JOURNAL OF CHEMICAL EDUCATION

Laboratory Experiment

pubs.acs.org/jchemeduc

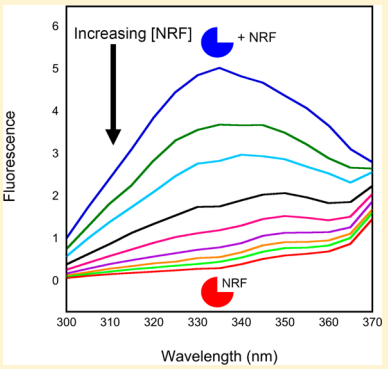
Measuring Norfloxacin Binding to Trypsin Using a Fluorescence Quenching Assay in an Upper-Division, Integrated Laboratory Course

Katherine A. Hicks*

Department of Chemistry, SUNY Cortland, Cortland, New York 13045, United States

Supporting Information

ABSTRACT: Fluorescence quenching assays are often used to measure dissociation constants that quantify the binding affinity between small molecules and proteins. In an upper-division undergraduate laboratory course, where students work on projects using a guided inquiry-based approach, a binding titration experiment at physiological pH is performed to analyze the interaction between the drug norfloxacin and the protein trypsin. The resulting nonlinear binding data are fitted using a scientific data analysis program to determine the dissociation constant (K_d value) describing this interaction. The experiment is especially of interest to students who plan to pursue careers in health-related aspects of biochemistry.



KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Biophysical Chemistry, Inquiry-Based/Discovery Learning, Hands-On Learning/Manipulatives, Noncovalent Interactions, Proteins/Peptides, Fluorescence Spectroscopy*

Characterizing binding affinity is an important biochemical measurement, especially for understanding the interaction between proteins and drug targets. To measure these interactions, fluorescence quenching assays are often employed.[1,2] In the chemical education literature, fluorescence quenching laboratories have been described to monitor the binding of the flavonoid quercetin to human serum album using anisotropy.[3] However, anisotropy experiments require specialized filters that might not be readily available in teaching laboratories. Other teaching laboratories have used fluorescence quenching to examine protein folding[4] and the binding of fluorescent dyes to proteins.[5]

Herein, a fluorescence quenching assay is described involving the binding of the drug norfloxacin (NRF) to trypsin, which was modeled on a recent paper.[6] NRF is a fluoroquinolone that is active against Gram negative pathogens and has been used clinically for decades.[7] However, trypsin is not the intended NRF target protein, and homology models suggest that NRF binds to a hydrophobic pocket on the protein.[6] Similar fluorescence quenching assays have been used to measure the binding between NRF and human serum albumin,[8] and between trypsin and other small molecules, including sulfathiazole[9] and bifendate.[10] Unlike human serum albumin, which is cost-prohibitive for use in an undergraduate teaching laboratory, trypsin is a commonly used biochemical reagent. Both trypsin and NRF are readily available from commercial sources, and this experiment provides a sensitive, quantitative measurement of binding affinities using a standard spectrofluorometer.

This experiment was developed as part of an upper-division, integrated science laboratory course that provides students with the opportunity to work on guided inquiry-based experiments. Guided inquiry-based courses like this one help convey a clearer understanding of the scientific process by developing students' troubleshooting, critical thinking, and problem solving skills.[11−15] For this specific experiment, the pedagogical goals are for students to (1) determine the appropriate experimental parameters, including fluorescence wavelengths and reagent concentrations, which accurately monitor equilibrium binding; (2) interpret data using curve fitting software; and (3) explain the relevance of their data to quantifying the affinity between NRF and trypsin.

EXPERIMENT

Students work in groups of 3 or 4 and require five, 3-h laboratory periods to complete the experiment. Prior to the start of the laboratory work, students are provided with a series of questions to guide their design of the experimental protocol (Supporting Information Section 5), which is prepared as a short, pre-laboratory report. Students prepare all stock solutions.

Binding Studies

Detailed protocols are available in the Supporting Information Section 6. On the basis of relevant biochemical literature, students are responsible for determining all experimental

DOI: 10.1021/acs.jchemed.5b00548
J. Chem. Educ. XXXX, XXX, XXX−XXX

parameters, including NRF and trypsin concentrations and an appropriate buffer system to measure binding at physiological pH. In a typical assay, students prepare a sample of 250−750 nM trypsin in 50 mM Tris (pH 7.0) buffer to which increasing concentrations of NRF are added systematically. In the laboratory handout (Supporting Information Section 5), students are told that their final NRF concentration should be at least five times the K_d to obtain an accurate endpoint fluorescence. Students are also instructed to excite tryptophan residues in trypsin at 280 nm, and monitor the resulting fluorescence emission between 300 and 450 nm in 5 nm increments. Depending on the available time, students also measure binding at pH values between 5 and 9.

