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A CURE Biochemistry Laboratory Module to Study Protein−Protein Interactions by NMR Spectroscopy

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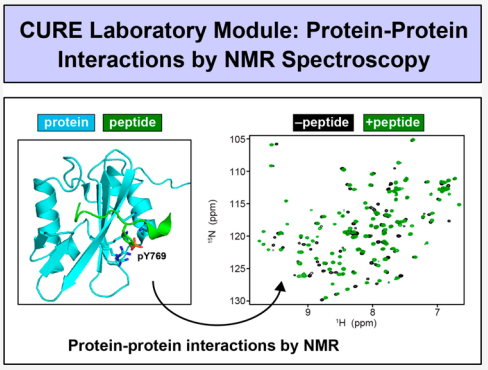
Supporting Information

ABSTRACT: The design of undergraduate laboratory courses that provide meaningful research-based experiences enhances undergraduate curricula and prepares future graduate students for research careers. In this article, a course-based undergraduate research experience (CURE) laboratory module was designed for upper-division undergraduate biochemistry and chemistry students. The laboratory module enabled students to build upon recently published data in the literature to decipher atomistic insight for an essential protein−protein interaction in human biology through the use of biomolecular NMR spectroscopy. Students compared their results with published data with the goal of identifying specific regions of the protein−protein interaction responsible for triggering an allosteric conformational change. The laboratory module introduced students to basic and advanced laboratory techniques, including protein purification, NMR spectroscopy, and analysis of protein structure using molecular visualization software.



KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Inquiry-Based/Discovery Learning, Biophysical Chemistry, NMR Spectroscopy, Proteins/Peptides*

INTRODUCTION

Motivating and preparing undergraduate students for Science, Technology, Engineering and Mathematics (STEM) careers is one of the central missions of academic life. Faculty are either directly involved in teaching undergraduate courses and/or indirectly exposed to the quality of undergraduate curricula through the mentorship of graduate students. The undergraduate teaching laboratory offers a unique opportunity for instructors to closely interact with students in a more hands-on manner than through lecture-based classes. The primary challenge in laboratory courses is ensuring experiments encourage student excitement, while balancing the need to teach laboratory skills and expose students to *real-life* research. Implementation of outdated experiments represents a lost opportunity for engaging the intellectual curiosity of students and faculty alike and can demotivate undergraduate students toward research-based careers and faculty toward engagement in teaching.[1] Innovative approaches to teaching laboratory courses can be categorized as skill-based,[2−5] project-based,[6,7] or inquiry-based.[8−10] A combined strategy integrating each type is referred to as course-based undergraduate research experiences (CUREs).[11,12] Goals of CUREs include exposing students to the scientific method and ensuring students have relevant experiences that resemble those found in a research laboratory.[13] Both are important objectives since they help establish a foundation for future graduate students to better understand the significance of a research project and to identify and frame unanswered questions in a field of study. Although no universal strategy exists for motivating every student, successful CUREs place an emphasis on (i) the importance of rationale and significance in the scientific method, (ii) data analysis and contextualizing findings relative to existing scientific literature, and (iii) incorporation of basic and advanced laboratory techniques, including state-of-the-art methods.

A key consideration for CURE laboratory modules is to place an emphasis on the significance of an experiment as it relates to a scientific field. Many beginning Ph.D. students pursuing biomedically relevant research often think that significance is strictly related to how a particular research project will cure or lead to therapeutics to treat diseases. While contributing knowledge or therapeutics to human disease is a motivating factor for biomedical research, it is important to emphasize that basic research is essential.[14] Therefore, it is critical that students are taught to think broadly about how an approach or experimental finding will advance a scientific field. Indeed, significance has been clearly defined by the National Institutes of Health: "*Does the project address an important problem or a critical barrier to progress in the field?*"[15] Advanced undergraduate laboratories offer an excellent place to teach students about significance. This can occur during a prelaboratory lecture or video when the rationale for the

