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An Introduction to Passive Ion Transport across Model Lipid Membranes for Undergraduate Students: Proton Permeation Measurements in Liposomes

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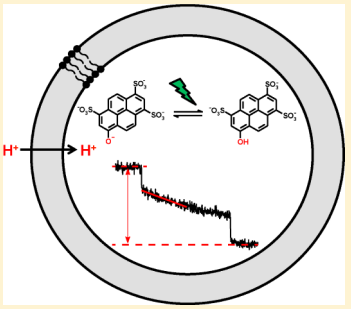
Supporting Information

ABSTRACT: In an upper-division biochemistry laboratory experiment, students use a variety of experimental techniques and apply several biochemical and biophysical concepts to measure and interpret proton permeation rates across model membranes. Model membranes in the form of liposomes are prepared from lipids by the extrusion method. The liposomes encapsulate the membrane-impermeable fluorescent pH-indicator pyranine, which facilitates pH detection in their aqueous interior. Rates of proton permeation across the lipid membrane in response to acidification of the external volume are measured by fluorescence spectroscopy. Data analysis introduces students to flux calculations and illustrates the concept that concentration differences are the driving force of diffusion processes such as passive ion transport across membranes. Students are given the task of formulating and then testing a hypothesis regarding the effects of bilayer thickness, liposome radius, magnitude of pH difference across the bilayer, and the presence of potential uncouplers on experimentally observable permeation rates.



KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Laboratory Instruction, Hands-On Learning/Manipulatives, Inquiry-Based/Discovery Learning, Biophysical Chemistry, Fluorescence Spectroscopy, Lipids, Membranes, Transport Properties*

Driven by the hydrophobic effect, liposomes are formed by the self-aggregation of amphiphiles, which arrange themselves in a spherical bilayer that completely encloses an aqueous volume.[1] Frequently, they serve as model systems for the study of the physical and chemical properties of biomembranes and embedded membrane proteins. Liposomes have also found numerous practical applications in industry, medicine, and research.[2,3] Despite their widespread use, their value as a teaching tool is often overlooked. The few examples describing their use in laboratory experiments for teaching purposes are dated and limited to narrowly defined topics, such as the encapsulation of dyes, the detection of the rapid penetration of bilayers by hydrogen peroxide, or the measurement of water and nonelectrolyte permeabilities.[4−6] Liposomes can be prepared readily by a variety of methods, such as extrusion, sonication, or solvent evaporation from relatively inexpensive starting materials and without the need for sophisticated equipment.[2]

The experiment described here is based on a well-established spectroscopic method of measuring the transport rates of protons across artificial bilayer membranes prepared as liposomes.[7−9] Most students in upper-division biochemistry classes are familiar with the concept that many physiological processes depend on membranes that act as an effective barrier to free diffusion of ions. This is particularly important for protons, whose transport across the inner mitochondrial membrane, for example, is key to ATP-generation during oxidative phosphorylation.[10] Unlike small polar molecules that cross a bilayer by the solubility-diffusion mechanism, protons are thought to diffuse through transient hydrated defects in the membrane that are produced by thermal fluctuations.[11] The pedagogic goals for this laboratory project encompass an introduction to students to the use of model bilayer membranes for proton transport studies and the teaching of several experimental techniques commonly used in biochemical research, such as the preparation of liposomes by extrusion, the application of size-exclusion chromatography, and the use of time-resolved fluorescence spectroscopy. Moreover, analysis of the obtained data reinforces basic kinetic principles, teaches the calculation of flux rates, and illustrates the behavior of a pH-sensitive fluorescent dye. After having mastered a typical permeation measurement, students are in a position to formulate hypotheses regarding experimentally observable effects on permeation rates caused by changes in bilayer composition, liposome size, and proton concentration difference across the membrane. Finally, students predict and experimentally test the effect of a potential protonophore on experimentally observed proton flux rates. Protonophores are compounds that facilitate proton transport, thereby greatly accelerating transmembrane transport rates. If present in the inner mitochondrial membrane, for example, protonophores can uncouple proton transport from ATP synthesis. In nature, the best-known example for this scenario is the protein thermogenin that exploits uncoupling for heat generation in hibernating or cold-adapted animals.[12]

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EXPERIMENTAL DETAILS

Equipment Needs

For the preparation of single-walled liposomes by extrusion through polycarbonate membranes with a defined pore diameter, a hand-held extruder, such as the one manufactured by Avanti Polar Lipids, is required. Time-resolved measurements can be conducted with any type of fluorimeter capable of recording time-resolved fluorescence intensities on a time scale of seconds. A stirred sample cell is advantageous to facilitate rapid mixing of solutions added to the sample. For the separation of pyranine from the liposomes, a small size exclusion column is needed, which can be purchased or self-made.

