Electron Paramagnetic Resonance (EPR) Spectroscopic Study of a Phospholipid Bilayer Membrane: An Undergraduate Laboratory Experiment

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Abstract: This laboratory experiment aims to introduce undergraduate students to the use of electron paramagnetic resonance (EPR) spectroscopy as a valuable tool for studying lipid bilayer membranes in laboratory experiments. This spectroscopic method utilizes a nitroxide spin-labeled stearic acid (5-DOXYL stearic acid) incorporated into a desired lipid bilayer membrane. Here, we highlight EPR's pivotal role in investigating the dynamic properties of various membrane lipid bilayers and provide hands-on laboratory experience to upper-level chemistry/physics undergraduate students to develop biophysical and biochemical research skills. Undergraduate students will be immersed in wet lab knowledge and learn skills on operating benchtop EPR instrument for EPR data acquisition, analyzing the data and writing professional lab report.

Keywords: Lipid bilayer membrane; Phospholipids; EPR spectroscopy; Undergraduate EPR laboratory experiment; Laboratory instruction; Membrane dynamics.

1. Introduction

Membranes are crucial biological structures, serving as barriers between cells and their external environment to protect cellular components from external stress, toxins or pathogens. Their fundamental role requires stable bilayers of lipids with hydrophobic and hydrophilic regions, making it essential for membranes to mimic those found in living organisms. Lipid bilayered vesicles provide stability to their constituents, such as membrane proteins, in their functional states. It is imperative to understand structural and dynamic properties of lipid bilayer membranes to understand their functions. Electron paramagnetic resonance (EPR) is a very useful and rapidly growing biophysical technique for studying structural dynamics of lipid bilayer membranes [1-3]. The thin-film rehydration method is one approach to preparing lipid bilayered vesicles in a laboratory setup, providing a close membrane mimic suitable for several biophysical experiments [4].

Electron paramagnetic resonance spectroscopy is a structural biology tool that detects and quantifies unpaired electrons in paramagnetic compounds [5-7]. It works by detecting the absorption of microwave radiation by the spin states of electrons in a magnetic field. EPR spectroscopy works on the basis of the principles similar to other spectroscopic techniques, such as nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy, which also use the interaction between electromagnetic radiation and matter to provide information about the structure and dynamic properties of molecules. However, unlike NMR, which detects the spin states of atomic nuclei in a magnetic field, EPR focuses on the spin states of electrons. In a typical continuous wave EPR experiment, EPR signal is generated by varying the applied magnetic field at a fixed microwave frequency. A benchtop CW-EPR spectrometer operating at X-band frequency, employed in this laboratory project, provides undergraduate students with a

valuable opportunity to learn about the structure and dynamic properties of biological molecules containing unpaired electrons.

EPR techniques have been widely applied to study structural and dynamic properties of many biological systems. However, undergraduate students have been poorly exposed to this technique in classroom laboratory settings. EPR-based undergraduate laboratory experiments have been previously reported in the literature [8-14]. In this laboratory experiment, undergraduate students prepare phospholipid bilayered vesicle samples using thin-film dehydration methods, conduct continuous wave (CW) EPR experiments, and perform EPR spectral lineshape analysis to determine spin-label mobility by measuring the central linewidth and spectral width. Spin-label mobility and spectral linewidth are important spectral parameters that provide information about the dynamic properties of spin-labeled phospholipids. By analyzing the EPR spectra of the nitroxide spin-probe, 5-DOXYL-stearic acid (5-DSA), in three different lipid bilayer membranes (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and 1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS)), we can gain insight into the dynamics and fluidity of bilayer membranes, which is crucial for understanding proper membrane functions. POPC and POPS are monounsaturated lipids, while DMPC is a fully saturated lipid. The chemical structure of POPC, DMPC, POPS, and 5-DOXYL-stearic acid is shown in Figure 1. However, undergraduate EPR laboratory experiments exposing students to biological systems, such as membrane systems, are still lacking in the undergraduate curriculum. In this novel experiment, students gain experience, build knowledge, and develop skills while working in a wet lab to prepare membrane samples for CW-EPR data acquisition, interpret data, and prepare

professional lab reports. These skills are valuable in academia as well as in industrial and research settings.

