Circular Dichroism: Theory and Applications

LEAH PANDISCIA, PHD
JASCO, INC.
JASCO: Our Products
Seminar Overview

I. Circular dichroism theory basics
II. Instrument design and components
III. Biological applications
IV. CD accessories

Second part of this webinar series will be at a later date and will discuss measurement parameter optimization, sample considerations, and how to acquire good CD data.
What is Circular Dichroism?

Difference in absorption of left and right circularly polarized light

CD signal observed when a chromophore is chiral (optically active)
- Intrinsically chiral
- Covalently linked to chiral center
- Placed in asymmetric environment
Can I use CD for my application?

- Protein structure
- Antibody structure
- DNA/RNA structure
- Protein - Protein interactions
- Protein - Nucleic Acid interactions
- Ligand binding (Induced CD)
- Carbohydrate structure
- Chemical stability studies
- Thermal stability studies
- Kinetic studies
- Stereochemistry
- Materials characterization
- Chiral Discrimination/Absolute configuration
Why use CD?

1. Uniquely sensitive to asymmetry.
2. Information on molecular and electronic structure.
3. Experiments are relatively quick and easy to perform.
4. Non-destructive (can recover most samples).
5. Solution phase.
   ▪ Crystallation process could change molecular structure.
6. Low concentrations (0.1 mg/mL).
   ▪ Doesn’t require a concentration which could change the system being studied.
Electromagnetic wave

- **Amplitude**: intensity of wave from tip of crest to central axis
- **Wavelength**: distance between two consecutive crests
- **Polarization**: directionality of electric and magnetic fields

Types of polarization

- **Linearly polarized light**: electric field components confined to a single plane (x,y and y,z)
  - Perpendicular, equal in amplitude

- **Circularly polarized light**: electric field components rotate along beam propagation
  - Perpendicular, equal in amplitude, 90° phase difference

[Link to Jasco website](https://www.jasco-global.com/principle/principles-of-cd-ord-1/)
Chiral molecules

(a) Chiral objects

(b) Achiral objects

Optical Rotation and the Cotton Effect

Optical rotation (a): rotation of plane of polarized light

CD: absorption band
ORD: change in refractive index
Circular birefringence

**Circular Birefringence**: velocity of the light passing through an optically active medium will differ depending on the medium's refractive index.
Elliptically polarized light

**Circularly polarized light**: electric field components rotate along beam propagation, perpendicular, *equal in amplitude*, 90° phase difference

**Elliptically polarized light**: electric field components rotate along beam propagation, perpendicular, *unequal in amplitude*, 90° phase difference

\[ \Delta A = A_L - A_R \]
Time = 0

Left Circularly Polarized Light

Right Circularly Polarized Light

Retarded and absorbed by Chiral Compound
Time = 1

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 2

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 3

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 4

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 5

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 6

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 7

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 8

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 0

Left Circularly Polarized Light

Retarded and absorbed by Chiral Compound

Right Circularly Polarized Light
Time = 1

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 2

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 3

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 4

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 5

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 6

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 7

Left Circularly Polarized Light

Right Circularly Polarized Light
Time = 8

Left Circularly Polarized Light

Right Circularly Polarized Light
Instrumentation
Principle of Measurement

Light Source → Unpolarized light → Prisms → Linearly polarized light → Double Monochromator → PEM → Circularly polarized light → Sample → RCP LCP → PMT Detector

Prisms

Light Source

Unpolarized light

Linearly polarized light

Double Monochromator

PEM

Circularly polarized light

Sample

RCP LCP

PMT Detector

Jasco
Optical Diagram of J-1500

M0 ~ M6: Mirrors
LS1 : Xenon lamp (for sample measurement)
LS2 : Mercury lamp (for the instrument inspection)
SH : Shutter
S1 ~ S3 : Slits
P1 : First prism (horizontal optical axis)
P2 : Second prism (vertical optical axis)

O-ray : Ordinary ray
E-ray : Extraordinary ray
L : Lens
F : Filter
CDM : Modulator (PEM)
D : PMT

Jasco
Double Monochromator and Stray Light

- CD is difference in absorption → very small signal
- Stray light: any light that does not fall under Gaussian distribution at a specific wavelength.
- Allow you to measure higher optical densities
- $3 \text{ Abs} = 0.001\% T$ → Less light transmitted through sample, more stray light effects
Light Source

- 150 W Xenon arc lamp (high pressure ~8 atm)
  - Broad spectral output (160-2000 nm)
  - Lasts ~ 1000 hours
Nitrogen Purge

- Lamp creates substantial amount of UV radiation in far-UV
  - When UV radiation strikes $O_2$ molecule, $O_3$ created.
  - $O_3$ oxidizes mirrors (lose reflectivity and S/N gets worse).
- Push out $O_2$ with $N_2$ so $O_2$ doesn’t absorb in far-UV.

