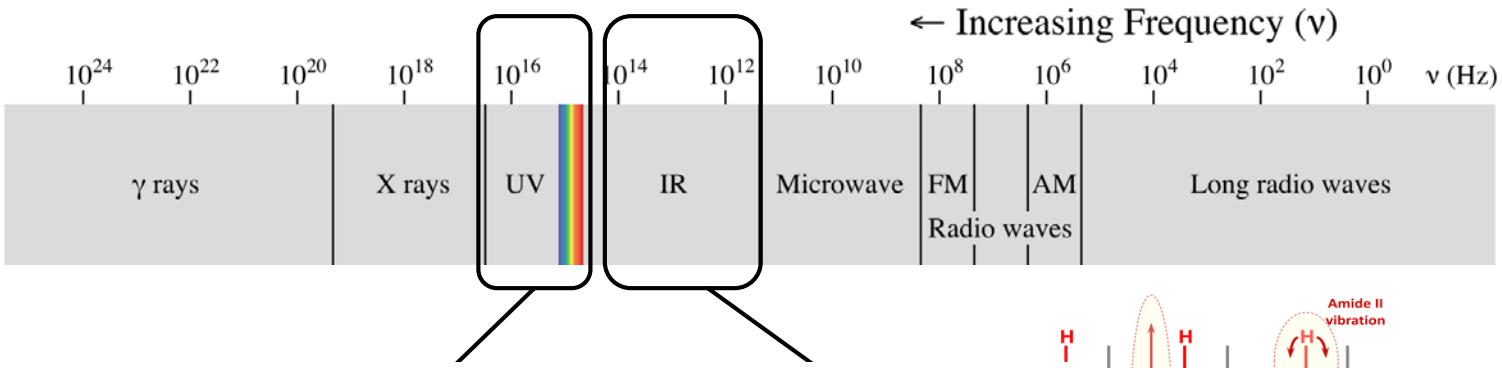
NANYANG TECHNOLOGICAL UNIVERSITY

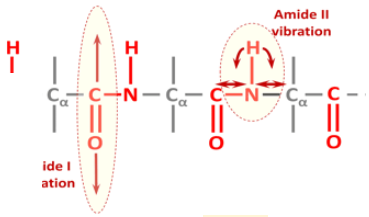
Circular dichroism and other spectroscopies

EMBO Global Exchange Lecture Course 'Structural and Biophysical methods for biological macromolecules in solution'

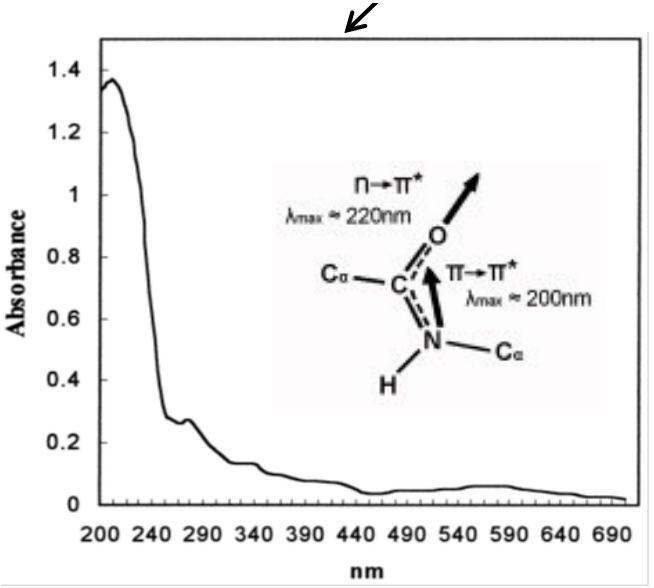
Jaume Torres NTU, 6-14 Dec 2017

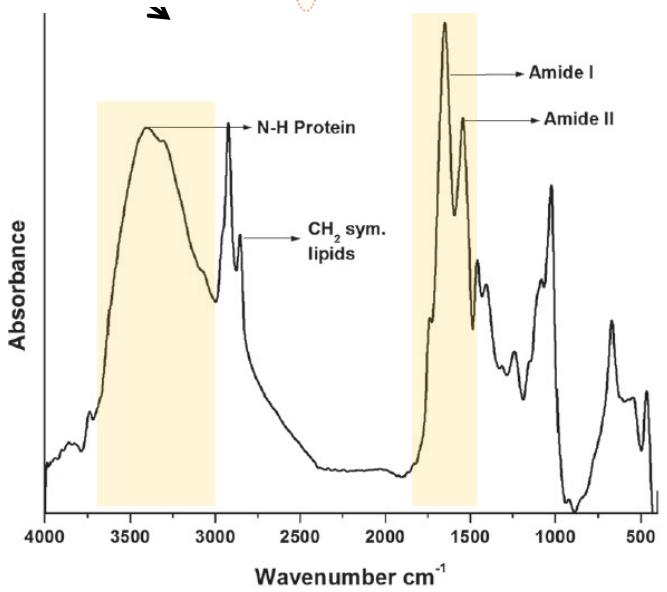
CD and IR spectroscopies – common chromophore





- **Low resolution information**
- **Sensitive to changes in native environments**

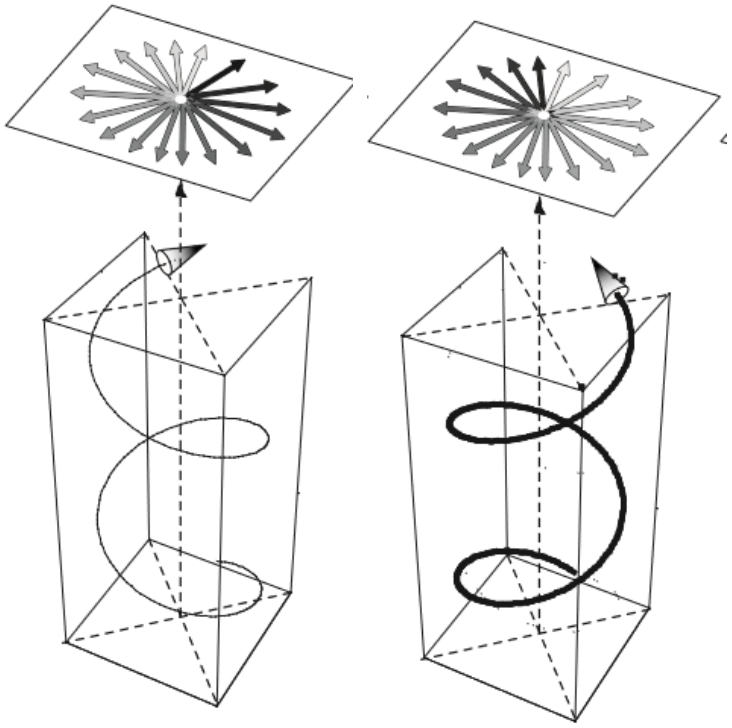




No water interference (>175 nm)

Water interference

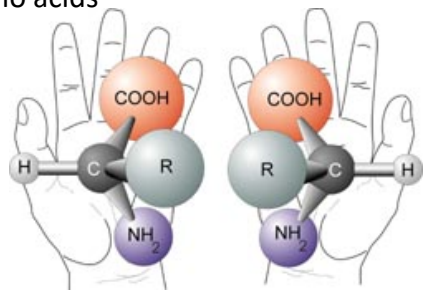
Right and left circular polarization



Left handed **Right handed**

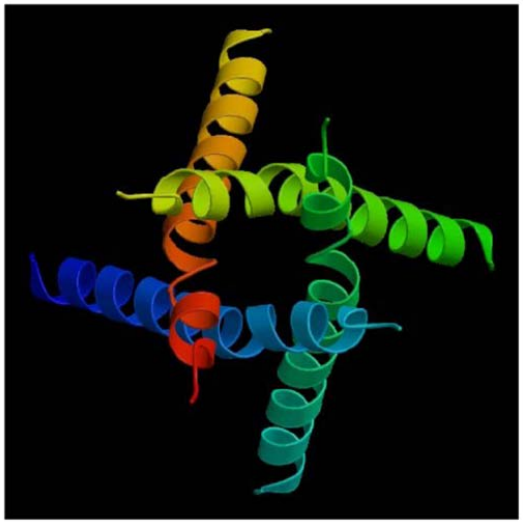
- **Mirror images**
- **Not superimposable**
- **Quiral**

- **Most biological molecules are chiral (proteins, DNA, sugars)**
  - Proteins contain only L-amino acids
  - DNA contains only D-sugars



In biological molecules, helicity is *another* source of chirality.

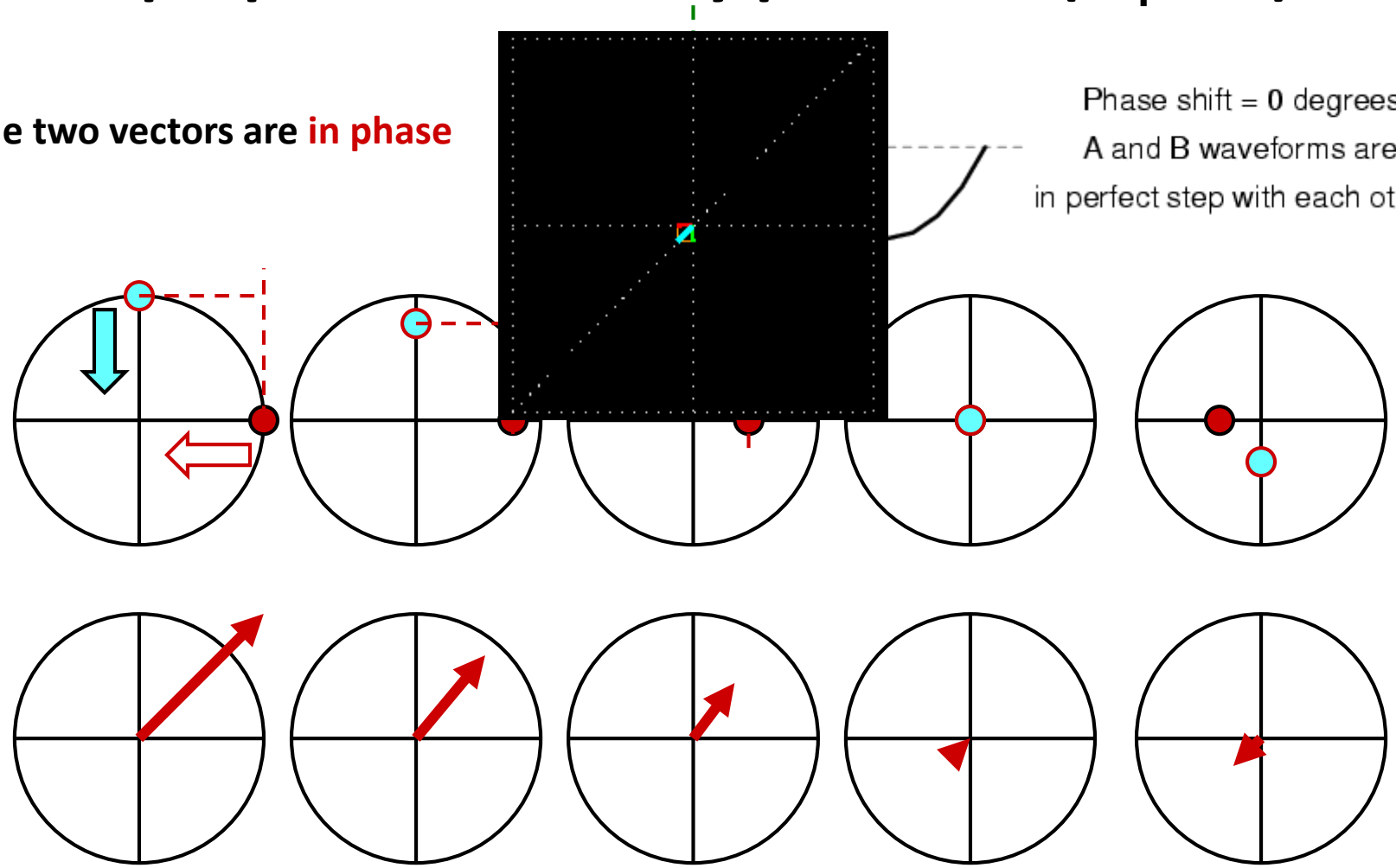
DNA



Proteins
(KCsa)

Superposition linearly polarized ($\Delta\phi = 0$)

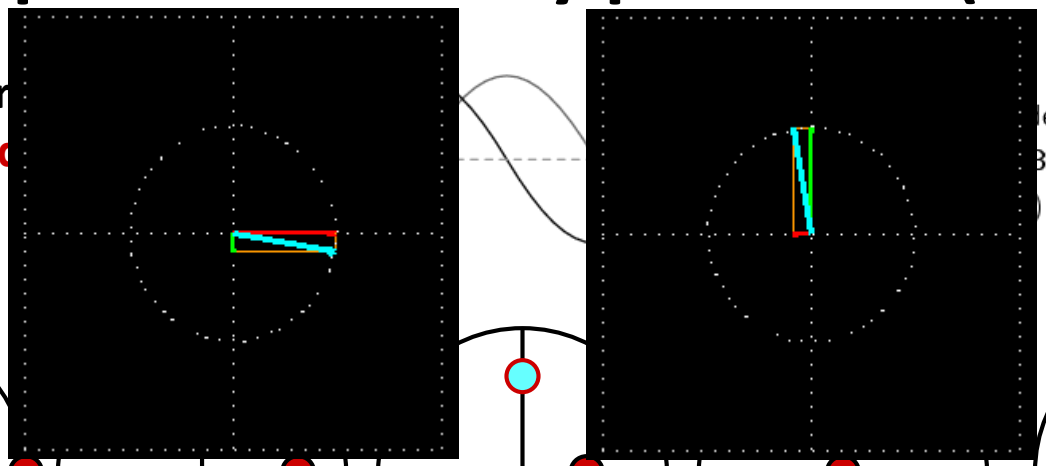
The two vectors are in phase



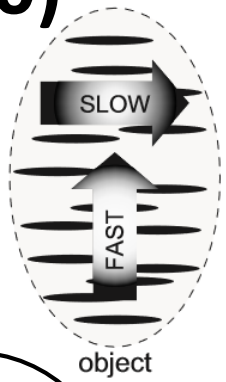
The resulting vector appears to move in a straight line (linearly polarized light).

