Vesicle Stability and Dynamics: An Undergraduate Biochemistry Laboratory

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ABSTRACT: A laboratory exercise is described that helps students learn about lipid self-assembly by making vesicles under different solution conditions. Concepts covering the chemical properties of different lipids, the dynamics of lipids, and vesicle stability are explored. Further, the described protocol is easy and cheap to implement. One to two laboratory periods of 4 h each are sufficient to perform the experiments.

KEYWORDS: Second-Year Undergraduate, Upper-Division Undergraduate, Biochemistry, Laboratory Instruction, Hands-On Learning/Manipulatives, Fatty Acids, Lipids, Chromatography

The chemical and physical properties of lipids and how these factors predispose many lipids to form bilayer membrane structures are too frequently neglected in undergraduate biochemistry courses. This is unfortunate since the forces that control lipid aggregate structure and dynamics are the same as for any biological molecule, including proteins. For example, the tertiary and quaternary features of protein structure are strongly influenced by what is commonly referred to as hydrophobic forces. That is, molecular organization is often guided by arrangements that minimize the unfavorable entropic effects resulting from the exposure of hydrophobic surfaces to water. Globular proteins increase water entropy by burying their hydrophobic amino acid side chains into the center of the protein structure. Similarly, lipids avoid unfavorable water interactions by assuming arrangements that place their hydrophobic parts into the air at air–water interfaces or by burying their hydrophobic regions by aggregating into monolayer micelles or bilayer vesicles (often referred to as liposomes) (Figure 1), in addition to other more complex structures. The types and properties of lipid aggregates formed are controlled by several factors, including lipid geometry, lipid hydrophobicity, lipid headgroup features, lipid concentration, salinity, and pH.

Several lipids, including single-chain (monoacyl) fatty acids and double-chain (diacyl) phospholipids, can form vesicles with similar morphology. Nevertheless, differences in stability can often be observed. For example, vesicles composed of fatty acids rapidly disassemble at high and low pH, whereas phospholipid vesicle stability is largely unaffected by pH.¹ Lipid dynamics and lipid shape are two factors that can be used to explain this stability difference. First, the dynamics of fatty acid and phospholipid membranes are different. Lipids within membranes can rotate, flip, laterally diffuse, and escape into solution.² This latter type of escape dynamics is strongly dependent upon the overall hydrophobicity of the lipid. For example, the energetic barrier for the escape of a single-chain lipid, such as a monoacyl fatty acid, from a membrane into solution is low. Therefore, single-chain lipids can jump between membranes and easily arrange in new ways giving rise to different aggregate structures in response to changing solution conditions. In other words, single-chain lipids behave more like a system under thermodynamic control³ that constantly seeks more energetically favorable structures as the environment changes. Conversely, double-chain lipids, such as diacyl phospholipids, of similar hydrocarbon length are not able to escape into solution from a membrane environment due to the high energetic cost of exposing their large hydrophobic surface area to water. The result is that, once double-chain phospholipids are arranged into bilayer membrane vesicles, they are kinetically trapped in that state and, thus, are less responsive to solution conditions. The second reason for the observed differences in stability between single-chain and double-chain lipids can be explained by the way in which the lipids pack against each other. Lipids can be described by geometric shapes, i.e., by their critical packing parameter, with cylindrical lipids naturally packing into bilayer membranes, and cones naturally packing into monolayer micelles⁴ (Figure 1).

Note that the critical packing parameter describes molecules in aggregates and is influenced by solution conditions. Double-chain glycerophospholipids with a phosphocholine headgroup fit well into the cylinder category and, thus, favorably pack into a bilayer arrangement. Conversely, single-chain lipids are often best categorized as cones, which tend to form micelles. Sodium dodecyl sulfate is one good example of such a cone-shaped single-chain lipid (Figure S1 in the Supporting Information). Under high pH solution conditions, fatty acids, too, behave as conical lipids and consequently form micelles. However, some fatty acids begin to show cylindrical properties and form...
vesicles at pH \( \sim 8.5 \). It is thought that this shift in behavior is
due to the formation of hydrogen bonding between fatty acids.
Although carboxylic acids typically have a \( pK_a \) of \( \sim 3 \), the \( pK_a \)
of the carboxylate headgroup of fatty acids is much higher in the
closely packed environment of a monolayer, which for oleic acid
is 8.5.\(^5\) Therefore, as the pH approaches the carboxylate
\( pK_a \) of the fatty acid, hydrogen-bonding links between the fatty
acids form,\(^6\) thereby generating lipid units that can be
represented as cylinders (Figure 1). In addition to these
extreme examples, lipid shape and, in particular, headgroup size
can be used to understand the lipid distribution between outer
and inner leaflets of a spherical bilayer membrane.

Several past laboratory experiences described in this Journal
and others are geared toward exposing students to vesicle
synthesis with double-chain phospholipids, purification by
chromatography,\(^7,8\) and the measurement of permeability.\(^9,10\)
The experiments described herein additionally expose students
to the influences of lipid dynamics on vesicle stability and on
the ability of single-chain lipids to form bilayer membranes.
The described protocol is easy and cheap to implement. One to
two laboratory periods of 4 h each is sufficient to perform the
experiments.

\section*{EXPERIMENTAL SECTION}

Students work in small groups of three or four. Students subject
lipid solutions to different pH conditions and observe the
effects of pH on vesicle formation by chromatography. More
specifically, students (1) use two different types of lipids and
two different vesicle generation procedures performed at
different pHs in the presence of a fluorescent dye, (2)
subsequently run the lipid solutions through a size-exclusion
column, (3) collect and analyze the fluorescence of the eluted
fractions, and (4) compare the results from each of the
conditions tested.

