# Measuring Binding Affinity of Protein−Ligand Interaction Using Spectrophotometry: Binding of Neutral Red to Riboflavin-Binding Protein

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Determination of the dissociation constant, $K_d$, or association constant, $K_a$, of the binding between a protein and ligand is an important analytical methodology that should be included in the fourth-year undergraduate and first-year graduate courses. The measurement enables researchers in biochemistry or chemical biology to assess the strength of protein−ligand interactions, which can be used to evaluate effects of substrates, inhibitors, or effectors on enzymes or receptors. The dissociation or association constant can be assessed by measuring the change in physical properties such as absorbance or fluorescence *(1)*. In the past, only a few lessons oriented to teach students how to measure $K_d$ of protein and ligand based on the change of physical properties have been developed and published: the binding of 8-anilino-1-naphtalenesulfonic acid and bovine serum albumin *(2)*, caffeine and human serum albumin *(3)*, 4,6-diamidino-2-phenylindole and DNA *(4)*, 2-*p*-toludinylnapthalene-6-sulfonate and calmodulin *(5)*, ethidium bromide and DNA *(6)*, and methyl orange and BSA *(7)*. The binding affinity based on fluorescence signal is used in references 2−6 and only reference *7* measures the value based on UV−vis spectroscopy. Generally, the signal from absorption spectroscopy is low when compared to that of fluorescence. However, spectrophotometers are widely available in the teaching laboratories; therefore, more experiments demonstrating how to measure binding affinity based on absorption signal are needed.

This laboratory experiment uses the binding of riboflavin-binding protein (RP) to neutral red (NR) to determine $K_d$. NR has been used in laboratory experiments to determine an acid dissociation constant using absorption spectrophotometry *(8)* and mathematical deconvolution techniques *(9)*, whereas RP has been used for determining the quantity of riboflavin with spectrofluorometric techniques *(10)*. The experiment presented here was used with first-year graduate students. On the basis of the class evaluation, the experiment is simple and efficient and can be finished within a 2.5 h period. Although RP can be purchased from a commercial source, students can purify RP from chicken egg white in reasonable quantity using a simple protocol (see the supporting information).

## Background

The physiological role of RP is to bind and transfer vitamin B2 to target tissues *(11)*. The binding of RP to the native ligand (vitamin B2) is strong with a $K_d$ value of $1.3 \times 10^{-9}$ mol $L^{-1}$ *(12)*. However, RP can also bind to other flavin derivatives such as flavin mononucleotide and flavin adenine dinucleotide *(12)*. Therefore, RP should be able to bind to any compound with structures similar to vitamin B2. We have shown that RP can bind to NR whose structure is similar to riboflavin. RP is rich in aromatic amino acids, especially around the binding site. Five tryptophan residues found at the binding site are speculated to be involved in the flavin binding *(13)*. NR is routinely used as a fluorescence probe and marker for biological system and also as a pH indicator dye *(14)*. At pH 9, an absorption spectrum of free NR in solution has a maximum wavelength at 450 nm. Upon binding to RP, the maximum wavelength of NR shifts to 545 nm and this absorption change is used as a signal indicating the binding of the dye to the hydrophobic environment in RP.

## Calculations of Dissociation Constant

Binding of NR to RP can be represented as follows

$$\mathrm{RP} + \mathrm{NR} \rightleftharpoons \mathrm{RPNR} \quad (1)$$

$K_d$, the dissociation constant of the binding, is defined as

$$K_d = \frac{[\mathrm{RP}][\mathrm{NR}]}{[\mathrm{RPNR}]} \quad (2)$$

[NR] and [RP] are the concentrations of free neutral red and free riboflavin-binding protein, respectively, and [RPNR] is the concentration of the complex. NR binds to RP with the ratio of 1:1. The ratio of the concentration of the complexed NR, [RPNR], versus the total concentration of NR, $[NR]_0$, is denoted

 
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as α:

$$\alpha = \frac{[RPNR]}{[NR]_0} = \frac{[RPNR]}{[NR] + [RPNR]} \quad (3)$$

In this experiment, $[NR]_0$ is fixed. From eq 2, [RPNR] = [RP][NR]/$K_d$, hence

$$\alpha = \frac{\left(\frac{[RP][NR]}{K_d}\right)}{[NR] + \frac{[RP][NR]}{K_d}} \quad (4)$$