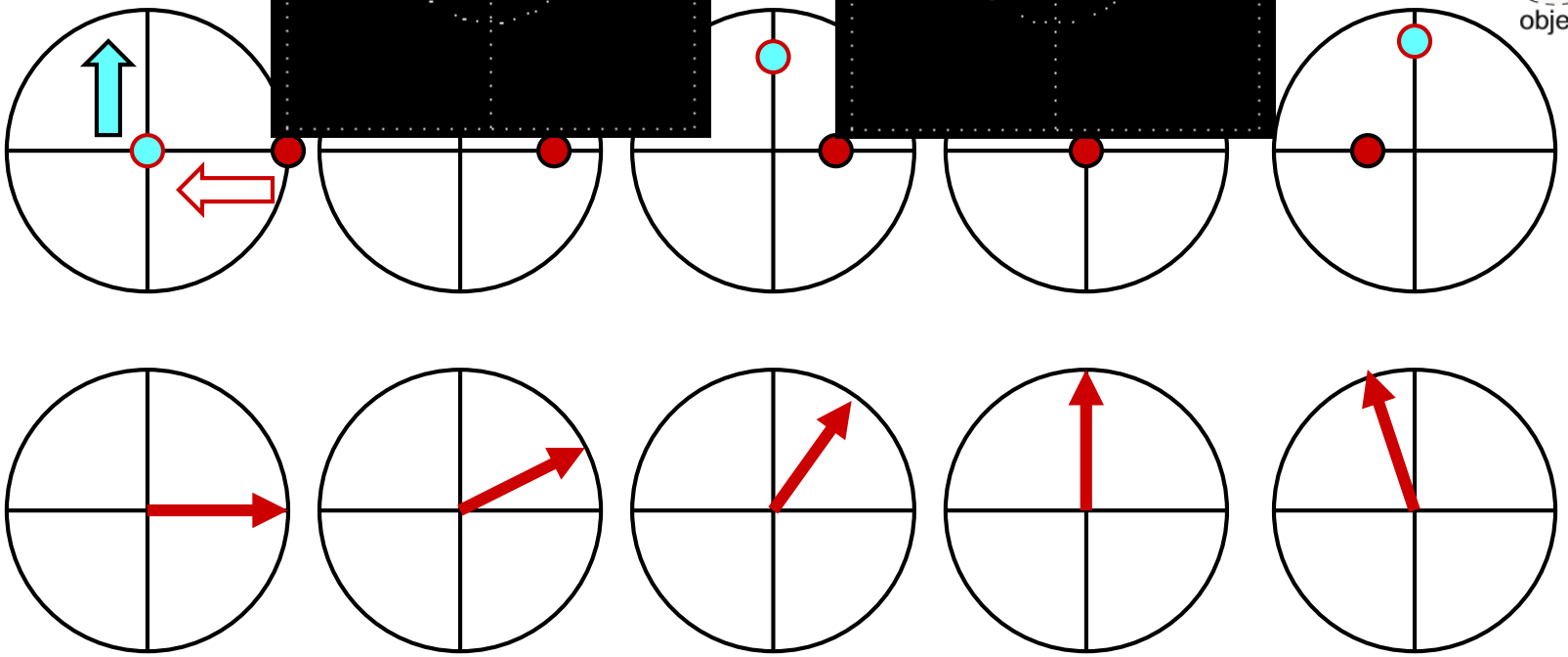
Superposition linearly polarized ($\Delta\phi = 90$)

The two vector
of phase by 90 d



legrees

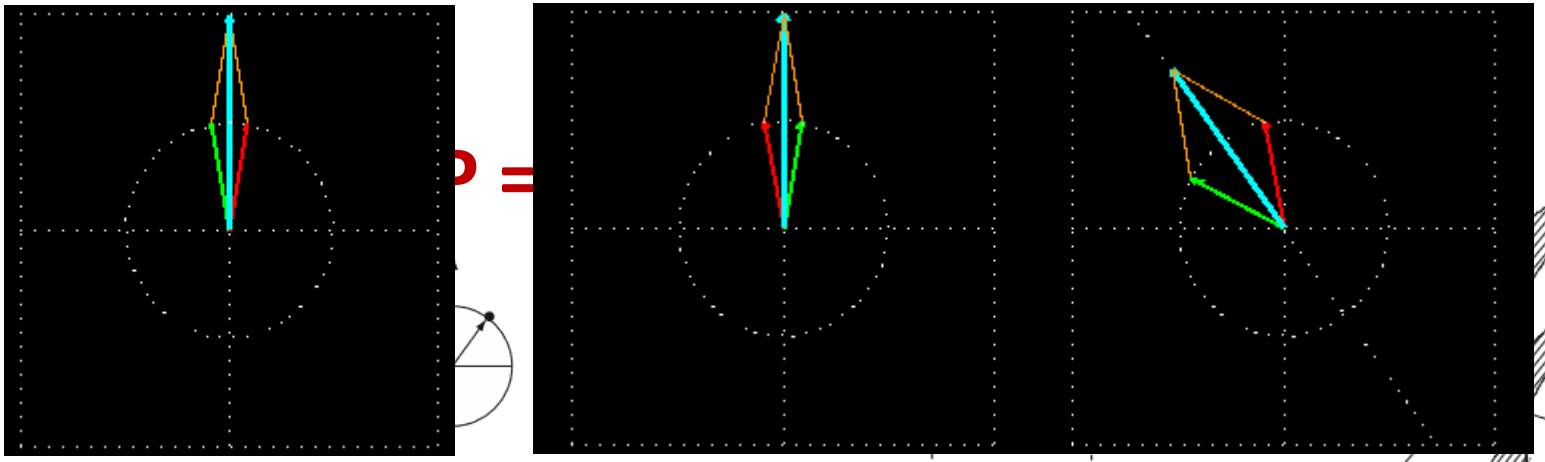


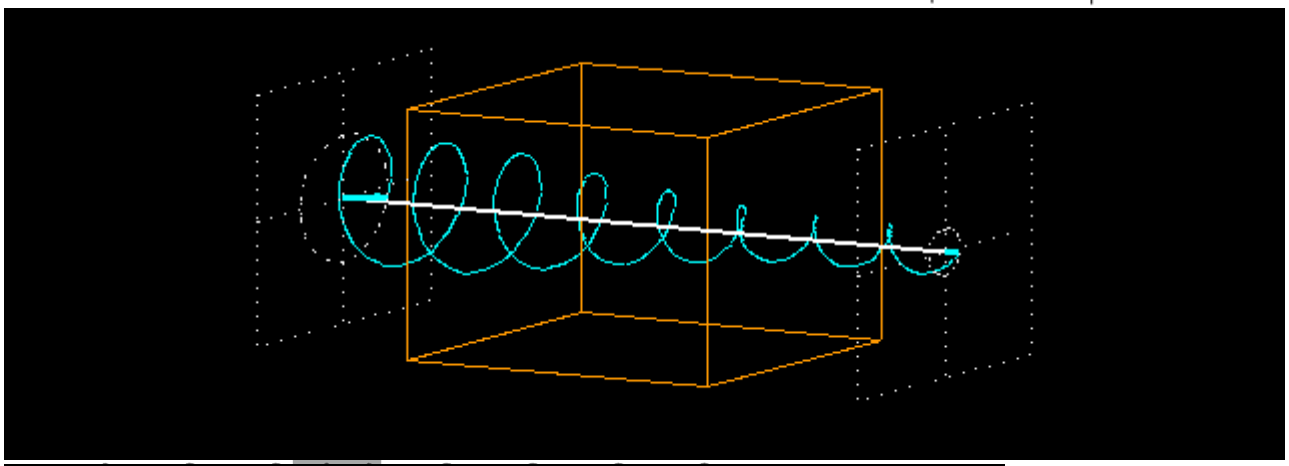


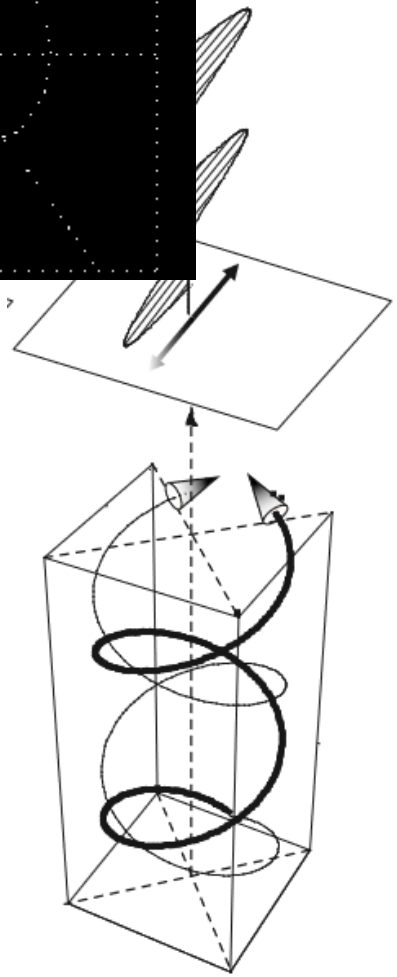
The resulting vector appears to move circularly (anticlockwise)

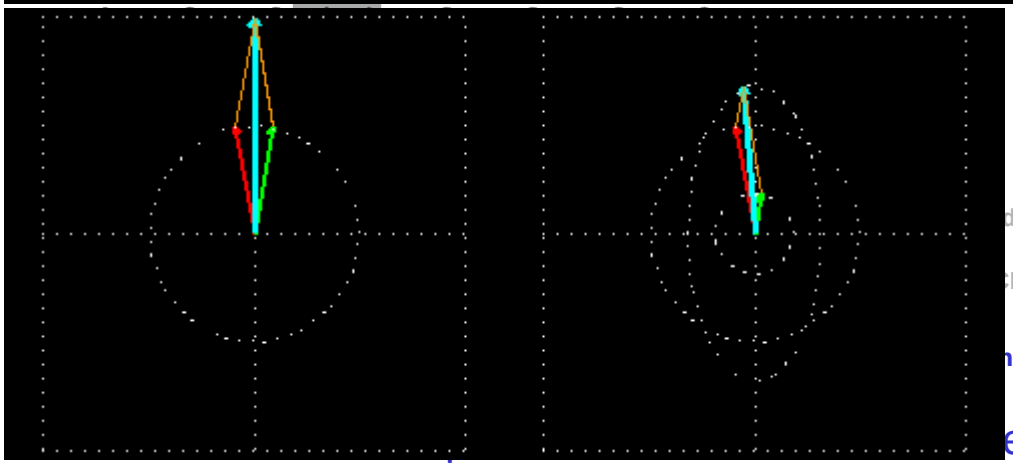
CP clockwise CP counterclockwise

This is how circular polarised light is generated in the CD spectrophotometer *(the relative phase of 2 LP can be shifted 90 or -90 degrees at high frequency)*



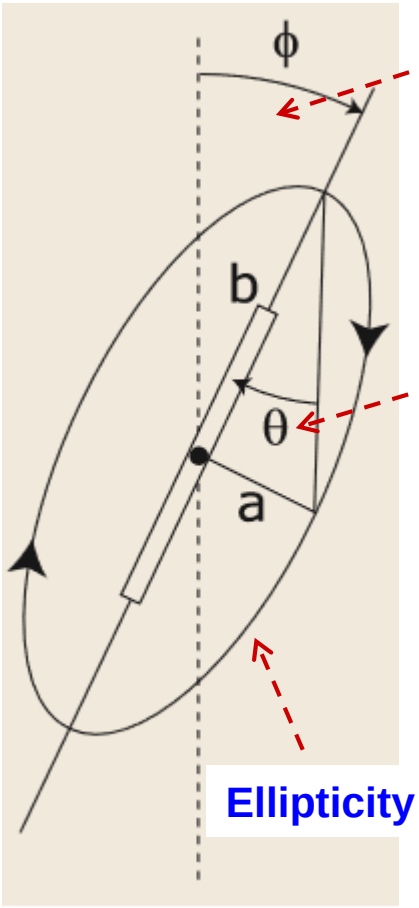






P and LCP
nd LCP
emo/

Ellipticity - degrees



rotation (due to $n_R \neq n_L$). This is the ORD effect.

$$\tan\theta^\circ = \frac{a}{b}$$

In practice, this ellipse looks almost like linearly polarized light)

Suppose **a/b = 0.0035**

$$\theta(\text{deg}) = \tan^{-1} 0.0035 = 0.2^\circ \ \ (200 \text{ mdeg})$$

Ellipticity (due to $A_R \neq A_L$). This is the CD effect.

Convert ‘ellipticity’ to ΔA

$$\theta(\text{deg}) = \frac{2.303(A_L - A_R)}{4} \cdot \frac{180^\circ}{\pi \text{ rad}} = 32.98 \cdot \Delta A$$

$$\Delta A = \frac{0.2^\circ}{32.98} = 6 \cdot 10^{-3} \text{ units of absorbance}$$

Example of calculation – normalization as Δε

Ellipticities or ΔA cannot be used for comparison because they depend on concentration and pathlength. To normalize results, extinction coefficients are compared.

$$\Delta\varepsilon = \frac{\Delta A}{c \cdot l}$$

However, the chromophore is the peptidic bond. Therefore the signal depends, not on the molar concentration of protein, but on molar concentration of amino acids (using the mean residue MW).

Protein conc: ~ 0.1 mM

$$\text{Mean residue MW} = \frac{10{,}000}{90} = 111.11 \frac{g}{mol}$$

Amino acid conc: 9 mM

$$\Delta\varepsilon = \frac{0.006}{0.009M \cdot 0.1cm} = 6.666\ M^{-1}cm^{-1}$$

$$[\theta] = \frac{0.2\,\text{deg}}{0.009 \frac{mol}{dm^3} \cdot \frac{1dm^3}{10^3 cm^3} \cdot 0.1cm \cdot \frac{10dmol}{1mol}} = 22{,}222\,\text{deg} \cdot cm^2 \cdot dmol^{-1}$$

Example:

$$\Delta A = 6 \times 10^{-3}$$

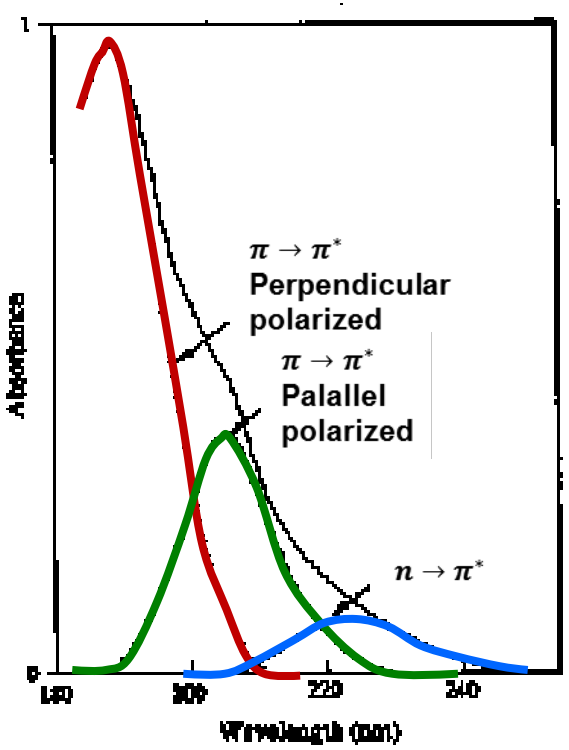
$$MW = 10{,}000$$

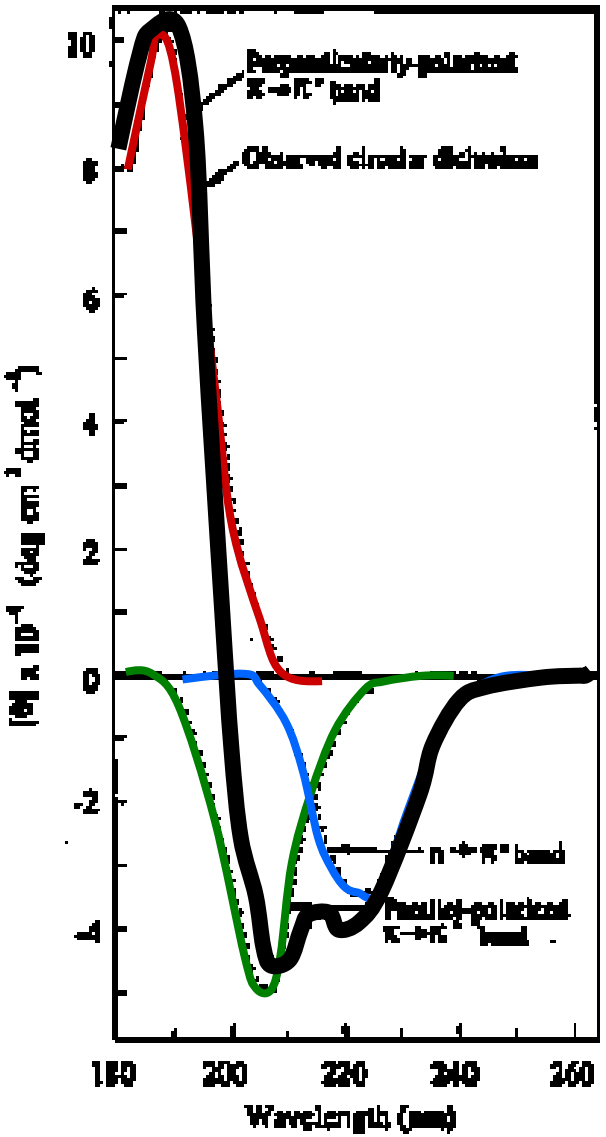
$$N_r = 90aa$$

$$c = 1mg/mL$$

$$l = 0.1cm$$

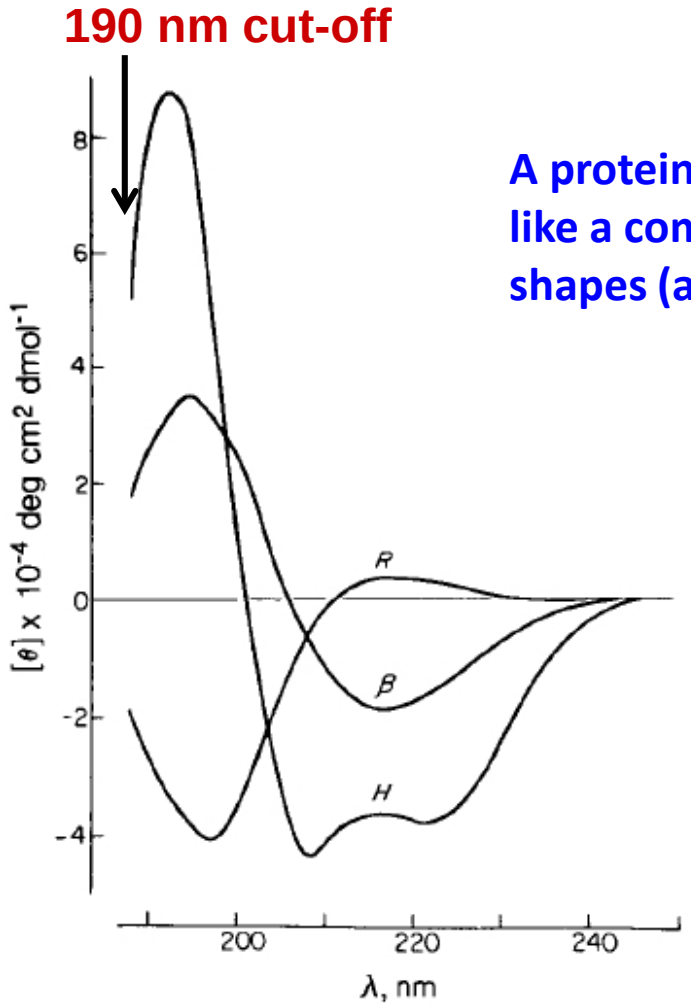
A CD spectrum is a difference spectrum





Polylysine spectra obtained in various experimental conditions

The lower the cut-off, the better (more information is available to discriminate similar shapes)

