SPECTROSCOPY EXERCISES

1. If a solution of a colored protein of 5 mg/mL yields an absorbance readout of 0.25 at 409 nm, what is the extinction coefficient of the protein at that wavelength? What would be the concentration of a hypothetical protein solution whose absorbance is 0.90?

For a given concentration of the protein is easy to calculate the extinction coefficient using the Lambert-Beer law. In this case, the molecular weight is unknown so the coefficient units can be presented in $mL/(mg * cm)$. Another possibility is to estimate the protein's molecular weight and obtain the corresponding coefficient in molar units.

$$A = \mathcal{E} * l * c \rightarrow 0.25 = \mathcal{E} * 1cm * \frac{5mg}{mL}$$

$$\mathcal{E} = 0{,}05 \frac{mL}{cm * mg}$$

I've supposed the cuvette length is 1cm, this length is the most commonly used and the biggest size of standardized spectroscopy sample cells.

For a hypothetical protein solution with an absorbance of 0.9 at the same wavelength for the same protein the concentration in mg/mL is easily obtained:

$$A = \mathcal{E} * l * c \rightarrow 0.9 = 0{,}05 \frac{mL}{cm * mg} * 1cm * c$$

$$c = 18 \frac{mg}{mL}$$

2. Propose an experimental strategy to determine the extinction coefficient of a protein at 280 nm if we know the amino acid sequence of the protein. If the protein presents a prosthetic group that absorbs at 280 nm, would this be a problem for the strategy that you have proposed before?

The more intuitive approach is to measure the solution of the protein with and without cofactor and the solution of the cofactor. For this purpose, we can remove the prosthetic group by precipitation in order to isolate both the ligand and the protein.

In order to be more precise, we can acknowledge the binding equilibrium constant in the calculation if we have that information. In this case:

$$A_p = \varepsilon_p * l * c_p$$
$$A_c = \varepsilon_c * l * c_c$$
$$A_{c+p} = \varepsilon_{c+p} * l * c_{c+p}$$

$$c_c = c_p = c_{c+p} - ([P] + [C]);\ k = \frac{c_{c+p}}{[P] * [C]};\ [P] = [C]$$
$$c_c = c_p = c_{c+p} - 2[C];\ k = \frac{c_{c+p}}{[C]^2} \rightarrow\ \ c_c = c_p = [C]^2 - 2[C]$$

$$c_{c+p} = k[C]^2 \rightarrow \ c_c = c_p = c_{c+p}/k - 2\sqrt{c_{c+p}/k}$$

Therefore, we can express the absorbance in terms of known concentration:

$$A_p = \varepsilon_p * l * \left(\frac{c_{c+p}}{k} - 2\sqrt{\frac{c_{c+p}}{k}}\right)$$

$$A_c = \varepsilon_c * l * \left(\frac{c_{c+p}}{k} - 2\sqrt{\frac{c_{c+p}}{k}}\right)$$

$$A_{c+p} = \varepsilon_{c+p} * l * c_{c+p}$$

3. Propose an experimental strategy to determine the extinction coefficient of a protein if it contains a non-covalently bound prosthetic group that absorbs in the visible region of the electromagnetic spectrum.

A possible strategy in this case is to make a spectrum and see if the two or more generated bands overlap each other. In order to quantify the signal, the maximum intensity wavelength for the protein will be optimal if they don't overlap:

$$\frac{A}{c * l} = \text{Ɛ}_{\lambda max}$$

If one band overlap with the other we can try to eliminate the interference caused by the cofactor with an appropriate ligand or chelate.

4. We want to study an enzymatic reaction. We mix 100 µL of a solution containing the substrate at 48 mM, 50 µL of a solution containing the enzyme and 650 µL of buffer solution. At time 0 min, the absorbance readout of the solution is 1.20 and after 12 min of reaction, the absorbance decreases up to 0.90. Determine the reaction rate (in concentration units).