Dividing both numerator and denominator of eq 4 by [NR] and multiplying by $K_d$ gives

$$\alpha = \frac{[RP]}{K_d + [RP]} \quad (5)$$

From eq 5, a plot of α versus [RP] gives a hyperbolic graph. When α = 0.5, [RP] = $K_d$. To determine the $K_d$, the increase of absorbance intensity at 545 nm upon adding RP ($\Delta A_{RP}$) was used as an indication of the formation of the RPNR complex. The value of α was determined from

$$\frac{\Delta A_{RP}}{\Delta A_{max}} = \alpha \quad (6)$$

where $\Delta A_{max}$ is the maximum change in the absorption intensity at 545 nm when the NR is saturated with RP. It represents a fraction of a limiting compound of the system (in this case NR) that binds to a compound with surplus concentration (in this case RP):

$$\frac{\Delta A_{RP}}{\Delta A_{max}} = \alpha = \frac{[RP]}{K_d + [RP]} \quad (7)$$

Because the experiment was performed using a concentration of NR in the $K_d$ range, $[RP] \approx [RP]_0$, the total concentration of RP, under this condition (see the details in supporting information). This allows a simple approximation by substituting [RP] with $[RP]_0$ and eq 7 can be modified to

$$\Delta A_{RP} = \frac{\Delta A_{max}[RP]_0}{K_d + [RP]_0} \quad (8)$$

A plot of $\Delta A_{RP}$ versus $[RP]_0$ is used to estimate $\Delta A_{max}$. Then, $\Delta A_{max}$ is used for calculating α values according to eq 7. The actual concentration of RP in each titration can be calculated from

$$[RP] = [RP]_0 - [RPNR] = [RP]_0 - \alpha[NR]_0 \quad (9)$$

From eq 3, [RPNR] = $\alpha[NR]_0$. $[NR]_0$ is known, thus, [RPNR] and [RP] can be calculated accordingly. The direct plot of α versus [RP] according to eq 7 is used to determine of $K_d$.

## Equipment

- Micropipets: 20, 100, and 1000 μL and tips (1 set per group)
- 1.5 mL quartz, glass, or plastic cuvette
- Parafilm sheet for covering a cuvette during mixing or a small plastic rod for stirring
- Shimazu UV−vis spectrophotometer (model UV 2501PC).

## Procedure

RP was purified from chicken egg white according to the protocol in reference *15* with slight modifications (see the supporting information). NR (1 mL of $8.4 \times 10^{-6}$ mol $L^{-1}$) in $50 \times 10^{-3}$ mol $L^{-1}$ Tris-HCl buffer, pH 9, was placed in a cuvette. An absorption spectrum of the NR solution was recorded with a UV−vis double-beam spectrophotometer. Then, 10 μL of $310 \times 10^{-6}$ mol $L^{-1}$ RP solution was pipetted into the NR cuvette. The solution was mixed and then the absorption spectrum was recorded. Additional aliquots of RP were added and the spectrum was recorded after each addition. The protocol was repeated until the absorption spectra did not change compared to that of the previous addition. The total volume of the RP solution added should not be more than 100 μL (10%) to avoid dilution effect.

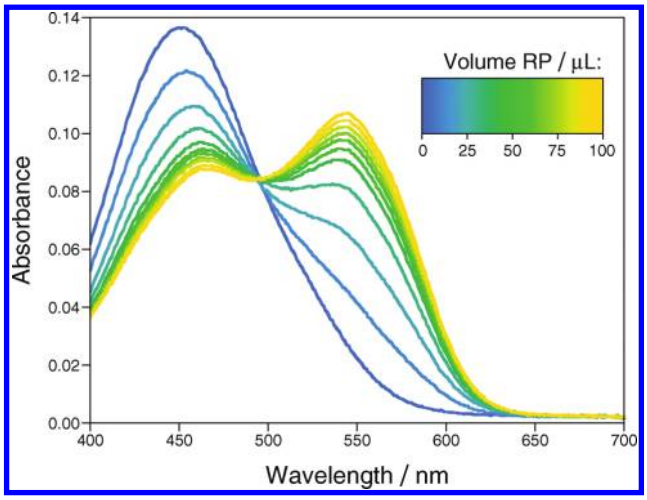


Figure 1. Absorption spectra of the titration: A solution of $8.4 \times 10^{-6}$ M NR (1 mL) in $50 \times 10^{-3}$ M Tris-HCl buffer, pH 9.0, was titrated with different concentrations of RP. The absorption peak changed to 545 nm when adding RP.