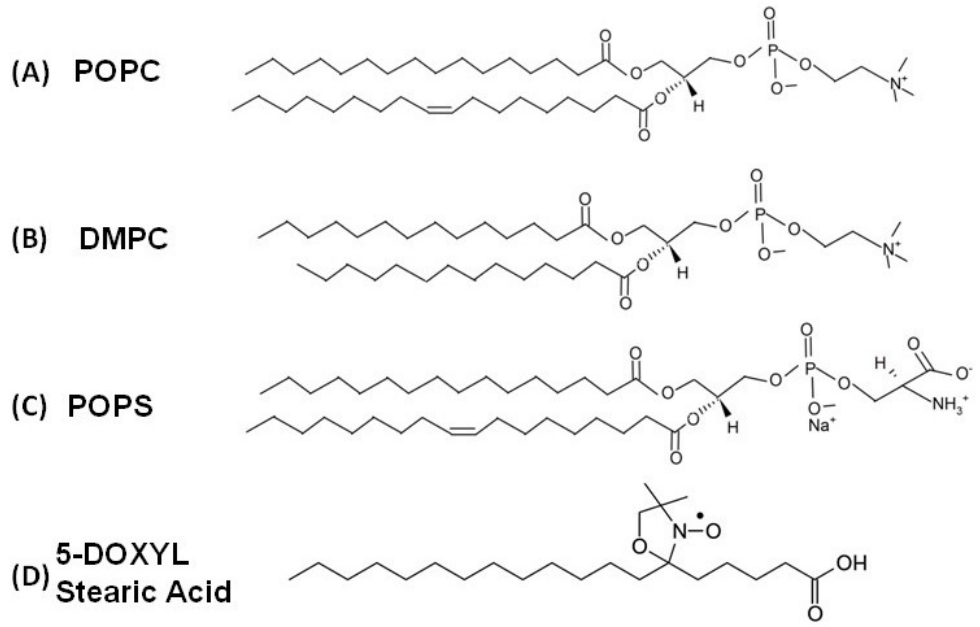


Figure 1. Chemical structure of POPC **(A)**, DMPC **(B)**, POPS **(C)**, and 5-DOXYL stearic acid **(D).**

2. Student Learning Goals:

This laboratory experiment is designed for undergraduate junior and senior chemistry and physics students. Following are the students' learning objectives:

1) Gain hands-on experience in using basic wet lab equipment to prepare nitroxide spin-labeled membrane bilayer samples.

2) Acquire knowledge of how to work with lipid samples in the laboratory setup.

3) Operate a benchtop EPR spectrometer.

4) Acquire CW-EPR data on membrane bilayer vesicle samples.

5) Analyze the CW-EPR spectral data using an analysis program (Excel/Igor) to calculate spectral parameters (e. g., central line width (ΔH) and spectral width (2Azz)).

6) Plot the spectral data using data analysis programs (Excel/Igor).

7) Interpret the experimental results and draw conclusions about the experiment.

8) Prepare a final laboratory report.

Teamwork, critical thinking, problem-solving, and student-centered laboratory experiences have been employed during this laboratory project.

3. Pedagogical Purposes

This novel student centered laboratory experiment contributes to the overall educational experience of students through hands-on approaches in achieving learning goals. Here are some key pedagogical purposes of this lab: it introduces students to applying advanced spectroscopic techniques as a practical context of analytical tool used in various disciplines. Students learn to design experiments, optimize conditions for EPR measurements, and develop critical thinking and problem-solving skills. By analyzing the data, students enhance their skills in drawing meaningful information from EPR spectra. Students work in groups of two. This helps them develop collaborative skills, which are valuable for solving complex problems in multidisciplinary scientific fields.

4. Feasibility

Students are engaged in a practical learning environment while conducting laboratory experiments. The wet lab procedure is simple and can be adapted at any arbitrary laboratory location in laboratory environment (see Supporting Information for the lab procedure). A detailed lab protocol, including pre-lab sample questions, solutions, example lab reports, and instructor instructions, is provided in the Supporting Information. The wet lab equipment utilized for the membrane EPR sample preparation is simple and can be easily setup in any arbitrary undergraduate science laboratory setting. The commercial benchtop Magnettech ESR5000 EPR spectrometer is user-friendly and requires very minimal effort to operate for data acquisition. Data analysis, interpretation, and report-writing procedures are also included in the Supporting Information. This lab has been incorporated into the upper undergraduate Physical Chemistry (CHE 454) and Biochemistry (CHE 461) laboratory courses and has been successfully conducted at the Natural Science Division, Campbellsville University.