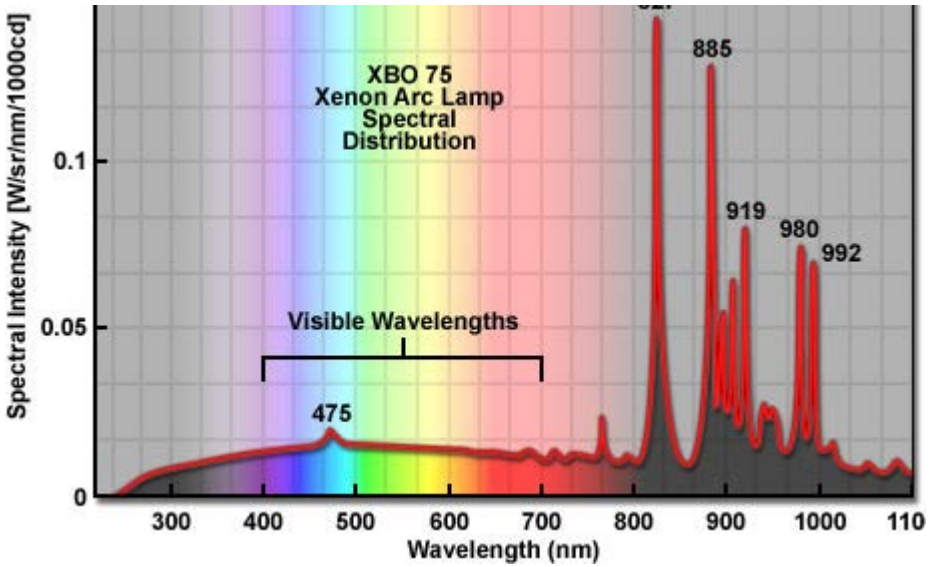


A protein spectrum will look like a combination of these shapes (and several others)

FIG. 1. CD spectra of the helix, β-form, and unordered form based on $(Lys)_n$ (M_r = 193,000) in water at 25°. Curves: R, unordered form at neutral pH; H, α-helix at pH 10.8; β, β-form at pH 11.1 after heating for 15 min at 52° and cooling back to 25°. Concentration of $(Lys)_n$: 0.07%. (From Yang and Kubota[20] with the permission of Plenum and copyrighted by Plenum.)

Xenon (Xe) Arc Lamps

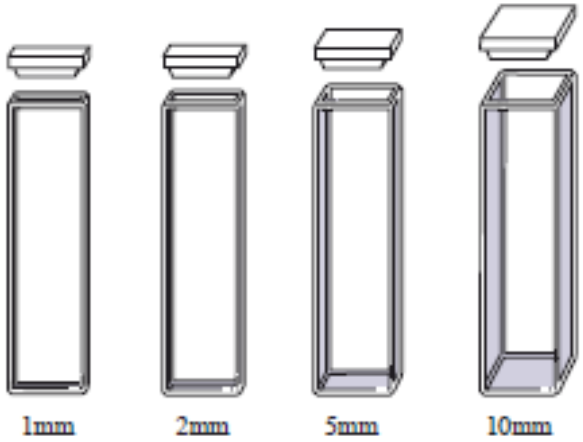
Conventional CD spectrometers are limited to >190 nm

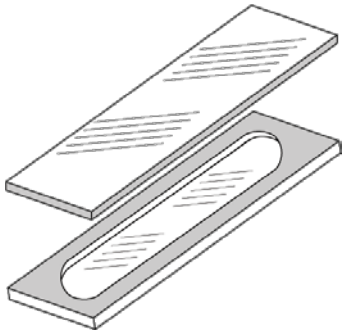


Problems at short wavelengths

- Low intensity below 190 nm from Xe arc lamp
- Buffers
- Salts
- Oxygen
- Scattering from large particles
- Water absorbs below ~ 175 nm

} Reduce pathlength while increasing protein concentration



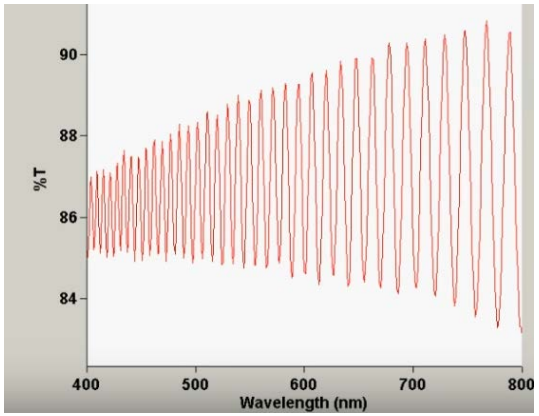


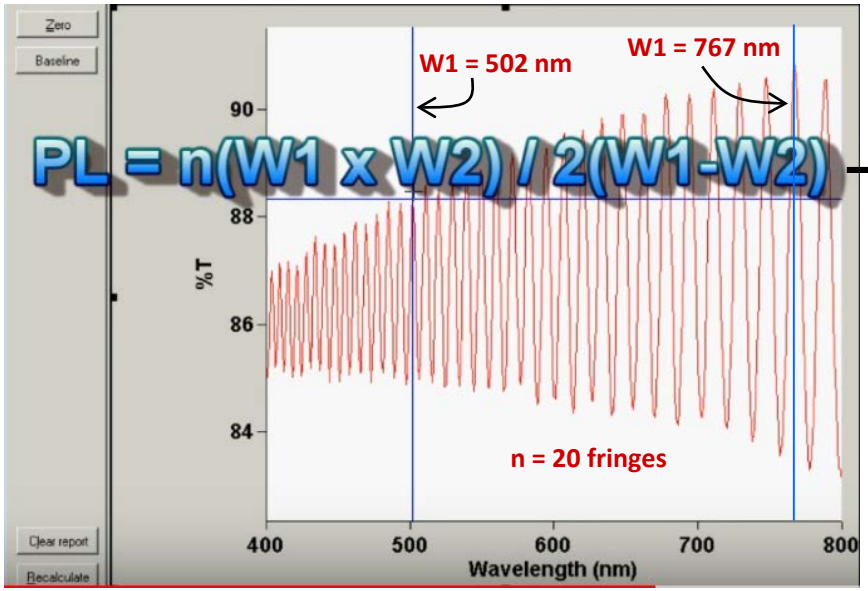
0.1 mm to 10 μm

UV Quartz (190-2,500nm) ⟶ CaF_2 (<190 nm)

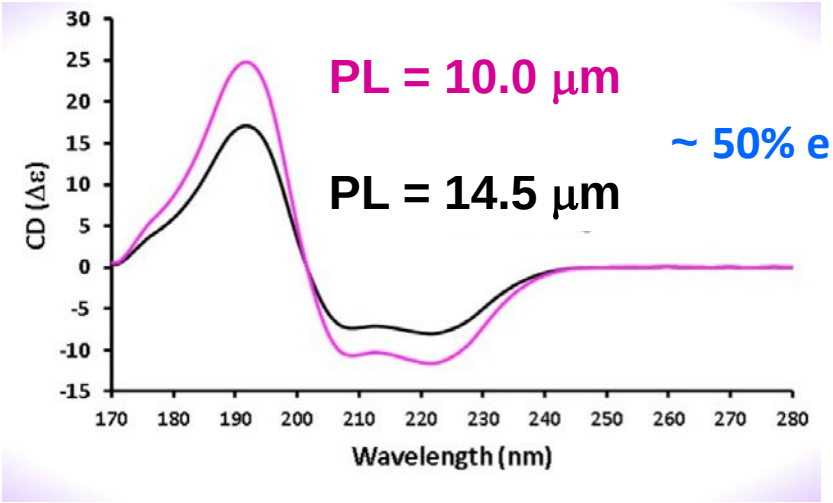
Pathlength determination (<100 μm)

Interference fringes in the transmission spectrum from an empty cell





14,530 nm (14.5 μm)



A. Miles (2017) ThePcddb https://www.youtube.com/watch?v=fCN7qWDmRLc

CD and secondary structure analysis

Protein CD data bank

Protein Circular Dichroism Data Bank

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- 2Struc
- DichroMatch
- CreateRDS
- ValiDichro
- PDB2CD

Current holdings (live data):
Released entries: 554
Entries in pre-release: 351

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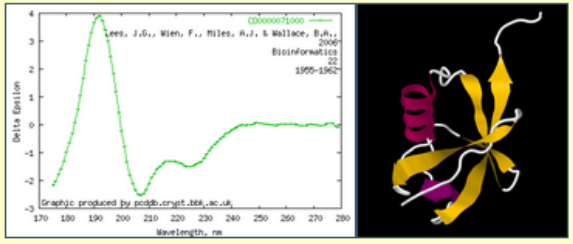
Whitmore, L., Miles, A.J., Mavridis, L., Janes, R.W. and Wallace, B.A., PCDDB: new developments at the Protein Circular Dichroism Data Bank. Nucleic Acids Research (2017) 45 (D1): D303-D307.

Featured Spectrum of the Month (November 2017)

CD0000071000 - Ubiquitin



Previous featured spectra

[YouTube video] Accurate measuring of the true pathlength of optical CD cells

For PCDDB feedback, please email : pcddb @ mail.cryst.bbk.ac.uk.

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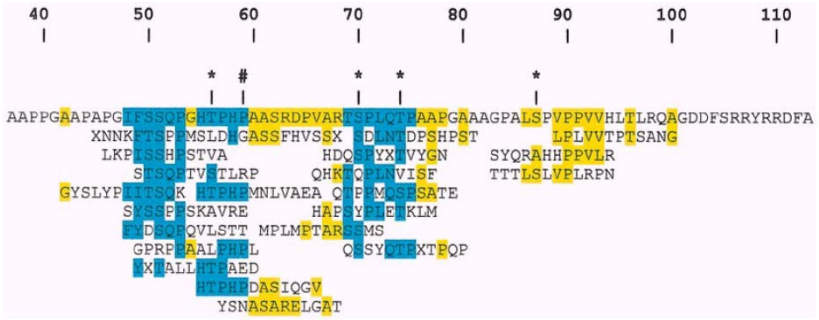
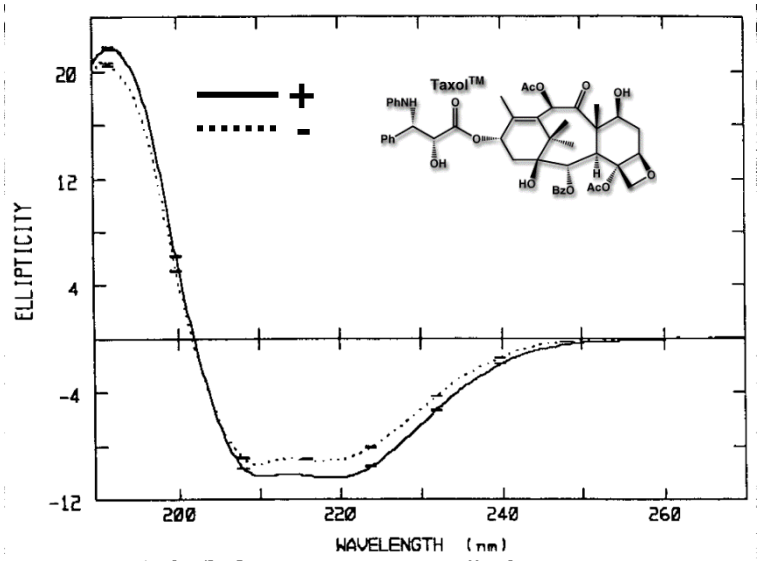
Protein-drug interaction

Random library of phage-displayed peptides screened for binding to a biotinylated derivative of anticancer drug paclitaxel (Taxol).
Affinity-selected peptides found similar to a loop region of anti-apoptotic human protein Bcl-2

~15 aa involved in binding



~4% change (~12 aa) in CD spectrum



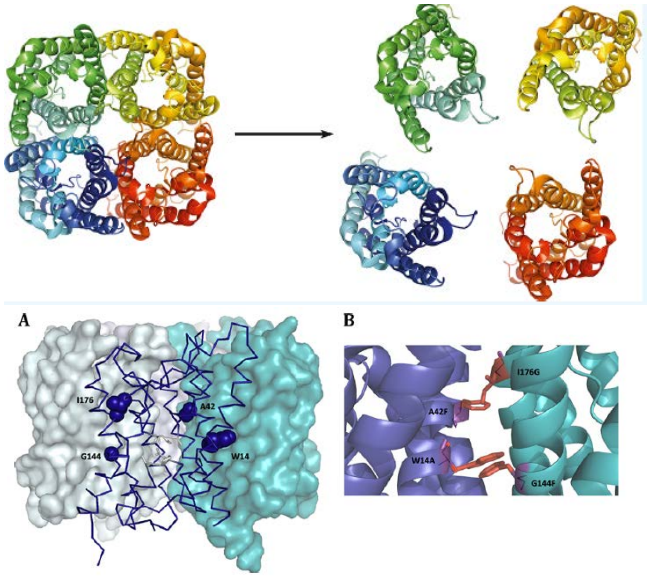
Conformational change of BcL-2 shown by CD.
In vivo, treatment with Taxol leads to Bcl-2 inactivation with phosphorylation (*) of residues in a disordered, regulatory loop region of the protein.

