Binding of Flavonoids to Bovine Serum Albumin: A Ligand-Binding Study

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Overview

Many biochemical processes work by the interaction of a small molecule (ligand) with a biological macromolecule (protein or nucleic acid). The degree to which the ligand binds to the biomolecule can be described by the binding constant (K_d). The goal of this experiment is to determine the binding constant (K_d) for the binding interaction between a flavonoid and a serum protein.

Teaching and Learning Goals

Upon completing this laboratory session, students will be able to:

- Correlate fluorophore concentration with fluorescence intensity
- Compute the binding constant (K_d) of a protein to its ligand from nonlinear data

During the pandemic, students will work independently, with designated instrument times.

Materials and Reagents

- Jasco FP-6300 or FP-8200 spectrofluorimeter with Spectra Manager™ software control; record the specific make/model used in your ELN
- Polymethylacrylate disposable cuvettes (4-optical windows)
- Buffer: 50 mM Sodium Phosphate buffer, pH 7.0
- Ligands *Record only the flavonoid tested in your ELN*
    - Apigenin, Sigma A3145, -20°C
    - Baicalein, Aldrich 465119, 2-8°C
    - (+) Catechin Hydrate, Sigma C1251, 2-8°C
    - Chrysin, Aldrich C80105, RT
    - Daidzein, Sigma D7802, -20°C
    - Diosmin, Sigma D3525, 2-8°C
    - (-) Epigallocatechin Gallate (EGCG), Sigma E4143, 2-8°C
    - Fisetin, Sigma F4043, -20°C
    - Flavone, Sigma F2003, RT
    - Genistein, Sigma G6649, -20°C
    - Hesperidin, Sigma H5254, 2-8°C
    - Morin Hydrate, Sigma M4008, RT
    - Naringenin, Sigma N5893, RT
    - Naringin, Sigma N1376, RT
    - Quercetin Anhydrous, Sigma Q4951, RT
    - Quercitrin Hydrate, Sigma Q3001, 2-8°C
    - Rutin Hydrate, Sigma R5143, RT
    - Silibinin, Sigma S0417, -20°C
    - Silymarin, Sigma S0292, -20°C
- Protein: Bovine Serum Albumin [**record source**]

Experimental Procedures

Albumin Concentration

How does albumin concentration affect the intrinsic fluorescence of albumin?

1. Prepare at least 20 mL of a 0.2 mg/mL BSA solution in buffer. Store on ice.

2. Compute the final concentration of albumin (in terms of mg/mL units and molarity units) in each cuvette based on the stock concentration and total volume. ***Show your mathematics in detail in your ELN.***

3. Generate a table like Table 1 and prepare samples according to Table 1.

Table #1. Role model of Sample Preparation for the Albumin Concentration Study

Final [BSA], nM or µM	Final [BSA], mg/mL	Volume of 0.2 mg/mL BSA (mL)	Volume of buffer (mL)	Fluorescence Intensity
		0.25	2.75	
		0.5	2.50	
		0.75	2.25	
		1.0	2	
		1.5	1.5	

4. Measure the fluorescence intensity of each sample and record and plot your data using Excel in your laboratory notebook using the following excitation and emission wavelengths. Be sure to blank instrument with buffer.

Excitation wavelength = 280 nm **Emission wavelength = 300 - 500 nm**

Record the following information and use these parameters for all subsequence measurements: *a screenshot of the parameters screen on the fluorometer is even better!*

a. Scanning speed
b. Step size
c. Excitation band width *adjust until "good" data is measurable*
d. Emission band width *adjust until "good" data is measurable*
e. Emission Wavelength Peak *use the cursor in the SpectraManager™ to identify the peak*

5. How does the data correlate? Describe the mathematical relationship between fluorescence intensity and [BSA] in your ELN.

Ligand-Binding Study

How does the binding of flavones affect the intrinsic fluorescence of albumin?
What is the estimated binding constant (K_d) for the flavone to albumin?

1. Select one flavone to test. You will need 10-15 mL of the selected flavone at a 0.5 mM concentration. *Note: Stock solutions will often be available at 10X concentration (i.e. 5 mM) from which students may dilute with buffer to generate a 0.5 mM working stock solution.*

   Record the specific flavonoid that you are testing, its source and actual working concentration in your ELN.

2. Prepare samples for the ligand binding study according to Table #2. Be sure to mix the protein before <u>each</u> sample is taken! Keep the working protein stock on ice.

