Homework for BCH 581 – biospectroscopy
Email your answers to klara.briknarova@umontana.edu by 10:30 am on Wednesday March 15. If you write some parts by hand and scan them, please make sure your writing is legible! Show your work and explain your reasoning. Be concise. If you use online resources (you may need to for some of the questions), make sure to cite them and explain what information you obtained from them.

1. (4 points) Protein X has the following sequence:
GSHMSPLVATSESVTEITASSFVVSWVSASDTVSGFRVEYELSEEGDEPQYLDLPSTATSVNIP
DLLPGRKYIVNVYQISEDGEQS
(a) Calculate the extinction coefficient of Protein X at 280 nm.

$\varepsilon(280\ nm) = n(Trp) \times \varepsilon(Trp) + n(Tyr) \times \varepsilon(Tyr) + n(disulfide) \times \varepsilon(disulfide)\ [M^{-1}\ cm^{-1}]$
$5{,}500 \qquad 1{,}490 \qquad 125$

$\varepsilon(280\ nm) = 1\ (5{,}500) + 4\ (1{,}490) = 11{,}460\ M^{-1}\ cm^{-1}$

You can also copy and paste the sequence on the ProtParam web site (https://web.expasy.org/protparam/), and it will count Tyr and Trp residues and calculate the extinction coefficient and other useful properties like molecular mass for you.

(see Fluorescence 2: p.10 and the output from ProtParam at the back of this key)

(b) Do you expect Protein X to exhibit fluorescence? If it does, what excitation wavelength will you use to observe the fluorescence? In what range of wavelengths do you expect to see fluorescence emission?

Yes! Protein X contains tryptophan, tyrosine and phenylalanine residues, which all exhibit fluorescence. Fluorescence spectra of proteins are usually dominated by signal from Trp because Trp residues have the highest extinction coefficient and quantum yield (see the table in Fluorescence 2: p.9). In addition, Tyr and Phe tend to undergo FRET to Trp, which decreases the signal from Tyr and Phe and increases the signal from Trp.
Fluorescence of Phe is for all practical purposes negligible and is therefore ignored unless the protein contains no Tyr and Trp.

Fluorescence spectra of proteins that contain Trp are typically measured using excitation at 280 nm (but other wavelengths in the 260-295 nm region will also work), and fluorescence signal can be observed from > 280 nm (~ 300 nm is a good start to avoid scattered excitation light) to approximately 400 nm.

(see Fluorescence 2: p.8-9 & 11-13)

Note that fluorescence signal from Trp in proteins may have a maximum at lower wavelength than ~ 350 nm because Trp is often buried and hence in a hydrophobic environment, which results in a blue shift. (see Fluorescence 2: p.12-13)

2. (4 points) You plan to study the interaction of Protein X with Protein Y (which is larger than Protein X) by fluorescence anisotropy. For this purpose, you label Protein X with fluorescein isothiocyanate (FITC).
(a) What excitation and emission wavelength will you use for this experiment? Explain your choice.

You should use excitation wavelength at or near the absorption maximum, and emission wavelength at or near the emission maximum.
According to the Molecular Probes Handbook (https://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook.html), the absorption and emission maxima are at 494 and 518 nm respectively (Tables 1.1 and 1.2). Similar values are obtained for FITC on the Chroma website (https://www.chroma.com/spectra-viewer). (see also Fluorescence 2: p.16)
The positions of the maxima depend on pH (Fig. 1.5.2 in the Molecular Probes Handbook) and may also be affected by conjugation to the protein, but excitation around 490 nm and emission around 520 nm should in general work well.

(b) You plan to measure fluorescence anisotropy as a function of Protein Y concentration while Protein X concentration is kept constant. If Protein Y binds to Protein X, do you expect the fluorescence anisotropy to increase or decrease when the concentration of Protein Y is increased? Explain.

Fluorescence anisotropy will increase.

Fluorescence anisotropy depends on the rate of tumbling (rotation) of the fluorescently labeled molecule. When polarized light (i.e. light in which the electric field vector has a single well defined direction) is used for excitation, it will preferentially excite molecules in a certain orientation. If all molecules have the same orientation when they emit light, the emitted light will be polarized. The less the molecules tumble between absorption of a photon and subsequent emission of the photon as fluorescence, the more similar their orientations will be when they emit the photon, and the more polarized the fluorescence emission will be.

