MOSBRI
Molecular-Scale Biophysics
Research Infrastructure

# BLS1: Quality control for Biology.

Lecture 4: UV and CD spectroscopy as qualitative and quantitative tools for proteins

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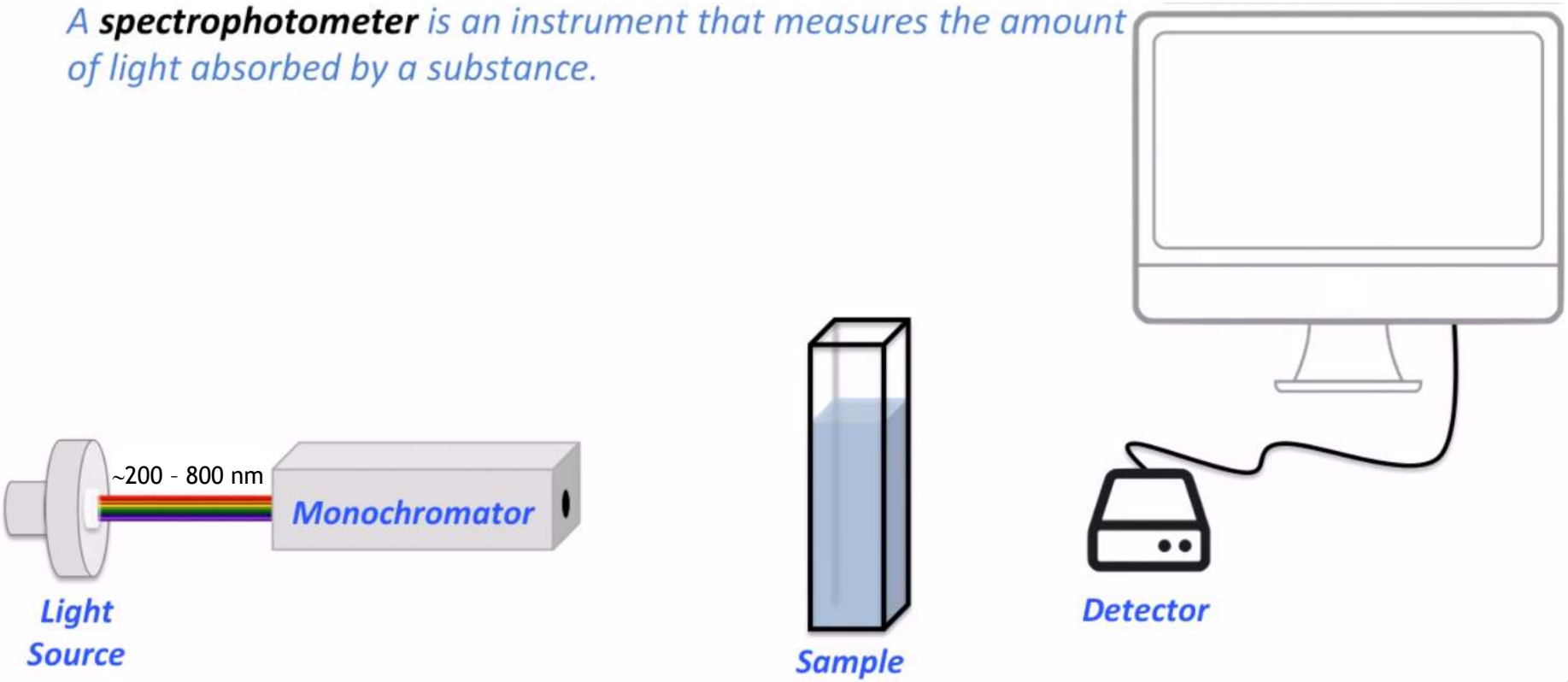
This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

# Principle of a spectrophotometer

*A **spectrophotometer** is an instrument that measures the amount of light absorbed by a substance.*



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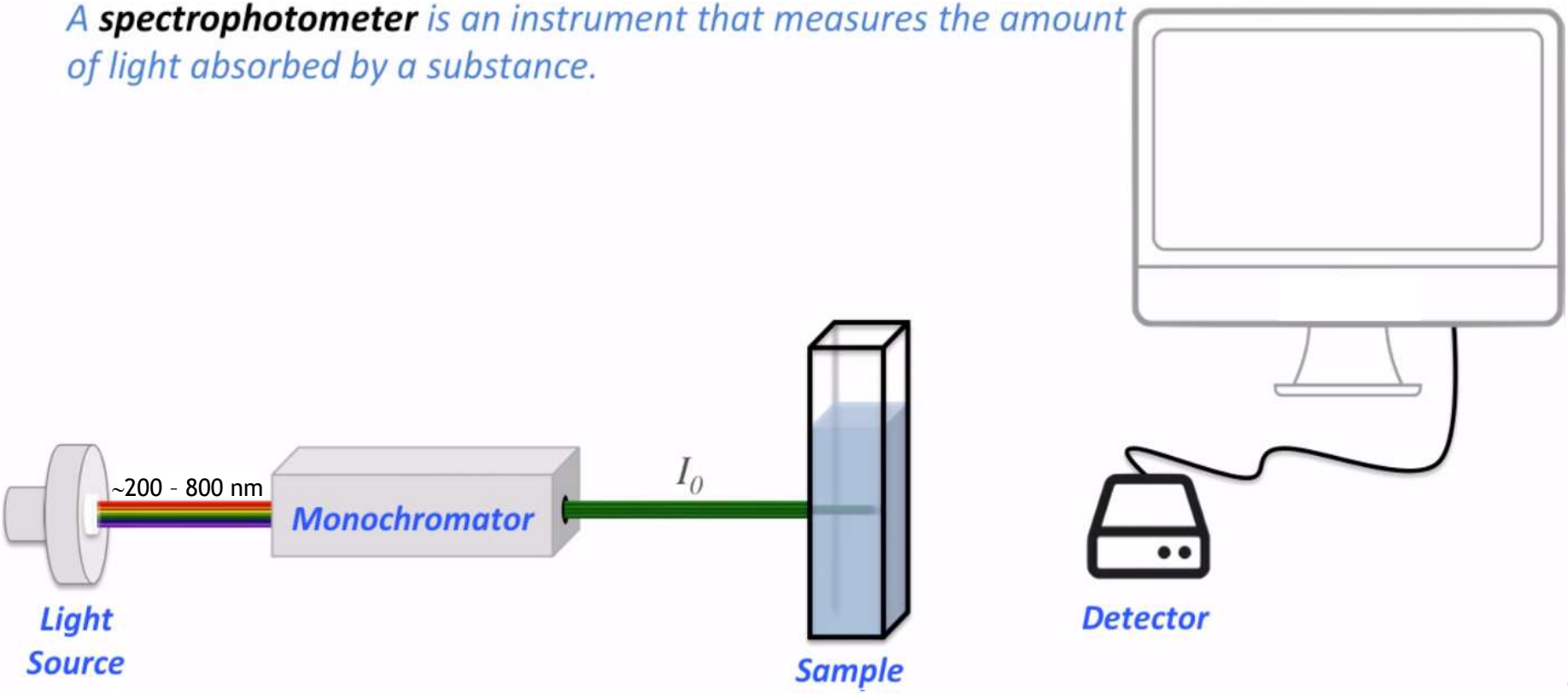




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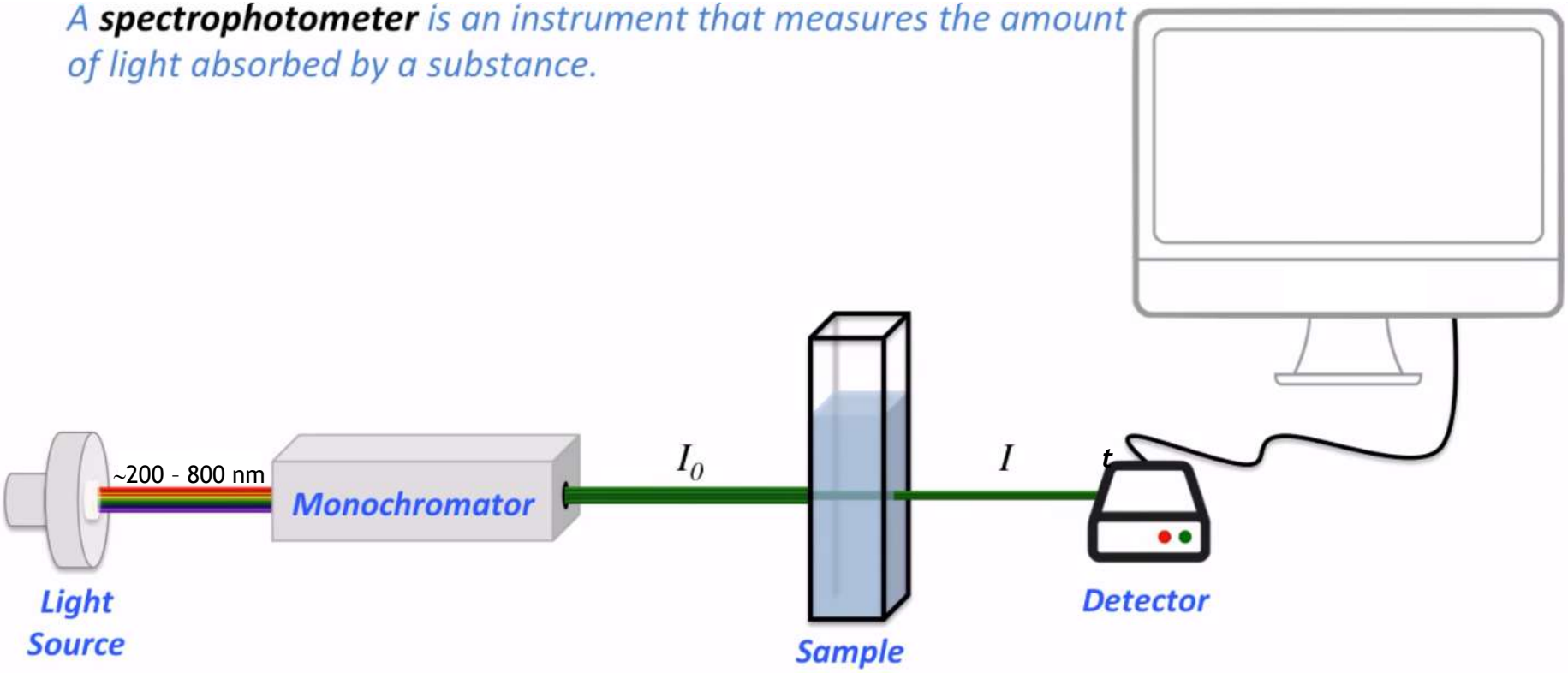