5. Experimental Overview

This experiment is designed to be completed by the group of two undergraduate students; however, the lab reports are expected to be submitted by each student to achieve students' learning goals. This is a two-day laboratory procedure (2.5 hours per day) to complete spin-labeled membrane sample preparation, EPR data acquisition, and data analysis. pre-lab assignments and related lab resources, including the lab protocol (Supporting Information), can be provided to students five days prior to the first day of the lab. The due date for the pre-lab assignment can be on the first day of the lab, and the final lab report can be due the following week. The pre-lab questions are designed to help students conduct the laboratory experiments

successfully. Below are experimental procedures used by students to prepare EPR active membrane samples and perform EPR spectral measurements.

Materials

All phospholipids POPC, DMPC, and POPS are purchased from Avanti Polar Lipids (Alabaster, AL). The 5-DOXYL-stearic acid spin-probe is obtained from Toronto Research Chemicals (TRC). N-[2-Hydroxyethyl] piperazine-N′-2-ethanesulfonic acid (HEPES) and chloroform are purchased from Sigma-Aldrich (St. Louis, MO, USA), and sodium chloride (NaCl) from Fisher Scientific (Pittsburgh, PA, USA).

Preparation of Lipid Bilayer Vesicles

Students prepare stock solution of POPC, DMPC or POPS lipid by dissolving the lipid powder in chloroform at a concentration of 20 mg/mL. The 5-DOXYL stearic acid spin-probe stock solution is prepared by dissolving the spin-probe powder in chloroform at a concentration of 25 mg/mL. Desired lipid solutions are mixed with the spin-probe solution at a concentration of 5 mol% in pear-shaped flasks. The use of chloroform is conducted in a properly functioning chemical fume hood. All samples are then evenly dried under a stream of nitrogen gas to form uniform thin-films. After drying, the flasks containing the thin-films are transferred into a desiccator and left overnight. The next day, the dried lipids are suspended in HEPES buffer (100 mM NaCl, 20 mM HEPES, pH 7.0) at a concentration of 100 mM lipids. The lipid-spin-probe samples are then vortexed and subjected to two freeze–thaw cycles to produce homogeneous lipid bilayered vesicles. The schematic diagram of the sample preparation procedure is shown in Figure 2.

CW-EPR Measurements

Students perform CW-EPR experiments at X-band on a Bruker Magnettech ESR5000 Benchtop EPR spectrometer. A 50 μL sample is loaded into a glass capillary (Bruker, Module # E4009) using capillary action or a 1 mL syringe with a needle and then placed inside the quartz sample tube (O.D. 3 mm, Module # E4300) within the EPR spectrometer resonator cavity. The CW-EPR spectrum is obtained by signal averaging 20 42-s field scans with a 3379 G central field and a 120 G sweep width, 9.48 GHz microwave frequency, 100 kHz modulation frequency, 1 G modulation amplitude, and 10 mW microwave power at room temperature. The CW-EPR spectral data are analyzed by students using a similar method of EPR spectral lineshape analysis reported previously to determine the motional properties of lipid bilayers bearing 5-DSA spin-probes [1, 15]. The central linewidth (ΔH) of the spectrum is calculated by measuring the width of the central line of the EPR spectrum (i. e., the width of the top peak and bottom dip of the central line of the first derivative EPR spectrum), using the scheme as shown in Figure 3A. The spectral width ($2A_{zz}$) is also calculated from the spectra, measured between the upper peak of the spectra in the lowest magnetic field line and the bottom dip of the spectra in the highest magnetic field line using the scheme as shown in Figure 3A. The mobility of the spin-probe incorporated into lipid bilayered vesicles is characterized by the width of the central line of the EPR spectrum, which is a very important spectral parameter widely used in studying bilayer membrane dynamics [1, 15]. CW-EPR data can be plotted using any plotting software such as Excel.