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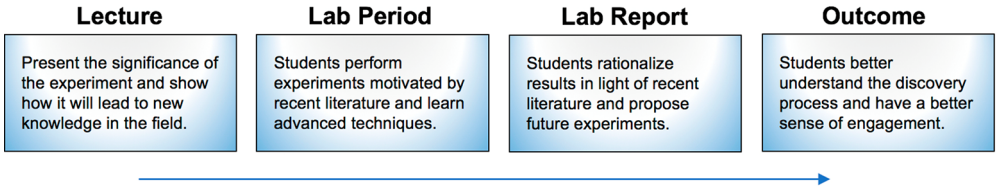

Figure 1. Workflow of a CURE laboratory module based on current literature, which exposes students to relevant basic and advanced techniques and methods. The figure depicts the experience of students and the goals and outcomes of the laboratory module.

experiment is presented (Figure 1). Topics include discussing knowledge that was established in the field over years or decades, and then articulating what are the unanswered questions in the field. Choosing a topic with controversy in the literature can also be an effective way to motivate student experiments. This approach is underscored in the philosophy of CUREs and is compatible with advancements in graduate student training that place a focus on developing a range of skills.[16]

With the goals of emphasizing significance in the undergraduate laboratory and teaching advanced techniques to students, a new CURE laboratory module was developed for upper-division undergraduate students majoring in biochemistry or chemistry. This laboratory module, which requires two 4 h sessions to complete, was successfully implemented at New York University and Spelman College, a Ph.D.-granting research institution and a primarily undergraduate college, respectively, where students investigated a protein−protein interaction using two-dimensional biomolecular NMR spectroscopy. The specific protein−protein interaction studied occurs within the phospholipase Cγ (PLCγ) cell signaling cascade and involves a phosphorylated peptide from the fibroblast growth factor (FGF) receptor with a Src homology 2 (SH2) domain from PLCγ. The results enabled students to decipher novel details of binding reactions including allosteric conformational changes propagating from protein−protein interfaces. This laboratory module represents the first published CURE to use NMR spectroscopy to study protein−protein interactions and demonstrates the feasibility of applying NMR to study other protein−protein interactions using NMR spectrometers that are available at most institutions (e.g., 400 MHz). Finally, perspectives are shared for future laboratory design based on the instructors' experience in developing the laboratory module.

LABORATORY MODULE

Motivation for the Laboratory Module: SH2 Domain of PLCγ Binding to a Phosphopeptide from the FGF Receptor

The broader themes of the laboratory module focused on protein−protein interactions and intracellular signaling through receptor tyrosine kinases (RTKs). These topics represent important material covered during biochemistry lecture courses and other classes such as molecular and cellular biology. A key feature within cellular signaling networks is the presence of protein−protein interactions that are required for propagating the signal, which ultimately leads to a functional response. To emphasize how protein−protein interactions can be studied using atomistic techniques, this laboratory module used NMR spectroscopy to probe a molecular interaction between the FGF receptor (an example RTK) and PLCγ (a substrate of the FGF receptor) that results in activation of PLCγ and formation of secondary messengers diacylglycerol and inositol 1,4,5-trisphosphate from the substrate phosphatidylinositol 4,5-bisphosphate.[17,18] While this specific laboratory module focused on interactions involving the FGF receptor with PLCγ, the approach described in this work to study protein−protein interactions is generalizable to other complexes on the basis of the interests of the instructor and students.

