A Determination of DNA-DAPI Binding using Fluorescence Spectroscopy

In this Laboratory Exercise, we will determine the binding constant $K_f$ for complex formation between 4′-6-diamidino-2-phenylindole (DAPI) and double stranded DNA using a Scatchard analysis of the complex's fluorescence. This analysis will also yield a measure of number of DNA nucleotides per bound DAPI ligand.

Luminescence involves emission of photons from excited atoms or molecules. Fluorescence and Phosphorescence, both luminescent processes, involve emission of photons from systems that have been excited by absorption of photons. In molecular Fluorescence Spectroscopy, an analyte molecule first absorbs a photon (excitation, $\Delta E_{\text{excite}}$) that leaves the analyte in an electronically and vibrationally excited state.

At this point, the molecule rapidly loses excess vibrational energy by non-radiatively relaxing to the ground vibrational level of the excited electronic state. This occurs because energy is transferred to solvent molecules as the analyte jostles against them. This relaxation process is very efficient and very rapid. Finally, the molecule can fluoresce ($\Delta E_{\text{relax}}$). Alternatively, the molecule can undergo a non-radiative transition (Internal Conversion) to the ground state. Molecules undergoing Internal Conversion transit to the Ground State without emitting radiation. This is an efficient relaxation process when higher vibrational states of the Ground Electronic State overlap with lower vibrational states of the Excited Electronic State.

Because of the non-radiative relaxation in the electronically excited state, the excitation energy is always larger than the relaxation energy.

$$\Delta E_{\text{excite}} > \Delta E_{\text{relax}} \quad \text{(Eq. 1)}$$
Since the energy of the photons involved in these transitions is inversely related to the photons' wavelength:

\[ E_{\text{photon}} = \frac{hc}{\lambda} \]  

(Eq. 2)

\( c \) is the speed of light and \( h \) is Planck’s constant, the wavelength of an exciting photon is always shorter than that of a photon emitted during relaxation:

\[ \lambda_{\text{excite}} < \lambda_{\text{relax}} \]  

(Eq. 3)

Fluorescence spectra can be measured using a Spectrofluorometer. Light from the source is dispersed and the excitation wavelength is selected using a monochromator. The excitation radiation impinges upon the sample, which then begins to fluoresce. Fluorescent radiation is itself dispersed and the spectrum is measured using an appropriate detector. In an actual spectrofluorometer, the dispersing element is usually a diffraction grating. In simpler fluorometers, wavelength selection is accomplished using filters.

The fluorescent intensity (\( F \)) of an analyte solution will be proportional to the radiant Power absorbed by the sample (\( P_0 - P \)):

\[ F = K' (P_0 - P) \]  

(Eq. 4)

Inserting Beer’s Law:

\[ \frac{P}{P_0} = 10^{\epsilon bc} \]  

(Eq. 5)
and expanding the exponential term, gives us:

\[ F = K' P_o \{2.3\varepsilon_{bc} - (2.3\varepsilon_{bc})^2/2 - \ldots\} \]  
(Eq. 6)

Provided the sample Absorbance is relatively low, we can truncate the expansion:

\[ F = 2.3 K' P_o \varepsilon_{bc} \]  
(Eq. 7)

When \( P_o \) is constant, we see the Fluorescent Intensity is proportional to the Concentration of the analyte:

\[ F = K c \]  
(Eq. 8)

This, then, provides a method for quantifying the amount of analyte in a system based on fluorescence measurements.

In this experiment we will determine the fluorescence of DAPI bound to DNA. DAPI was originally synthesized in 1971 as a potential treatment for typanosomiasis. Although the treatment proved to be ineffective, it was found DAPI's fluorescence increases significantly when bound to DNA. Thus, DAPI acts as an effective fluorescent staining agent for DNA.

It has been found that DAPI binds to the minor groove of A:T DNA sequences and by intercalation. Minor groove binding is much stronger, by several orders of magnitude, than binding by intercalation.
Association between the DNA and DAPI molecules can be written as:

\[
\text{DNA Binding Site} + \text{DAPI} \rightleftharpoons \text{Complex}
\]

The Complex's formation constant is then given by:

\[
K_f = \frac{[\text{Complex}]}{[\text{DNA Site}][\text{DAPI}]} \quad \text{(Eq. 9)}
\]

Then, the ratio of the DNA's occupied sites \( n_{oc} \) to its total number of sites \( n \) is:

\[
\frac{n_{oc}}{n} = \frac{[\text{Complex}]}{[\text{Complex}]+[\text{DNA Site}]} \quad \text{(Eq. 10)}
\]

In terms of \( K_f \), then:

\[
n_{oc} = n \frac{K_f [\text{DAPI}]}{K_f [\text{DAPI}]+1} \quad \text{(Eq. 11)}
\]

Appealing now to our fluorescence measurements, where \( F_{obs} \) is the observed fluorescence of the DAPI-DNA complex, \( F_L \) is the fluorescence of the free DAPI and \( F_{max} \) is the maximal fluorescence of the DAPI-DNA complex for the concentrations used, the fraction of DAPI bound to the DNA \( f \) is:

\[
f = \frac{F_{obs} - F_L}{F_{max} - F_L} \quad \text{(Eq. 12)}
\]
In terms of \( f \), we can now write:

\[
\begin{align*}
n_{oc} &= \frac{f [DAPI]_0}{[DNA]_0} \quad \text{(Eq. 13)}
\end{align*}
\]

where \([DAPI]_0\) and \([DNA]_0\) represent the total concentration of each species.