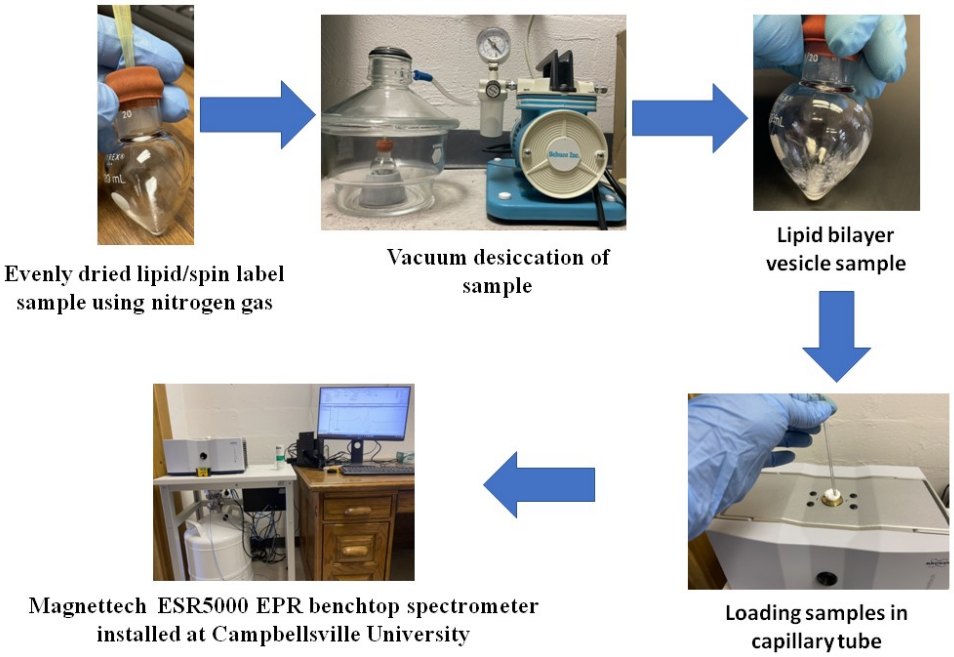


Figure 2. Experimental methods: sample preparation of lipid bilayered vesicles and CW-EPR spectral measurement.

6. Result and Discussion

6.1 Student Learning Outcomes

The lab has been incorporated into upper-level undergraduate Physical Chemistry (CHE 454) and Biochemistry (CHE 461) laboratory courses at Campbellsville University. A total of twelve students were enrolled in these courses and participated in this laboratory experiment. Teaching guides and rubrics (see Supporting Information) were developed and employed to evaluate students' learning gains. The instruction manual and guidelines provided sufficient support to troubleshoot issues and address questions that arose during the laboratory work. Based on students' performance in the laboratory experiments, interactions with instructors, students' evaluations and feedback, we believe that the instruction manual and guidelines effectively supported students' success in the lab. An illustration of major learning outcomes is presented in

Table 1. Responses to pre-lab questions, performance in the laboratory, and students' final laboratory reports were evaluated to measure student success. A summary of students' evaluations and feedback regarding the laboratory experiments is provided in the Supporting Information. These evaluations and feedback data were collected through an anonymized survey, in which students rated the lab on a scale of 1 to 5 (1 = strongly disagree, 5 = strongly agree) and provided additional comments for improvement. To protect students' identities and avoid bias, a fellow student distributed and collected the surveys in the instructor's absence, ensuring that all responses were anonymized. Additionally, each student completed a consent form at the beginning of the class to allow their feedback and ratings to be used for research and publication purposes. Three criteria (Unsatisfactory, Satisfactory and Excellent) were used during the evaluation of this lab. Students met most learning outcomes with satisfactory or excellent ratings. An unsatisfactory rating represents either not attempting or providing nonscientific answers. A satisfactory rating represents that the students correctly answered most questions but made minor scientific errors or failed to provide in-depth explanations of the concepts. An excellent rating represents that student fully demonstrated the learning objectives and provided reasonable explanations.