The first step is to know the extinction coefficient that allows to calculate concentration and therefore its evolution with time. It's easy to do it knowing the initial concentration of substrate.

$$c_1 = \frac{0{,}048\ mol * 0{,}1 * 10^{-3} l}{1l * 0{,}8 * 10^{-3} l}$$

$$1.2 = \text{Ɛ} * 1cm * c_1 = \text{Ɛ} * 1cm * 0{,}006M$$

$$\text{Ɛ} = 2000 \frac{l}{mol * cm}$$

$$0.9 = 2000 * 1cm * c_2 \rightarrow c_2 = 0{,}00045M$$

$$reaction\ rate = \frac{\Delta c}{\Delta t} = \frac{0{,}00555M}{12min} = \frac{0{,}0004625M}{min} = \frac{0{,}02775M}{s}$$

5. Ferredoxin-NADP(+) reductase (FNR) is an enzyme that catalyzes the reduction of NADP+ ($\varepsilon_{340nm} = 0\ mM^{-1}cm^{-1}$) to NADPH ($\varepsilon_{340nm} = 6.3\ mM^{-1}cm^{-1}$). The process is reversible. FNR can also reduce the compound 2,6-dichlorophenolindophenol, DCPIP, that is blue-colored in its oxidized state ($\varepsilon_{620nm} = 21\ mM^{-1}cm^{-1}$) and does not show any color in its reduced state ($\varepsilon_{620nm} = 0\ mM^{-1}cm^{-1}$). With this information, propose an experimental strategy to measure the enzymatic activity of FNR. How would you calculate K_m and k_{cat}?

The strategy consists on having the two substrates in the same medium so the FNR can also participate in an irreversible process in order to make first reaction run:

$$\boldsymbol{FNR + NADP^{+} \leftrightarrow NADP^{+}FNR \rightarrow NADPH + FNR}$$

$$\boldsymbol{FNR + DCPIP \rightarrow DCPIP(reduced) + FNR}$$

Initial velocities method will be good for the determination of the constants Km and Kcat in the 340nm wavelength.

6. RNA and DNA solutions at 2.10^{-5} M concentrations of nucleotides present the following differential absorbances (A_L: absorbance with left-handed circularly polarized light; A_R: absorbance with right-handed circularly polarized light):

Wavelength (nm)	A_L-A_R, DNA	A_L-A_R, RNA
240	$-4.4x10^{-4}$	0
260	0	$12.0x10^{-4}$
280	$6.0x10^{-4}$	$3.2x10^{-4}$
300	$0.2x10^{-4}$	$-1.0x10^{-4}$

a. Determine εL- εR for each wavelength for RNA and DNA.

$$(\mathcal{E}_L - \mathcal{E}_R)lc = A_L - A_R \rightarrow (\mathcal{E}_L - \mathcal{E}_R) = \frac{(A_L - A_R)}{lc}; l = 1cm, c = 2 * 10^{-5}M$$

For the DNA

$$(\mathcal{E}_{L,240} - \mathcal{E}_{R,240}) = -22\ \frac{L}{mol * cm}$$

$$(\mathcal{E}_{L,260} - \mathcal{E}_{R,260}) = 0\ \frac{L}{mol * cm}$$

$$(\mathcal{E}_{L,280} - \mathcal{E}_{R,280}) = 30\ \frac{L}{mol * cm}$$

$$(\mathcal{E}_{L,300} - \mathcal{E}_{R,300}) = 10\ \frac{L}{mol * cm}$$

For the RNA

$$(\mathcal{E}_{L,240} - \mathcal{E}_{R,240}) = 0\ \frac{L}{mol * cm}$$

$$(\mathcal{E}_{L,260} - \mathcal{E}_{R,260}) = 60\ \frac{L}{mol * cm}$$

$$(\varepsilon_{L,280} - \varepsilon_{R,280}) = 16\ \frac{L}{mol * cm}$$
$$(\varepsilon_{L,300} - \varepsilon_{R,300}) = -5\ \frac{L}{mol * cm}$$

b. Determine the molar ellipticity for RNA and DNA at each wavelength.

$$(\varepsilon_L - \varepsilon_R) * 3298 = [\theta]$$

ΔA, DNA	ΔA, RNA	ΔƐ, DNA	ΔƐ, RNA	[θ], DNA	[θ], RNA
-4,40E-04	0,00E+00	-22,00	0,00	-72556	0
0,00E+00	1,20E-03	0,00	60,00	0	197880
6,00E-04	3,20E-04	30,00	16,00	98940	52768
2,00E-05	-1,00E-04	1,00	-5,00	3298	-16490

These [θ] values are in deg*cm^2/mol.

c. A mixture of RNA and DNA has the following differential absorbances:

Longitud de onda (nm)	A_L-A_R
240	$-0.53x10^{-4}$
260	$4.0x10^{-4}$
280	$1.05x10^{-4}$
300	$-0.31x10^{-4}$

Determine the concentration of RNA and DNA in the mixture.