- Flowrates: shorter wavelengths, higher flowrate
  - $>185$ nm: 2 L/min
  - $<180$ nm: 5 L/min
Prisms

- Dispersive element used for wavelength selection
  - Doesn’t produce second order effect, which is a source of stray light.
- Creates linearly polarized light.
Lens

- Collimates light to constant width.
- PMT will collect more light if there are two parallel beams.
Filter

- Unpolarized light incident on birefringent material splits into two rays: O- and E-ray.
  - Filters out any E-ray that gets through the slit.
- In far-UV, slit is more open (more dispersion of light, need more light throughput).
Photo-elastic Modulator (PEM)

- Converts linearly polarized light to circularly polarized light.
- Voltage is applied to vibrate piezoelectric element at resonance frequency (~50 kHz).
- Stresses and bends quartz attached to element, which induces birefringence.
- Linearly polarized light components travel through the birefringent quartz piece at different speeds.
Photo-elastic Modulator (PEM)

- When quartz compressed, polarization parallel to modulation axis travels faster than vertical component.
- When quartz stretched, parallel component lags behind vertical component.
- Modulates between left- and right-handed CPL at different times.
Photomultiplier Tube Detector

Absorption of photon $\rightarrow$ emission of electron

Every time electron hits plates, more electrons fall off, amplifying the number of electrons
Since CD is the *difference* absorption in left- and right CPL, the signal is very small
- Detector measures difference \(A_L - A_R\)

- Lock-in amplifier is tuned to the resonance frequency of the PEM (i.e. the voltage applied to the crystal to create CPL)

- CD signal can now be detected from an extremely noisy environment
Circular Dichroism
Applications
Circular Dichroism Applications

I. Structural Characterization of proteins
II. Antibody stability evaluation
III. Thermal stability study
IV. DNA and G-quadruplex structure
V. Stopped flow folding study
VI. Microassay methods
Structural Information Obtained from CD Measurements

**Far-UV (< 260 nm)**
Secondary Structure

**Chromophore: Peptide Bond**
Sensitive to changes in the protein backbone bond angles and can be used to estimate secondary structure components.

**Near-UV (< 340 nm)**
Tertiary Structure

**Chromophore: Aromatic Amino Acid Residues**
Probes solvent environment and interactions of aromatic acid side chains, as well as the disulfide bonds.

**UV-Visible**

**Chromophore: Prosthetic Group**
At visible wavelengths, structural information can be obtained for prosthetic groups such as heme in hemoglobin, although these are not strictly proteins.
Far-UV CD spectra of protein solutions
Far-UV CD spectra of protein solutions

Peak Wavelengths (nm)

<table>
<thead>
<tr>
<th>Structure</th>
<th>Wavelengths</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helical</td>
<td>222 (-)</td>
</tr>
<tr>
<td>β-sheet</td>
<td>215 (-)</td>
</tr>
<tr>
<td>Turn</td>
<td>225 (-)</td>
</tr>
<tr>
<td>Random</td>
<td>195-200 (-)</td>
</tr>
</tbody>
</table>
Far-UV CD spectra of protein solutions

<table>
<thead>
<tr>
<th>Peptide Structure</th>
<th>Peak Wavelengths (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helical</td>
<td>222 (-) 208 (-) 190 (+)</td>
</tr>
<tr>
<td>β-sheet</td>
<td>215 (-) 195-200 (+)</td>
</tr>
<tr>
<td>Turn</td>
<td>225 (-) 200-205 (+) 180-190 (-)</td>
</tr>
<tr>
<td>Random</td>
<td>195-200 (-) 235 (-)</td>
</tr>
</tbody>
</table>
Far-UV CD spectra of protein solutions

<table>
<thead>
<tr>
<th>Peak Wavelengths (nm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helical</td>
<td>222 (-) 208 (-) 190 (+)</td>
</tr>
<tr>
<td>β-sheet</td>
<td>215 (-) 195-200 (+)</td>
</tr>
<tr>
<td>Turn</td>
<td>225 (-) 200-205 (+)</td>
</tr>
<tr>
<td>Random</td>
<td>195-200 (-) 235 (-)</td>
</tr>
</tbody>
</table>
Far-UV CD spectra of protein solutions