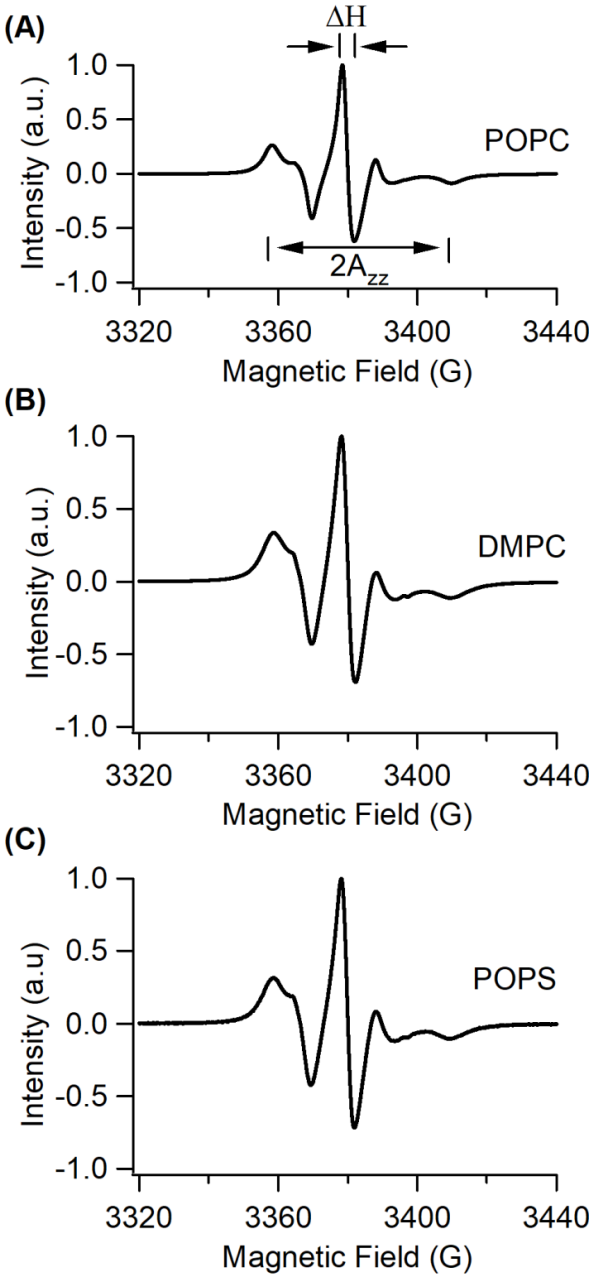
Learning Outcomes	**Unsatisfactory**	**Satisfactory**	**Excellent**
Answering the pre-lab questions in a logical manner		33%	67%
Understanding EPR spectroscopic technique		33%	67%
Wet lab equipment, EPR Instrument operation, and data acquisition		17%	83%
Calculation of EPR spectral parameters: central		25%	75%

linewidth and spectral width			
Illustration of data with graph and table		25%	75%
Accessing the dynamic properties of spin-labeled membrane for drawing the conclusion		33%	67%
Preparation of lab report	8%		92%

Table 1. An illustration of the accomplishments of student learning outcomes. Twelve students participated in this laboratory experiment.

6.2 Experimental Results

CW-EPR spectra of molecules containing unpaired electron spin-probes are sensitive to changes in the motion of spin-probe side chains [16-19]. In this laboratory experiment, over the period of two days (2.5 hours/day), students prepare the EPR active membrane samples and operate a benchtop EPR spectrometer to acquire experimental data. Students characterize the motion of the lipid bilayered vesicles using CW-EPR spectral lineshape analysis. Representative students' CW-EPR spectra collected on POPC, DMPC, and POPS lipid bilayered vesicles, as well as a control sample without lipid bilayers, each containing the 5-DOXYL stearic acid spin-probe are shown in Figure 3. The experiments were replicated three times.


(A)
ΔH
1.0
0.5
0.0
-0.5
-1.0
Intensity (a.u.)
POPC
2A$_{zz}$
3320
3360
3400
3440
Magnetic Field (G)
(B)
1.0
0.5
0.0
-0.5
-1.0
Intensity (a.u.)
DMPC
3320
3360
3400
3440
Magnetic Field (G)
(C)
1.0
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-0.5
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Intensity (a.u)
POPS
3320
3360
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Magnetic Field (G)

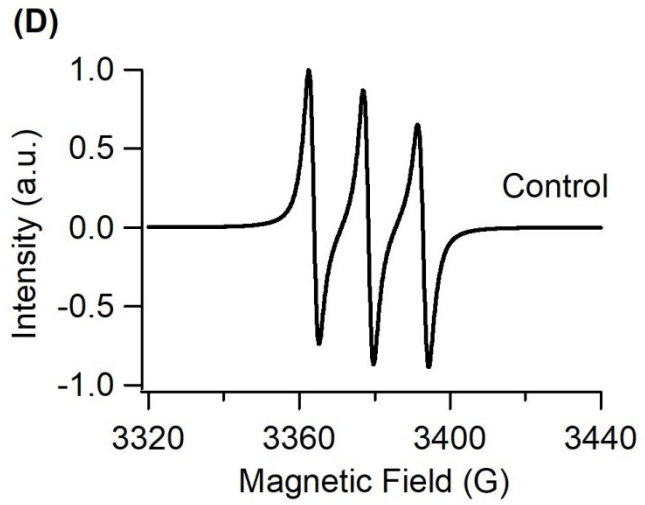

Figure 3. Representative students' CW-EPR spectral data from their lab reports on 5-DOXYL stearic acid (5-DSA) spin-probe incorporated into POPC **(A)**, DMPC **(B)**, and POPS **(C)** lipid bilayered vesicles, and chloroform without lipid bilayers (control) **(D)** at room temperature. The spectra were normalized to maximum intensity. The scheme for measuring the central linewidth (ΔH) and spectral width ($2A_{zz}$) is shown in Figure 3A. The experiments were replicated three times.