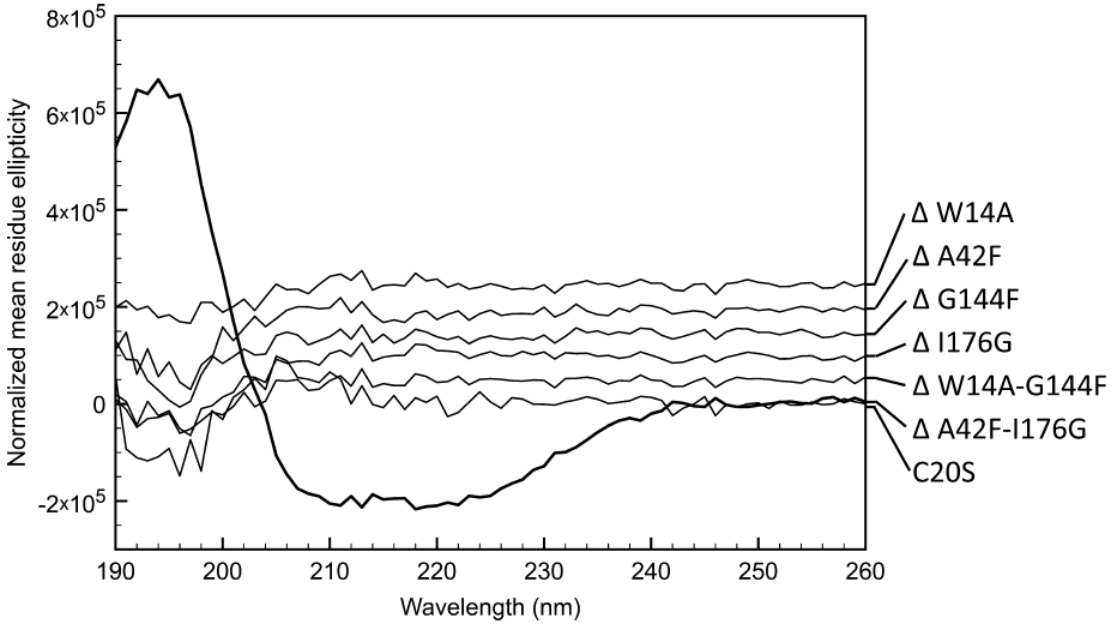
Paclitaxel Directly Binds to Bcl-2 and Functionally Mimics Activity of Nur77. Ferlini et al. (2009) Cancer Res. DOI: 10.1158/0008-5472.

Rodi et al., (1999) J. Mol. Biol. 285, 197-203

Investigation of residues important for packing in a membrane protein.

Mutations introduced at the hydrophobic interfaces on the structure and function of the tetrameric Escherichia coli water channel aquaporin Z (AqpZ).

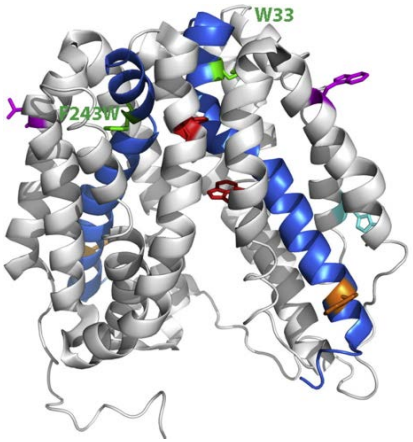


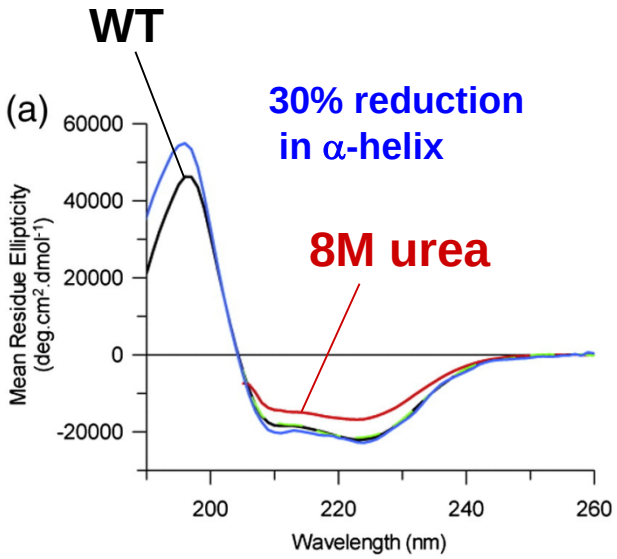


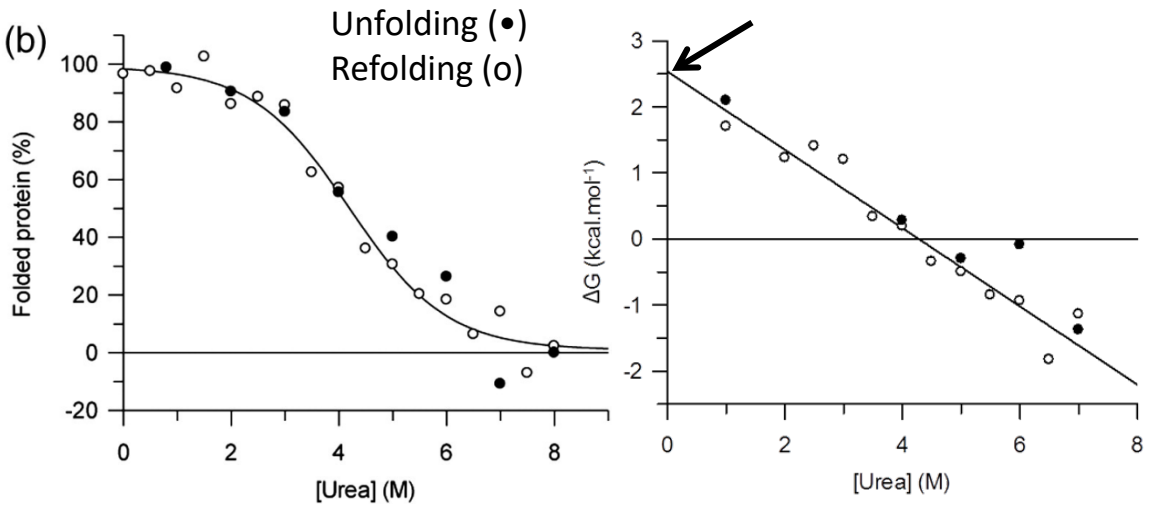
CD spectra of AqpZ proteins in detergent DDM.

Schmidt and Sturgis (2017) DOI: 10.1021/acsomega.7b00261. ACS Omega , 2, 3017–3027

Protein stability- free energy of unfolding of Lactose permease (LacY) in DDM detergent





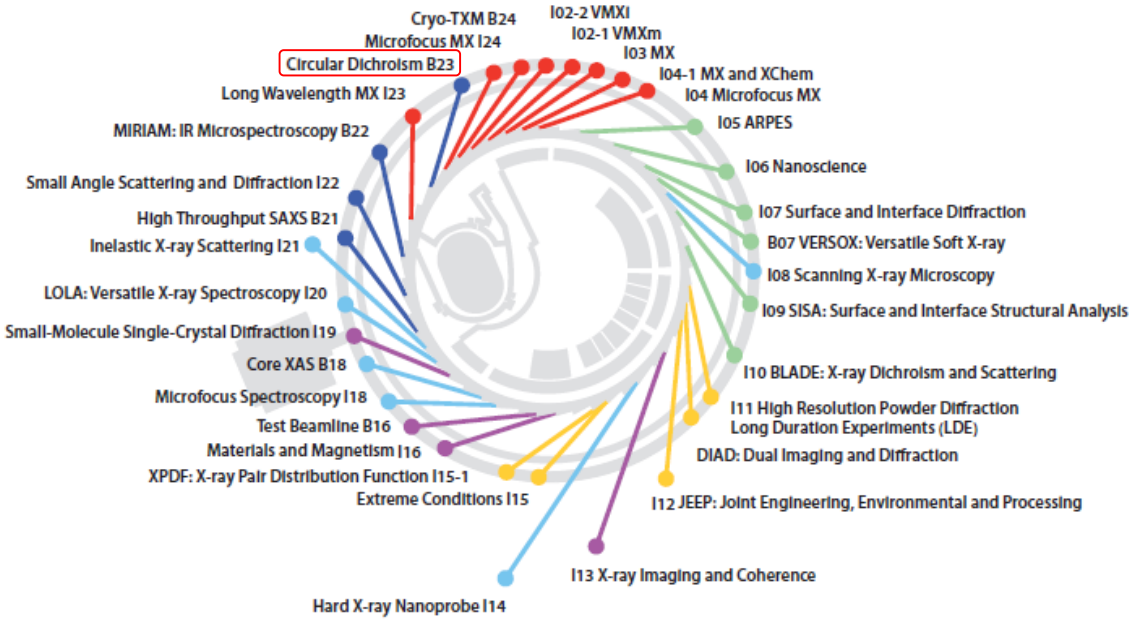


ΔGU_{H2O}, of + 2.5 ± 0.6 kcal mol^{-1}

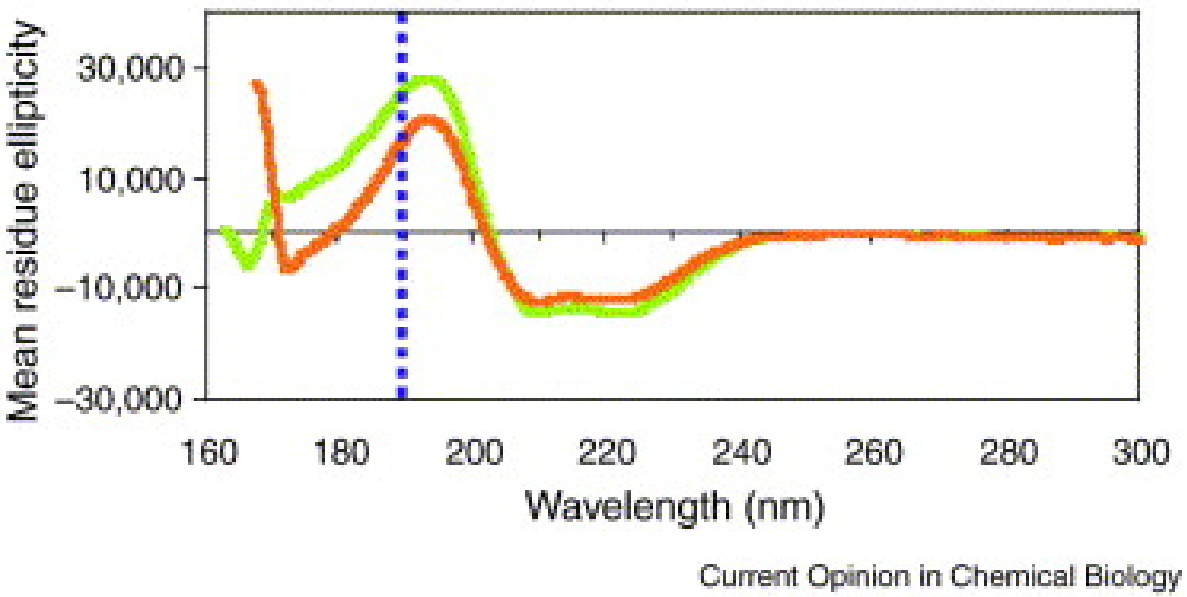
Harris et al., (2014) J. Mol. Biol. 426, 1812-1825

Sinchrotron Radiation Circular Dichroism (SRCD)

Synchrotrons accelerate electrons to near light speeds and emit high brilliance light These bright beams are then directed off into ‘beamlines’. Diamond Beamline B23



SRCD: Spectral discrimination at short wavelengths



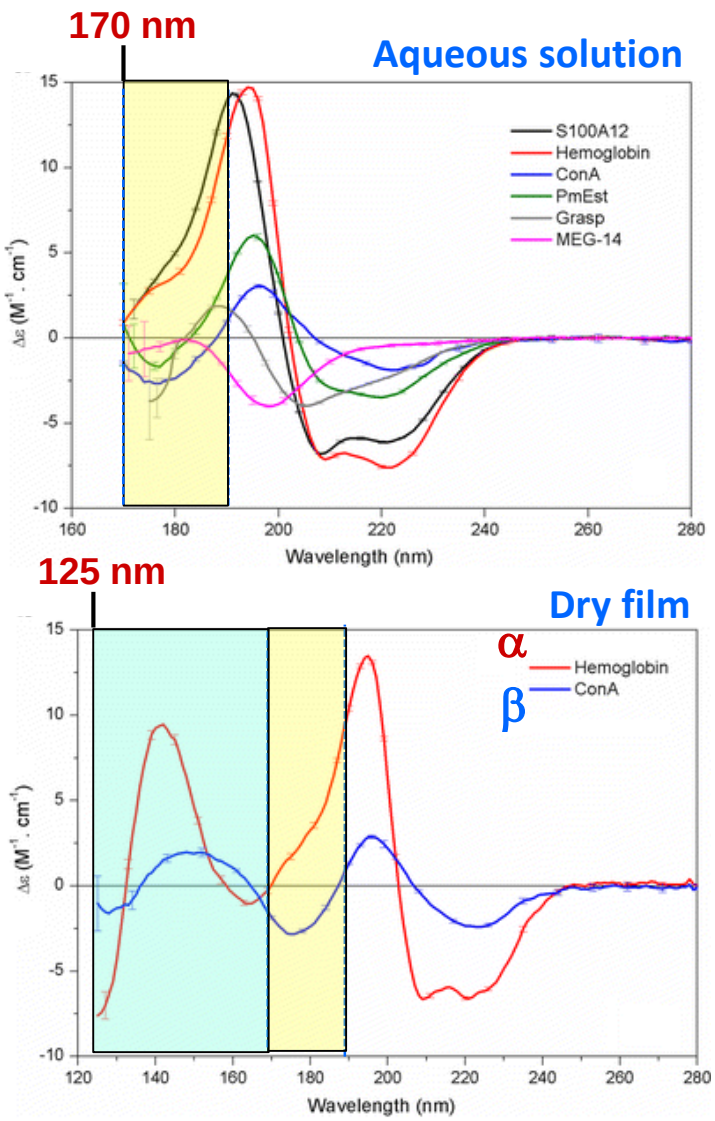
SRCD spectra of two proteins

74% helix, 0% sheet, 10% turn, 16% other
48% helix, 5% sheet, 16% turn, 31% other

Only when the low-wavelength data (left of the vertical line) are considered, differences are obvious.

Wallace & Janes Curr. Op. Chem. Biol. (2001) 5, 567-571

SRCD advantages



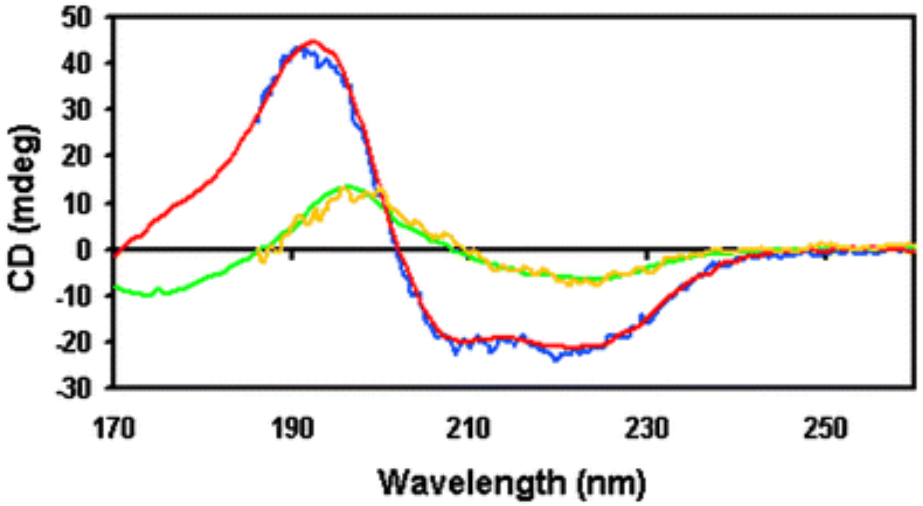
High flux of photons and collimated beam (~2 mm^2). Intensity of SRCD beam (VUV region, <190 nm) is > 10^3 times those of conventional CD.