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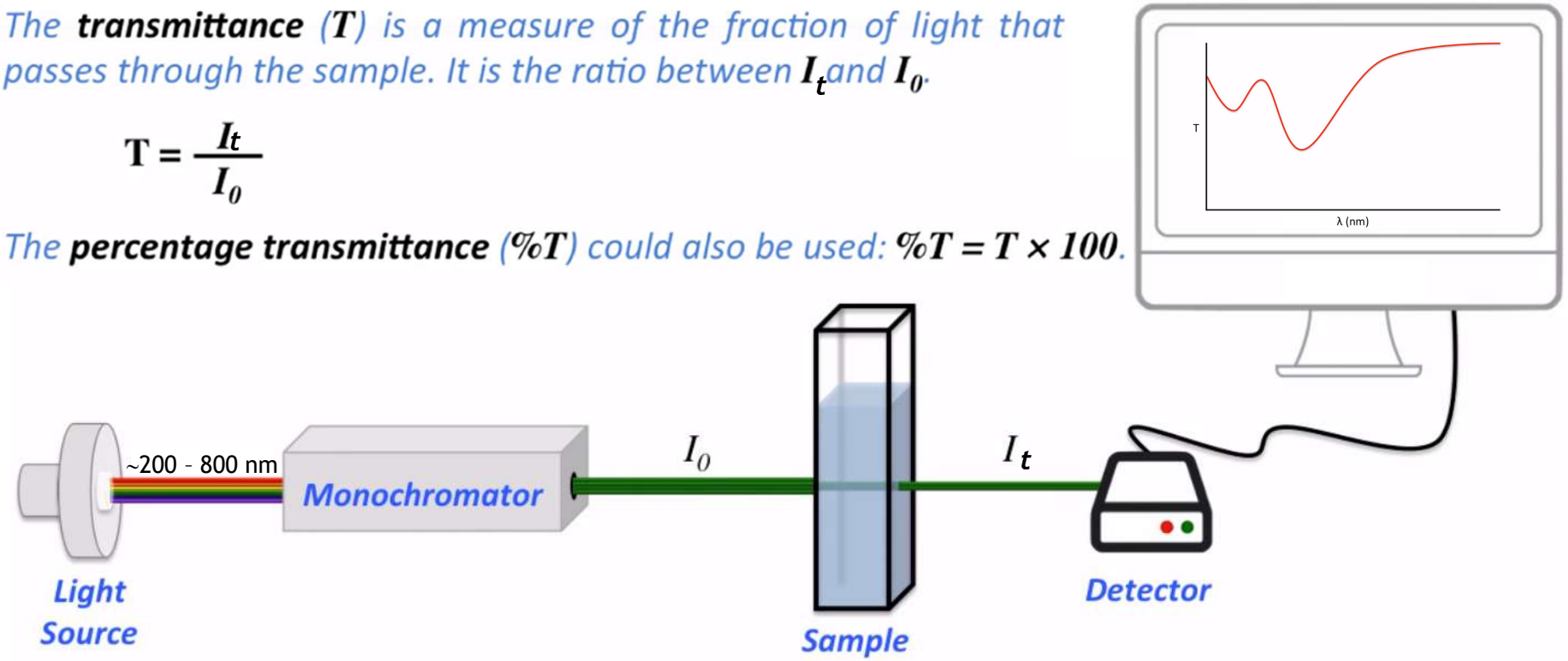
# Principle of a spectrophotometer

*The* ***transmittance*** *(**T**) is a measure of the fraction of light that passes through the sample. It is the ratio between* $I_t$ *and* $I_0$.

$$T = \frac{I_t}{I_0}$$

*The* ***percentage transmittance*** *(**%T**) could also be used:* $\%T = T \times 100$.



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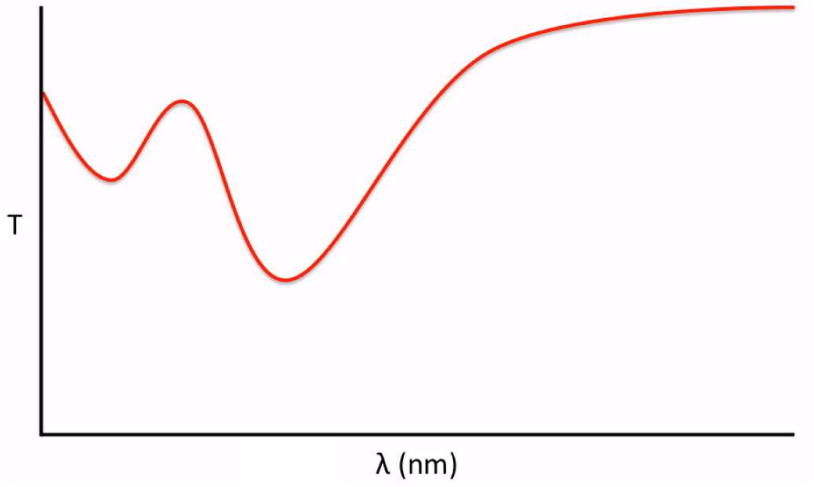
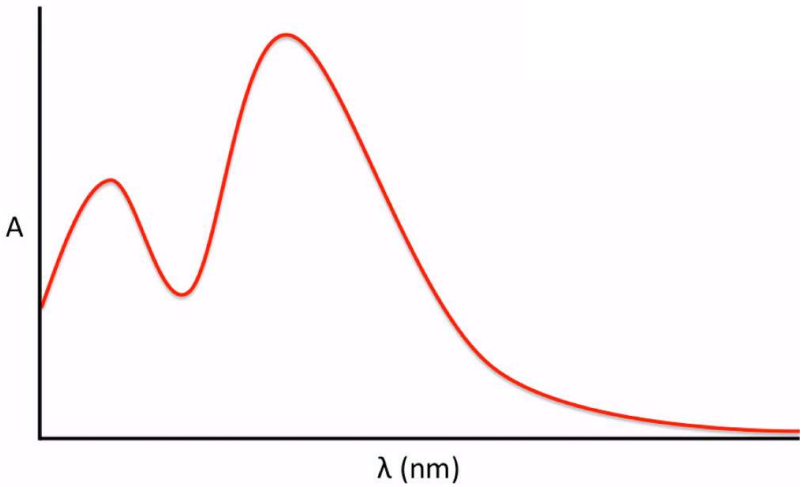
# Principle of a spectrophotometer

Transmittance spectrum → Absorbance spectrum




*The **absorbance** (**A**) is the amount of light absorbed by the sample.* $\mathbf{A = -\log T = -\log \frac{I_t}{I_0}}$



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# Beer-Lambert Law


$$C = A_\lambda / (\varepsilon_\lambda . l)$$

A = Absorbance at specific wavelength λ (no units).
ε = Molar extinction coefficient at specficic wavelength ($L.mol^{-1}.cm^{-1}$).
l = pathlength (cm).
C = Concentration ($Mol.L^{-1}$).

ε is the capacity of a molecule to attenuate (absorb) light at a given wavelength (intrinsic property)

1 molecule => 1 epsilon!

λ is the distance from one wave cycle to the next



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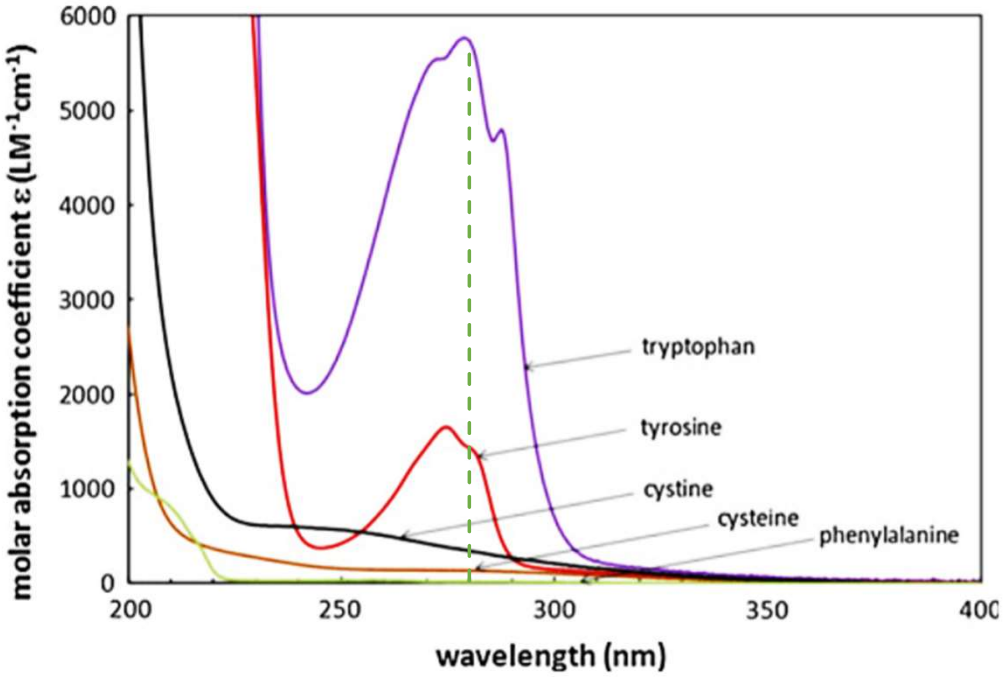
# Where does the proteins UV contribution at 280 nm come from?




**Keith R. Millington. (2012)** Diffuse reflectance spectroscopy of fibrous proteins. *Amino acids*

Contribution of 1 W at 280 nm in water:
3.7 times more than Y
45 times more than cystines

$\varepsilon_{280\ nm}$= 5500 (# of Trp) + 1490 (# of Tyr) + 125 (# of Cystines)

**Pace CN et al. (1995)** How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **11**, 2411-2423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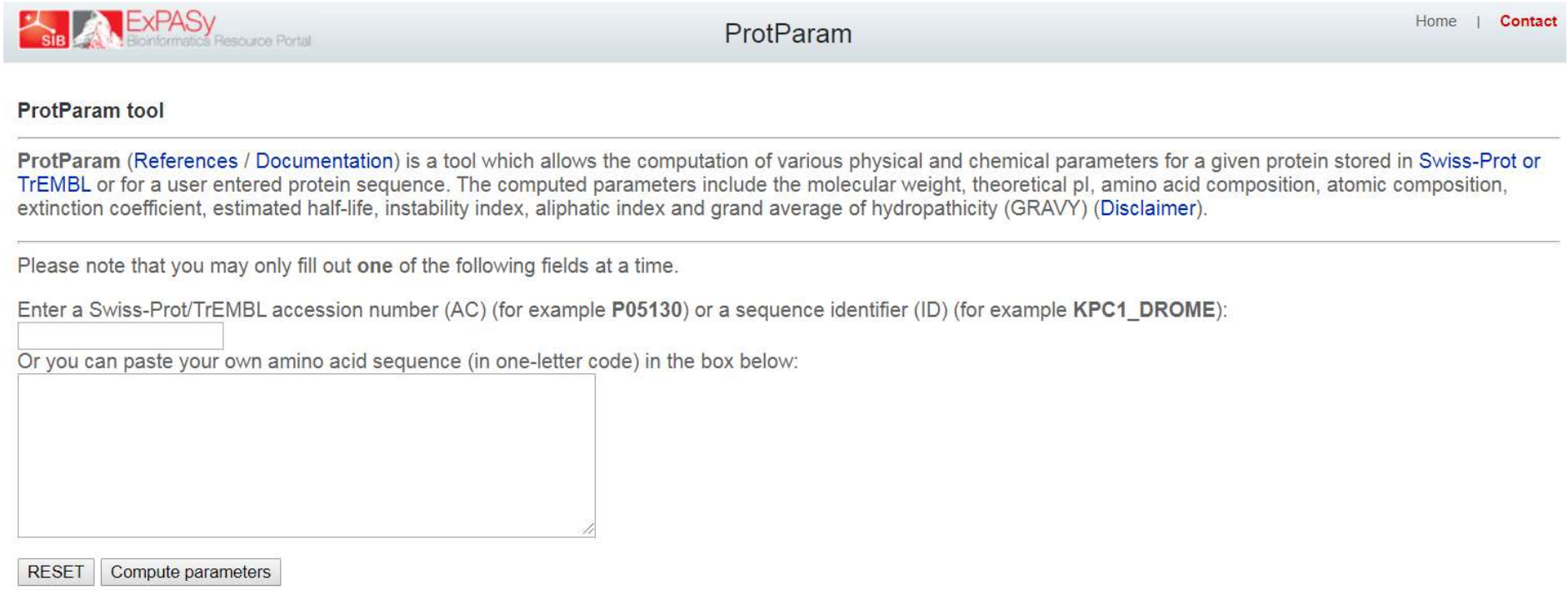
# Tool to calculate epsilon at 280nm




https://web.expasy.org/protparam/



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# Tool to calculate epsilon at 280nm