Liposome Preparation and Proton Permeation Measurements

Students prepare liposomes containing the fluorescent pH-indicator pyranine. Removal of dye outside the liposomes is accomplished by passage through a size-exclusion column pre-equilibrated with dye-free buffer. Proton permeation is initiated by acidification of a liposome sample and permeation rates are measured by monitoring the fluorescence of pyranine trapped inside the liposomes (Figure 1).

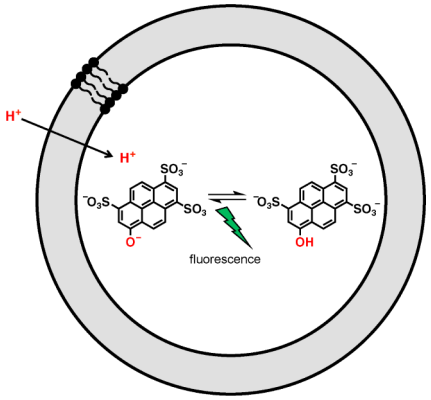

Figure 1. Schematic depiction of proton permeation through a lipid bilayer and response of the encapsulated dye pyranine. Upon protonation, the pyranine absorbance maximum shifts from 460 nm to a shorter wavelength, causing the fluorescence signal at 515 nm to decline.

After having mastered the basic permeation measurement with egg-phosphatidylcholine (egg-PC; an inexpensive mixture of lipids with mostly 16 or 18 carbon atoms in their chains) liposomes, students proceed to conduct a selection of inquiry-based experiments. In each case, a factor that has a potential impact on the observable proton permeation rate is varied. Students formulate a hypothesis on the anticipated effect and then conduct a variation of the standard flux measurement to test it. The possibilities are listed below.

- *the influence of bilayer thickness*: can be assessed in two separate experiments using lipids that differ in the length of their alkyl chains. Good choices are pure samples of dioleoyl-PC and dimyristyl-PC, whose chains have 18 and 14 carbon atoms, respectively
- *the effect of liposome radius*: can be explored by extruding through polycarbonate filters of varying pore size, such as 50, 100, and 200 nm, which yields liposomes of different sizes
- *the impact of the pH-difference across the bilayer*: is dictated by the amount of acid added and is thus readily modified
- *the presence of a potential protonophore*: students develop a hypothesis as to whether a compound presented to them can act as a protonophore and thus as an uncoupler of oxidative phosphorylation. Several possible uncouplers are provided in the Supporting Information. The hypothesis should be based on inspection of the chemical structure of the compound in question. In general, neutral weak acids with negatively charged conjugate bases that are moderately hydrophobic and capable of delocalizing negative charge are good candidates.[13] For experimental testing, the basic flux experiment with egg-PC is conducted with an injection of a small aliquot of the test compound halfway through the run to assess if there is an observable effect on the fluorescence decay rate.

HAZARDS

Chloroform is toxic and should only be handled in a well-ventilated fume hood by individuals wearing personal protective equipment. The uncouplers nigericin, 2,4-dinitrophenol, carbonyl cyanide-*m*-chlorophenylhydrazone, tyrphostin, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone, and pentachlorophenol are also toxic and their stock solutions should be prepared by the instructor only. They need to be handled wearing personal protective equipment, including nitrile gloves. Skin contact with hydrochloric acid and potassium hydroxide can cause burns and needs to be avoided.

RESULTS AND DISCUSSION

Figure 2 shows a typical student-generated trace for a proton-permeation experiment in which the fluorescence of pyranine

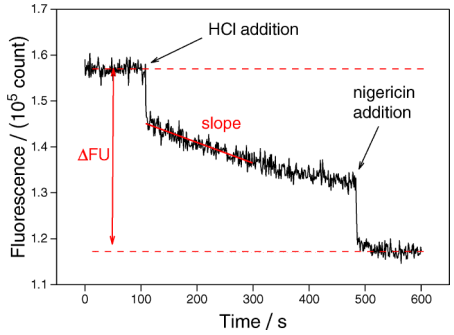

Figure 2. A student-generated fluorescence versus time trace obtained for egg-PC liposomes (λ_{ex} = 460 nm; λ_{em} = 515 nm). The red line represents the initial slope as obtained by linear regression and ΔFU is the difference in fluorescence intensity between beginning and end of the experiment.

