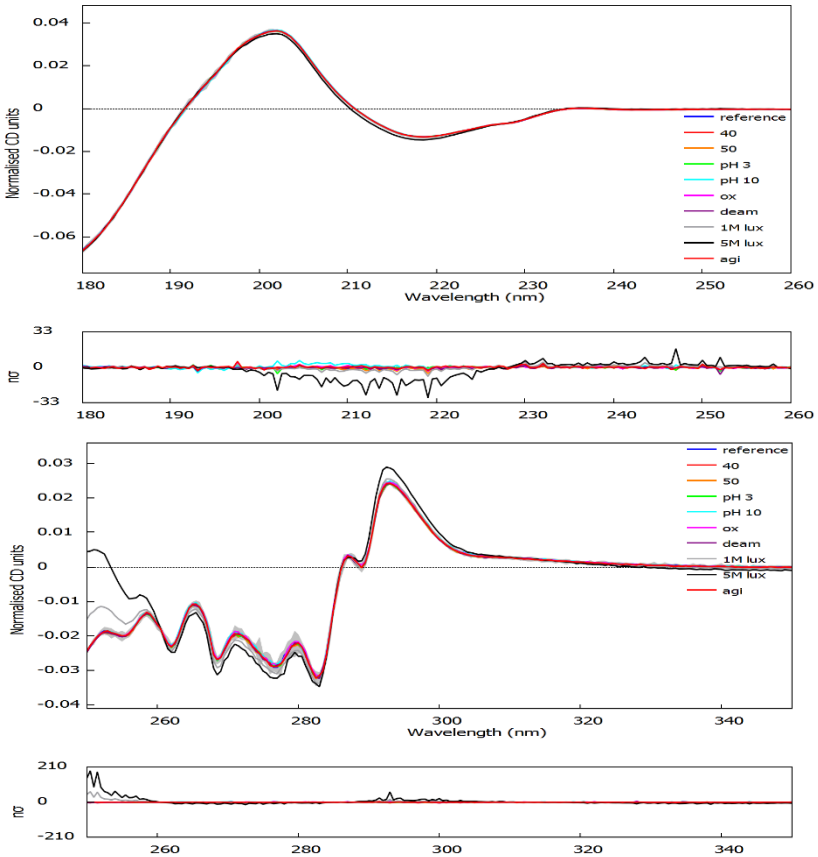
- No obvious visible differences
- Light stressed samples are statistically significant at 95% confidence

Sample	p-value
40 °C	0.233
50 °C	0.821
pH 3	0.497
pH 10	0.226
Oxidation	0.804
Deamidation	0.552
1 Mlux hour	0.006
5 Mlux hour	0.000
Agitation	0.936

Near UV

- Light stressed samples very obviously different
- Additional samples are statistically significant at 95% confidence

Sample	p-value
40 °C	0.063
50 °C	0.503
pH 3	0.382
pH 10	0.001
Oxidation	0.044
Deamidation	0.682
1 Mlux hour	0.000
5 Mlux hour	0.000
Agitation	0.314



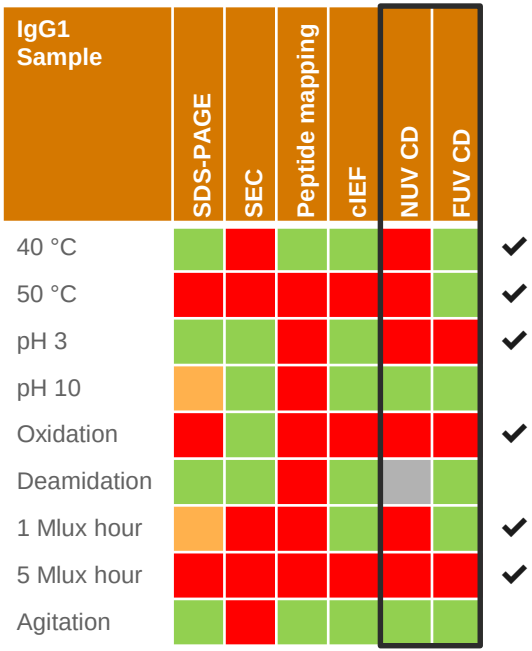
Orthogonal testing – are the CD results meaningful?

How sensitive is CD to the types of modifications introduced?

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

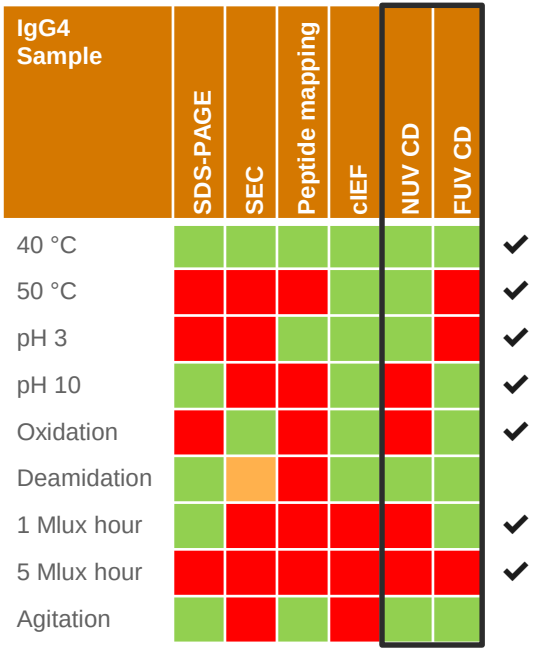
- Mass spectrometry
  - Peptide mapping
  - Intact mass
- Size exclusion chromatography
- SDS-PAGE
- Capillary isoelectric focusing

Correlation of results – IgG1



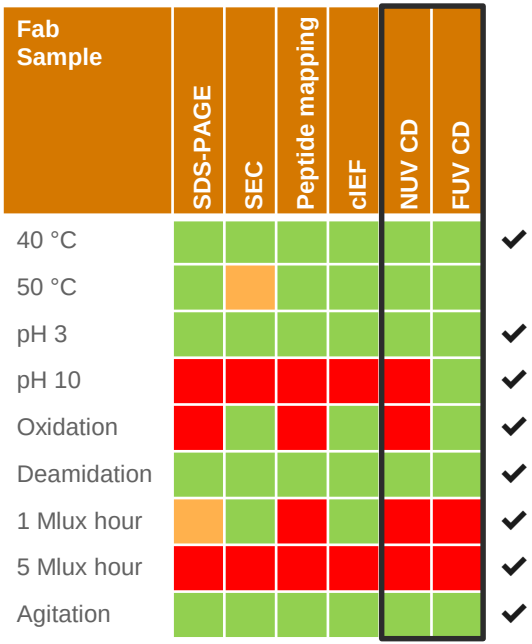
- Near UV CD seems to have better sensitivity than far UV CD
  - Tertiary structure more perturbed by small modifications
- Higher levels of modifications and/or other effects required before the effects are seen in the far UV CD
  - 100% methionine oxidation levels under oxidation and light stress
  - 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

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

Correlation of results – Fab



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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https://www.photophysics.com/

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