## BSA

**Number of amino acids:** 583

**Molecular weight:** 66432.96

**Theoretical pI:** 5.60

**Amino acid composition:** CSV format

| | | |
|---|---|---|
| Ala (A) | 47 | 8.1% |
| Arg (R) | 23 | 3.9% |
| Asn (N) | 14 | 2.4% |
| Asp (D) | 40 | 6.9% |
| Cys (C) | 35 | 6.0% |
| Gln (Q) | 20 | 3.4% |
| Glu (E) | 59 | 10.1% |
| Gly (G) | 16 | 2.7% |
| His (H) | 17 | 2.9% |
| Ile (I) | 14 | 2.4% |
| Leu (L) | 61 | 10.5% |
| Lys (K) | 59 | 10.1% |
| Met (M) | 4 | 0.7% |
| Phe (F) | 27 | 4.6% |
| Pro (P) | 28 | 4.8% |
| Ser (S) | 28 | 4.8% |
| Thr (T) | 33 | 5.7% |
| Trp (W) | 2 | 0.3% |
| Tyr (Y) | 20 | 3.4% |
| Val (V) | 36 | 6.2% |
| Pyl (O) | 0 | 0.0% |
| Sec (U) | 0 | 0.0% |
| (B) | 0 | 0.0% |
| (Z) | 0 | 0.0% |
| (X) | 0 | 0.0% |

**Total number of negatively charged residues (Asp + Glu):** 99
**Total number of positively charged residues (Arg + Lys):** 82

**Atomic composition:**

| | | |
|---|---|---|
| Carbon | C | 2934 |
| Hydrogen | H | 4615 |
| Nitrogen | N | 781 |
| Oxygen | O | 897 |
| Sulfur | S | 39 |

**Formula:** $C_{2934}H_{4615}N_{781}O_{897}S_{39}$
**Total number of atoms:** 9266

**Extinction coefficients:**

Extinction coefficients are in units of $M^{-1}$ $cm^{-1}$, at 280 nm measured in water.

Ext. coefficient 42925
Abs 0.1% (=1 g/l) 0.646, assuming all pairs of Cys residues form cystines

Ext. coefficient 40800
Abs 0.1% (=1 g/l) 0.614, assuming all Cys residues are reduced

**Estimated half-life:**

The N-terminal of the sequence considered is D (Asp).

The estimated half-life is: 1.1 hours (mammalian reticulocytes, in vitro).
3 min (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

**Instability index:**

The instability index (II) is computed to be 40.11
This classifies the protein as unstable.

**Aliphatic index:** 76.14

**Grand average of hydropathicity (GRAVY):** -0.475



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# What's the solution for proteins w/o tryptophan?

## 2nde example: no W

**Number of amino acids:** 236

**Molecular weight:** 25163.13

**Theoretical pI:** 5.48

**Amino acid composition:** CSV format

| | | |
|---|---|---|
| Ala (A) | 24 | 10.2% |
| Arg (R) | 17 | 7.2% |
| Asn (N) | 6 | 2.5% |
| Asp (D) | 13 | 5.5% |
| Cys (C) | 1 | 0.4% |
| Gln (Q) | 12 | 5.1% |
| Glu (E) | 12 | 5.1% |
| Gly (G) | 29 | 12.3% |
| His (H) | 2 | 0.8% |
| Ile (I) | 10 | 4.2% |
| Leu (L) | 26 | 11.0% |
| Lys (K) | 5 | 2.1% |
| Met (M) | 10 | 4.2% |
| Phe (F) | 9 | 3.8% |
| Pro (P) | 7 | 3.0% |
| Ser (S) | 7 | 3.0% |
| Thr (T) | 14 | 5.9% |
| Trp (W) | 0 | 0.0% |
| Tyr (Y) | 4 | 1.7% |
| Val (V) | 28 | 11.9% |
| Pyl (O) | 0 | 0.0% |
| Sec (U) | 0 | 0.0% |
| (B) | 0 | 0.0% |
| (Z) | 0 | 0.0% |
| (X) | 0 | 0.0% |

**Total number of negatively charged residues (Asp + Glu):** 25
**Total number of positively charged residues (Arg + Lys):** 22

**Atomic composition:**

| | | |
|---|---|---|
| Carbon | C | 1108 |
| Hydrogen | H | 1810 |
| Nitrogen | N | 314 |
| Oxygen | O | 330 |
| Sulfur | S | 11 |

**Formula:** $C_{1108}H_{1810}N_{314}O_{330}S_{11}$
**Total number of atoms:** 3573

**Extinction coefficients:**

This protein does not contain any Trp residues. Experience shows that
this could result in more than 10% error in the computed extinction coefficient.

Extinction coefficients are in units of $M^{-1}$ $cm^{-1}$, at 280 nm measured in water.

5960
0.237, assuming all pairs of Cys residues form cystines


5960
0.237, assuming all Cys residues are reduced

:

ıe sequence considered is Q (Gln).

life is: 0.8 hours (mammalian reticulocytes, in vitro).
10 min (yeast, in vivo).
10 hours (Escherichia coli, in vivo).

**Instability index:**

The instability index (II) is computed to be 22.38
This classifies the protein as stable.



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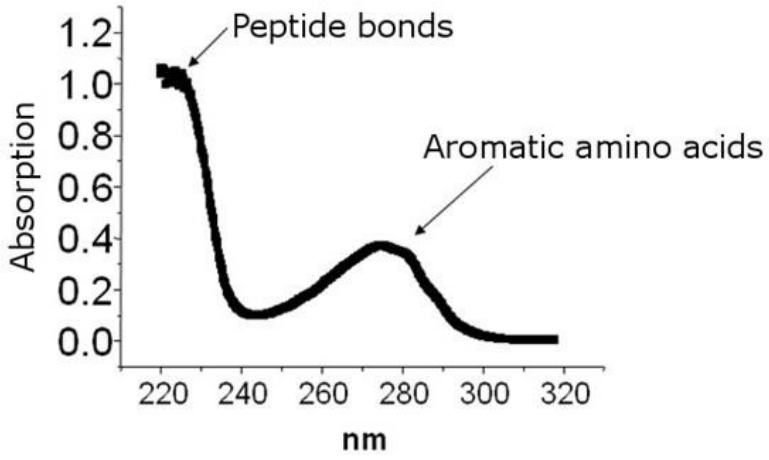
# Proteins w/o W: monitoring at 205 or 214 nm




Many buffer incompatibilities!!!

Buffer and sample must be diluted 50-100 times in water to be in accepted abs range and to reduce buffer saturation.

## CALCULATION OF EXTINCTION COEFFICIENTS AT 205 AND 214 NM

Sequence:

*Protein concentration can be determined by the absorbance at 205 or 214 nm. It is especially useful when absorbance at 280 nm cannot be used in the lack of Trp and Tyr residues. CD samples can be directly measured at these wavelengths due to the high extinction coefficients. If the spectropolarimeter is capable of converting the HT values to absorbances, then the concentrations can be determined right from the CD measurements after subtracting the baseline absorptions. Extinction coefficients at 205 and 214 nm can be calculated from the sequence.*

**References:**
205 nm: Prot. Sci. 2013, 22, 851-858.
214 nm: J. Agric. Food Chem. 2007, 55, 5445-5451.

Number of S-S bonds:
0

https://bestsel.elte.hu/extcoeff.php

Please, type the characters above or use your password.

Submit Reset



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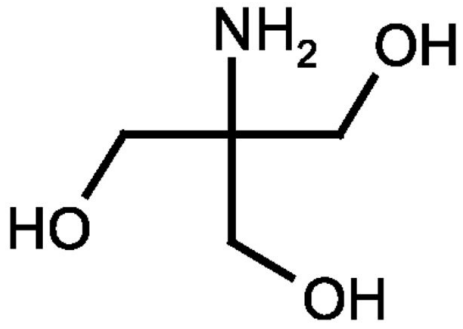
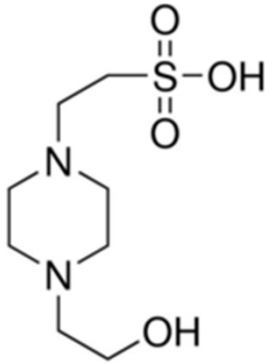
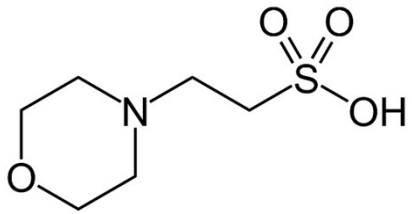
# Buffer compatibility at 280 nm



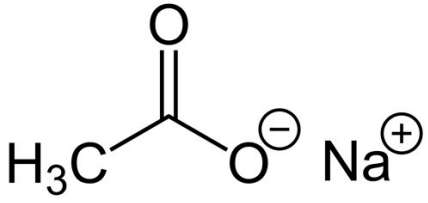

TRIS



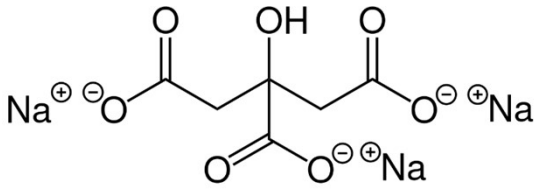

HEPES




MES




ACETATE




CITRATE

**These buffers are compatible with the 280 nm detection**



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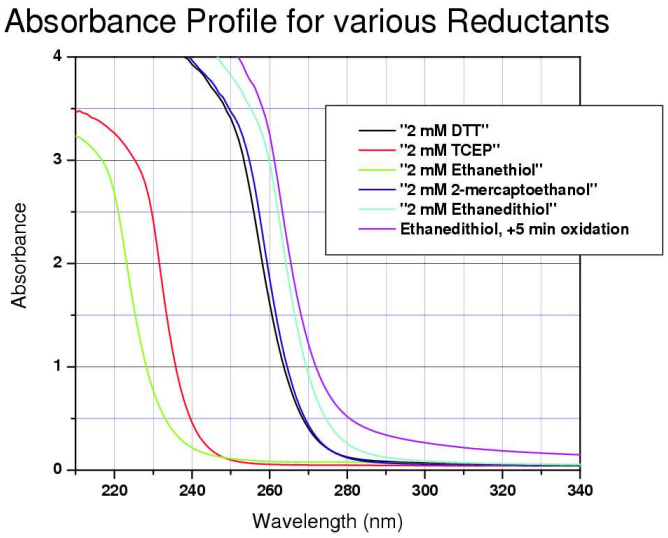
# Are all molecules compatible at 280 nm?