To deduce which wavelength corresponds to the binding of NRF, students examine the fluorescence emission as a function of wavelength and determine that the fluorescence emission at 335 nm is a direct measure of the trypsin·NRF complex.[6] Students also consider how the background fluorescence from free NRF and the light attenuation due to NRF absorbance at 280 nm and 335 nm should be corrected.

Determination of Binding Constants

The adjusted fluorescence emission at 335 nm is plotted as a function of NRF concentration. In the lab handout, students are provided with a modified direct binding eq (eq 1) that is used to calculate the K_d value.[16]

$$\Delta Fl = \frac{IF}{1 + [NRF]/K_d} + EP \quad (1)$$

where ΔFl is the change in the adjusted fluorescence, IF is the initial fluorescence, EP is the endpoint fluorescence, [NRF] is the concentration of NRF, and K_d is the dissociation constant describing the binding between NRF and trypsin. The derivation to this equation is provided in Supporting Information Section 7. We used the program KaleidaGraph (Synergy Software) for these analyses, which provides students with exposure to nonlinear fitting programs that they will encounter if they continue in the field. Other curve fitting programs that can fit arbitrary functions include SigmaPlot (Systat Software) and Prism (GraphPad Software), which could also be used for these analyses.

HAZARDS

The chemicals used in this experiment can cause skin and eye irritation. In addition, in rare cases, contact reactions with NRF are possible due to allergic reactions. These compounds should all be used in a well-ventilated area equipped with an eyewash and shower. In addition to wearing long-sleeve shirts, closed-toe shoes, and pants, it is recommended chemical goggles and protective gloves be worn when working with these reagents. If contact occurs, the affected area should be washed immediately with soap and water. Additional medical treatment should be sought, if needed.

RESULTS AND DISCUSSION

This experiment was performed four times by 16 students as part of an upper-division Advanced Laboratory I course (one classroom, two laboratory periods per week) consisting of four inquiry-based experiments (see the Supporting Information for a further description of the course). A one-semester Foundations of Analytical Chemistry course, which also used inquiry-based experiments, was a prerequisite so students were familiar with this pedagogical approach. During each offering of the course, this experiment was performed by two groups of students.

A representative plot of fluorescence as a function of wavelength is shown in Figure 1. The fluorescence data were

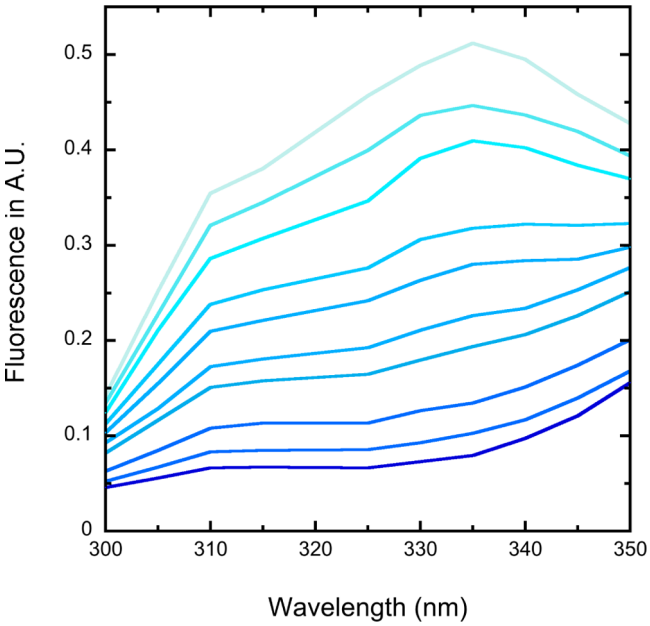


Figure 1. Emission spectra of the NRF binding experiment. NRF was titrated into a cuvette containing 250 nM trypsin and 50 mM Tris (pH 7.0). The intensity of the color corresponds to increasing NRF concentrations from 0 to 30 μM.

then adjusted to correct for background fluorescence and light attenuation. Figure 2 is a plot of adjusted fluorescence at 335 nm as a function of NRF concentration fitted to eq 1. A K_d