The inspection of the CW-EPR spectra indicated a broader spectral lineshape close to a rigid limit regime for the lipid bilayer samples when compared to that of the control sample. The EPR spectral lineshape of the control sample exhibited three sharp peaks, indicating an isotropic motion of the spin-probe in the absence of lipid bilayers. This suggests a restricted motion of the 5^{th} carbon of the acyl chain for all these lipid bilayered vesicles when compared to that of the control sample without lipid bilayers [15, 20, 21]. Students further explored the mobility of the spin-probe of the lipid chain by calculating spectral parameters such as the central linewidth (ΔH), and spectral width ($2A_{zz}$) [15]. The value of these parameters generally increases with a

decrease in the motion of the motional freedom of the spin-probe. The acyl chain mobility behavior of lipid bilayers can be determined using a fatty acid spin-probe using EPR spectroscopy.

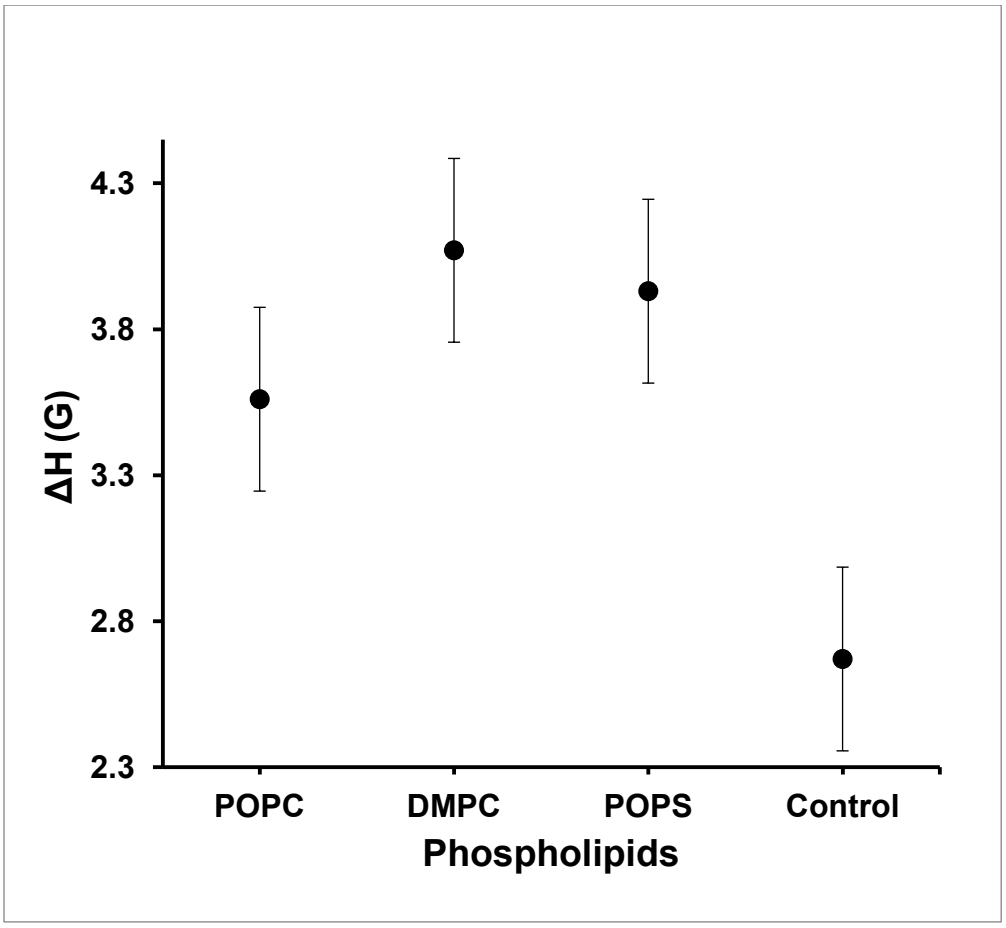

Figure 4. Plot of the central linewidth (ΔH) calculated from EPR spectra as a function of phospholipids. The control sample represents 5-DSA spin-probes in chloroform without lipid bilayers. The error bars represent standard error.