**No!**



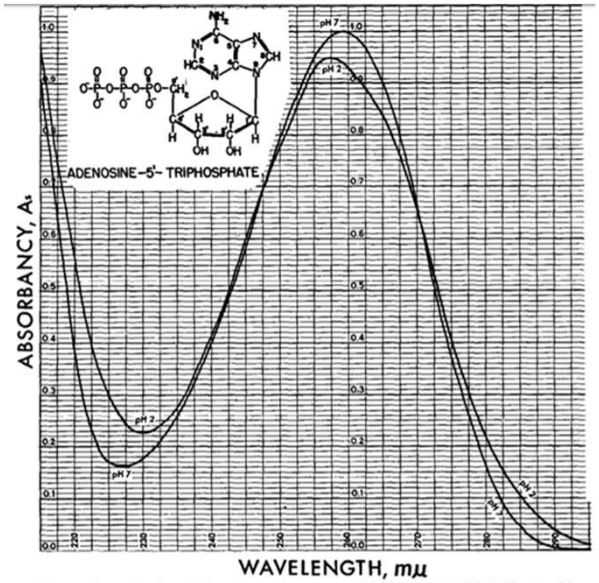




FIG. 4. Sample ultraviolet absorption curves for $6.5 \times 10^{-5}$ *M* ATP. At this concentration the spectrum at pH 11.3 is the same as that at pH 7 to within 0.01 unit of $A_s$.

65 µM ATP at 280 nm = 0.15 Abs

1 mM ATP (common concentration) Abs= 2.3!

65 µM ATP at 260 nm = 1 Abs

1 mM ATP (common concentration) Abs= 15.4!!!

R.M.Bock et al. **(1956)** Ultraviolet absorption spectra of adenosine-5′-triphosphate and related 5′-ribonucleotides. *Archives of Biochemistry and Biophysics*. Volume 62, Issue 2, June 1956, Pages 253-264



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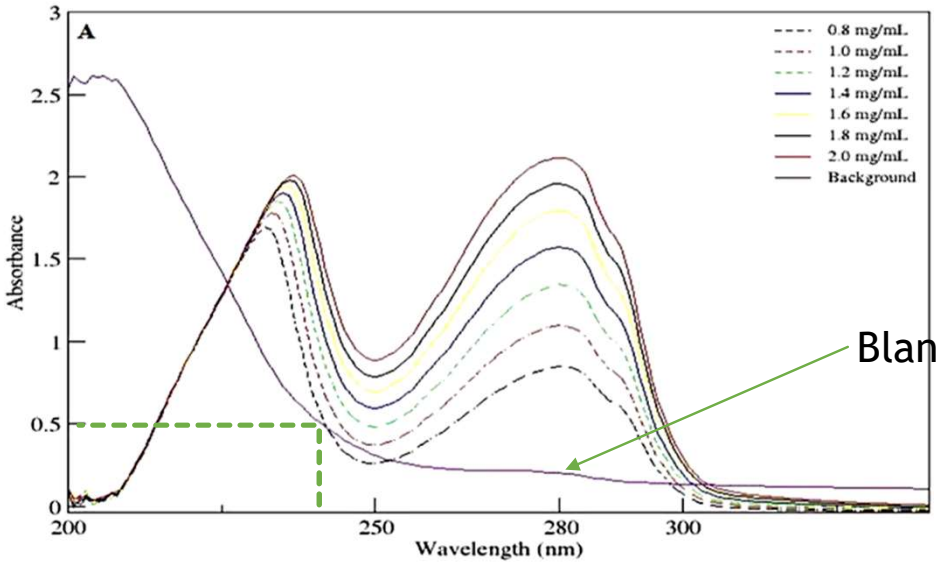
# How to confirm any buffer limitations at 280 nm?

Do a blank on your buffer and visualize the transmittance signal.
or Do a blank on air and use your buffer as sample and monitor the absorbance signal.




A = 2-log %T

| Blank absorbance | Blank transmittance |
|---|---|
| 0 | 100% |
| 1 | 10% |
| 2 | 1% |
| 3 | 0.1% |
| 4 | 0.01% |
| 5 | 0.001% |

=> Get more than 30% of transmittance signal or less than 0.5 Abs to avoid saturation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# Few things to do and check before a 280 nm measurement

$\varepsilon$

Go on Protparam or Bestsel to get extinction coefficient and molecular mass.

Molecular mass must be confirmed by MS

W


Presence of Tryptophan and buffer compatiblity?



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# Do you know the difference between a 280 nm measurement and a UV spectrum?





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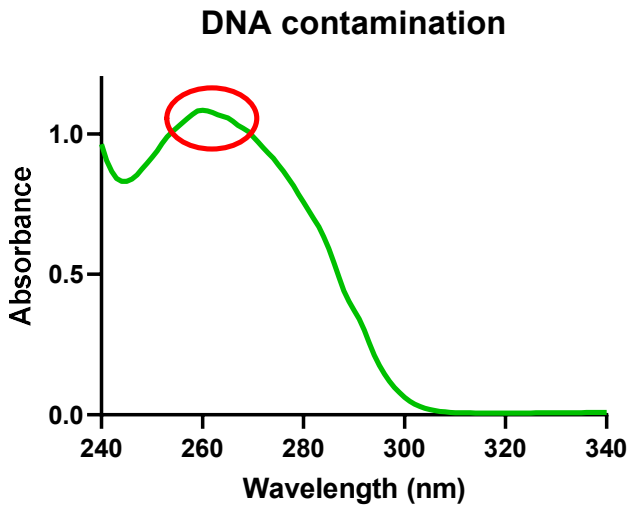
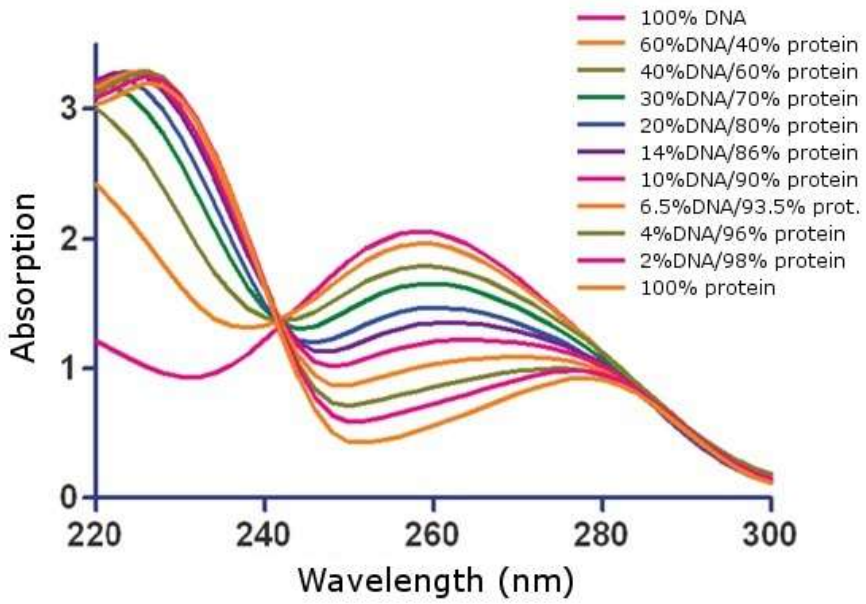
# Advantages of the full UV spectrum




(Abs 260nm/Abs 280nm) < **0.6**

Glasel, J.A. (**1995**) Validity of Nucleic Acid Purities Monitored by A260/A280 Absorbance Ratios. *Biotechniques*, 18, 62-63.



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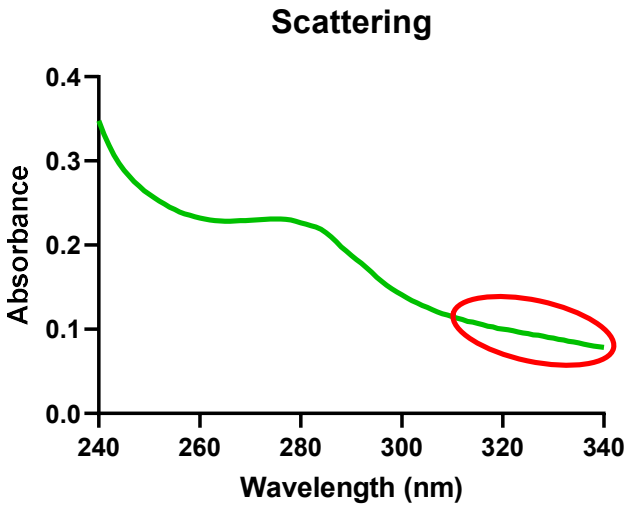
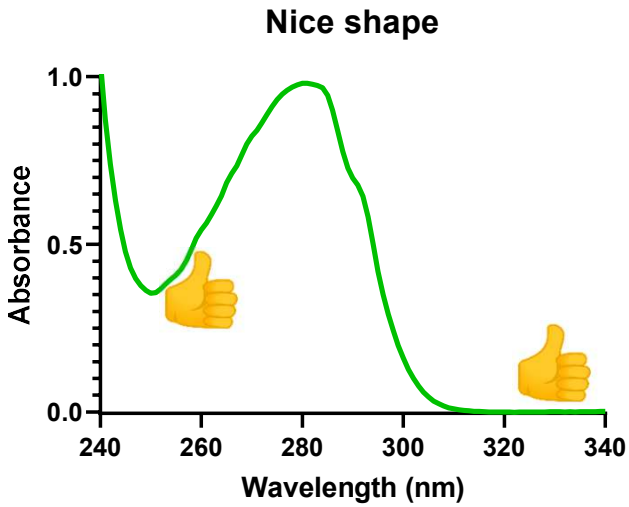
# Advantages of the full UV spectrum




Aggregation Index (AI)
=100 x  Abs 340/(Abs 280-Abs 340) < **2**

In presence of scattering, sample must be spun.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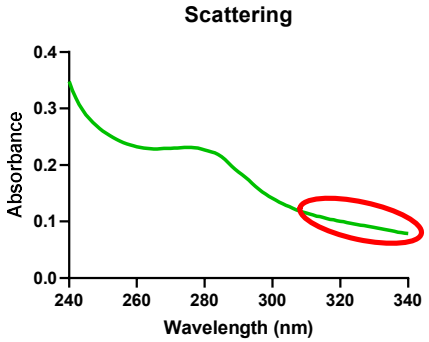
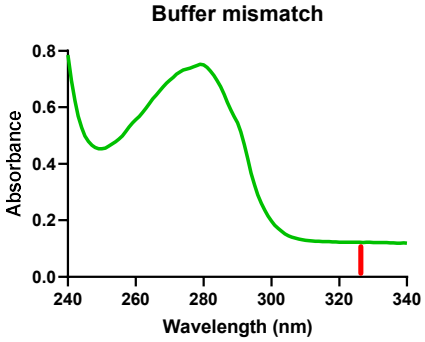
# Well quantify in presence of scattering or buffer mismatch