Equating our expressions for \( n_{oc} \) and rearranging, we get:

\[
\begin{align*}
\frac{[DNA]_0}{f} &= \frac{[DAPI]_0}{n K_f} + \frac{[DAPI]_0}{n} \quad \text{(Eq. 14)}
\end{align*}
\]

Finally, expressing \((1 - f)\) as:

\[
1 - f = \frac{[DAPI]}{[DAPI]_0} \quad \text{(Eq. 15)}
\]

allows us to form the Scatchard Equation:

\[
\begin{align*}
\frac{[DNA]_0}{f} &= \frac{1}{n K_f (1 - f)} + \frac{[DAPI]_0}{n} \quad \text{(Eq. 16)}
\end{align*}
\]

A plot of \([DNA]_0/f \) vs. \(1/(1 - f)\), a Scatchard Plot, allows us to determined \(K_f\) and \(n\).

If the DNA has multiple and distinct types of binding sites for the DAPI, then the Scatchard analysis becomes significantly more complex. Two types of binding sites will yield a Biphasic Scatchard Plot.

A plot of \([DNA]_0/f \) vs. \(1/(1 - f)\), a Scatchard Plot, allows us to determined \(K_f\) and \(n\).

A discussion of this case is beyond the scope of the current project.
All fluorescence measurements will be made using the Photon Technology International system pictured below. You should examine this Spectrofluorometer and identify all the major components that comprise the system.

Thus, a measurement of the fluorescence increase of DAPI when it binds to the minor groove of DNA will yield the complex formation parameters $K_f$ and $n$. 
Procedure

These procedure steps are taken from the Supplementary Student Handout of "Quantitative Determination of DNA-Ligand Binding Using Fluorescence Spectroscopy: An Undergraduate Biochemistry Experiment" by Eamonn F. Healy J. Chem. Ed. 84 (2007) 1304.

**Preparation of the Buffer**

Prepare 1 L of 0.02 M NaOH and 1 L of 0.02 M Sodium Dihydrogen Phosphate. Add 40 mL of the NaOH solution to 50 mL of the Phosphate solution and dilute to 100 mL. This yields a buffer at pH = 7.4

**Preparation of Reagents**

Prepare a solution of 50 µg/mL of DNA in Phosphate Buffer by dissolving a 1 mg vial of Calf-Thymus DNA in 20 mL of the Phosphate Buffer prepared above.

Measure the UV Absorbance of the solution in a 1 cm quartz cuvette at 260 nm and 280 nm. A pure solution of native double-stranded DNA gives a $A_{260/280}$ ratio of 1.9.

Prepare a solution of 400 µg/mL of DNA in Phosphate Buffer by dissolving a 2 mg vial of Calf-Thymus DNA in 5 mL of the Phosphate Buffer prepared above.

Prepare a 1.10 x $10^{-6}$ M DAPI solution by dissolving 1 mg of DAPI in 30 mL of Ethanol. This is the Stock DAPI solution. This solution should be stored in a refrigerator.

Prepare a 2.10 x $10^{-6}$ M DAPI solution in Phosphate Buffer by diluting 2 mL of the DAPI stock solution to 100 mL using the Phosphate Buffer prepared above.

Prepare a 1.10 x $10^{-6}$ M DAPI solution in Phosphate Buffer by diluting 1 mL of the DAPI stock solution to 100 mL using the Phosphate Buffer prepared above.

**Fluorescence Measurements: Determination of $F_{max}$**

The fluorospectroscope should be set at $\lambda_{\text{excite}} = 360$ nm and $\lambda_{\text{emit}} = 450$ nm.

Prepare 3 cuvettes as follows:

- **#1**: 3 mL of the 50 µg/mL DNA solution.
- **#2**: 3 mL of the 1.10 x $10^{-6}$ M DAPI solution.
#3: 1.5 mL of the $1.10 \times 10^{-6}$ M DAPI & 1.5 mL of $2.10 \times 10^{-6}$ M DNA solutions.
(Vortex stir for 30 seconds to ensure complete complex formation.)

Obtain fluorescence readings for each of the above solutions.

The results of cuvette #1 demonstrate DNA does not fluoresce under these conditions. The results of cuvette #2 give the fluorescence of free DAPI, $F_L$. The results of cuvette #3 represent the maximal fluorescence of the DNA-DAPI complex, $F_{max}$.

**Fluorescence Measurements: The Saturation Curve**

Prepare a cuvette containing 3 mL of the $1.10 \times 10^{-6}$ M DAPI solution and add 10 µL of the 400 µg/mL DNA solution. Vortex stir for at least 30 seconds. Determine the fluorescence of the solution. This represents the fluorescence of the DAN-DAPI complex, $F_{obs}$.

Repeat for a total of 18 additions.
Data Analysis

For the following analysis you can use the average molecular weight for a nucleotide pair as 650 g/mol.

1. Prepare a table of $F_{\text{obs}}$ vs. $f$, $1/(1-f)$, $[\text{DNA}]_o$ and $[\text{DNA}]_o/f$.

2. Using only values of $1/(1-f) < 10$ (why?) prepare a Scatchard Plot and determine $K_f$ and $n$. You should provide error estimates for both values.
References


Kubista, Mikael; Bjorn, Akerman and Norden, Bengt "Characterization of Interaction between DNA and 4'-6-Diamidion-2-phenylindole by Optical Spectroscopy" Biochemistry 26 (1987) 4545.