trapped inside liposomes was monitored as a function of time. After a short period of baseline acquisition, the sample was acidified and the rate of proton entry into the liposomes was measured for several minutes. The presence of the ionophore valinomycin facilitated counter-transport of potassium ions out of the liposomes and, thus, prevented hindrance of proton movement by electrostatic effects. Toward the end of the run, the protonophore nigericin was added, which resulted in the immediate collapse of the remaining pH difference across the bilayer and, therefore, permitted the calibration of the

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fluorescence signal. The procedure and relevant equations for calculating the proton flux J and the permeability coefficient P are detailed in the student handout available in Supporting Information. Figure 3 illustrates the meaning of flux, the number of protons passing a unit area per second.

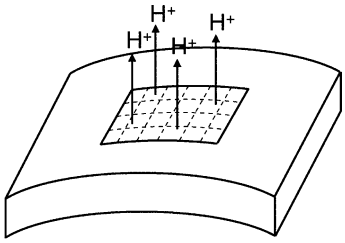

Figure 3. Illustration of proton flux (J) through a bilayer membrane. The flux is defined as the number of protons passing through an area unit in 1 s.

Typical results for the inquiry-based experiments conducted by students can be summarized as follows.

- *bilayer thickness:* As shown previously, both J and P decreased with increasing thickness of the bilayer since a thicker bilayer represents a greater diffusion barrier.[14]
- *liposome radius:* Smaller liposomes yielded faster fluorescence decays (larger slope) because their larger surface/volume ratio allowed for a quicker pH equilibration, but this effect was canceled by the smaller radius in eq 3b (see the student handout in Supporting Information) consistent with a faster dissipation of the proton concentration gradient so that both J and P remained unchanged.
- *pH-difference:* Increasing the pH-difference, the driving force of proton permeation, accelerated J but did not change P, an instrinsic property of the lipid. As evident from eq 4 (student handout in Supporting Information), the changes in numerator and denominator mutually compensated.
- *presence of a potential uncoupler:* An uncoupler accelerated the fluorescence decay or, if sufficiently potent, caused the signal to drop to its final level (as nigericin in Figure 2). In contrast, a compound incapable of acting as an uncoupler had no noticeable effects on the rate of fluorescence decay.

The described laboratory experiment has been performed over a period of three years by a total of 16 students enrolled in the upper-division general biochemistry laboratory course (CHE483L) offered by the chemistry department at Northern Kentucky University. Working in pairs of two, students typically completed the project in two 3-h sessions. The experiment was generally well received by students, particularly the inquiry-based project parts. The assessment of student performance was based on a detailed formal report. Main evaluation criteria were the quality of the generated data as judged by comparison to literature data and the proper formulation of a hypothesis, along with a feasible plan for its experimental test. Student-generated results were mostly of good quality, reproducible, and in agreement with literature data. For example, reported values for P for egg-PC liposomes are typically found at approximately 10^{-4} cm/s[7,8,14−16] and the average permeability coefficient obtained by students over a time period of two years was $(8.4 \pm 2.5) \times 10^{-5}$ cm/s. Likewise, the average student-determined P value for liposomes prepared from DMPC, a shorter lipid, was about 1 order of magnitude higher (8.8×10^{-4} cm/s), as expected. These findings suggested that the pedagogic goals were reached and that the experiment was well suited for the skill levels of students in an upper-division biochemistry laboratory course.

CONCLUSION

This experiment entailed the measurement of proton permeation rates across model membranes prepared as lipid bilayers and inquiry-based variations of experimental conditions that may have an effect on observable permeation rates. It offered the unique advantage of training students in a variety of methods, among them fluorescence spectroscopy, liposome preparation, kinetic measurements, size-exclusion chromatography, and the calculation of ion transport rates and permeability coefficients. Because it also included an advanced inquiry-based part, it was well suited for an upper-division laboratory course biochemistry.

ASSOCIATED CONTENT

Supporting Information

A student handout, including a detailed protocol for liposome preparation, permeation measurements, and data analysis, as well as several references for further reading; instructor notes containing a list of all required chemicals (with CAS numbers), necessary instrumentation, advance preparations, and general practical advice as how to conduct the experiment successfully. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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