Protein Fluorescence and Quenching

Materials
* Potassium Phosphate Buffer
  -10 mM potassium phosphate (K$_2$HPO$_4$), pH 7.5

*Quenchers
-5 M potassium iodide (KI) in 10 mM potassium phosphate (K$_2$HPO$_4$), pH 7.5

-5 M CHCl$_3$ (chloroform) in isopropyl alcohol

*BSA Solution
-0.23 mg/ml Bovine Serum Albumin in 10 mM potassium phosphate (K$_2$HPO$_4$), pH 7.5

Overview
Fluorescence will be measured for increasing concentrations of a reagent that is able to quench the excited state of the fluorophore (tryptophan).

Sample Preparation

- Aliquot ~1.1 ul of BSA solution into 10 separate 1.5 ml microfuge tubes.
- Centrifuge all five tubes for ~2 min at ~13,000 RPM.

Absorbance and fluorescence can be taken in any order and it does not matter which data are collected first.
Fluorescence

To observe tryptophan's fluorescence emission the detector will be set to record over the range of 300-400 nm, and the excitation wavelength will be set to 295 nm. The fluorescence spectrum of the sample will be measured in the absence of quencher, then additional fluorescence spectra will be collected after each addition of a quencher (either iodide or chloroform).

- Fluorescence will be performed in the instrument room (CP227). When you are ready to collect fluorescence data you will need to bring the following to the instrument room:
  a. 4 of the 10 samples you prepared
  b. USB jumpdrive/storage device (the instruments are NOT on the web)
  c. Lab notebook and writing implement.

- While collecting fluorescence data you will have an opportunity to familiarize yourself with key parameters of the instrument. Please make an effort to record parameter settings in your notebook, ask questions, and think critically about data acquisition.

1. Pipette 1 ml from two prepared samples into the fluorescence cuvette
   \textit{Notebook observation: Notice anything different about the cuvette?}

2. Record fluorescence

3. Add 2.5 ul of, quencher mix by pipetting or inversion, and record fluorescence.

4. Repeat step 3.

5. Add 5 ul quencher, mix, and record fluorescence.

6. Add 20 ul quencher, mix, and record fluorescence.

7. Discard sample (sink) and clean the cuvette (water then acetone then water).

8. Familiarize yourself with the spectral analysis software.

9. Use the software to calculate the area from 340-355 nm (The area will simultaneously be calculated for all spectra shown).

10. Export data

   \textit{File} \rightarrow \textit{Export} \rightarrow \textit{Sheet} \rightarrow \textit{Save As}
Absorbance

The absorbance will increase upon addition of the quencher. Absorbance of the sample at the excitation (295 nm) and the emission (350 nm) wavelengths will be used to account for the loss of fluorescence intensity due to internal absorption (inner filter effect):

\[ I_{corr} = I_{app} \times \left( \frac{A_{295} + A_{350}}{2} \right)^2 \]  

Eq. 1

Where \( I_{corr} \) is the corrected intensity, \( I_{app} \) is the apparent or measured intensity, \( A_{295} \) is the absorbance at 295 nm, and \( A_{350} \) is the absorbance at 350 nm.

1. Transfer 3 ml of buffer to a quartz cuvette to use as a blank.
2. Load 3 ml of your centrifuged BSA solution into another cuvette.
3. Measure absorbance from 295 nm to 350 nm from the blank (wavelength scan). (You may also choose to write in your notebook the \( A_{295} \) and \( A_{350} \))
4. Repeat the absorbance scan and data records for the BSA solution.
5. Add 5 ul of quencher to the BSA solution in the cuvette. To mix, use the 1000 µl pipette and gently pump the liquid up and down 2-3 times.
6. Repeat step 3 (measure and record).
7. Repeat step 4 (add 2.5 ul)
8. Repeat step 3 (measure and record)
9. Repeat step 4 (add 2.5 ul)
10. Repeat step 3 (measure and record)
11. Repeat step 4 but using 5 ul of quencher
12. Repeat step 3 (measure and record)
13. Repeat step 4 but using 20 ul of quencher
14. Repeat step 3 (measure and record)
15. The series of measurements can be used to make a standard curve to relate quencher concentration to absorbance. The standard curve can be used to obtain the \( I_{corr} \) to account for the loss in fluorescence intensity.

Analysis

1. Use your observed fluorescence intensities at 350 nm in conjunction with your standard absorptions curves and calculate corrected fluorescence intensities for each concentration of quencher (including zero).
2. Also calculate the change in fluorescence
\[ \Delta I = I_{0,corr} - I_{corr} \]
where $I_{corr}$ is the corrected fluorescence intensity at a given concentration of quencher and $I_{0,corr}$ is the corrected fluorescence intensity in the absence of quencher.

3. Plot $\frac{I_{0,corr}}{I_{corr}}$ vs. the concentration of quencher $[Q]$. Is your maximum quencher concentration saturating? (See figure 7.7.6 A in Pain).

4. Plot $\frac{I_{0,corr}}{\Delta I}$ vs. $1/[Q]$. Do your data conform to the expectations of the Stern-Volmer model? (In the simple case, Stern-Volmer predicts a straight line $\frac{I_{0,corr}}{\Delta I} = \frac{1}{f_A K_D [Q]} + \frac{1}{f_A}$ with intercept $1/f_A$ where $f_A$ is the fraction of the tryptophans accessible to quencher (fraction of total fluorescence that is eventually quenched at infinite $[Q]$). The slope then yields $K_D$, the Stern-Volmer quenching constant for the quencher and fluorophore in question. (See figure 7.7.6 B in Pain).

5. Find the emission maximum (the wavelength at which emission is strongest) for each of your spectra and tabulate the values obtained in the absence of quencher and in saturating quencher. Compare your values with those reported by classmates using the same protein upon quenching with a different quencher.

5a. Which is more quenched by chloroform: short-wavelength emission or longer-wavelength emission? Does this make sense? Why?

5b. Which is more quenched by iodide: short-wavelength emission or longer-wavelength emission? Does this make sense? Why?

6. Repeat 1. through 4. for quenching by the other quencher (whichever one you did not measure yourself), using data from your classmate performing the experiments on the same day.

Questions
Draw a Jablonski diagram including the processes of absorption, non-radiative decay, fluorescence and quenching. For each, write a sentence or two saying what information it provides in this lab exercise and explaining how (the logic as well as the physics).

How many Trp residues are there BSA?
Based on your experiments, how many Trp appear to be exposed to iodide? to chloroform?

References