- lower sample concentration/volumes
- Fast collection (kinetic studies)
- High S/N ratio = minute differences detected
- Use of scattering samples
- Use of absorbing buffers

Longer spectral range for data collection: aqueous solutions to 160 nm, dry films to 125 nm (more information)

- more precise secondary structure determination
- More structural motifs can be discerned

SRCD: higher S/N ratio, especially at short λ



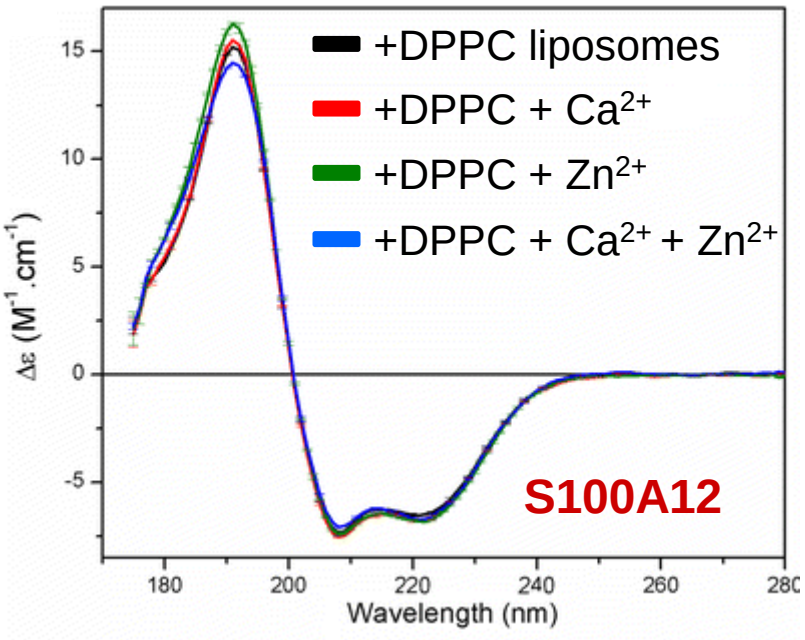
Myoglobin
Myoglobin SRCD

Concanavalin
Myoglobin SRCD

Miles and Wallace (2006) Chem. Soc. Rev., 35, 39-51

Protein-partner interactions by SRCD

Protein and liposomes

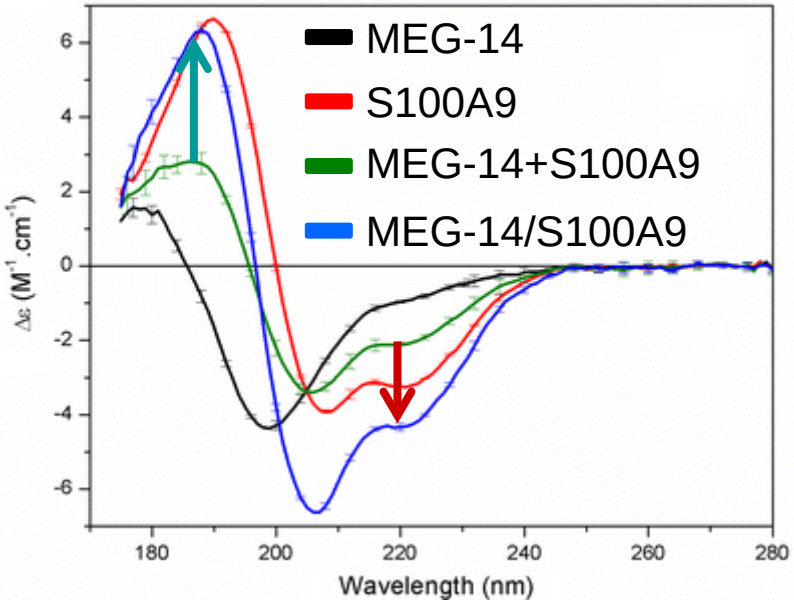


High photon flux of SRCD allows studies in presence of scattering (e.g., liposomes, LUVs).

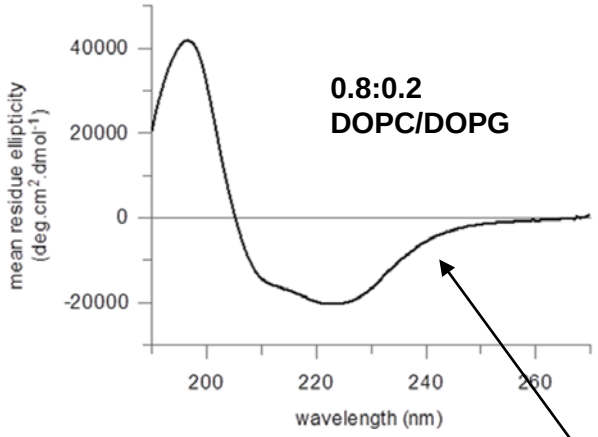
This can also be done with in-house CD, but access to lower λ allows more accurate determination of the changes taking place at the complex:

Transition disordered ⟶ α-helix

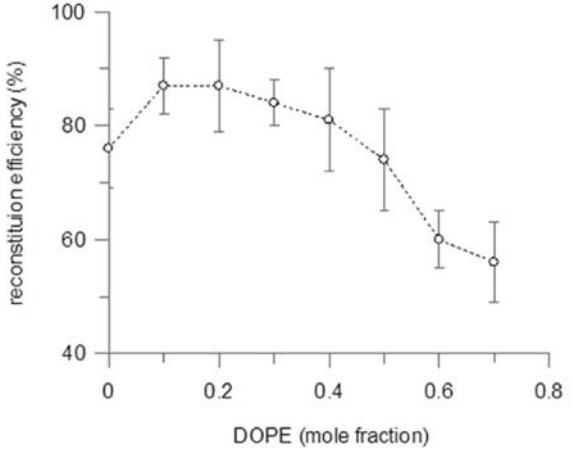
Protein-protein interactions

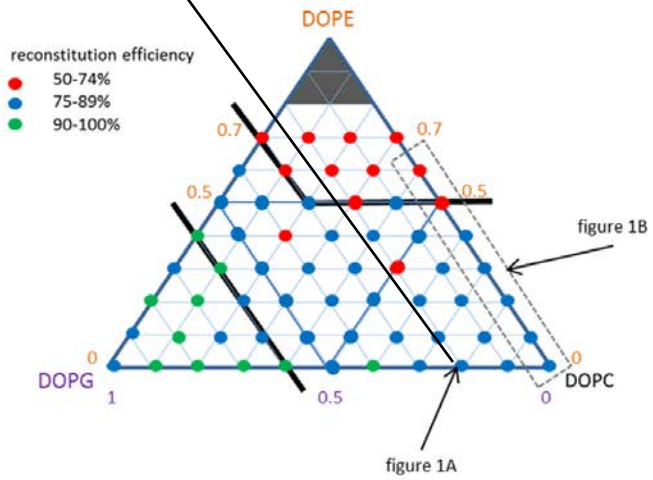


Effect of lipid composition of reconstitution folding, stability of lactose permease (LacY)



0.8:0.2 DOPC/DOPG



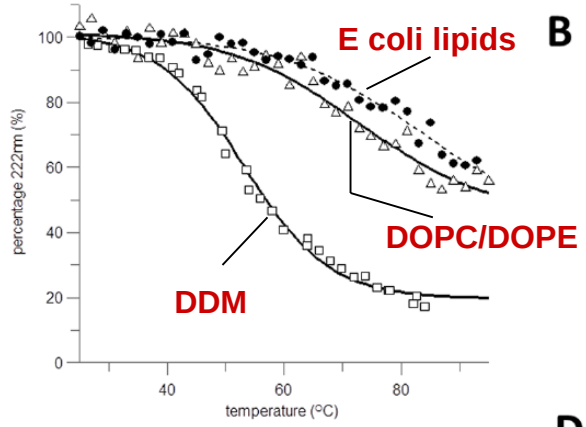


Efficiency of reconstitution into liposomes + OG from DDM micelles

Findlay and Booth (2017) Scientfic Reports, 7, 13056

Effect of lipid composition of reconstitution folding, stability of lactose permease (LacY)

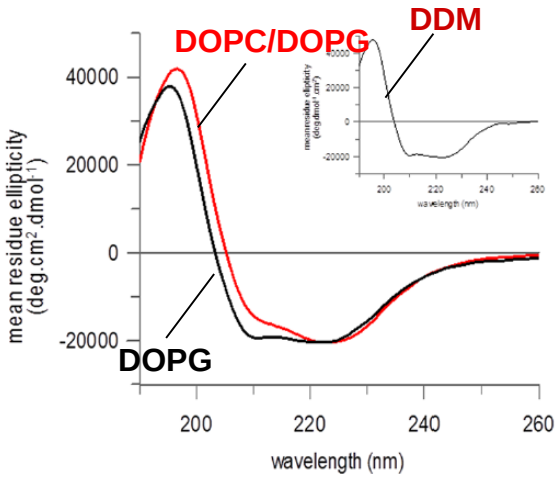
A



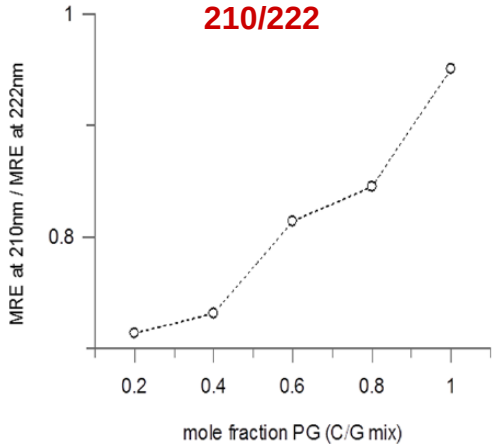
B

	Tm
DDM	53
PC	67
0.8/0.2 PC/PE	70
0.6/0.4 PC/PE	71
0.4/0.6 PC/PE	74
E. coli	81

C

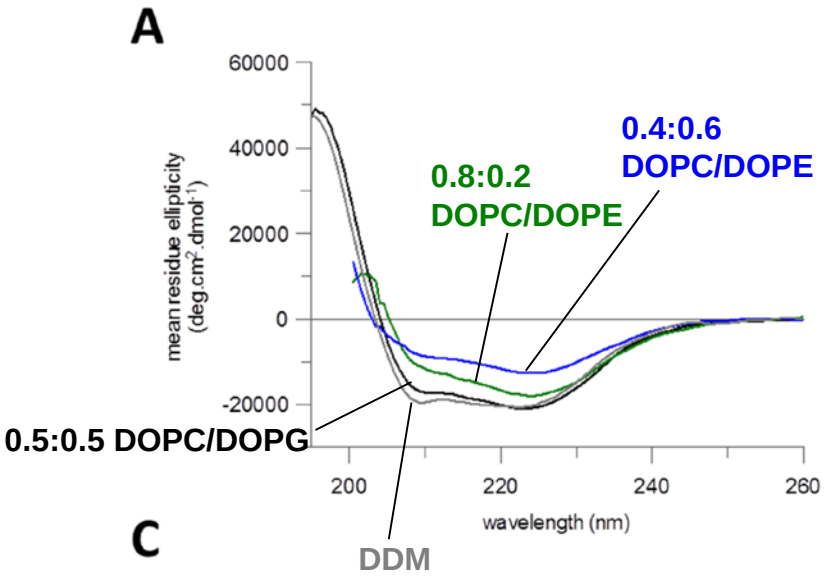


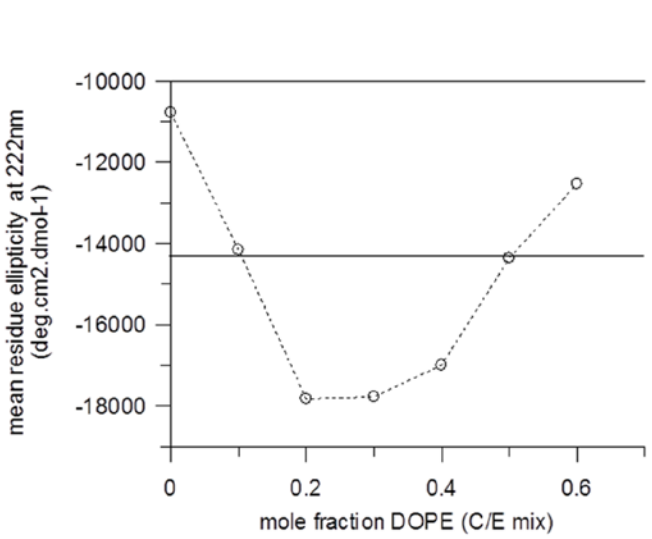
D



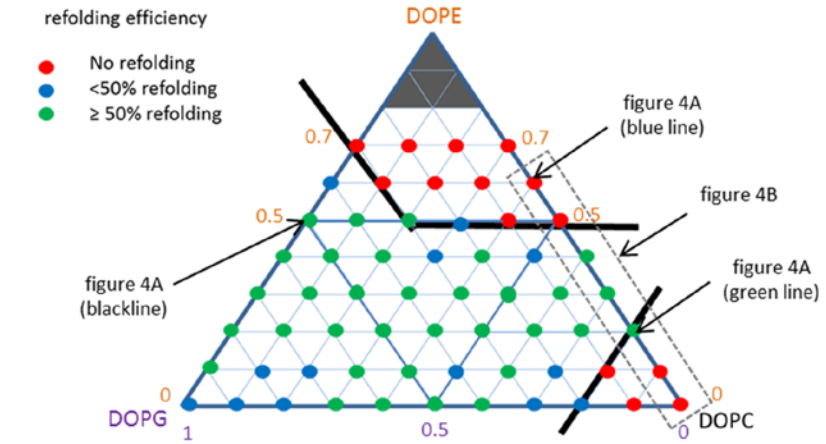
Findlay and Booth (2017) Scientfic Reports, 7, 13056

Effect of lipid composition of reconstitution folding, stability of lactose permease (LacY)





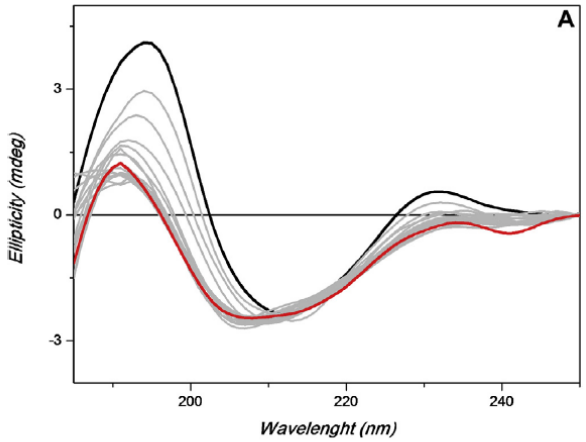
Denat.