In both cases, 280 nm abs overestimated


Spectragryph used to read raw data and process the data (scattering and buffer mismatch subtraction, etc.)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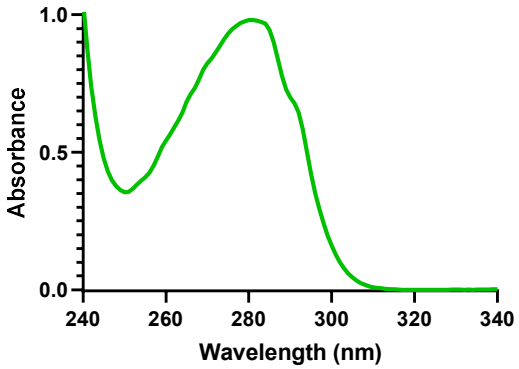
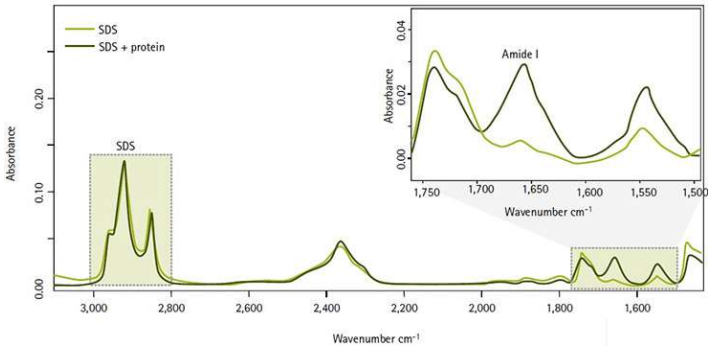
# Other approaches to quantify your protein


Bradford assay

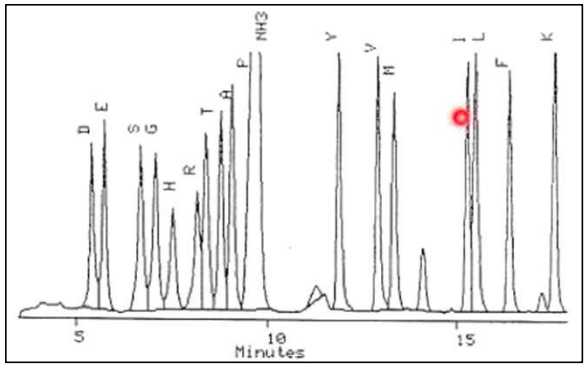



Full UV spectrum

You have to find the good technique for your system (excipient & amino acids compatibility).




Infra-red measurement




Amino acid analysis



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# 2nd part: Introduction to Circular Dichroism

**Definition:** $\text{Circular dichroism} = \Delta A(\lambda) = A(\lambda)_{LCPL} - A(\lambda)_{RCPL}$

Left circularly-polarized light (LCPL)
Right circularly-polarized light (RCPL)

**Requirement:**

**CD active molecules**

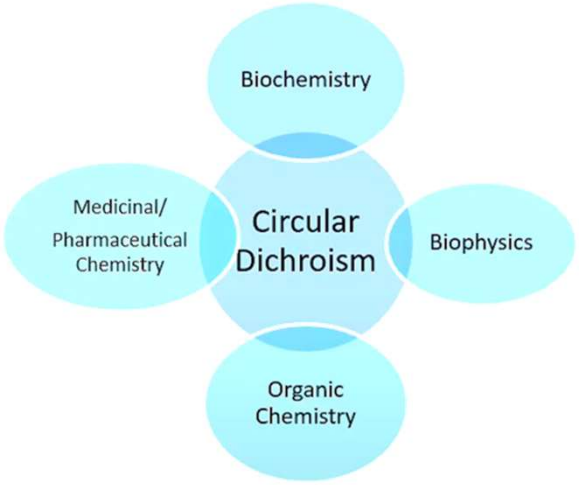
- Structurally asymmetric
- Exhibit absorbance

- Chiral molecules
- Non-chiral molecules attached to a chiral molecule
- Non-chiral molecule in a asymmetric environment

**Applications for proteins:**

**Proteins**

- Secondary structure analysis
- Tertiary structure fingerprint
- Detection of conformational changes
  - Structural comparison of variants (WT vs mutants)
- DNA, quadruplexes, small molecules etc.





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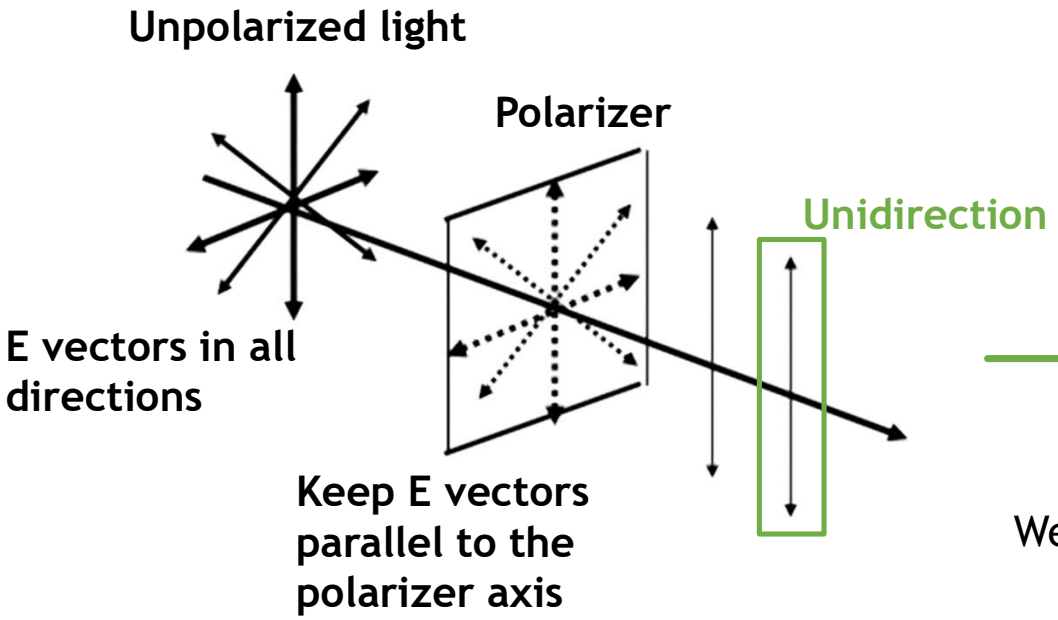
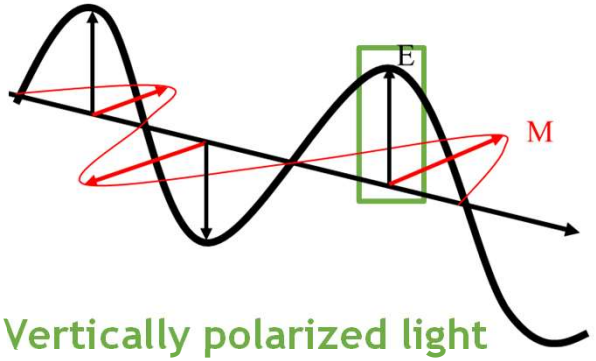
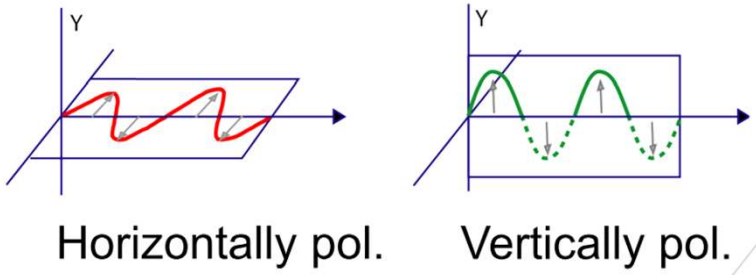
# How to get circularly polarized light?

Any electronic wave consists of an **electric** field (plane of polarization) and a **magnetic** field component.




We recover both hor. and ver. polarized light…



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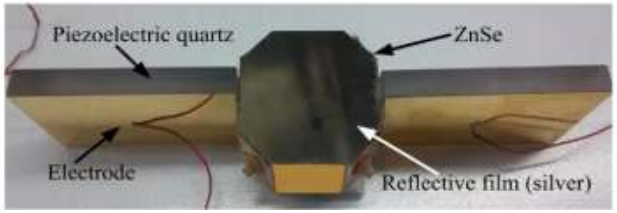
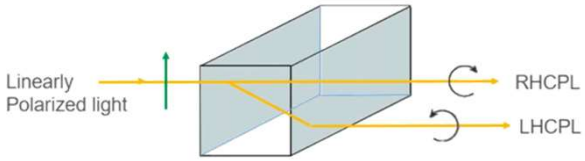
# How to get circularly polarized light?

PEM converts linearly polarized light to circularly polarized light

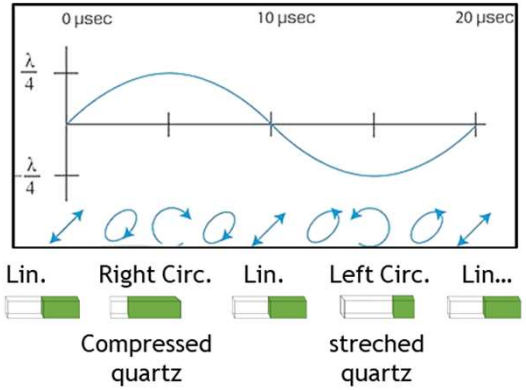



Photoelastic Modulator (PEM)

When PEM vibrates at a specific resonnance frequency (50 kHz), this stress induces **birefringence** to the quartz.




=>Under vibration, parallel and perpendicular light travels at different speed in the quartz

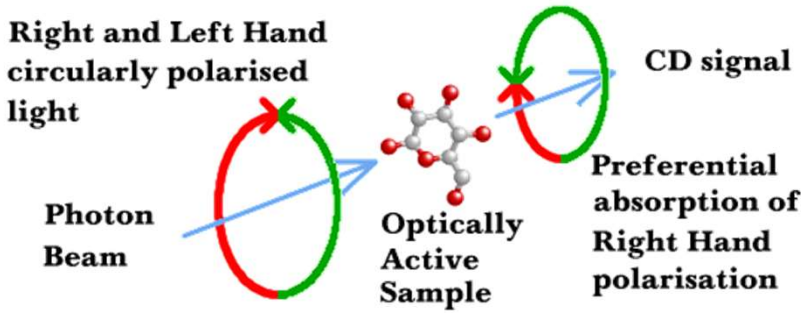


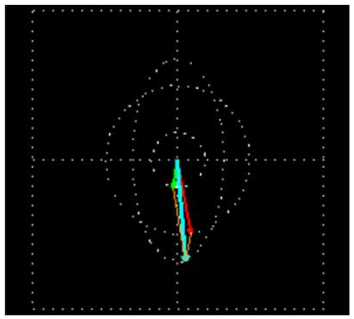

The modulation allows to compensate the difference in phase velocity.


This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

# How to get circularly polarized light?

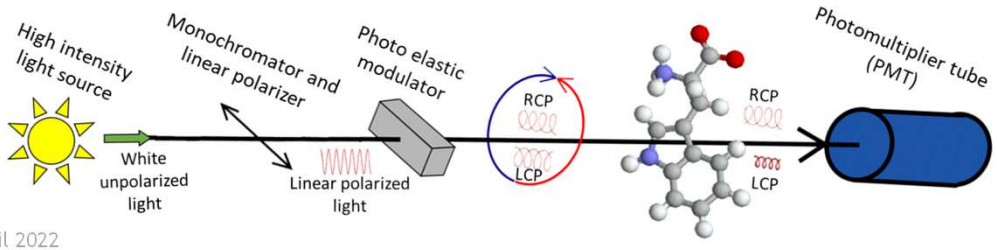
Any electronic wave consists of an **electric** field (plane of polarization) and a **magnetic** field component.