<table>
<thead>
<tr>
<th>Structure</th>
<th>Peak Wavelengths (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helical</td>
<td>222 (-) 208 (-) 190 (+)</td>
</tr>
<tr>
<td>β-sheet</td>
<td>215 (-) 195-200 (+)</td>
</tr>
<tr>
<td>Turn</td>
<td>225 (-) 200-205 (+) 180-190 (-)</td>
</tr>
<tr>
<td>Random</td>
<td>195-200 (-) 235 (-)</td>
</tr>
</tbody>
</table>
Far-UV CD spectra of protein solutions

- α-helix
- β-sheet
- Random coil
- turn
Protein Secondary Structure Estimation

The CD spectrum of a protein can be expressed as the sum total of its CD spectra component (α-helix, β-sheet, turn, and random coil), multiplied by their respective abundance ratios.

\[ [\theta]_\lambda = f_\alpha[\theta]_\alpha + f_\beta[\theta]_\beta + f_t[\theta]_t + f_u[\theta]_u \]

\[ \theta_{\lambda,i} \]: ellipticity at each wavelength of each \( i \)th secondary structure component (CD Spectra)

\( f_i \): fraction of each secondary structure

Secondary structure abundance ratios are determined using different component analysis and regression techniques to ensure the minimum distance between measured and calculated spectra.
A calibration model for secondary structure abundance ratios obtained by X-ray crystallography and CD is produced using PCR or PLS, which is then used to estimate the unknown secondary structure of proteins.
Peptide titration with dilute sulfuric acid
Near-UV CD spectra of protein solutions

Factors that influence Near-UV Spectra

- Solvent environment (hydrogen bonding, polar groups)
- Interactions with aromatic amino acid residues (distance)
- Rigidity of protein
- Number of aromatic amino acid residues in protein
Chemical denaturation of apo-α-lactalbumin

The spectral changes suggest that the aromatic amino acids in the interior of the protein were exposed as a result of protein unfolding under denaturant conditions.

Denatured by 3.44 M GdmCl

Non-denatured

: Aromatic amino acid
Chemical denaturation of apo-α-lactalbumin

![Graph showing chemical denaturation of apo-α-lactalbumin](image_url)
Circular Dichroism Applications

I. Structural Characterization of proteins
II. Antibody stability evaluation
III. Thermal stability study
IV. DNA and G-quadruplex structure
V. Stopped flow folding study
VI. Microassay methods
VHH Antibodies

- Highly stable with respect to heat, pH, and denaturing agents
- High affinity and specificity due to long CDR3
- Low molecular weight (~15 kDa) increases mass production efficiency
- Unique binding capacity to small cavities
- High solubility (good imaging agents)
- Easily modified

The effects of ambient environmental factors such as temperature, pH, salt concentration may cause antibody drugs such as VHH and IgG to undergo a change in their higher order structure, so that they lose their activity and function.
Evaluation of VHH structural changes due to pH and salt concentration

High Throughput CD System

Automatic measurements of multiple samples
- Sample aspirating, measurement, and flow path washing and drying are fully automated
- Automatic measurement of up to 192 samples
- Runs all night for high operational efficiency

Spectrum QC Test program

Quantification and judgement of changes in spectra
- Automated judgement whether or not there have been changes in spectra based on statistical methods
- Evaluation of stability and structural comparability of antibodies, peptides, and nucleic acid drugs
Quantifying Spectral Differences with the Spectrum QC Test program

Step 1: Square the difference spectrum

\[ (U_i - \bar{R}_i)^2 \]

Step 2: Weighting

\[ \frac{\sigma_i}{(U_i - \bar{R}_i)^2 / \sigma_i} \]

Step 3: Quantitation

\[ I = \frac{1}{n} \sum_{i=\lambda_1}^{\lambda_n} \frac{(U_i - \bar{R}_i)^2}{\sigma_i} \]

- \[ U_i \]: Denatured CD spectrum
- \[ \bar{R}_i \]: Average of native spectra
- \[ \sigma_i \]: Standard deviation of noise for unknown spectrum

Instrument noise is larger at shorter wavelengths where there is less light throughput, so the effects of noise must be reduced to detect slight changes.