To give students the necessary background on the interaction between PLCγ and the FGF receptor, two structure-based papers were introduced to the class through a prelaboratory module lecture. These articles proposed alternative binding interactions occurring between a phosphotyrosine within the C-terminal tail of the FGF receptor and an SH2 domain within PLCγ. Namely, Bae et al.[19] solved a crystal structure (PDB ID 3GQI) of a tandem SH2 domain composed of N- and C-terminal SH2 domains (nSH2 and cSH2, respectively) where the nSH2 domain was bound to the receptor. On the basis of these data and others, the authors concluded the nSH2 interactions led to autophosphorylation by the same kinase (i.e., in *cis*). Subsequent to this article, Huang et al.[20] published a crystal structure showing that the cSH2 domain binds to the receptor and is phosphorylated by a second phosphorylating kinase (i.e., in *trans*). Thus, the latter work proposed a 2:1 RTK:substrate complex and proposed a reason why receptor dimerization is required beyond kinase activation (Figure 2A). The introductory class period took place during the lecture session that is associated with the laboratory module. The lecture at New York University was delivered by the graduate student working on this area of research as part of his Ph.D. thesis (W. M. Marsiglia), and a separate lecture at Spelman College was given by the faculty investigator (N. J. Traaseth). Lectures were delivered a few days prior to the laboratory period which allowed students to review the material presented and assigned literature relevant to the laboratory module. Note that if a dedicated lecture period is unavailable for the laboratory course, it is recommended to record a lecture and make it available to students prior to the laboratory module, which is commonly employed in flipped classrooms.

Following introduction to the PLCγ and FGF receptor system, students were taught how a biomolecular complex can be studied at atomic detail using NMR spectroscopy. One of the most common ways this is accomplished is to perform chemical shift perturbation (CSP) analysis, which is a method to quantify the extent of chemical shift changes upon binding to a molecule (e.g., substrate, ligand, protein, nucleic acid, etc.). A schematic of the CSP experiment is shown in Figure 3A. A series of spectra can be acquired in a sequential fashion to infer residues involved in binding or to determine a binding constant. The student laboratory module was designed to build upon previously published findings in the Huang et al.[20] study by having students perform CSP analysis between the cSH2 domain of PLCγ and a C-terminal region from the FGF

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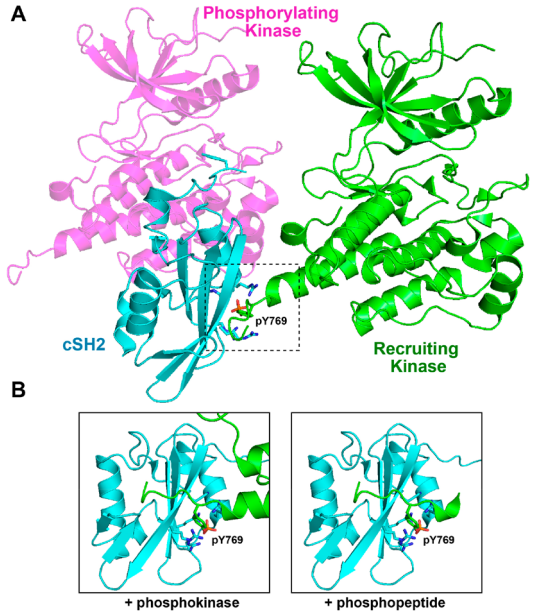

Figure 2. Protein−protein interactions studied in the laboratory module. (A) View of the crystal structure of the cSH2 domain from PLCγ (cyan) binding to the C-terminal tail containing the phosphotyrosine of the recruiting kinase (green) (PDB ID 5EG3). In addition, Tyr771 of the C-terminal tail of the cSH2 domain is bound to the phosphorylating kinase (purple) that occurs through crystal contacts within the lattice (PDB ID 5EG3). (B) Zoomed-in view of the interaction between the recruiting kinase and the cSH2 domain (left) and a schematic view of the C-terminal tail peptide of the FGF receptor used in the laboratory module (right).

receptor containing a phosphotyrosine (Figure 2B). The published work reported CSP values from an experiment involving ^{15}N-labeled cSH2 (i.e., NMR-active) and natural abundance FGF receptor composed of the kinase domain with a phosphotyrosine in the C-terminal region (i.e., NMR-inactive). It was reported that CSPs extended beyond the canonical SH2 phosphotyrosine binding pocket to the C-terminal region of the cSH2 domain of PLCγ. These long-range changes supported a role for allostery that may lead to increased accessibility of tyrosine residues within PLCγ that are subsequently phosphorylated by the FGF receptor kinase. The student laboratory module involving the shorter construct of the FGF receptor allowed students to determine whether the allosteric structural changes could be induced at the C-terminal region of the cSH2 domain with a smaller peptide or whether the kinase domain was essential to inducing these changes.