ΔƐ, DNA	ΔƐ, RNA	ΔA	c (M) DNA	c (M) RNA
-22	0	-5,30E-05	2,41E-06	0,00E+00
0	60	4,00E-04	0,00E+00	6,67E-06

7. A typical structural motif of proteins that bind DNA is the "leucine zipper" motif, that is created by the dimerization of two specific leucine-rich sequences of 30-35 residues that fold into a right-handed α-helix structure. The two α-helices dimerize and fold into a coiled left-handed helix. A number of studies of this protein structural motif have been carried out typically using peptide models. In one of such studies, the CD spectrum of the peptide model at a 0ºC showed an intense positive peak at 195 nm and two negative minima at 208 nm and a 222 nm. This spectrum was assigned to a fully helical structure.

a. At 10ºC the molar ellipticity at 222 nm is -33000 deg cm^2 dmol^{-1}. At 80ºC the molar ellipticity increases to zero. What has happened?

At 80ºC there are 1/3 of the total number of domains are in monomers with right α-helices, and 2/3 of the total number of domains of proteins are forming dimers creating coiled left-handed helices.

b. At 55ºC the molar ellipticity at 222 nm is -16000 deg cm2 dmol-1. What percentage
of the peptide is now in α-helix?

If we consider the value of the molar ellipticity at 10ºC the minimum value of this parameter:

$$\frac{-16000}{-33000} = 0{,}4848$$

$\rightarrow$ *there is a* 48,5% *of left handed helices, plus the inital ones at ellipticity* 0

That means there is a $\frac{48{,}5+48{,}5}{48{,}5+48{,}5+51{,}5} = 65{,}3\%$ of the 'extra' monomer regions forming also dimers.

The total monomers that are dimerized is 2/3 plus 65,3% of 1/3, this means an 88,43%. In this case a 11,57% of the peptide is in alfa-helix.

c. For some peptide models, the temperature at which the ellipticity is zero increases
with the peptide concentration. What is the interpretation of this result?

This means that the energy required for the formation of dimers increases with concentration. As is logical that higher concentrations require higher energy $\Delta G = \Delta G_f{}^*n$, with $\Delta G>0$.

8. Estrogen receptors are transcription factors that are activated upon binding to ligands that mediate the effects of the female sex hormones during the DNA transcription. The interaction of these receptors with a DNA fragment has been studied following CD. The analysis of the CD spectrum indicated that the receptor presented 75% α-helix, 3% β-sheet, 10% turns and 12% random coil. In the presence or absence of DNA and estradiol, the ellipticity at 222 nm was:

Ligand	[θ], deg cm^2 $dmol^{-1}$
none	-25000
5 µM DNA	-32000
5 µM estradiol	-22000

a. Assume that the signal at 222 nm only reflects α-helical structure and that all the receptor molecules are bound to ligand. Propose an explanation for the behavior of the receptor and calculate the percentage of α-helix when each ligand is bound to it.

It is clear that the binding of DNA and/or estradiol stabilizes or destabilizes the α-helix, the DNA binding increases the stabilization of this structure while the estradiol destabilizes it.

$$\frac{25000}{75} = \frac{32000}{\alpha\ helix\ \ \%, DNA} = \frac{22000}{\alpha\ helix\ \%, estr.}$$

$$\alpha\ helix\ \ \%, DNA = 96\ \%$$
$$\alpha\ helix\ \%, estr.\ \ = 66\ \%$$

b. When the temperature increases, the ellipticity at 222 nm is close to zero. Explain this behavior.

When temperature rises the helices start to unroll and these regions remain unfolded so they can't interact with circularly polarized light.

c. The temperature at which the difference between the initial and final ellipticity is half of the maximum difference, Tm, is 38°C in absence of ligand, but it increases to 43.6°C and 46.1°C in the presence of 5 µM of estradiol or DNA, respectively. Explain this behavior.