Figure 4 shows the plot of the central linewidth calculated from the CW-EPR spectra (Figure 3) as a function of phospholipids by students. The control sample contains 5-DSA in chloroform without lipid bilayers. Figure 4 demonstrates that the central linewidth for all three

lipid samples is higher than that of the control sample without lipid bilayers. It further indicates that the central linewidth for the POPC lipid is slightly lower than that of the DMPC lipid samples. Similarly, the spectral width ($2A_{zz}$), calculated from the EPR spectra and plotted as a function of phospholipids by students, is shown in Figure 5. The error bars in Figures 4 & 5 represent standard errors. The standard error bars were calculated using Excel's built-in Standard Error function under the Error bars option in the main menu. The standard error measures how spread out the values are in the dataset. The standard error bars are calculated by dividing the standard deviation by the square root of the number of data points. Students found it easier to use this Error Bars function to estimate errors in these datasets. Figure 5 reveals that the spectral widths for all three lipid samples are similar and higher than that of the control samples without lipid bilayers. These results interpreted by students suggest that the motion of 5-DSA spin-probes in these three lipid bilayers is restricted when compared to the control sample without the lipid bilayers. The restricted motion of the spin-probe in the lipid chain may be due to the local environment of the spin-probes in these lipid samples. The restricted motional behavior of 5-DSA spin-probe in the lipid bilayer environment is expected and consistent with previously published literature [15]. The nitroxide spin-probe incorporated at the 5^{th} position of the acyl chain can provide information about the fluidity of the lipid bilayer membrane. We employed 5-DSA as a spin-probe to study the dynamic behavior of three different lipid bilayers. POPC and POPS are monounsaturated lipids, while DMPC is a saturated lipid with a shorter acyl chain. The head groups of POPC and DMPC lipids are similar, both containing choline, making the lipid overall neutral at physiological pH, whereas the POPS head group contains serine, making the lipid overall negatively charged. The minor decrease in the central linewidth of the EPR spectrum for POPC lipids suggests a minor increase in lipid bilayer fluidity. This might be

due to the mobility behavior of the acyl chain in the presence of monounsaturated POPC lipids when compared to the acyl chain behavior in fully saturated DMPC lipids. The EPR laboratory results are very helpful for undergraduate students to understand the dynamic behavior of different lipid bilayered vesicles as shown in Student Learning Outcomes in Table 1 and student evaluations and feedback (Supporting Information).

