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Quantitative Circular Dichroism (*q*CD) for the Comparison of the Higher-Order Structures of Monoclonal Antibodies

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Introduction

Understanding or preventing degradation is essential when developing a complex biotherapeutic. This study uses a novel approach to HOS analysis — quantitative circular dichroism — to detect and quantify statistically significant changes in HOS of IgG1 for important degradation pathways.

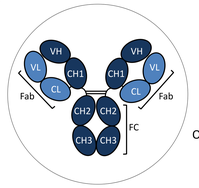
Use of a Chirascan-auto qCD system provided a rapid, highly productive, auditable method for HOS comparisons with:

- High turnaround of multiple samples
- Unattended, reproducible operation during spectral acquisition
- Objective quantification of subtle differences not discernible by eye
- Elimination of subjective visual comparisons and human error

Forced degradation study of IgG1

Deamidation of asparagine and glutamine residues: Induced by heat/high pH

Isomerization of aspartic acid residues: Induced by heat



Glycation of, for example, lysine residues: Induced by glucose

Oxidation of, for example, methionine residues: Induced by H_2O_2

Sample	Condition
Buffer	10mM Na_3PO_4, 137mM NaCl, pH7.4
Aliquot 1	Stored at pH 8.5 and at 40°C for 1 week to promote asparagine deamidation / aspartic acid isomerization
Aliquot 2	50 mM glucose at 20°C for 24 hours to promote glycation (glucose subsequently removed by dialysis)
Aliquot 3	2 M glucose at 40°C for 1 week to promote glycation (glucose subsequently removed by dialysis)
Aliquot 4	0.3% H_2O_2 for three hours at 20°C to promote methionine oxidation (H_2O_2 subsequently removed by dialysis)
Aliquot 5	4°C for seven days, served as a control
Aliquot 6	4°C for seven days, served as a reference

Experimental set-up prior to unattended operation

- Five aliquots from each treatment (#1-5) were presented on a 96-well plate.
- Aliquots of buffer were included for each sample (B).
- Three repeat Near UV CD and absorbance spectra were measured for each well using a 5 mm pathlength quartz flow cell. An equivalent Far UV experiment was performed at 0.1 mm pathlength.
- 180 CD and 180 absorbance spectra were measured in unattended operation for 18 hours (Near UV) or for 12

	A	B	C	D	E	F	G	H
1								
2								
3	B	5	B	6				
4	B	1	B	2	B	3	B	4
5	B	5	B	6				
6	B	1	B	2	B	3	B	4
7	B	5	B	6				
8	B	1	B	2	B	3	B	4
9	B	5	B	6				
10	B	1	B	2	B	3	B	4
11	B	5	B	6				
12	B	1	B	2	B	3	B	4

Equipment

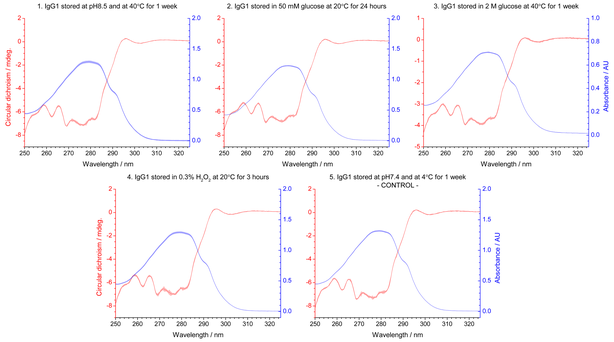
Samples were run and analyzed on a Chirascan-auto qCD. This fully automated system enabled repeat measurements of multiple samples thereby increasing signal:noise and enabling the generation of quantitative, statistically significant data.

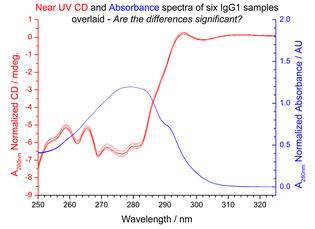
Proprietary DichOS™ calibration, standardized operating procedures and comprehensive statistical analysis combine to provide a novel, information-rich approach to biophysical characterization of proteins and peptides by circular dichroism.

Chirascan-auto qCD is the highest sample turnaround system in the Chirascan™ platform

Results prior to statistical analysis

To the naked eye, many of the overlaid spectra appear similar. Near UV CD difference spectra indicate that treatments designed to induce deamidation/ isomerization, glycation and oxidation may have resulted in small structural differences. However, statistical analysis is required to quantify the significance of these differences.





Objective statistical analysis provided quantitative results

Two well-established statistical methods[1,2], correlation coefficient (Pearson coefficient) and weighted spectrum difference (WSD), were applied:

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

x_i = signal from reference spectrum
y_i = signal from sample spectrum
n = number of data points
Characteristics: Pearson: 0 < r < 1, 1 = identical
WSD > 0, 0 = identical

Similarity scores were calculated from a reference set of spectra and from sets of sample spectra. A Student's *t*-test was used to calculate the probability that the two populations of similarity scores were representative of the same population.

If p < 0.05, then difference is significant at the 95% confidence level.

Analysis of Near UV CD Da-

Sample	Condition	Scores for comparison with control (aliquot 6)			
		Correlation coefficient		Weighted spectral difference	
		Similarity	p-value (t-test)	Similarity	p-value (t-test)
Aliquot 1	pH8.5 and at 40°C for 1 week	0.99995	0.024	0.074	0.001
Aliquot 2	50 mM glucose at 20°C for 24 hours	0.99998	0.312	0.032	0.524
Aliquot 3	2 M glucose at 40°C for 1 week	0.99993	<0.001	0.050	0.011
Aliquot 4	0.3% H_2O_2 at 20°C for 3 hours	0.99994	<0.001	0.180	<0.001
Aliquot 5	Control	0.99998	0.923	0.027	0.807

Green = Similar (2σ significance)
Red = Dissimilar (2σ significance)

Analysis of Far UV CD Data:

Sample	Condition	Scores for comparison with control (aliquot 6)			
		Correlation coefficient		Weighted spectral difference	
		Similarity	p-value (t-test)	Similarity	p-value (t-test)
Aliquot 1	pH8.5 and at 40°C for 1 week	0.99983	0.289	0.090	0.236
Aliquot 2	50 mM glucose at 20°C for 24 hours	0.99993	0.103	0.052	0.193
Aliquot 3	2 M glucose at 40°C for 1 week	0.99987	0.28	0.079	0.236
Aliquot 4	0.3% H_2O_2 at 20°C for 3 hours	0.99976	0.638	0.134	0.103
Aliquot 5	Control	0.99987	0.418	0.076	0.358

Conclusions

- The affects of all but one of the treatments designed to induce deamidation/ isomerization, glycation and oxidation in IgG1 were detected in the Near UV CD datasets and shown to be significant at the 2σ confidence interval.
- Significant differences in the equivalent Far UV datasets were not detected.
- Together, these results suggest that treatments caused changes to the local environment of aromatic side chains (tertiary structure), but did not cause changes to the secondary structure content.
- The ability to generate truly quantitative data will substantially strengthen the role of CD analysis throughout biotherapeutic development programs and help fulfil regulatory demands.

References
1. Dinh, N.N. et al. Quantitative spectral comparison by weighted spectral difference for protein higher order structure confirmation, Analytical Biochemistry 464 (2014), 60-62.
2. Teska, B.M. et al. Comparison of quantitative spectral similarity analysis methods for protein higher-order structure confirmation, Analytical Biochemistry, 434.1 (2013), 153-165

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