Although estradiol destabilizes alfa-helices, the protein is stabilized by its presence, so other structures such as beta-sheets can be stabilized in the presence of this ligand. In the case of DNA binding, the stabilization of alfa-helices, the main structure, contributes to protein stability that's why protein stability is higher.

d. In the presence of 750 nM estradiol, the Tm is 40.8°C. Explain this result and estimate the dissociation constant of the receptor-estradiol complex, assuming that the concentration of ligand is much higher than that of the receptor. At 40.8°C, the ratio between the denatured and native protein was determined to be 1.20 in absence of ligand.

There's a possibility that the ligand joins also to the unfolded state, this way in excess of estradiol unfolded proteins are also stabilized. This fact is related to the low percentage of alfa-helices in the presence of estradiol.

$$\Delta G^0 = -RTln(K) \rightarrow -8{,}314 * 313{,}95 \ln(1{,}2) = -475{,}89\ J$$

$$\Delta G = \Delta G^0 + RTln(1 + K_{estradiol} * [0{,}000750]) = \ -RTln(K)$$

$$-475{,}89 + 8{,}314 * 298{,}15 * \ ln(1 + K_{estradiol} * [0{,}000750]) =$$

$$= \ -8{,}314 * 313{,}95 ln(1)$$

$$ln(1 + K_{estradiol} * [0{,}000750]) = \frac{475{,}89}{8{,}314 * 298{,}15}$$

$$\frac{\left(e^{\frac{475{,}89}{8{,}314*298{,}15}} - 1\right)}{0{,}000750} = K_{estradiol}\ = 282{,}20 M^{-1}$$

$$K_{d,estradiol}\ = \frac{1}{282{,}20 M^{-1}} = 3{,}54 * 10^{-3} M$$

9. Pirenomale imide is a convenient fluorescent probe for the labeling of sulfhydryl residues in proteins. The fluorescence lifetime of pyrene in the sulfhydryl of a protein was measured by determining the relative fluorescence intensity after the excitation of pyrene with a light pulse. The data obtained are given below:

Fluorescencia relativa	Tiempo (ns)
0.716	20
0.513	40
0.367	60
0.264	80
0.189	100

a. Determine the fluorescence lifetime.

relative fluor.	time (ns)	lifetime (ns)
0,716	20	14,32
0,513	40	20,52
0,367	60	22,02
0,264	80	21,12
0,189	100	18,9

*Relative fluorescence*time =lifetime*

b. If the quantum yield is 0.7, determine the natural lifetime.

relative fluor.	time (ns)	lifetime (ns)	natural lifetime (ns)
0,716	20	14,32	10,024
0,513	40	20,52	14,364
0,367	60	22,02	15,414
0,264	80	21,12	14,784
0,189	100	18,9	13,23

*Lifetime*0,7=fluorescence lifetime*

c. When the protein binds a ligand, the fluorescence lifetime decreases 10%. Why?

The ligand is an external quencher, the excitation energy of the protein is given to the ligand so a dark complex is formed and the fluorescence decreases its intensity and lifetime.

10. Bovine rhodopsin is a protein photoreceptor that forms an integral part of the discs of the rod membranes and plays a critical role in vision. It has a molecule of 11-cisretinal attached which has a strong absorbance at 500 nm. As part of the vision cycle, absorption decreases by conversion to the trans form of retinol. The protein has a molecular weight of 28000-40000 Da. Three sites of the protein were labeled with a fluorescent probe, A, B and C. The fluorescence resonance energy transfer was

measured between these three sites and the retinal, and between the three sites themselves. Some of the results are summarized in this table:

Donor	Acceptor	Efficiency of energy transfer	R_o (Å)
A	11-cis-retinal	0.09	51
B	11-cis-retinal	0.36	52
C	11-cis-retinal	0.12	53
A	B	0.90	51
A	C	0.92	48
B	C	0.92	47

a. Calculate the distance between these three sites

Donor	Acceptor	Efficiency	R0 (angstroms)	r (angstroms)
A	B	0,9	51	18
A	C	0,92	48	17
B	C	0,92	47	16

$$r = \left(\sqrt[6]{\frac{1+E}{E}} \right) * R$$

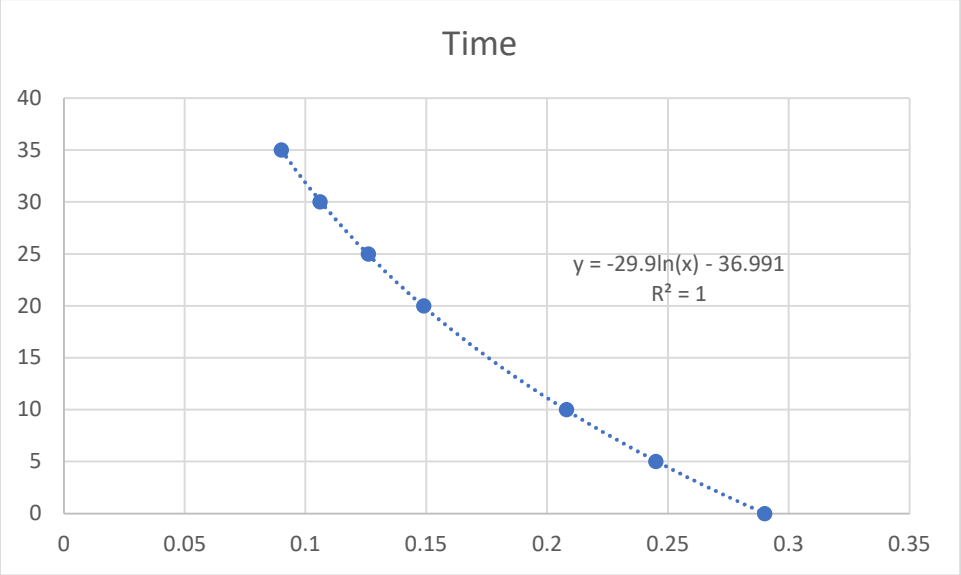
b. A 28-40 kDa protein that is spherical has a radius of 40-45 Å. What can you say about the shape of rhodopsin? Schematize a model for the molecule based on the distances that have been measured.

Distances between points A, B and C are similar so the shape can be ruled by a circumference. The diameter of this hypothetical sphere will be ruled by the circumscribed circle of an equilateral triangle of 17 angstroms which has a diameter of 17 angstroms. This suggest that the third dimension is longer so the form is a cylinder with length $(4/3*pi*45^3)/(2*pi*8{,}5^2) = 840$ angstroms.

11. The system for the transport of mannitol through the membrane of E. coli involves a membrane enzyme that couples the phosphorylation of the sugar with its translocation through the membrane. Measurements of the lifetime and fluorescence anisotropy of tryptophan have been performed on this enzyme prepared in detergent micelles. The drop in fluorescence anisotropy of one of these tryptophans has two components. The fastest component, with a rotational correlation time of 1 ns, is due to the local movement of the tryptophan. The decrease in the anisotropy of the slowest component is approximated by the following data:

Anisotropy	Time (ns)
0.290	0
0.245	5
0.208	10
0.149	20
0.126	25
0.106	30
0.090	35

a. Calculate the rotational correlation time



$$r(t) = r + (r_0 - r)e^{-\frac{t}{\tau}}$$

$$t = -\tau \ln(r(t) - r) - \ln(r_0 - r)$$

If r≃0; being r the anisotropy in time = ∞.

$$\tau \simeq 29{,}9\ ns$$

b. The expected rotational correlation time for a macromolecule with the same molecular weight is 120-140 ns. How can you explain the discrepancy between values?

The molecule is very heterogeneous in its structure.

c. When the protein is phosphorylated, the rotational correlation time increases to 51 ns. What could be the reason for this change?

The phosphorylation process changes the protein structure.

12. For a single donor and acceptor pair, if the distance between them can be varied, what is the efficiency of the energy transfer if the donor and the acceptor are separated by 0.5 Ro, Ro and 2 Ro? (Ro, Förster distance)

$$E1 = \frac{1}{1 + 0{,}5^6} = 0{,}985$$

$$E2 = \frac{1}{1 + 1^6} = 0{,}5$$

$$E3 = \frac{1}{1 + 2^6} = 0{,}015$$