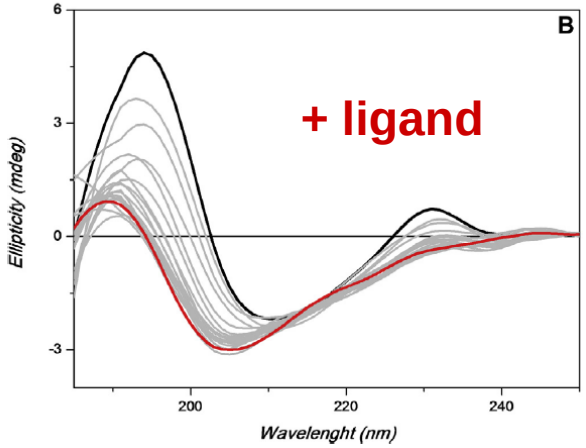


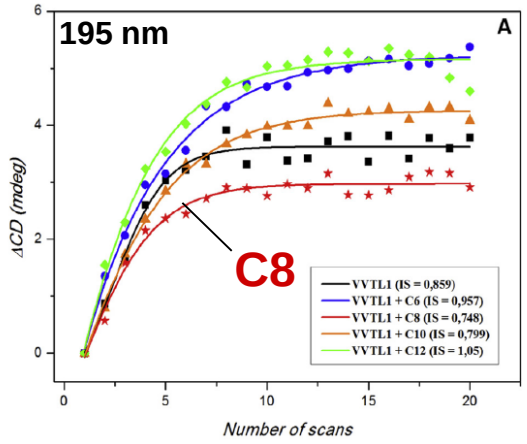
Refolding from urea into lipid vesicles

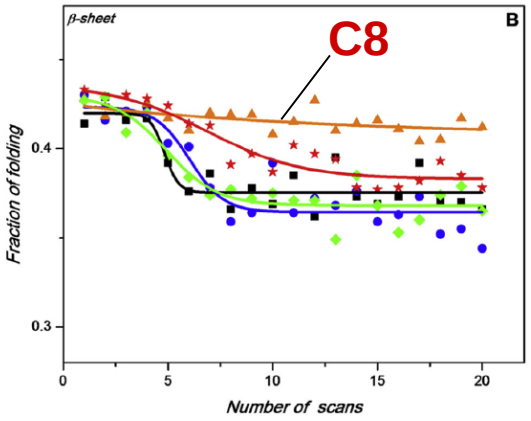
Ligand binding- photo and thermal denaturation assays

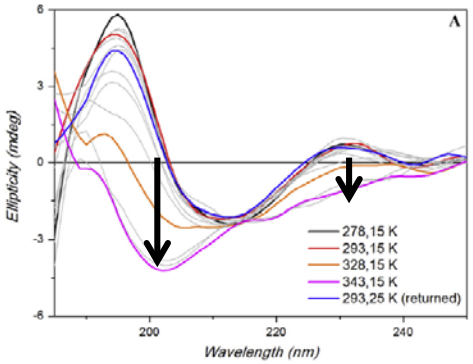
Interaction of ethyl esters with proteins in wine

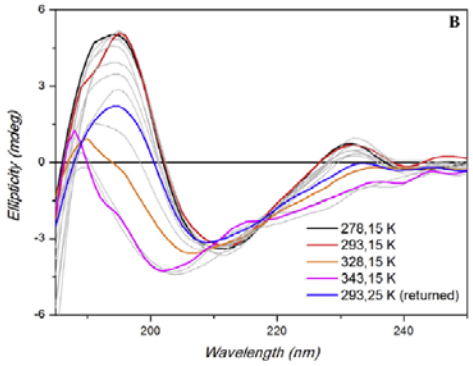


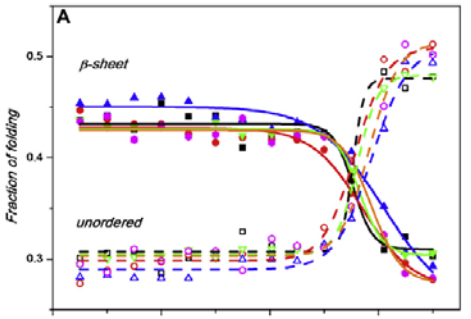










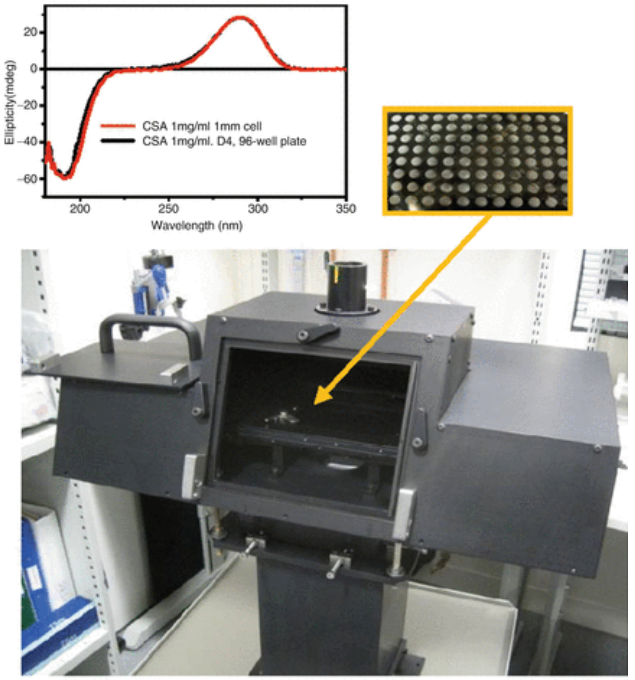


Di Gaspero et al. (2017) 217, 373-378

HT-CD: Quality Control of Protein Folding

- assessing protein folding in solution
- effect of buffer conditions on secondary structure, which informs on how a protein sample behaves in crystallization trials
- screening of the binding properties of the proteins in e.g., crystallization buffers.
- Batch variability

Chirascan-auto qCD (liquid handling robot)



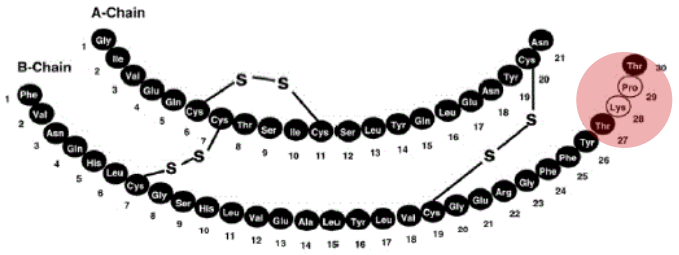
SRCD 96 or 284 well plates (beam scans the plate)

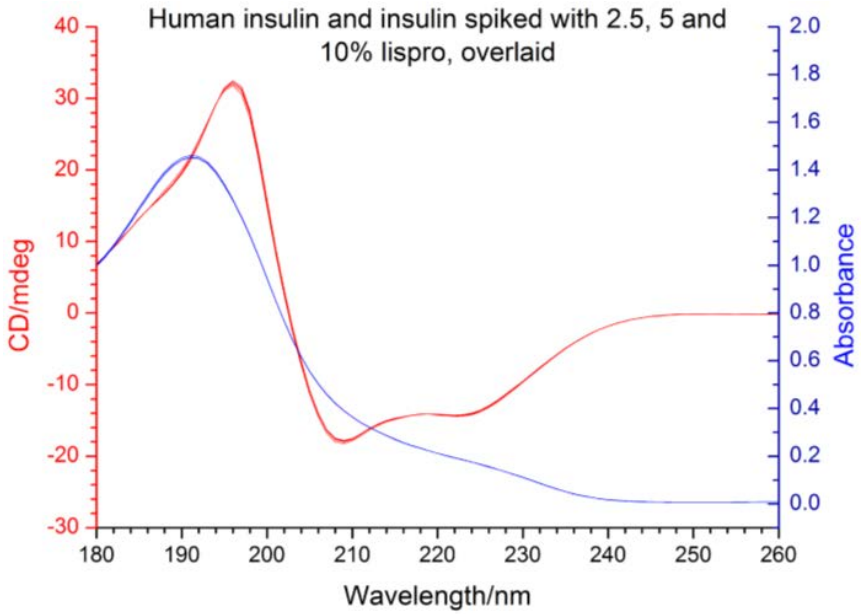
Siligardi and Hussain (2014) Structural Proteomics MIMB, 1261, 255-276

qCD-resolves small differences in spectra

Biotherapeutics, comparison of higher order structures of proteins. Control of systematic error and random error to achieve accuracy and precision. qCD eliminates or correct systematic error (e.g. multipoint CD calibration) to achieve reproducible results and quantification.

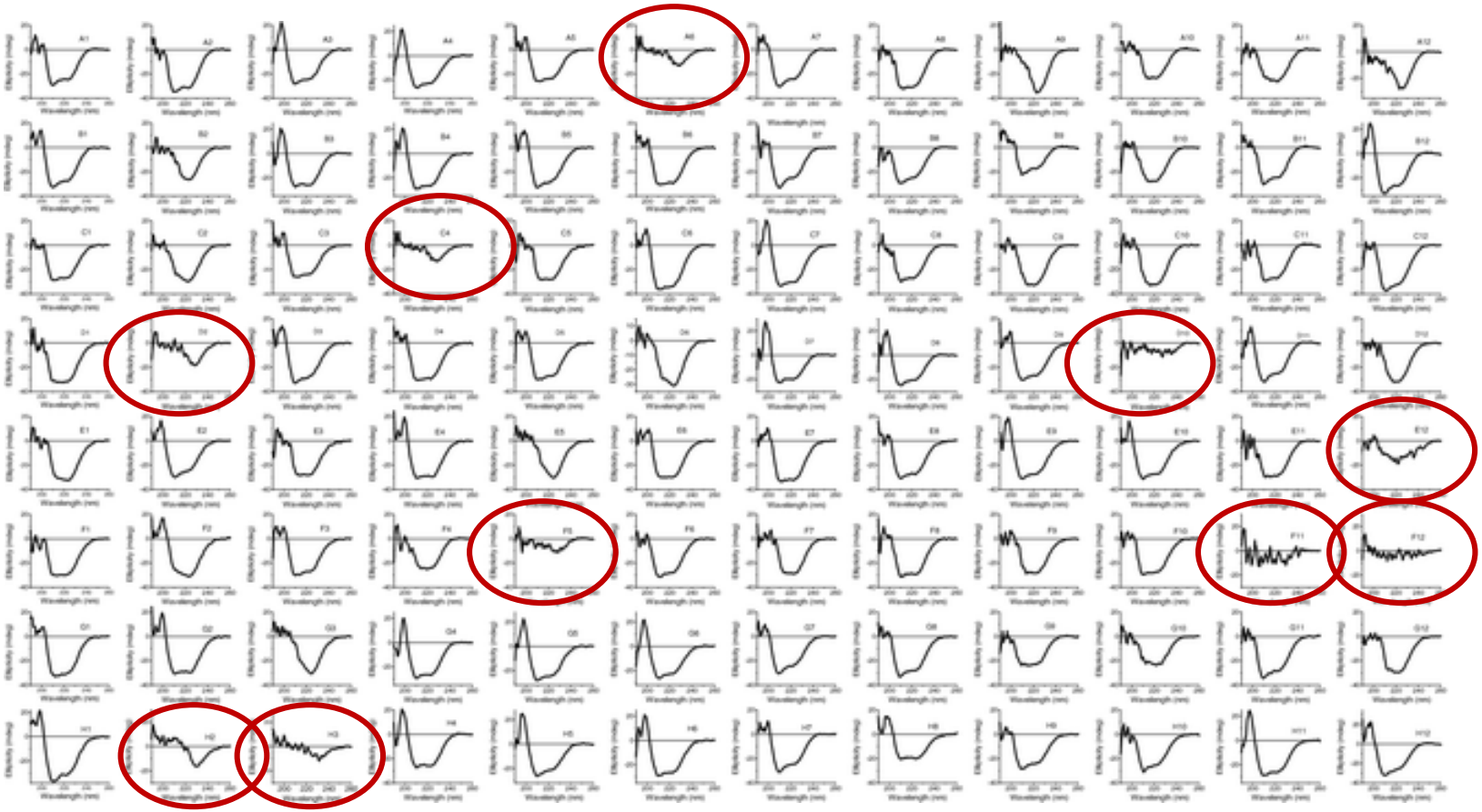
- **One single automated experiment**
- **4 protein samples (human insulin + 2.5, 5 and 10% lispro analog**
- **12 alliquotes of each**
- **farUV CD and absorbance collected**
- **Spectra scored for similarity**





Applied Photophysics (www.photophysics.com)

HT-CD: Quality Control of Protein Folding

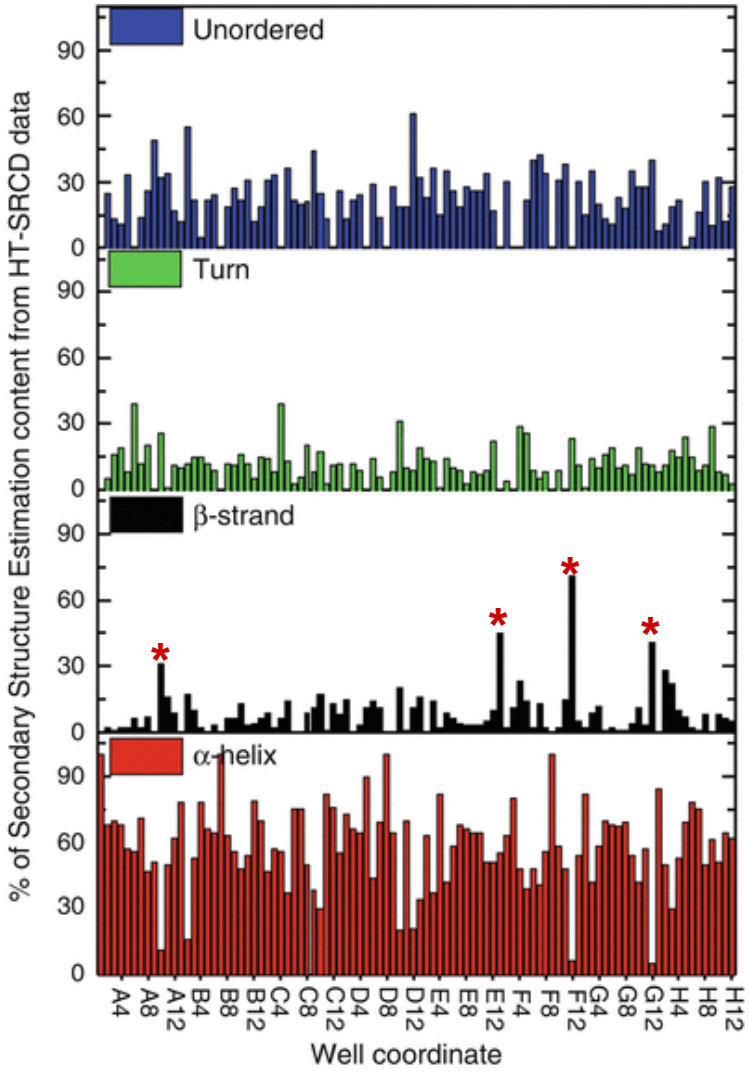


SRCD spectra of 96 myoglobin solutions prepared from 96 crystallization buffer conditions of MemGold2™

High salt may interfere with % helix quantification

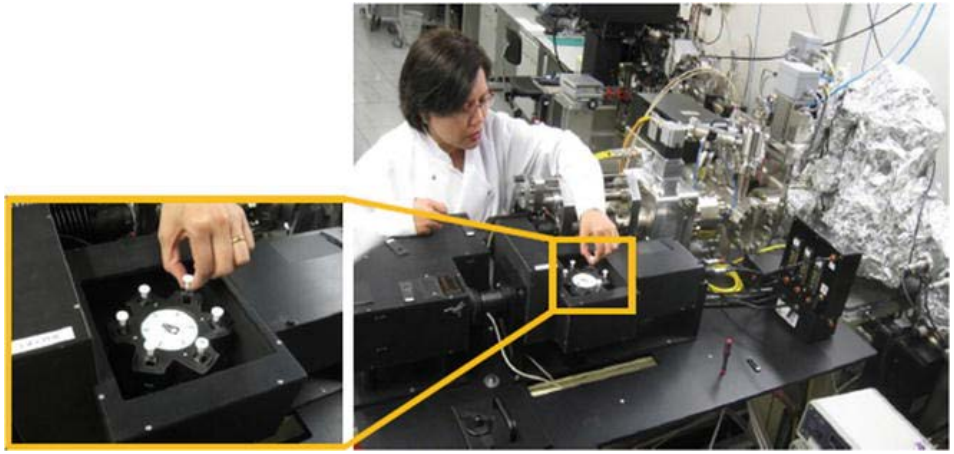
Siligardi and Hussain (2014) Structural Proteomics MIMB, 1261, 255-276