After going through a CD-active molecule → **Elliptically polarized light**

As the sample is optically active, there is a **tiny change** between RCPL and LCPL




- Right circularly-polarized component (RCPL) has been partly absorbed.
- Left circularly-polarized component (LCPL) has a larger amplitude than the right one
- Superposition of the two waves of different amplitude forms an **ellipse.**

**Raw data in mdeg° of ellipticity**



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MOSBRI Molecular-Scale Biophysics Research Infrastructure

# Unit used for CD measurement

| From ↓ To → | Absorbance[1] | Milliabsorbance[2] | Molar Extinction[3] | Degrees[4] | Millidegrees[5] | Molar Ellipticity[6] |
|---|---|---|---|---|---|---|
| (A) | A | A*1000 | A*M/(C*L) | A*32.98 | A*32980 | A*M*3298/(L*C) |
| (mA) | mA/1000 | mA | A*M/(C*L*1000) | mA*0.03298 | mA*32.98 | mA*M*3.298/(L*C) |
| ($\varepsilon$) | $\varepsilon$*C*L/M | $\varepsilon$*C*L*1000/M | $\varepsilon$ | $\varepsilon$*C*L*32.98/M | $\varepsilon$*C*L*32980/M | $\varepsilon$*3298 |
| (°) | °/32.98 | °/0.03298 | °*M/(C*L*32.98) | ° | °*1000 | °*M*100/(L*C) |
| (m°) | m°/32980 | m°/32.98 | m°*M/(C*L*32980) | m°/1000 | m° | m°*M/(10*L*C) |
| [Θ] | [Θ]*C*L/(3298*M) | [Θ]*C*L/(3.298*M) | [Θ]/3298 | [Θ]*C*L/(100*M) | [Θ]*C*L*10/M | [Θ] |

[1]Units are Absorbance (Abs)
[2]Units are milliabsorbance (mAbs)
[3]Units are A*L/mol*cm
[4]Units are degrees (°)
[5]Units are millidegrees (m°)
[6]Units are deg*cm2/dmol

C is concentration in g/L
M is average molecular weight (g/mol)
L is path length of cell (cm)

1. Raw data in mdeg (angle)
2. Convert mdeg in Delta epsilon or in molar ellipticity to be independent of pathlength and concentration
3. For proteins, we usually divide Delta epsilon by number of residues.

Knowing the concentration, pathlength protein sequence and purity is highly important!!!

Before CD, researchers must check their protein by UV spectroscopy and mass spectrometry



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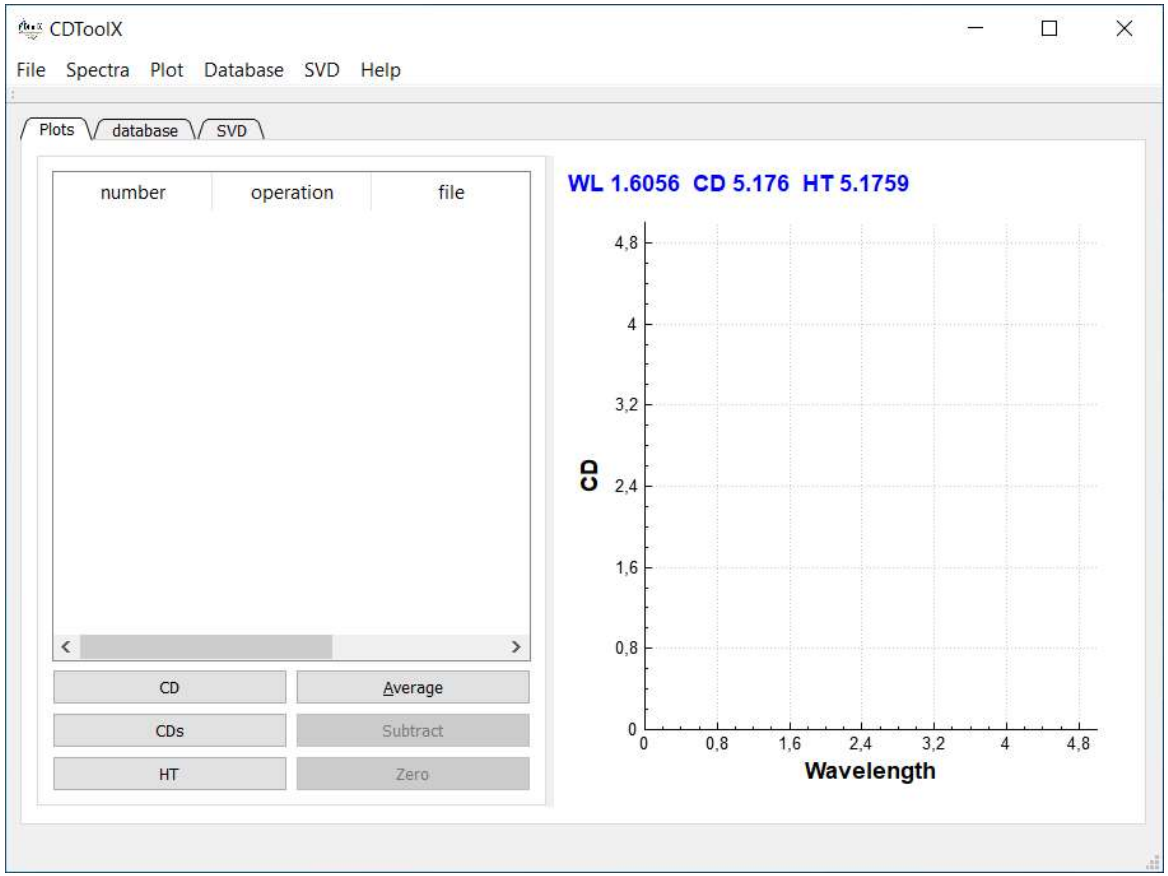
# Data processing

Machine softwares are usually not user friendly
Raw data are easily processed with CDToolX (sub, smmothing zero, CSA cal etc.)



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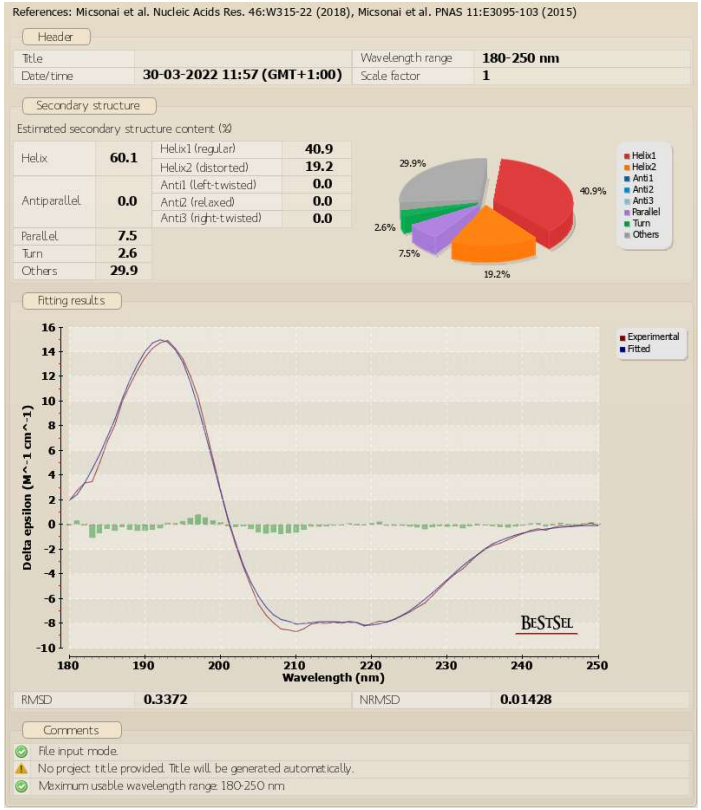
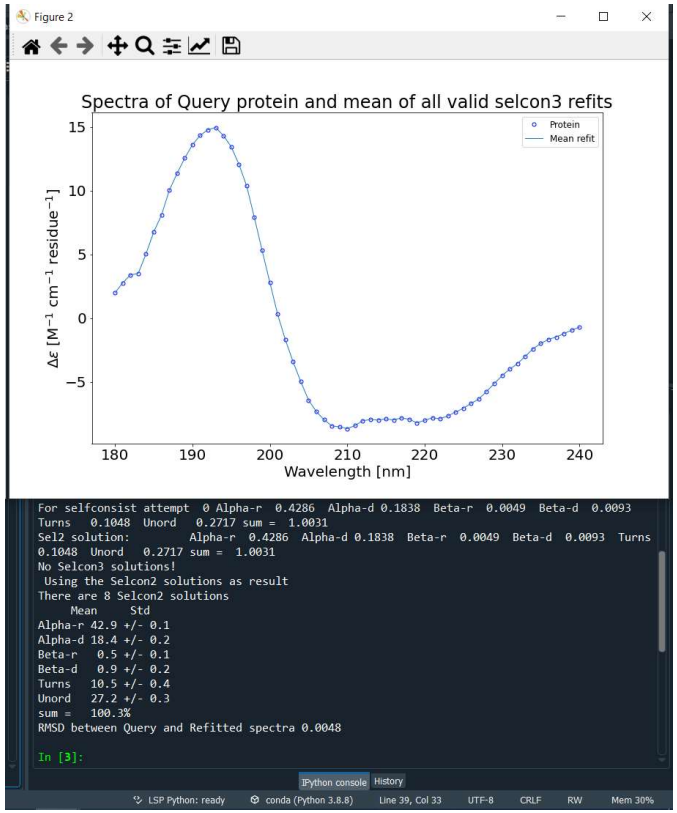
# Analysis softwares

Two main algorithms: BestSel and Selcon175 but other algorithms are available (See Dichroweb)



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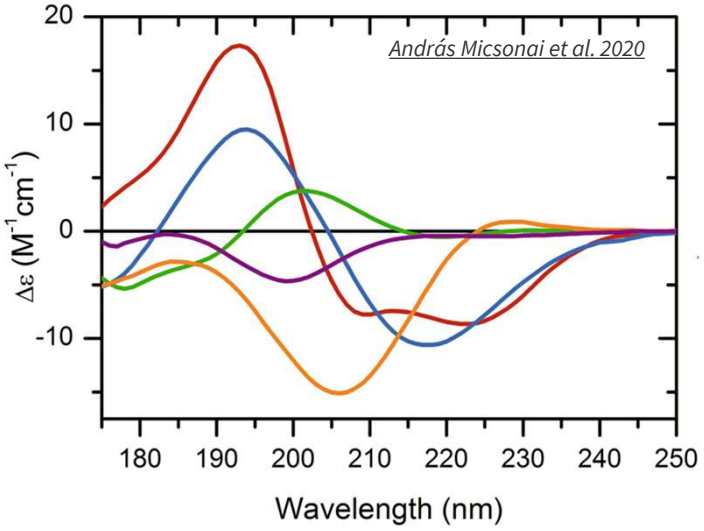
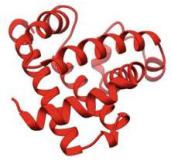
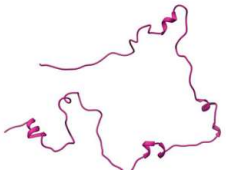
# The peptide bond and secondary structure

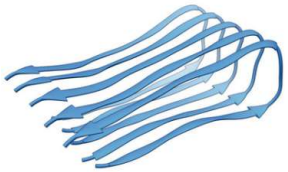


α -helix (red)