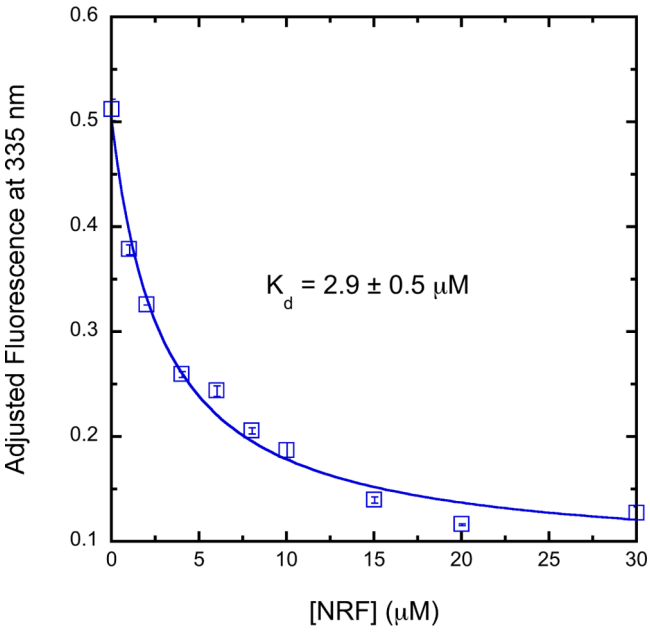


Figure 2. Measurement of K_d for the trypsin·NRF complex using fluorescence quenching. The assay contained 50 mM Tris (pH 7.0) and 250 nM trypsin. The sample was titrated with NRF (0−30 μM). The K_d value quantifying the interaction was determined by a fit of eq 1 to these data.

DOI: 10.1021/acs.jchemed.5b00548
J. Chem. Educ. XXXX, XXX, XXX−XXX

value of 2.9 ± 0.5 μM was calculated for the trypsin·NRF binding interaction, which is within 2-fold of the literature value (6.7 μM).[6]

Students who performed the experiment first presented their results in an approximately 30 min group meeting style presentation. In these presentations, students provided their classmates, who would be performing the experiment next, with valuable insights into the project, including potential obstacles and suggestions. The second group of students presented their results in a written lab report in the style of the *Journal of the American Chemical Society*. Students wrote two drafts of the lab report. In the second version, changes were incorporated based on the instructor's suggestions.

At the beginning of the course, most students could not design a binding experiment, and many students assumed that these experiments were measuring kinetic constants rather than equilibrium binding constants. By asking a series of guided questions, the instructor assisted students in clarifying these misconceptions (Supporting Information Section 8). Preliminary evaluations suggest that this guided-inquiry approach is successful. Before completing the experiment, 80% of the students thought that binding assays provide information about the kinetics of an enzyme-catalyzed reaction, while after the laboratory this number decreased to 10%. When asked in a post-lab survey if this experiment provided information on how binding experiments can be used to explain biological phenomena, 70% of students agreed or strongly agreed.

SUMMARY

Using a guided inquiry-based approach, students measured the binding of NRF to trypsin using equipment and reagents that are readily available at most institutions. Students working in groups of 3 or 4 were able to complete the laboratory in five, 3-h laboratory periods. After completing the laboratory, 90% of students were able to accurately explain that this experiment did not measure kinetic constants, a common student misconception. This experiment could easily be adapted to measure the binding of other commercially available drugs, such as sulfathiazole and bifendate, to trypsin.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00548.

Additional information including student handouts and notes for the instructor (PDF, DOCX)

AUTHOR INFORMATION

Corresponding Author

*E-mail: katherine.hicks@cortland.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The author would like to thank all of the Advanced Laboratory I students for their comments about this experiment, especially Francesco Langella, Samuel Lothridge, Gregory Simone, Meigan Yuen, John Chodkowski, Joseph Guttilla, and Melissa Sullivan who provided their data for the figures. Jeffrey Werner is thanked for helpful discussions on experimental design. Frank Rossi, Jeffrey Werner, Maureen Smith, and Megan Sikowitz Erb are also thanked for their helpful and thoughtful comments on the manuscript.

REFERENCES

(1) Zhu, T.-H.; Cao, S.-W.; Yu, Y.-Y. Synthesis, Characterization and Biological Evaluation of Paeonol Thiosemicarbazone Analogues as Mushroom Tyrosinase Inhibitors. *Int. J. Biol. Macromol.* **2013**, *62*, 589−595.