HT-CD: Quality Control of Protein Folding

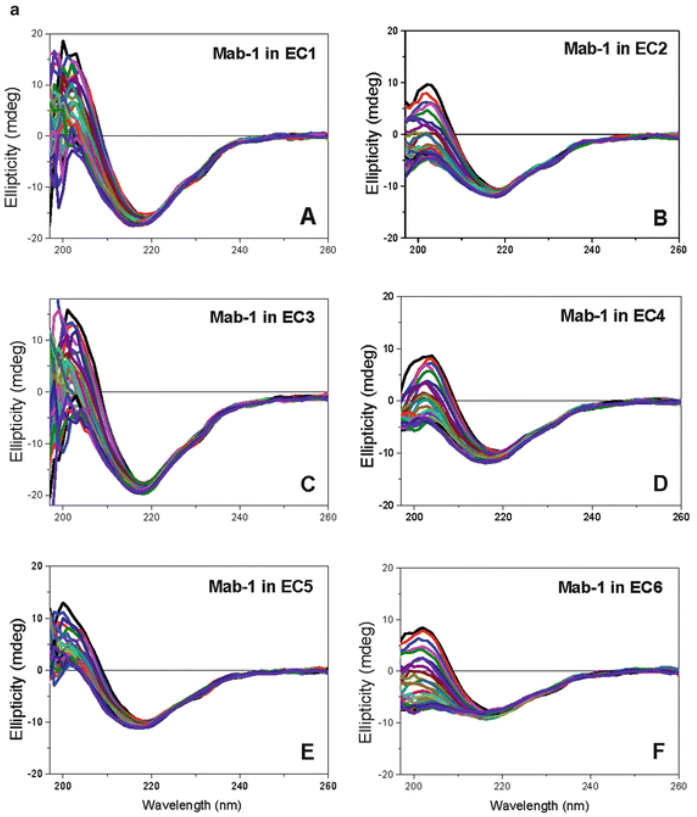


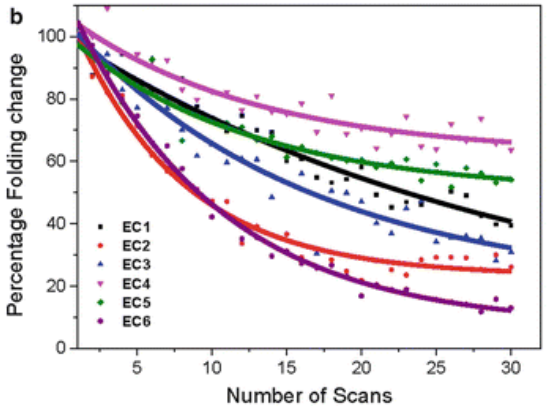
Siligardi and Hussain (2014) Structural Proteomics MIMB, 1261, 255-276

HT-CD: Quality Control of Protein Folding



6-Cell Turret of Diamond B23 module B beamline used for SRCD UV-protein denaturation or variable temperature measurements in the 5–95 °C range



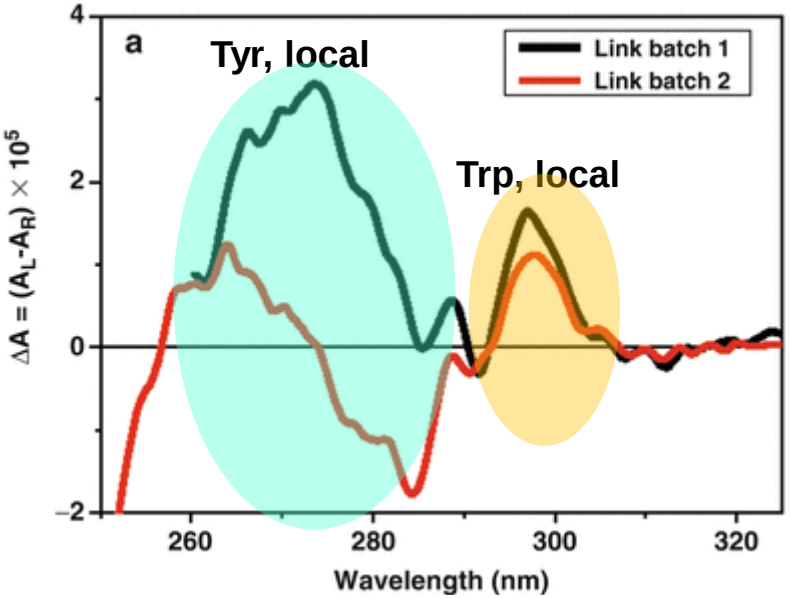


SRCD UV-denaturation assay in the far-UV region of a monoclonal antibody (Mab1) in six different formulations (EC1 to EC6).

Siligardi and Hussain (2014) Structural Proteomics MIMB, 1261, 255-276

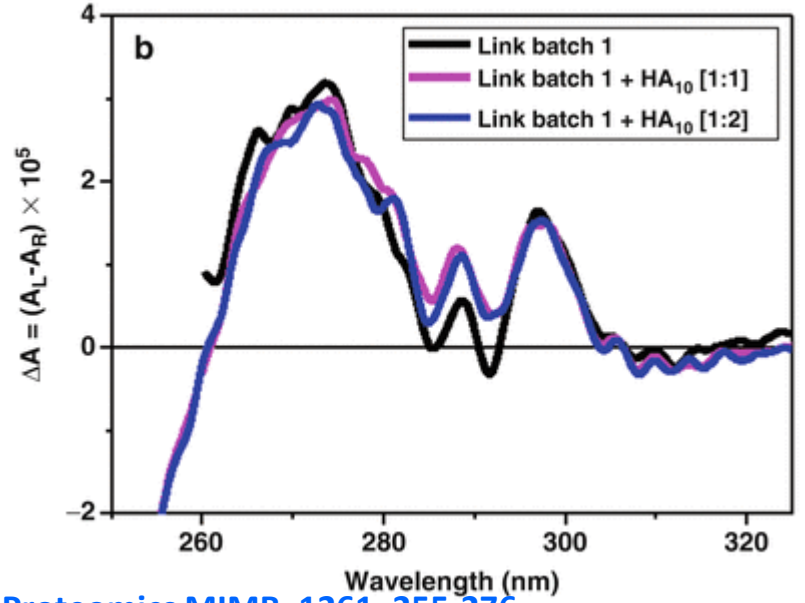
SRCD: QC protein folding and ligand binding

The Link module of human TSG-6 glycoprotein is involved in the formation of the extracellular matrix and cell migration by interacting with hyaluronan 10 (HA_{10}).



Near-UV CD of two batches of TSG-6 Link Module protein.

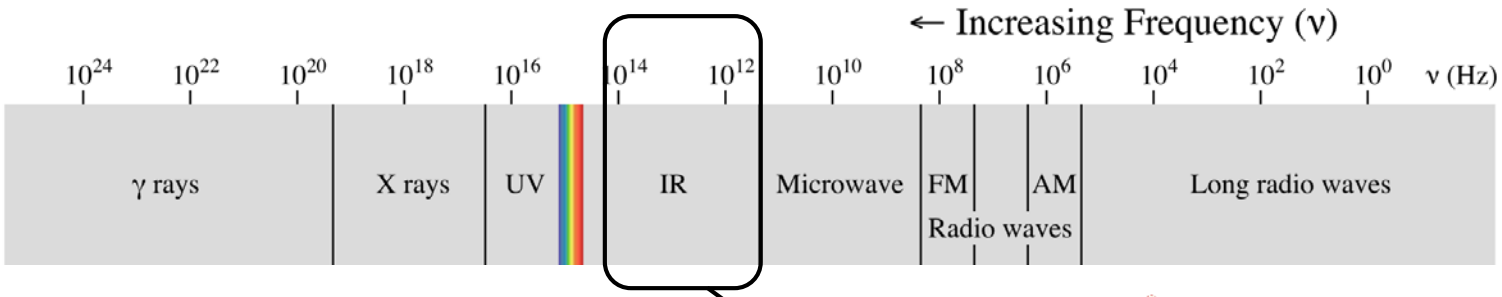
No major involvement of aromatics in binding, consistent with NMR

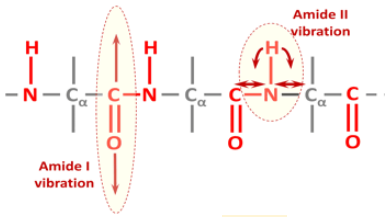


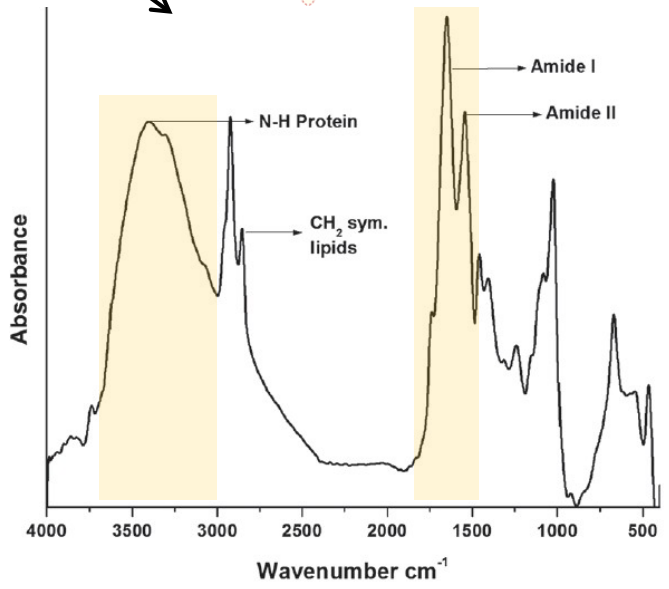
Addition of binder, hyaluronan 10 (HA10).

Siligardi and Hussain (2014) Structural Proteomics MIMB, 1261, 255-276

CD and IR spectroscopies – common chromophore

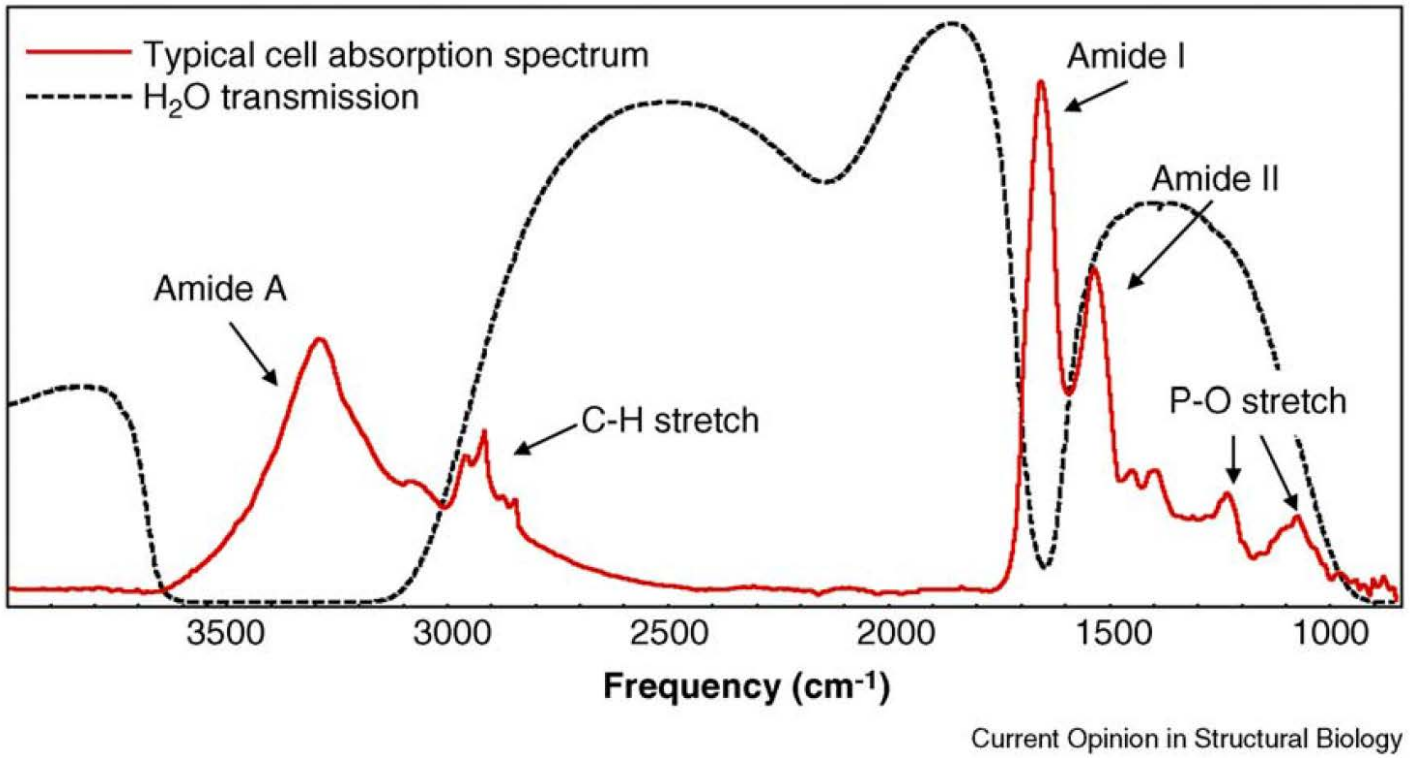






Water interference

IR spectroscopy - water has a high absorption in the IR and obscures amide A and I

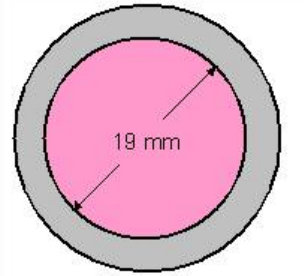


Miller and Dumas (2010) Curr. Opin. Struct. Biol. 20:649–656

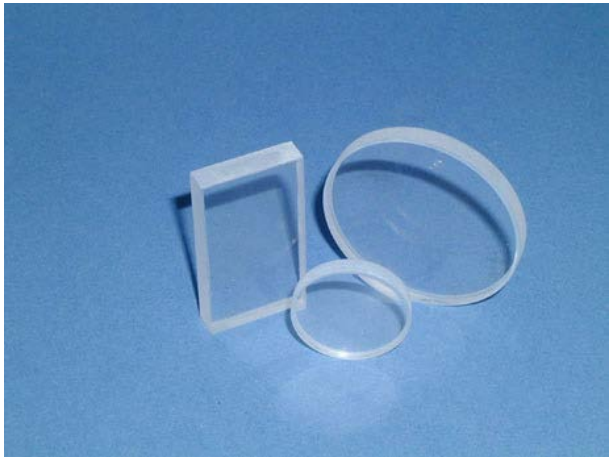
Transmission and ATR modes

Water contribution can be subtracted when using short pathlengths.