The fatty acid and phospholipids used in this protocol are
oleic acid and lecithin, respectively. A small impermeable
fluorescent molecule (8-hydroxypyrene-1,3,6-trisulfonic acid or
HPTS) is encapsulated within oleic acid and lecithin vesicles
simply by adding the fluorophore during the vesicle generation
procedure. The solution is briefly sonicated to break down
larger vesicles into smaller vesicles and to improve sample
homogeneity. The resulting, more homogenized solution is
then incubated with tumbling for 20 min. Mechanical agitation
for both oleic acid and lecithin vesicle formation is necessary,
since an input of energy into the system is required to assemble
the lipids properly. In other words, vesicle formation is not
truly spontaneous. A detailed description of vesicle preparation
is provided in the Supporting Information.

\section*{HAZARDS}

Students should follow standard laboratory safety rules. The
chloroform stock solution of lecithin should be prepared by the
instructor and kept under a chemical hood. Chloroform can
cause irritation to eyes and could have carcinogenic effects.
HPTS and oleic acid are eye and skin irritants. Also, the rotary
evaporator should be used with the supervision of the
instructor. Students should always wear safety glasses and
gloves.

\section*{RESULTS}

Oleic acid and lecithin vesicles formed if the solution
conditions were conducive to vesicle formation. Vesicle
formation was easily observable by the appearance of turbidity.
Since larger particles scatter more light than smaller particles,
large vesicles scatter much more light than micelles or
monomeric, free lipid. Similarly, the turbidity of the vesicle
solution should decrease after sonication. It is also important to
note that the samples should not be agitated until all of the
required components are added. In this way, premature vesicle
formation that does not contain within its interior all of the
desired molecular components is avoided.

To demonstrate if vesicles were produced or not, the sample
was run through a size-exclusion column. If vesicles were
present, two colored bands could easily be observed by eye.
The band that eluted first represented vesicles with entrapped
HPTS fluorophore. Since free fluorophore is much smaller than
vesicles, free, unencapsulated fluorophore molecules eluted as
the second band. If stable vesicles were absent, then only one
band was observed. Vesicle formation was quantified by
fluorescence spectroscopy. Students then plotted their data
(fluorescence intensity versus fraction number) to compare the
eletion profiles of different vesicle preparations (Figure 2).

Note that the single elution peak of the pH 10 sample was
not identical to the elution of free HPTS. This may reflect ionic
interactions with the lipid headgroup, entrapment within
nonvesicular lipid aggregates, or even entrapment within
vesicles that were not stable over the duration of the
chromatographic separation. Analogous samples that were not
sonicated did not show the presence of vesicles at pH 10 by

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Fatty acids and phospholipid aggregate structures. \textit{A} fatty
acid molecule, such as oleic acid (panel \textit{A}), can be geometrically
represented by a cone and packs well into a spherical micelle (panel
\textit{D}). Two hydrogen-bonded fatty acid molecules (panel \textit{B}) and one
molecule of the diacyl phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-
phosphocholine, a major component of egg yolk lecithin (panel \textit{C}),
each can be viewed as a cylindrically shaped structure that can pack
into a bilayer membrane of a vesicle (panel \textit{E}). Cones and cylinders
are represented here as two-dimensional triangles and rectangles,
respectively.}
\end{figure}
The experiment can be used with second-year and advanced undergraduate students. The protocol was used in four different years of a biochemistry class (spring semester of 2010, 2011, 2012, and 2013). Each class was composed of approximately 60 students that were divided into small groups (2–3 students per group). Within the same class period some groups worked with fatty acids and other groups worked with lecithin at one defined pH (e.g., pH 8.5). The following day the groups switched and used vesicle generation procedures with different lipids and at a different pH (e.g., pH 10). In this way, each student group had an opportunity to work with fatty acids and lecithin. However, this protocol is highly flexible and easily modifiable. For example, within the same lab period, some groups could also make vesicles starting from fatty acids using different buffered solutions (pH 8.5 and 10), while other groups work with lecithin vesicles (pH 8.5 and 10). To facilitate different laboratory arrangements, different versions of the protocol are provided in the Supporting Information.

No particular problems were encountered during this laboratory experience. An example of the data obtained by the students is shown in Figure 2. Puriﬁed vesicles with entrapped HPTS eluted between fractions 4 and 7, while the free fluorophore eluted after fraction 10. Small changes in the elution proﬁle were observed between different runs and different columns, due in part to small differences in resin packing, column volume, and the skill of students in loading samples onto the column. Much of this variability can be reduced by demonstrating proper chromatographic techniques at the beginning of the laboratory session. For both types of vesicles, i.e., for oleic acid and lecithin vesicles, two peaks were observed at pH 8.5. At pH 10, two peaks were observed for the lecithin sample and only one broad peak was detected for the oleic acid sample. Since oleic acid vesicle stability is greatly diminished at pH 10, a sharp vesicle peak was not observed. Plotting the data gave students an additional visual means of grasping the inﬂuences of pH on vesicle integrity as a function of lipid composition.

**CONCLUSIONS**

This laboratory experience was useful in dispelling several misconceptions of students. First, biochemistry is more than just the study of proteins and nucleic acids. Unfortunately, lipids are a neglected biomolecule, and this laboratory experience gave students a hands-on interaction with lipids. Also, lipids are governed by the same chemical–physical forces as any molecule. As this laboratory showed, hydrophobicity is a powerful organizing force. Similarly, the assembly of vesicles was easy and not nearly as complex as students expected. By the end of the semester students were able to answer questions pertaining to lipid dynamics, geometry, vesicle stability, and molecular encapsulation. For example, 80% of the students were able to propose methods for encapsulating pharmaceuticals into vesicles for drug delivery on a final exam.

**REFERENCES**