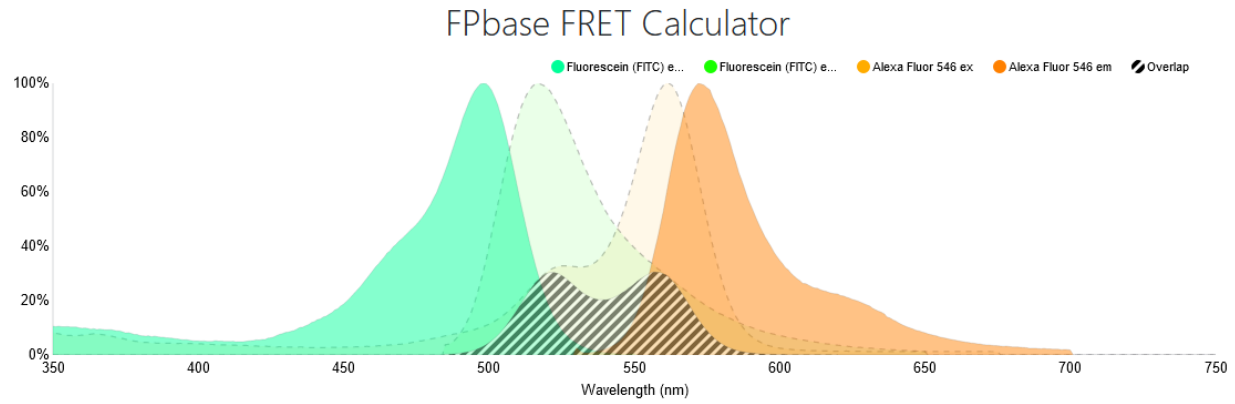
The complex formed by Protein X and Protein Y is larger than free Protein X and will tumble more slowly. The fluorescence anisotropy of Protein X bound to Protein Y will therefore be higher than anisotropy of free protein X. As the concentration of Protein Y is increased, more Protein X will be bound to Protein Y, and the fluorescence anisotropy of the mixture will increase. (see NMR 1: p.5-6)

3. (6 points) You also plan to study the interaction of Protein X with Protein Y using FRET. For this experiment, you will use Protein X-FITC that you prepared previously (#2 above), and label Protein Y with Alexa Fluor 546.
(a) Which dye will serve as the donor, and which one as the acceptor?

FITC will serve as the donor, and Alexa Fluor 546 as the acceptor (see spectra in part b).

(b) You plan to measure fluorescence intensity as a function of Protein Y concentration while the concentration of Protein X is kept constant. What excitation and emission wavelength will you use? Explain your choice.

The normalized absorption and emission spectra of FITC and Alexa Fluor 546 are shown below (https://www.fpbase.org/fret/).




| $QY_{Donor}$ | $EC_{Acc.}$ ($M^{-1}$ $cm^{-1}$) | $QY_{Acc.}$ | J(λ) (*1e15 $M^{-1}$ $cm^{-1}$ $nm^4$) | $R_0$(Å) | $R_0 \times QY_A$ |
|---|---|---|---|---|---|
| 0.92 | 112,000 | 0.79 | 3.36 | 62.22 | 49.16 |

You should use excitation wavelength around 490 nm, which is where the donor (FITC) absorbs. There are two possible choices for the emission wavelength:

I. Monitor fluorescence of the donor around 520 nm.
II. Monitor fluorescence of the acceptor around 570 nm.

(c) If Protein X binds to Protein Y, do you expect the fluorescence intensity that you measure to increase or decrease when concentration of Protein Y is increased? Explain.

The interaction between FITC-Protein X and Alexa 546-Protein Y will bring the two fluorophores close to each other. When the donor (FITC) is excited, the excitation will be transferred via FRET to the acceptor (Alexa 546), and a photon will be emitted by the acceptor (Alexa 546) instead of the donor (FITC). Binding of Protein X to Protein Y and the resulting proximity of FITC to Alexa 546 will therefore lead to:

I. Decrease of donor fluorescence.
II. Increase of acceptor fluorescence.

(see Fluorescence 2: p.25-26)

ProtParam output for sequence in Question 1:

Expasy ProtParam

## ProtParam

**User-provided sequence:**

```
        10         20         30         40         50         60
GSHMSPLVAT SESVTEITAS SFVVSWVSAS DTVSGFRVEY ELSEEGDEPQ YLDLPSTATS

        70         80
VNIPDLLPGR KYIVNVYQIS EDGEQS
```

References and documentation are available.

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**Number of amino acids:** 86

**Molecular weight:** 9301.13

**Theoretical pI:** 3.94

**Amino acid composition:** CSV format

| | | |
|---|---|---|
| Ala (A) | 4 | 4.7% |
| Arg (R) | 2 | 2.3% |
| Asn (N) | 2 | 2.3% |
| Asp (D) | 5 | 5.8% |
| Cys (C) | 0 | 0.0% |
| Gln (Q) | 3 | 3.5% |
| Glu (E) | 9 | 10.5% |
| Gly (G) | 5 | 5.8% |
| His (H) | 1 | 1.2% |
| Ile (I) | 4 | 4.7% |
| Leu (L) | 6 | 7.0% |
| Lys (K) | 1 | 1.2% |
| Met (M) | 1 | 1.2% |
| Phe (F) | 2 | 2.3% |
| Pro (P) | 5 | 5.8% |
| Ser (S) | 15 | 17.4% |
| Thr (T) | 6 | 7.0% |
| Trp (W) | 1 | 1.2% |
| Tyr (Y) | 4 | 4.7% |
| Val (V) | 10 | 11.6% |
| 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):** 14
**Total number of positively charged residues (Arg + Lys):** 3

**Atomic composition:**

| | | |
|---|---|---|
| Carbon | C | 408 |
| Hydrogen | H | 629 |
| Nitrogen | N | 101 |
| Oxygen | O | 145 |
| Sulfur | S | 1 |

**Formula:** $C_{408}H_{629}N_{101}O_{145}S_1$
**Total number of atoms:** 1284

**Extinction coefficients:**

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

| | |
|---|---|
| Ext. coefficient | 11460 |
| Abs 0.1% (=1 g/l) | 1.232 |