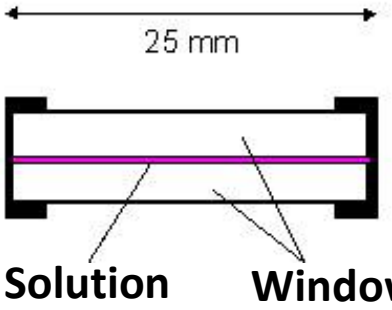
To further avoid water absorption, samples can also be measured dissolved in deuterated water (D_2O), which absorbs a different part of the spectrum



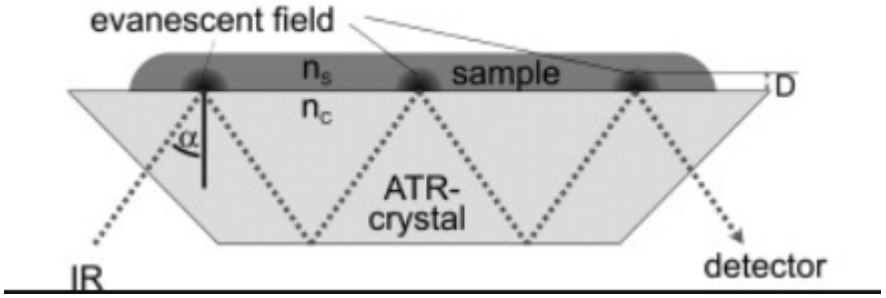
Top view



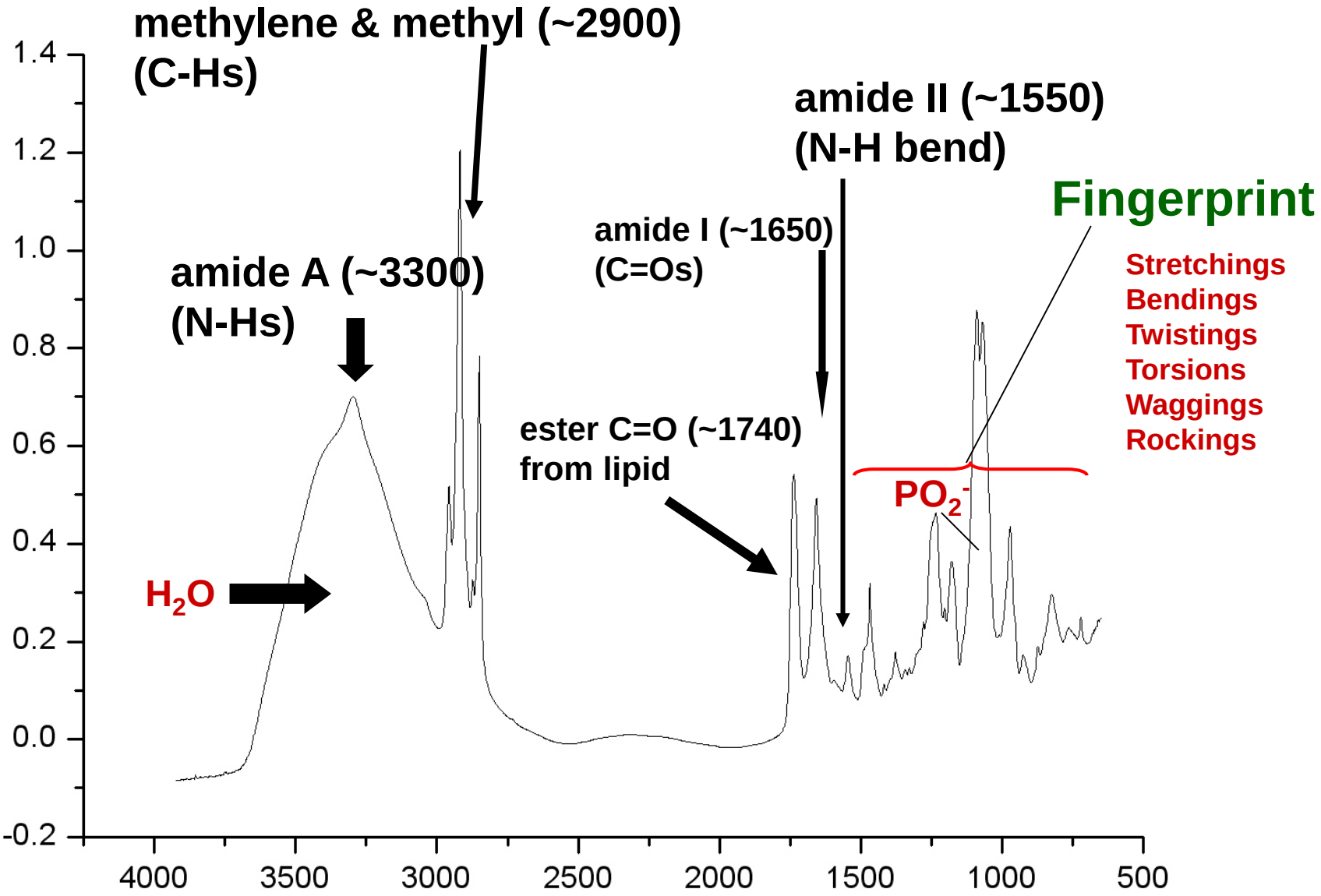
Transmission cell



ATR



Mid IR spectrum, mixture of protein and lipid



Protein bands used in secondary structure determination

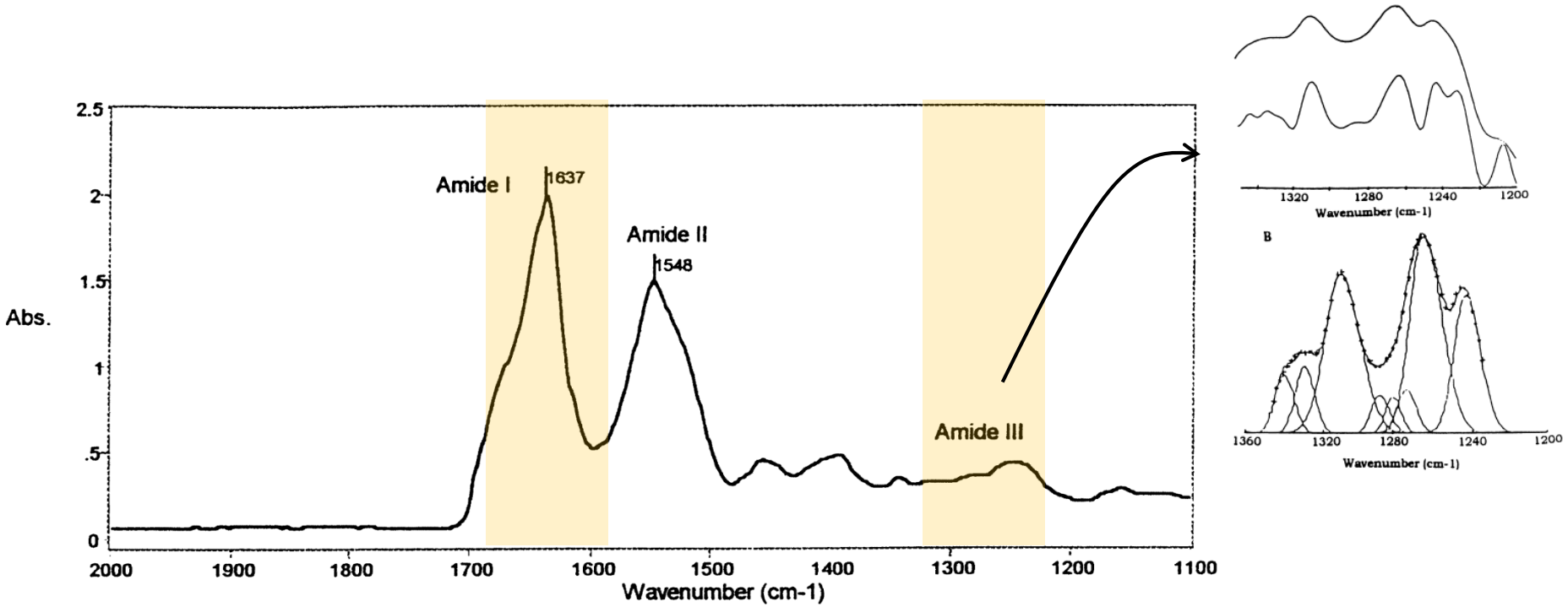
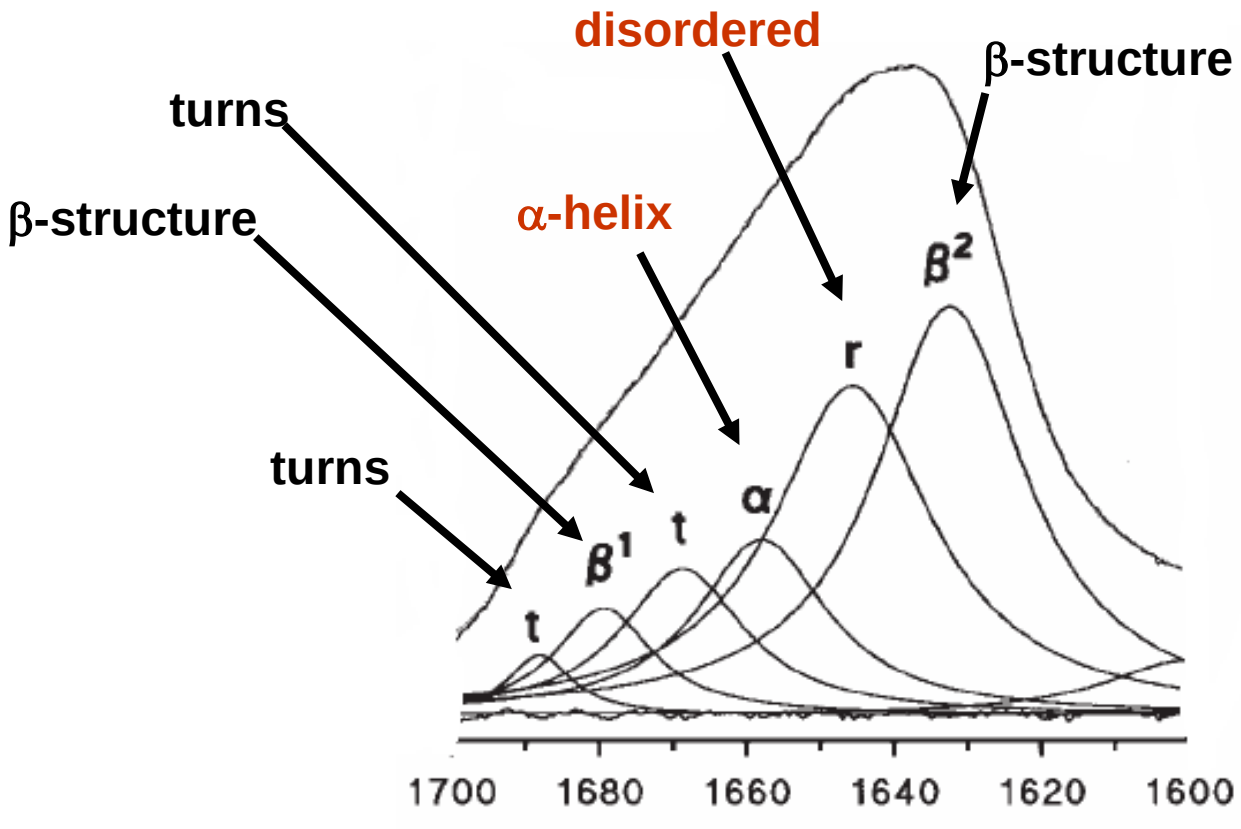


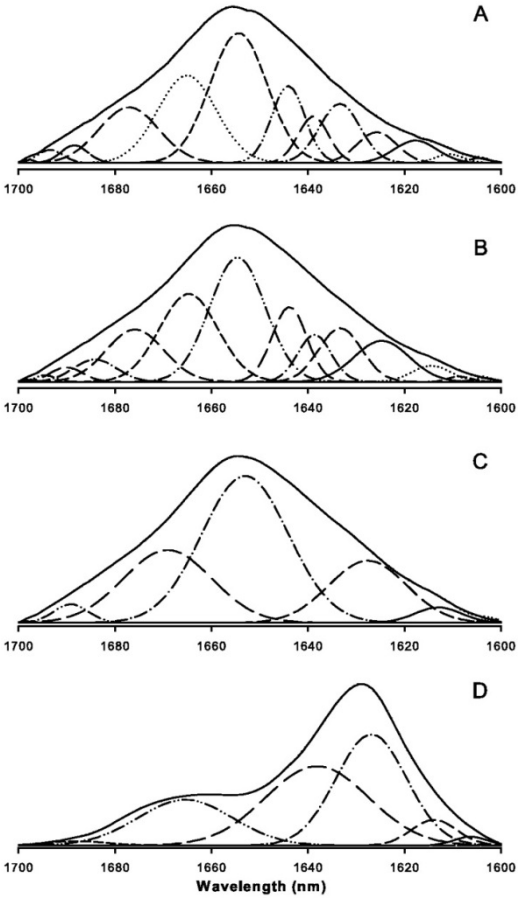
Figure 1. IR spectrum of α-chymotrypsin. The amide I region (1600-1700 cm^{-1}) corresponds to the C=O stretch weakly coupled with C-N stretch and N-H bending. The amide II region (1500-1600 cm^{-1}) represents C-N stretch strongly coupled with N-H bending. The amide III region (1200-1350 cm^{-1}) is N-H in-plane bending coupled with C-N stretching and also includes C-H and N-H deformation vibrations.

Singh; Infrared Analysis of Peptides and Proteins ACS Symposium Series; American Chemical Society: Washington, DC, 1999

Secondary structure from amide I band

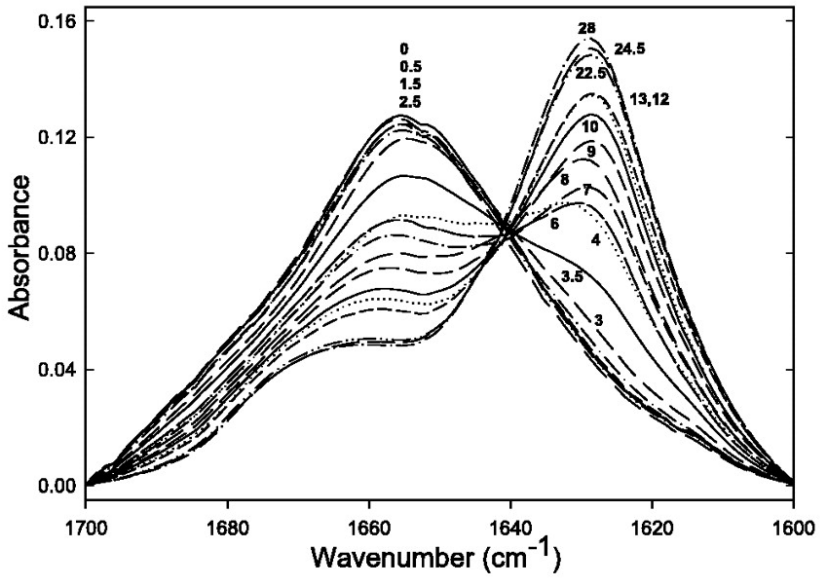
The amide I band is usually a smooth envelope. Here it has been fitted with Lorentzian bands (each band represents a different secondary structure)



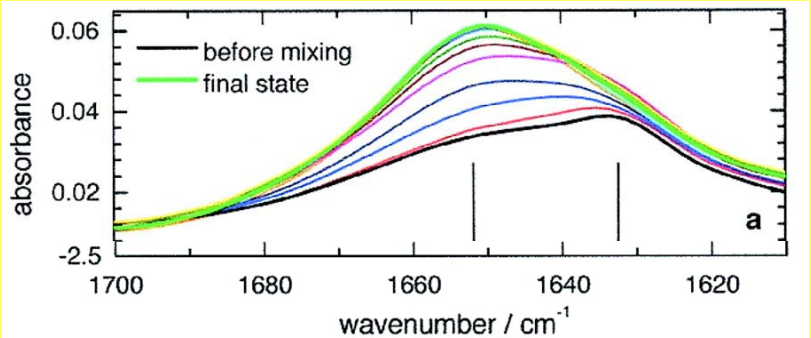


The proximity between α-helix and disordered structure makes it difficult to distinguish between these two (CD is better in this case). But IR is better to monitor and quantify β-structure

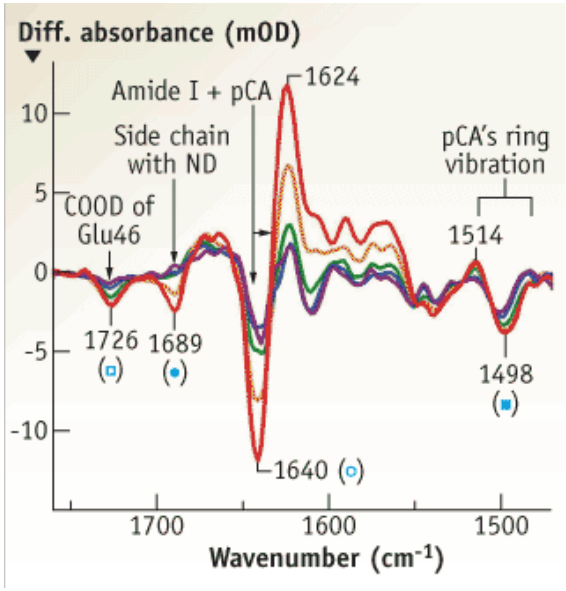
IR is useful in monitoring conformational changes



Aggregation of insulin. Conversion of α-helical insulin (peak at 1654 cm^{-1}) into a β-sheet peak at 1628 cm^{-1}. The numbers represent the time of incubation in hours.



Time-resolved IR spectra of β-lactoglobulin mixed with TFE (helix inducer). Spectra taken at 0, (black), 1.1, 3.4, 5.7, 10.2, 21.6, and 103 ms (green).



Conformational changes after proton transfer

Summary

CD and IR can be used to

- determine secondary structures of proteins and peptides.

- monitor conformation and stability under a wide range of conditions.

- Kinetic studies.

- Quality control.

- Rapid screening conditions, e.g., in X-ray and NMR.

- Ligand binding