Table #2. Role Model of Sample Preparation for the Ligand Binding Study Data

Sample #	Final [Flavonoid],	Volume of Stock Flavonoid	Vol. of 0.1 mg/mL BSA	Vol. of Buffer, mL	Fluorescence Intensity
1		0.1 mL	2	1.9	
2		0.2 mL	2	1.8	
3		0.3 mL	2	1.7	
4		0.4 mL	2	1.6	
5		0.5 mL	2	1.5	
6		0.6 mL	2	1.4	
7		0.7 mL	2	1.3	
8		0.8 mL	2	1.2	
9		0.9 mL	2	1.1	
10		1.0 mL	2	1.0	

3. Cover each cuvette with parafilm and mix well prior to each fluorescence

measurement.

4. Measure the intrinsic fluorescence of albumin using the same excitation and emission wavelengths from the concentration study. Record your data in your laboratory notebook. Also record all other parameters used for measurement (i.e. scanning speed, step size, band widths, etc.) Use the same parameters for all measurements.

DATA ANALYSIS

[BSA] *Answer these questions in the Results section of your ELN*

- What is the final concentration of BSA used in the Ligand Binding study?
- Where does your [BSA] used in the ligand binding study lie with respect to the data produced from the Albumin Concentration study?
- What are the advantages of the [BSA] used in the ligand-binding study?

Estimation of K_d

1. Graph the fluorescence intensity (from Table 2) as a function of the concentration of your flavonoid. Use a "connect-the dot" graphing option for showing the data trend.

2. Determine ΔF - the value over which your fluorescence intensities span: $F_{max} - F_{min}$

3. Determine ½ ΔF.

4. Estimate the K_d by visual inspection.

Scatchard Plot Analysis of K_d

5. Determine $\frac{F}{[L]}$

6. Generate a Scatchard plot as modeled in Figure 1 where $\frac{[Ligand_{bound}]}{[Ligand_{free}]} = \frac{F}{[L]}$

7. Fit the data to a line.

8. Use the slope to determine the K_a value (the association constant).

9. Invert the K_a to determine the K_d value.

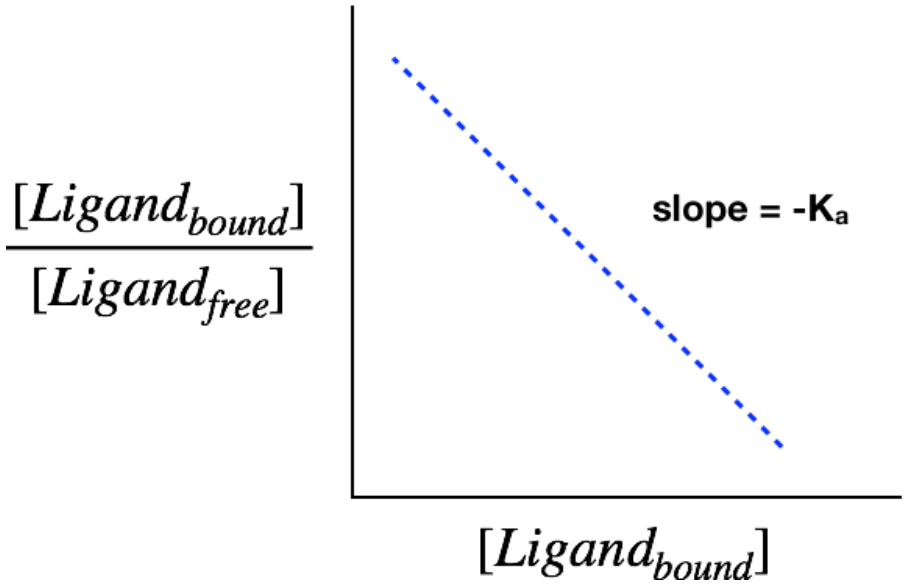

Figure 1. Generic illustration of a Scatchard Plot. Be sure to include units and raw data points in your graph. The dotted line represents the negatively sloped, linear trendline expected. K_d may be deduced from the determination of the slope.

NOTES

#1 – If your flavonoid quenched the fluorescence, then you will need to transpose your data as described below:

- Take the negative of each Fluorescence intensity
- Add an arbitrary/constant value to each fluorescence intensity measurement in order to make each fluorescence intensity positive
- Then proceed with the Scatchard plot analysis of K_d above (Data Analysis Steps 5-9)

#2 – If your ligand binding profile (i.e. the graph from Data Analysis Step 1) does not flatline, then generate more samples to extend Table 2 until the data indicates saturation

Electronic Notebook Maintenance Reminders

- Document sources of reagents.
- Document the instrument model used.
- If any unexpected data is observed, then explain its meaning.
- To exclude a given data point you must perform some kind of statistical analysis for justification.
- Feel free to take photos of your samples for your notebook record. Make observations about your experiment.