Specifics of the Laboratory Module

Protein expression and purification are widely utilized in biochemical experiments and these skillsets can be broadly applied to other proteins of interest. In order to purify the cSH2 domain from PLCγ, students were provided with bacterial lysate corresponding to the expression of this protein in ^{15}N minimal media in *Escherichia coli*. Each student group of two or three was given the equivalent of ∼50 mL bacterial culture, which amounted to 50 mg of $^{15}NH_4Cl$ at a cost of $0.82 per student group. Lysate was provided to minimize the time demands of bacterial growths and the need to accommodate other experiments during the semester. Starting from the lysate, students purified the cSH2 domain using cation-exchange chromatography in a manual fashion with a

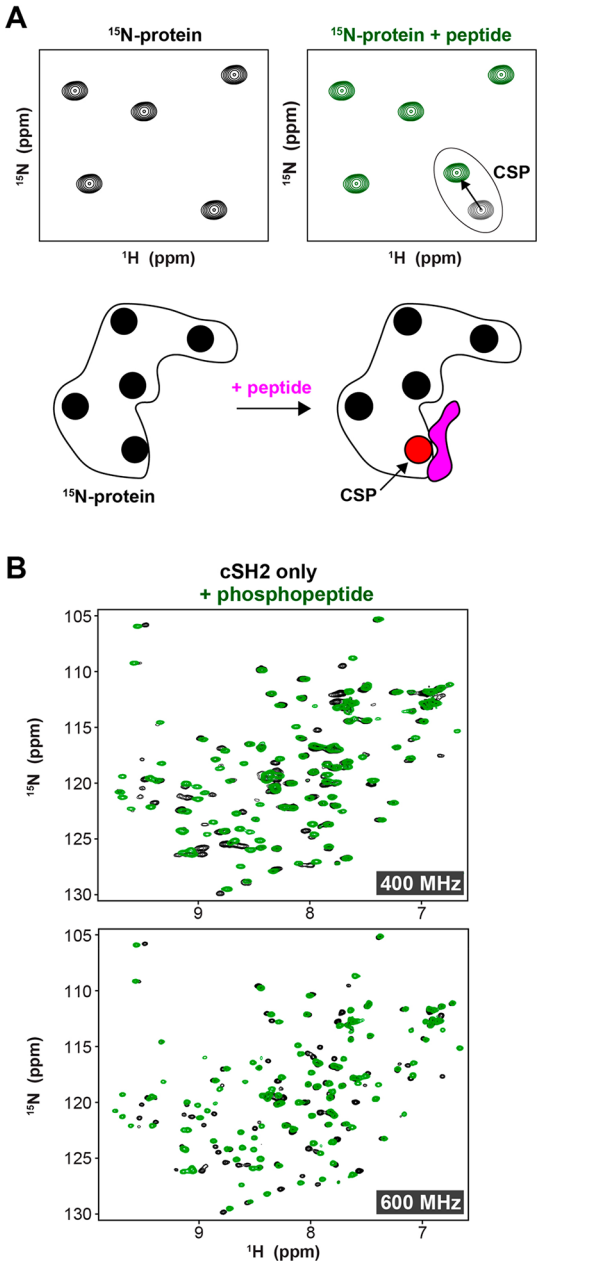

Figure 3. (A) Schematic of the protein−protein CSP experiment involving addition of natural abundance peptide (magenta) into a ^{15}N-labeled protein (white). The peak with the indicated CSP is near the location of peptide binding. (B) Overlay of experimental ^{15}N-HSQC spectra at 400 MHz (top) and 600 MHz (bottom) comparing chemical shifts for the free cSH2 domain (in black) and the cSH2 domain bound to the phosphopeptide (in green).

syringe attached to the column. A detailed procedure of the protein purification for the laboratory module is provided in the Supporting Information. The cost of each column was $28, and it can be reused several times. Students analyzed SDS-PAGE results to determine the fractions containing cSH2 to concentrate for NMR spectroscopy. The efficient expression of cSH2 samples resulted in NMR samples of 0.5 mL at a concentration of ∼0.5 mM. ^{1}H/^{15}N HSQC experiments were acquired in the absence and presence of a 10-residue phosphopeptide corresponding to the C-terminal tail of the FGF receptor (TNEEpYLDLSQ). The cost of the synthetic peptide was $3.72 per student group.