## Hazards

Neutral red at a very high concentration may cause skin and eye irritations. Phenol is a strong oxidizing agent and contact can cause sever burns. Hydrochloric acid is corrosive and may cause sever damage to the skin and eyes. Riboflavin may cause eye and skin irritation.

## Results and Discussion

Titration of RP with 1 mL of $8.4 \times 10^{-6}$ mol $L^{-1}$ NR was monitored by absorption spectroscopy as shown in Figure 1. The titration was performed at pH 9 and 25 °C. Upon increasing RP concentration, the absorption peak of NR shifted to 545 nm. The change of absorption spectra of NR is likely to be due to the increase of hydrophobic environment upon binding. A single isosbestic point at 490 nm was observed, indicating a simple change between bound and unbound states. The increase in the absorbance intensity at 545 nm was used to determine the $K_d$. A directed plot of $\Delta A_{RP}$ versus $[RP]_0$ was used to calculate $\Delta A_{max}$ by fitting the hyperbolic graph with nonlinear regression analysis. The value of $\Delta A_{max}$ was calculated to be 0.113. The value of $\Delta A_{RP}/\Delta A_{max}$ or α was used for calculating the quantity of the bound complex in each titration and then the concentration of free RP in each titration was obtained by subtracting the bound fraction from the total concentration of RP as described in eq 9. The value of $K_d$ was

 

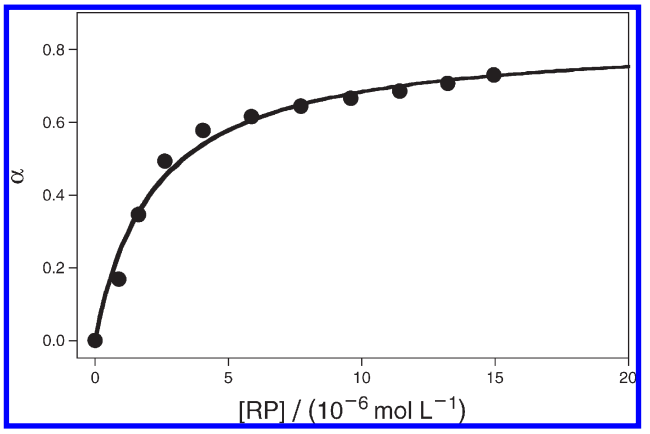


Figure 2. A plot of α versus free RP.

determined from the plot of α ($\Delta A_{RP}/\Delta A_{max}$) versus the concentrations of free RP as shown in Figure 2. The data were analyzed according to eq 7 using nonlinear regression analysis. The $K_d$ value for the binding of NR and RP is $2.2 \pm 0.3 \times 10^{-6}$ mol $L^{-1}$.

## Conclusion

This experiment is simple and allows students with various backgrounds (graduate students in biochemistry and toxicology or fourth-year undergraduate students in chemistry) to gain hands-on experience in the determination of $K_d$ and use of spectrophotometry. They can finish the experiment within 2.5 h. Their results yielded $K_d$ values between $2 \times 10^{-6}$ and $10 \times 10^{-6}$ mol $L^{-1}$, which are similar to the value shown in Figure 2 ($2.2 \pm 0.3 \times 10^{-6}$ mol $L^{-1}$). RP also provides learning interest because the protein source, egg, is a common item that students can relate to.

Data from questionnaire, lab report, pretest, and posttest indicated that most students found the experiment to be interesting and motivating. They acquired the understanding of how to determine $K_d$ and skills with spectroscopic titration. This experiment can be adapted or directly used for teaching undergraduate students in biochemistry or chemistry or first-year graduate students in biochemistry and physical chemistry.

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## Supporting Information Available

Instructions for the students including postlab exercises; notes for the instructor. This material is available via the Internet at http://pubs.acs.org.

 