(2) Ribeiro, M. M. B.; Franquelim, H. G.; Castanho, M. A. R. B.; Veiga, A. S. Molecular Interaction Studies of Peptides Using Steady-State Fluorescence Intensity. Static (De)Quenching Revisited. *J. Pept. Sci.* **2008**, *14* (4), 401−406.

(3) Ingersoll, C. M.; Strollo, C. M. Steady-State Fluorescence Anisotropy To Investigate Flavonoids Binding To Proteins. *J. Chem. Educ.* **2007**, *84* (8), 1313−1315.

(4) Schlamadinger, D. E.; Kats, D. I.; Kim, J. E. Quenching Of Tryptophan Fluorescence In Unfolded Cytochrome C: A Biophysics Experiment For Physical Chemistry Students. *J. Chem. Educ.* **2010**, *87* (9), 961−964.

(5) Huisman, A. J.; Hartsell, L. R.; Krueger, B. P.; Pikaart, M. J. Thermodynamic Exploration Of Eosin−Lysozyme Binding. *J. Chem. Educ.* **2010**, *87* (3), 299−302.

(6) Lu, Y.; Wang, G.; Lu, X.; Lv, J.; Xu, M.; Zhang, W. Molecular Mechanism Of Interaction Between Norfloxacin And Trypsin Studied By Molecular Spectroscopy And Modeling. *Spectrochim. Acta, Part A* **2010**, *75* (1), 261−266.

(7) Appelbaum, P. C.; Hunter, P. A. The Fluoroquinolone Antibacterials: Past, Present And Future Perspectives. *Int. J. Antimicrob. Agents* **2000**, *16* (1), 5−15.

(8) Azimi, O.; Emami, Z.; Salari, H.; Chamani, J. Probing The Interaction Of Human Serum Albumin With Norfloxacin In The Presence Of High-Frequency Electromagnetic Fields: Fluorescence Spectroscopy And Circular Dichroism Investigations. *Molecules* **2011**, *16* (12), 9792−9818.

(9) Gökoğlu, E.; Yılmaz, E. Fluorescence Interaction And Determination Of Sulfathiazole With Trypsin. *J. Fluoresc.* **2014**, *24* (5), 1439−1445.

(10) Li, H.; Pu, J.; Wang, Y.; Liu, C.; Yu, J.; Li, T.; Wang, R. Comparative Study Of The Binding Of Trypsin With Bifendate And Analogs By Spectrofluorimetry. *Spectrochim. Acta, Part A* **2013**, *115*, 1−11.

(11) Kirk, S. R.; Silverstein, T. P.; Holman, K. L. M.; Taylor, B. L. H. Probing Changes In The Conformation Of $tRNA^{Phe}$: An Integrated Biochemistry Laboratory Course. *J. Chem. Educ.* **2008**, *85* (5), 666−673.

(12) Macdonald, G. Teaching Protein Purification And Characterization Techniques. A Student-Initiated, Project-Oriented Biochemistry Laboratory Course. *J. Chem. Educ.* **2008**, *85* (9), 1250−1252.

(13) Newton, T. A.; Tracy, H. J.; Prudenté, C. A Research-Based Laboratory Course In Organic Chemistry. *J. Chem. Educ.* **2006**, *83* (12), 1844−1849.

(14) Russo, S.; Gentile, L. Preparation, Purification, And Secondary Structure Determination Of Bacillus Circulans Xylanase. A Modular Laboratory Incorporating Aspects Of Molecular Biology, Biochemistry, And Biophysical Chemistry. *J. Chem. Educ.* **2006**, *83* (12), 1850−1852.

(15) Johnson, R. J.; Hoops, G. C.; Savas, C. J.; Kartje, Z.; Lavis, L. D. A Sensitive And Robust Enzyme Kinetic Experiment Using Microplates And Fluorogenic Ester Substrates. *J. Chem. Educ.* **2015**, *92* (2), 385−388.

(16) Hartman, H. L.; Hicks, K. A.; Fierke, C. A. Peptide Specificity Of Protein Prenyltransferases Is Determined Mainly By Reactivity Rather Than Binding Affinity. *Biochemistry* **2005**, *44* (46), 15314−15324.

DOI: 10.1021/acs.jchemed.5b00548
J. Chem. Educ. XXXX, XXX, XXX−XXX