- Change is evaluated as small where noise is larger, and large where effects of noise are smaller.
- Slight changes in sample differences are detected with high sensitivity.
Measurement conditions and CD spectra

The CD spectra of VHH at various pH and salt concentrations were measured. Differences in the spectra from the native spectra were quantified.
Quantifying changes in the CD spectra

The larger the Z-score, the greater the distance or difference between the native and measured spectra.
Circular Dichroism Applications

I. Structural Characterization of proteins
II. Antibody stability evaluation
III. **Thermal stability study**
IV. DNA and G-quadruplex structure
V. Stopped flow folding study
VI. Microassay methods
Method for evaluating the thermal stability of proteins

<table>
<thead>
<tr>
<th></th>
<th>$T_m$ (°C)</th>
<th>$\Delta H$ (kJ/mol)</th>
<th>$\Delta S$ (J/mol·K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>47.40 ± 0.067629</td>
<td>820.558 ± 44.389</td>
<td>2559.83 ± 138.477</td>
</tr>
<tr>
<td>Sample 2</td>
<td>47.61 ± 0.065995</td>
<td>782.463 ± 40.3946</td>
<td>2439.37 ± 125.932</td>
</tr>
</tbody>
</table>
Thermal stability and reversibility of VHH
Thermal stability of VHH

- At 50ºC, there is a slight change in the CD spectrum.
- At 90ºC, the spectrum changes greatly after the temperature is raised and lowered once, indicating that the secondary structure has changed.
At 50ºC, the slope increases sharply from the 16th cycle, indicating that the structure changed as a result of repeated increases and decreases in temperature, even though 50ºC was lower than the denaturation temperature.
Circular Dichroism Applications

I. Structural Characterization of proteins
II. VHH Antibody stability evaluation
III. Thermal stability study
IV. DNA and G-quadruplex structure
V. Stopped flow folding study
VI. Microassay methods
Inducing chirality into an electronic transition: DNA

- CD observed because chiral sugar units bonded to base and phosphate groups.
- Measure CD induced into transition of bases as a result of coupling with backbone transitions.
- Spectrum arises from $\pi \rightarrow \pi^*$ transitions of stacked bases (200-300 nm).
DNA Structure Studies

- A-DNA
- B-DNA
- Z-DNA

Graph showing CD signal vs. Wavelength (nm) with different DNA structures: A, B, and Z.
G-quadruplex topology

Parallel

Hybrid

Antiparallel

Metal Ion

Guanine Base

220 240 260 280 300 320
Wavelength (nm)

220 240 260 280 300 320
Wavelength (nm)

220 240 260 280 300 320
Wavelength (nm)
Circular Dichroism Applications

I. Structural Characterization of proteins
II. VHH Antibody stability evaluation
III. Thermal stability study
IV. DNA and G-quadruplex structure
V. Stopped flow folding study
VI. Microassay methods
Stopped flow method
Tracking the refolding of cytochrome c

Denatured State \[\xrightarrow{\text{Buffer}}\] Native state

The rate constant is smaller for the near-UV than for the far-UV, indicating the tertiary structure of the protein refolds slower than the secondary structure.
Circular Dichroism Applications

I. Structural Characterization of proteins
II. VHH Antibody stability evaluation
III. Thermal stability study
IV. DNA and G-quadruplex structure
V. Stopped flow folding study
VI. Microassay methods
Structural characterization using a microsampling disc

1. Drop the sample on the disk with micropipette
2. Put the cover in place
3. Place the disk in the sample compartment

2 µL: pathlength 0.2 mm → 1 µg/sample
10 µL: pathlength 1 mm → 1 µg/sample
Thermal denaturation measurement using a capillary cell

1. Sample is drawn into a capillary
2. The end of the capillary is closed with sealant

Approximately 10 µL (0.5 mm pathlength) → ~2 µg/sample

S/N is lower than rectangular cell because smaller aperture but the melting temperature can be obtained from the melting curve.
JASCO Educational Resources

Upcoming Webinars:
• Vibrational Circular Dichroism
• FTIR Theory, Instrumentation, and Techniques
• FTIR Microscopy
• Circular Dichroism Measurement Optimization
• Raman Microscopy and Imaging
• SFC Theory and Applications

E-books and Tips and Tricks Posters
• Raman
• Fluorescence
• FTIR
• CD

KnowledgeBase

NEXT WEBINAR WILL BE ON
VCD THEORY AND APPLICATIONS

DR. CARLOS MORILLO
TUESDAY APRIL 21TH AT 2:00 PM EDT
Thank you for attending our CD Webinar Part 1!

ANY QUESTIONS?