Disordered chain (purple)

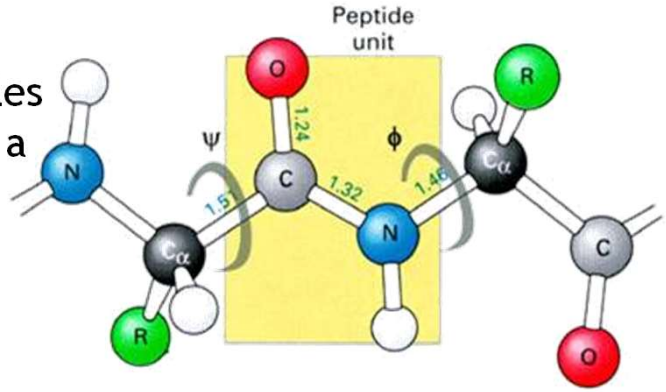


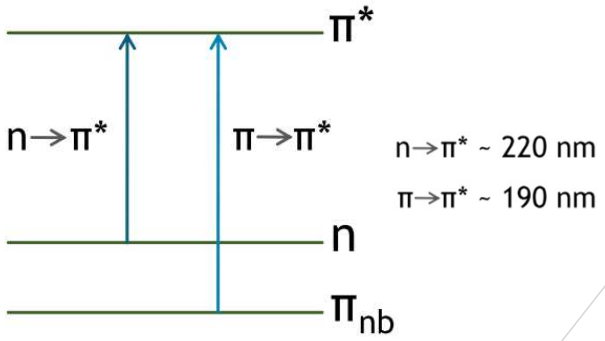
parallel ß -sheet (blue)

antiparallel ß -sheet (green)

polyproline-helix (orange)

Phi (φ) and Psi (ψ) angles define the structure of a protein's backbone.



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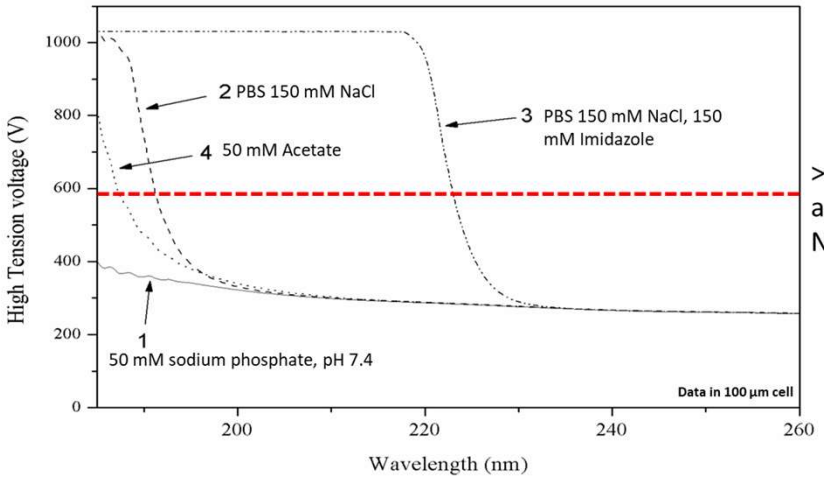
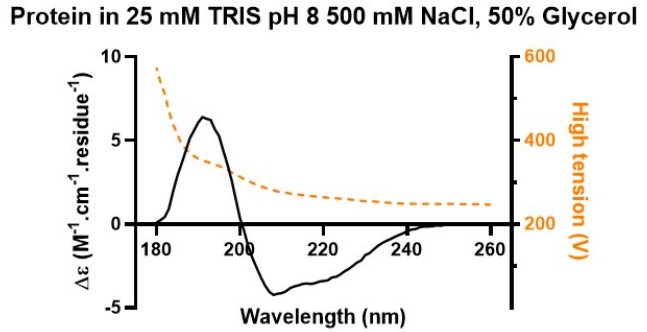
# In CD, buffer limitations are more important than in UV!

Several compounds like chloride, DTT, Imidazole, DMSO, TRIS, HEPES, etc. strongly absorb in the far-UV range




>600V, too much absorption.
Non-analyzable data

High Tension voltage (HT) is used as a gain to keep the DC of 1V in the PMT detector
HT is monitored along the spectrum to valid the CD signal just acquire




Working with high concentration (1-10 mg/ml) in low pathlength cuvettes (<100µm) reduces the contribution of the buffer signal in the far-UV range.

Protein at 10 mg/ml in a 10 µm cell.
⇒ With small pathlength, it is possible to go down to 180 nm in 25mM TRIS pH8 500 mM NaCl and 50% glycerol
Against 195 nm for 150 mM NaCl in 100 µm!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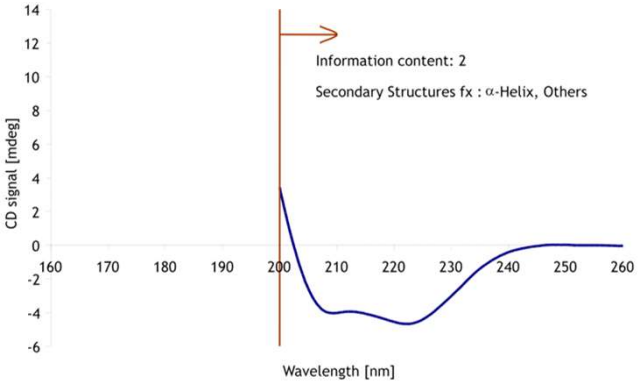
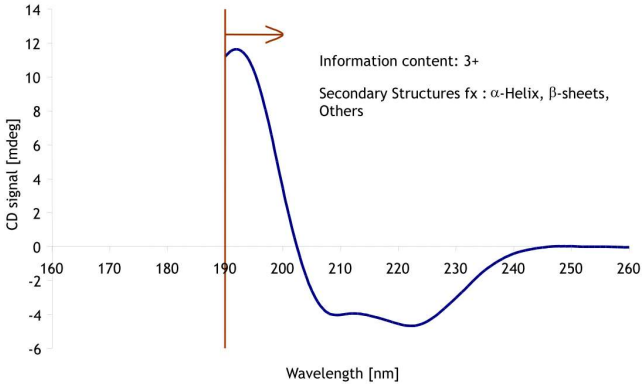
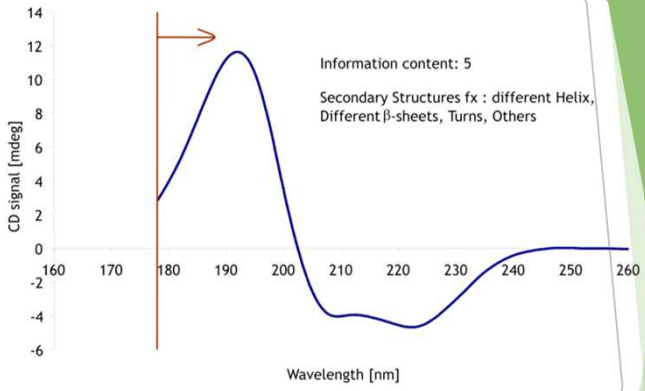
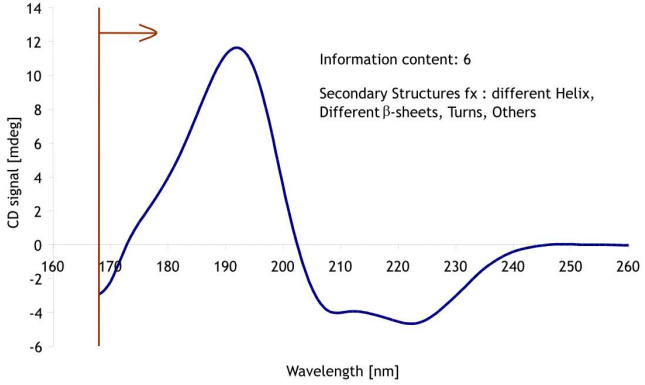
# What's the good range of analysis?

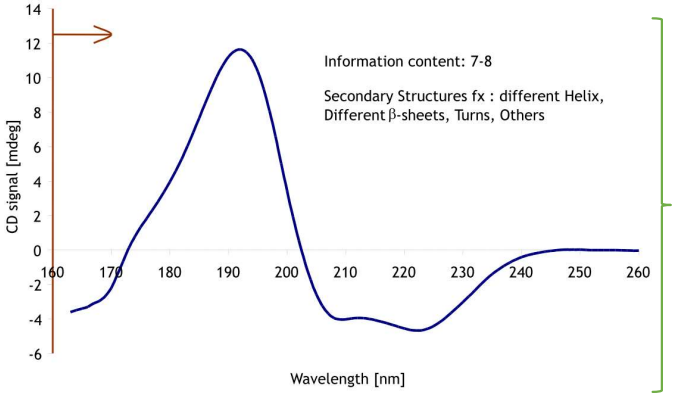
Basically, more you go down in FAR-UV, better it is for the analysis




Only possible with a synchrotron radiation circular dichroism

Søren Vrønning Hoffmann' slide



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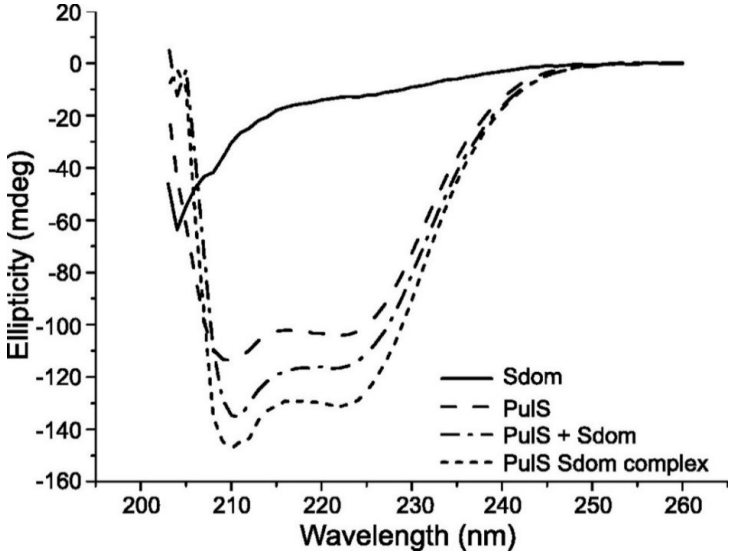
# A few results obtained at the Molecular Biophysics platform
## Acquisition of secondary structure upon complex formation




**Dual cuvette circular dichroism analysis shows an increase in secondary structure upon PulS-Sdom complex formation**

**TABLE 2**

Secondary structure of PulS, Sdom, and the PulS-Sdom complex

| Sample | Relative secondary structure[a] | | | | $T_m$[b] | $\Delta H$[b] |
|---|---|---|---|---|---|---|
| | $\alpha$ | $\beta$ | Turn | Unordered | °C | kcal/mol |
| PulS | 84 | 0 | 7 | 8 | 77.3 ± 0.1 | 60.8 ± 1.0 |
| Sdom | 17 | 19 | 19 | 46 | NA[c] | NA |
| PulS + Sdom[d] | 54 | 5 | 12 | 30 | NA | NA |
| Complex | 63 | 3 | 11 | 24 | 80.6 ± 0.1 | 70.2 ± 1.0 |

[a] Relative secondary structure was estimated from the far-UV CD spectra using the CDSSTR routine of the DICHROWEB server run on the SP175 reference dataset, see "Experimental Procedures."