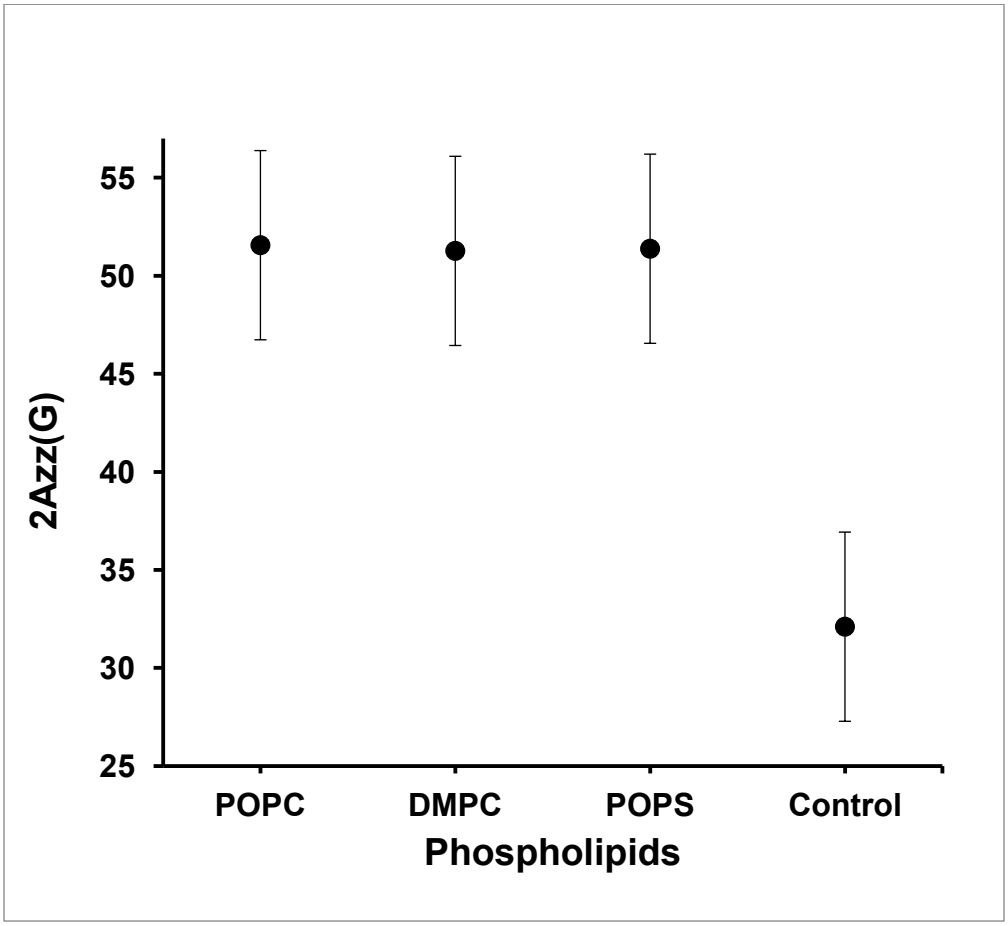

Figure 5. Plot of the spectral width ($2A_{zz}$) of EPR spectra as a function of phospholipids. The control sample represents 5-DSA spin-probes in chloroform without lipid bilayers. The error bars represent the standard error.

7. Limitations and Challenges

Some challenges associated with this laboratory experiment include: 1) Preparation of the homogeneous thin-film on the wall of the pear-shaped flask. Care should be taken while blowing the lipid-spin-probe solution using nitrogen gas. 2) Loading EPR samples in glass capillaries and placing the sample tube inside the resonator cavity of the EPR spectrometer. The sample tubes should be placed carefully to prevent breaking the tube inside the cavity. 3) Measurement of the differences in central linewidth and spectral width from the EPR spectra. Proper care should be taken when choosing the upper peak and lower dip positions during the calculation of the central linewidth. Similarly, this also applies when choosing the left upper peak and right lower dip during the calculation of the spectral width. 4) The EPR instrument operation instruction manual should be followed properly to avoid damage to the spectrometer. 5) Sample contamination during the preparation of the lipid and spin-probe mixture. Proper labeling of each pear-shaped flask is necessary to avoid any confusion regarding sample names while working in the wet lab. 6) Use of organic solvents during sample preparation. Proper precautions should be taken when handling chloroform. Chloroform should be used under a properly functioning chemical hood to avoid exposure. Reagents, such as lipids and 5-DSA spin probes, should be measured using a digital balance whenever possible to ensure accurate measurements. Overall, these factors can contribute to uncertainties and may affect the precision and accuracy of the measured motional parameters of the spin-probe.

8. Conclusion

We have designed a laboratory experiment to provide hands-on experience in utilizing the structure biology technique of EPR spectroscopy for studying the dynamic properties of

phospholipid bilayer membranes. This experiment is intended for junior and senior undergraduate students to enhance their laboratory experience. Basic wet lab facilities and a commercial benchtop EPR spectrometer can be utilized in this experiment. EPR spectroscopy is a very useful technique for studying structural and dynamic properties of biomolecules.

Hazards

There are no significant hazards expected with this experiment. However, students are expected to follow proper lab protocols (see Supporting Information) during sample preparation and EPR data collection. Proper care should be taken when handling organic solvents, such as chloroform, and chemical reagents, including phospholipids and 5-DOXYL stearic acid. Organic solvents, such as chloroform, should be used under a properly functioning chemical hood to avoid exposure. Additionally, students need to be careful while loading the EPR sample tube inside the resonator cavity during the EPR data acquisition.

Notes

The authors declare no conflict of interest.

ASSOCIATED CONTENT

Supporting Information

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

Prelab questions, Sources of reagent and equipment, Handout for student, Laboratory protocol, Format of the final lab report, Students' evaluation and feedback about the laboratory experiment, Rubric for the lab

Instructor's guide: Objectives, Beginning lab instructions, Solution to prelab questions, An example of final lab report

Example of CW-EPR data: Sample_Data_POPC_5DSA.txt, Sample_Data_DMPC_5DSA.txt, Sample_Data_POPS_5DSA.txt, Sample_Data_Control_5DSA.txt

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