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Across all semesters, students successfully acquired high quality 2D HSQC spectra with one failed experiment resulting from improper usage of the cation-exchange column (i.e., no purified protein). Note that the laboratory module was implemented for three different semesters with class sizes varying from 7 to 10 students at Spelman College and for four different semesters with class sizes varying from 4 to 49 students at New York University. At Spelman College, spectra were acquired with a 400 MHz NMR spectrometer equipped with a room temperature probe that enabled 2D data sets to be collected in 15 min (Figure 3B, top panel). At New York University, spectra were acquired with a 600 MHz NMR spectrometer equipped with a cryogenic probe that enabled 2D data sets to be collected in 5 min (Figure 3B, bottom panel). Peaks within 2D spectra had average signal-to-noise ratios of ∼28/1 and ∼47/1 at 400 and 600 MHz, respectively. These data sets were generally of publishable quality and enabled students to analyze the majority of nonproline peaks in the spectrum.

It is important to emphasize that the biomolecular NMR approach described in this laboratory module can be applied to other protein−protein interactions. The key considerations when selecting a suitable complex are the total size of the system to be studied and the achievable protein concentration. The former is limited to ∼25 kDa using a similar isotopic labeling approach described in this work. The latter is an important consideration for acquiring spectra using NMR spectrometers available at most undergraduate institutions (e.g., 400 MHz and without cryogenic probes) and stems from the need to achieve sufficient signal-to-noise to acquire 2D HSQC spectra. It is recommended that a minimum concentration of ∼0.3 mM of the isotopically enriched protein is obtained in order to study a protein−protein complex using a 400 MHz spectrometer in the absence of a cryogenic probe.

Data Interpretation from NMR Experiments

The primary data sets that students analyzed were HSQC spectra of cSH2 in the absence and presence of the phosphopeptide (Figure 3B). Spectral data obtained on student cSH2 samples were consistent among various student groups within the class and across different semesters. Using these HSQC spectra, students quantified the CSPs and plotted these on the available crystal structure (PDB ID 5EG3).[20] Students compared their calculated CSP values with published results that used an FGF receptor construct that contained both the kinase domain and the C-terminal tail. To facilitate the CSP analysis, chemical shift assignments were provided to students for the cSH2 domain in the apo state[20] (see Supporting Information). A Sparky tutorial and additional insights into NMR data interpretation were delivered by the principal investigator (N. J. Traaseth), who has extensive experience in NMR spectroscopy. This offered students an opportunity to interact with and learn from someone directly in the scientific field. Evaluation of student laboratory reports revealed that the majority of students correctly identified CSPs induced upon addition of the phosphopeptide corresponding to the C-terminal tail of FGF receptor. Note that tutorials to interpret NMR spectra using Sparky[21] and subsequent analysis with PyMOL[22] are provided in the Supporting Information. These tutorials are applicable to other protein−protein interactions studied using biomolecular NMR spectroscopy.

The laboratory module incorporated a variety of skills, including protein purification using ion-exchange chromatography, evaluation of protein purity using gel electrophoresis, concentration of protein samples for structural biology, biomolecular NMR spectroscopy, and analysis of protein structure with molecular visualization software.