[b] The thermal denaturation profiles were analyzed by a nonlinear least squares fit assuming a two-state transition and were used to calculate the melting temperature ($T_m$) and enthalpy of unfolding ($\Delta H$); see under "Experimental Procedures."

[c] NA means not applicable.

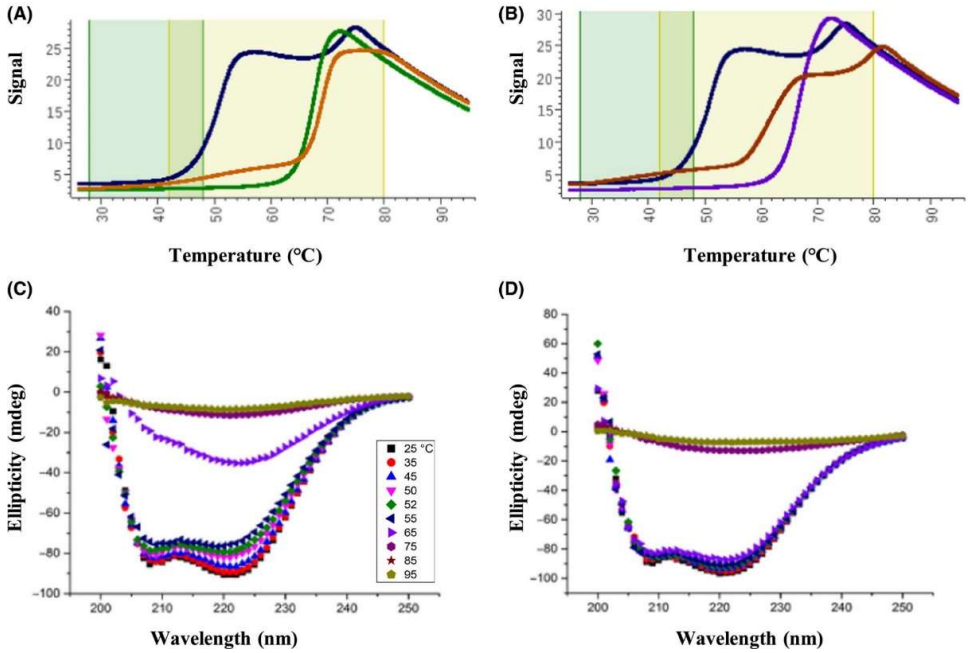
[d] Theoretical spectrum of an equimolar mixture of PulS + Sdom was calculated as a mean of each individual spectrum weighted according to their masses and the number of each amino acid in each protein and deconvoluted to give the predicted secondary structure.

**Nickerson et al. J Biol Chem 2011**



This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

# A few results obtained at the Molecular Biophysics platform
# Comparison of thermal denaturation monitored by CD and TSA




Thermal denaturation of *M. tuberculosis* UMP kinase. (A and B) TSA melting curves of *M. tuberculosis* UMP kinase. Apo enzyme (blue) in both panels. In the presence
A) of GTP (1 mM, green) or UTP (1 mM, orange)
B) of UMP (1 mM, brown) or Mg-ATP (7 mM, purple)

Far-UV circular dichroism spectra over a temperature range of 25–95 °C of
C) the apo enzyme
D) in the presence of 1 mM of Mg-ATP (D).

**Walter et al. FEBS J 2022**

=>Here, MgATP improves the thermal stability of UMPK both by looking at the secondary structure or tertiary environment.



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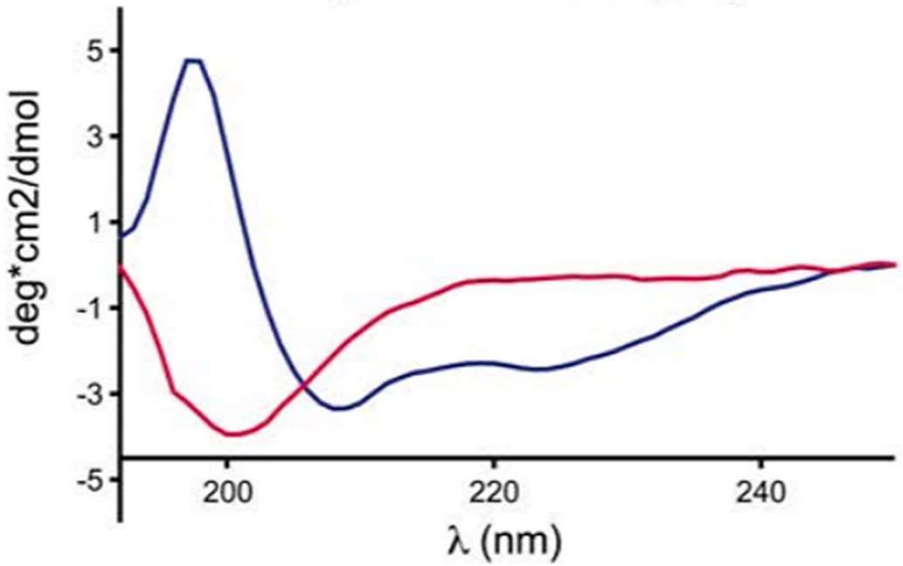
# A few results obtained at the Molecular Biophysics platform
## Peptide stability with SUVs




SepFM peptide in solution behaves as a random coil and mainly folded into an α-helix upon interaction with SUVs, a behavior like that seen for *B. subtilis* SepF.

**SepFM peptide-membrane interactions**
Circular dichroism spectra of SepFM in the absence (red) and presence (blue) of SUVs.

**Sogues et al. *Nat. Commun*. 2020**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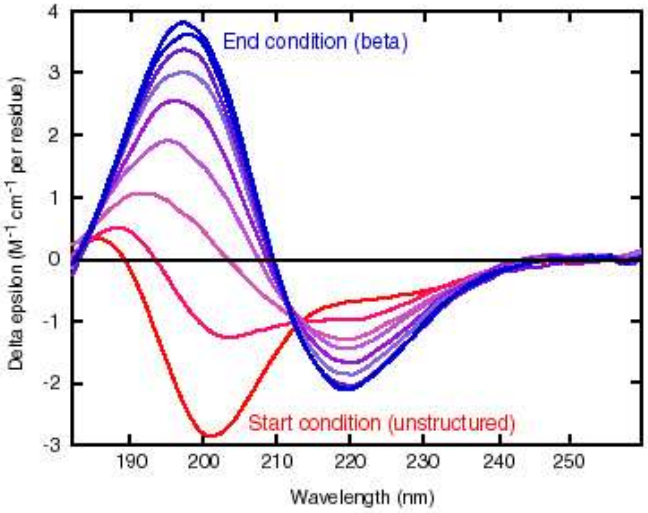
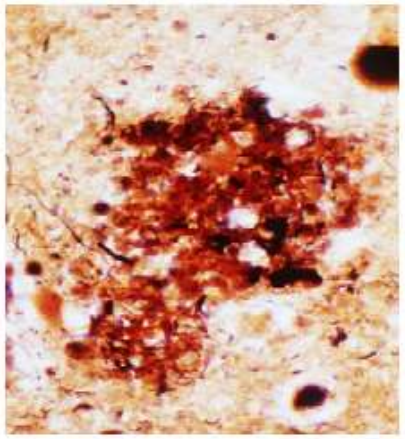
# Use of SRCD facility for amyloid diseases

A number of diseases (e.g. Alzheimer's, CJD, BSE) involve the folding of proteins and peptides into beta-sheet structures which can polymerise, forming insoluble plaques in nerve tissue (below right).

A model for the Alzheimer's peptide is LRRN, which forms spontaneously into gels with a β-sheet structure.



SRCD spectra* (left) taken during the polymerisation of LRRN peptide show that the rate of polymerisation varies with substitution of a single amino acid residue.

*Collaboration with N.Gay and M. Symmons, Cambridge University

The SRCD data provide important information about the processes involved in polymerisation, and may lead to the development of drugs to treat these diseases.

Alexandre Chenal' slide



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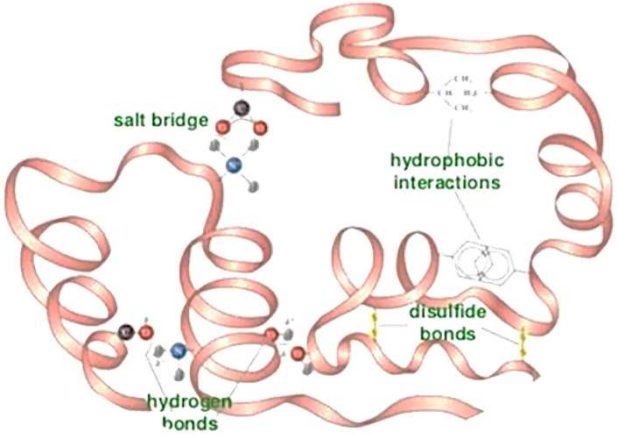
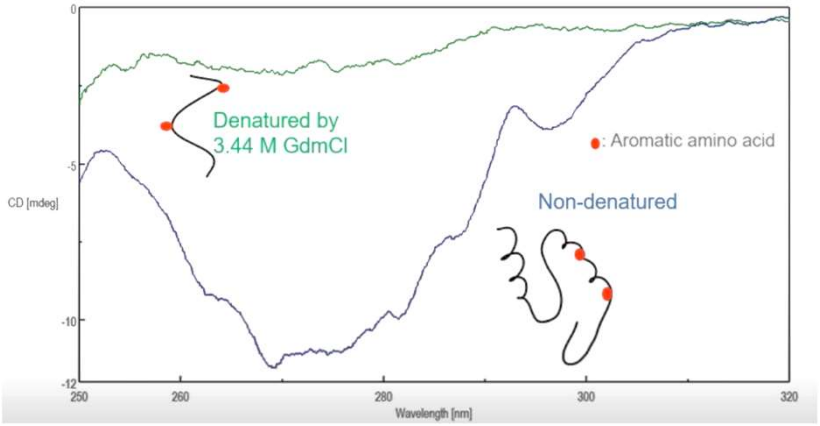
# Near-UV CD spectra of protein solutions (tertiarty structure)

- Mainly monitor aromatic residues environment.




Factors of influence:

- Solvent environment?
- Interactions with aromatic amino acid residues?
- Protein rigidity?




- Effect of Guanidinium chloride on the protein
=> loss of tertiary environment.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# Why use CD?

- Simple and quick experiments
- No extensive preparation
- Measurements on solution phase
- Relatively low concentrations/amounts of sample
- Microsecond time resolution
- Any size of macromolecule



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# Practicalities

- CD is based on measuring a very small difference between two large signals must be done carefully
- the Abs must be reasonable max between ~0.5 and ~1.5.
- Quartz cells path lengths between 0.0001 cm and 10 cm.
- Have to be careful with buffers high UV abs
- Measure cell base line with solvent
- Then sample with same cell **inserted same way around**
- **For accurate 2ndry structure estimation must know concentration of sample**



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# Summary

- CD is a useful method for looking at secondary structures of proteins and peptides.
- It is an adaptation standard absorption spectroscopy in which the difference in the abs between left and right hand circularly polarized light is measured.
- CD can be measured under a wide range of conditions - e.g., good for membrane proteins.
- CD can also be used to measure tertiary structure changes.
- CD compliments other more detailed techniques such as crystallography.



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