Perspectives about the Laboratory Module and Future CUREs

The design of the laboratory module was approached with the following central paradigm: *what motivates basic research can also inspire students in the teaching laboratory classroom*. This approach served two primary goals: (i) to motivate undergraduate students to participate in the process of scientific discovery[1,11,13,23] and (ii) to motivate instructors' natural passion for scientific discovery which leads to a mutual excitement from students.[24] The instructors found that the laboratory module encouraged a greater sense of participation from the students relative to more traditional laboratory experiences where the results were well-established. Students commented to the instructors that they enjoyed working directly with researchers in the field and showed a high level of enthusiasm for learning how NMR data sets were collected and used to study protein−protein interactions. Positive responses from student course evaluations included the following:

> *"Conducting larger experiments throughout the course of the semester is one of the strong points of this class, as it mimics the multi-step nature of "real research" and allowed us to explore questions that have not been answered yet (as opposed to most lab courses, where you know exactly what outcome to expect)".*
>
> *"If I were not graduating, I would definitely be interested in another class or lab similar to this one."*

On the basis of the instructors' experience in developing the laboratory module, the following design principles were viewed as important for incorporating into future modules. Note that these points have been supported by academic literature and suggest improved student knowledge retention and participation.[23,25]

- **Emphasis on the scientific rationale for carrying out the experiment**. Evaluation of CUREs shows that students benefit from the process of motivating a scientific question by understanding literature and why carrying out an experiment will advance a field of study.[11] The prelaboratory lecture delivered to students is provided in the Supporting Information.
- **Incorporate techniques as part of the laboratory, but not as the goal for the laboratory.** Introducing students to techniques forms an integral component of undergraduate experiments. The laboratory module was designed to teach several skills while at the same time placing the emphasis on a scientific question.
- **Engage students with ongoing research within the institution.** It is our opinion that undergraduate students in the natural sciences should be as familiar with ongoing science within the institution as their sports teams. This knowledge and exposure can build a sense of pride within the research enterprise.
- **Introduce students to advanced techniques.** Undergraduate lecture-based courses in biochemistry and molecular biology routinely teach students about classical experiments that form the basis for understanding protein and nucleic acid structure−function relationships.[26] It is more challenging to incorporate advanced techniques into the laboratory.[3−5] However,

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exposure to these techniques can better prepare aspiring Ph.D. students for graduate school research in interdisciplinary fields and can reduce the *fear factor* surrounding advanced techniques that can be generated in lower-division courses.

- **Involve graduate students and postdoctoral associates in the design and implementation of laboratory modules (if possible).** Recent evidence suggests that involving graduate students and postdoctoral associates in evidence-based teaching strategies benefits research production and improves overall communication skills.[27]
- **Incorporate new laboratory modules/directions.** Similar to basic research, classroom experiments can be designed such that student results seed new experiments.[28] This further underscores to students the discovery process and provides an opportunity to contextualize findings relative to scientific literature. One aspect of new experiments is the possibility of unanticipated hurdles and failed experiments; however, similar to academic research, negative results are a part of science and students often learn as much from failures as successes.[29]

With regard to future laboratory modules, signal transduction pathways offer the opportunity to conduct several structure−function studies due to the abundance of enzymes and protein−protein interactions. Future years will also explore the possibility of diversifying the classroom by giving individual student groups separate projects. If selected carefully, student groups can interpret data collectively to derive conclusions beyond what would be achievable in a single laboratory module.

CONCLUSION

A CURE undergraduate laboratory module was developed to obtain atomistic insights into a protein−protein interaction using biomolecular NMR spectroscopy. In the process, students gained knowledge of protein purification, NMR spectroscopy, and analysis of structural data by using molecular visualization software. The overall design principle was to pursue objectives in the same manner as that of an academic laboratory. This focus on significance harnessed the natural tendency from research-active instructors to be energized by acquisition of new experiments with the goal of obtaining new knowledge. Class evaluations and student feedback suggested that students enjoyed learning advanced techniques and being involved in the discovery of new knowledge.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.9b00364.

Appendix describing the experiment in a protocol-style manner including a tutorial for NMR data analysis (PDF)

Prelaboratory lecture that illustrates the background and motivation for the laboratory module (PDF)

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Notes

The authors declare no competing financial interest.

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