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On the Long and Winding Road to a Perfect Membrane Model

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Molecular interactions at and within cellular membranes are at the heart of many biological processes. Studying these interactions in their native environment is often challenging because of the inherent complexity of the cellular milieu. Model systems can fill a crucial niche by simplifying the interactome to a few key components, enabling elucidation of the functional contribution of different factors by systematically varying parameters of interest.

Designing an appropriate model requires finding the right balance between simplicity and biological relevance. To study membrane biology, researchers have relied on various approaches to recapitulate key features of cell membranes (Fig. 1). Some used liposomes prepared from total lipid cell extracts to study their interactions with proteins. Liposomes made of synthetic lipids have also been widely used as simpler alternatives to the native membrane protein environment, allowing for better control of membrane components. However, liposomes prepared by traditional methods lack a critical feature of the limiting cellular membrane (i.e., the plasma membrane, or PM), namely the asymmetric distribution of most lipid species between the two membrane leaflets. Life has evolved a sophisticated and carefully orchestrated protein machinery to control this lipid asymmetry and utilize it for various cellular functions. Moreover, this asymmetric transverse organization of lipid components has its own biophysical signature. For example, asymmetry uniquely affects the lipid packing of the two leaflets (1,2), the lateral pressure distribution across the bilayer (3,4), and the membrane electrostatic potential (5). Membrane asymmetry has thus emerged as an important parameter in model studies, especially those focusing on the structure of the PM and its interactions with proteins. However, because of technical hurdles, very few labs have investigated the effects of asymmetry on protein function.

In this issue of *Biophysical Journal*, Markones et al. open new doors in this line of research by describing a general protocol for the preparation of asymmetric extruded liposomes with a reconstituted large multipass transmembrane protein (6). In their study, titled "Stairway to Asymmetry: Five Steps to Lipid-Asymmetric Proteoliposomes," the authors build on previous work on the preparation of lipid-only asymmetric vesicles (7) by incorporating the functional integral membrane protein NhaA (a bacterial Na^+/H^+ antiporter) and demonstrating a long-lasting stable membrane asymmetry. This study is notable because it outlines, discusses, and follows general principles for the design and preparation of a model asymmetric proteoliposome system:

1) Although it is generally accepted that an appropriate model bilayer would mimic key components of the native membrane composition, a less appreciated but critically important consideration is the range of workable conditions set by practical constraints. Some lipid compositions may not form stable liposomes in vitro or may exist in a nonfluid state at the temperature of preparation, assay, or storage, rendering them unsuitable and requiring some tradeoffs. Thus, Markones and colleagues emphasize the importance of a model design that meets both scientific and technical criteria. The well-documented information about lipid physical properties (8), the increasing availability of detailed lipidomics data (9), and the growing palette of techniques for preparing asymmetric liposomes (7,10–13) can all guide the search for the most appropriate asymmetric proteoliposome system.
2) Although making asymmetric liposomes may involve multiple steps, most of them start from symmetric liposomes in which the protein of interest is reconstituted. As discussed in the article, complete removal of the solubilizing

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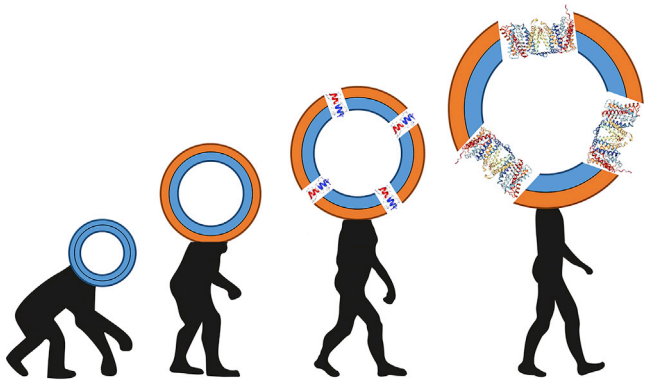

FIGURE 1 Evolution of cell membrane models. To see this figure in color, go online.

detergents used to stabilize large transmembrane proteins becomes essential for the generation of stable asymmetry. Moreover, considerations of the nonuniform protein orientation in the membrane must be taken into account in any measurements. Alternatively, special care must be taken to ensure single sidedness of the embedded proteins. Interestingly, a study cited by the authors shows that some proteins may change their topology in the presence of membrane asymmetry (14), although this may happen at the cost of increased rates of lipid flip-flop.

3) Once the protein is reconstituted, generation of leaflet asymmetry involves replacing some or all of the outer leaflet lipids with those of the desired lipid composition and then validating the composition and asymmetric distribution of the lipid components. Because no single general method exists for the preparation and assay of an asymmetric system with arbitrary lipid composition, in addition to presenting their own approach, Markones et al. provide references to alternative protocols that can be used according to the desired model system design.
4) After the asymmetry of the model membrane is established and verified, protein functionality must be assayed. For antiporter proteins such as NhaA, activity can be measured with a membrane potential sensitive dye. For ion channels like gramicidin, fluorescence-based assays can be applied (15). Although not directly discussed in the article, it is important to note that if asymmetry is hypothesized to negatively affect protein function, a negative control must also be used (e.g., replacing the outer leaflet lipids of the symmetric liposome with an identical lipid composition or scrambling the asymmetric membrane to regain function) to rule out any undesired artifacts of the vesicle preparation.

Markones et al. beautifully illustrate the above principles by preparing asymmetric liposome models of the native NhaA environment, namely the inner bilayer of *Salmonella typhimurium*. The model liposomes contain a mixture of phosphatidylethanolamine, phosphatidylcholine, and cardiolipin in both leaflets, and phosphatidylglycerol confined to the outer leaflet. Asymmetry is assayed by means of ζ potential, with calibration curves allowing determination of the abundance and distribution of phosphatidylglycerol in the liposomes. This particular approach is specific for membranes with an asymmetric distribution of a single type of charged lipid but provides the ability to perform robust quantification on the degree of asymmetry in the proteoliposomes. Special controls are performed to ensure complete removal of the detergent while both asymmetry and protein function are monitored over time. The study, whose design and execution are explained in detail and whose results are very clear, illustrates the larger set of considerations (relative to symmetric membranes) that need to be taken into account when working with asymmetric proteoliposomes.

One assay of particular interest is the time evolution of the extent of asymmetry. Many transmembrane proteins have been hypothesized to act as nonspecific scramblases, accelerating lipid translocation between leaflets by various mechanisms. Thus, protein-rich asymmetric liposomes may be expected to gradually lose their asymmetry, as has been shown for the ion channel gramicidin (15). The changes in leaflet compositions would change bilayer properties, which could, in turn, affect the conformation and function of the resident proteins. Measuring the kinetics of potential scrambling activity would inform on the time window available for experiments that rely on stable leaflet compositions.

The apparent loss of asymmetry can also be affected by additional factors not related to the protein. For example, the presence of any residual detergent from protein reconstitution could speed up lipid flip-flop and requires a separate control of a protein-free asymmetric liposome prepared with the same detergent protocol as the proteoliposome. The authors also raise an interesting point about "structural lipids" that stabilize the conformation and function of membrane proteins in their native membrane environment. If such structural lipids are absent in the model system, this could affect protein reconstitution and interaction with the asymmetric bilayer, including the apparent acceleration of lipid flip-flop. Performing all necessary controls and ensuring the lack of any artificial contributions to transverse lipid movement is therefore paramount to the accurate determination of a protein's ability to scramble lipids.

It is important to note that most measurements of the degree of lipid asymmetry, including the one used by Markones et al, report on the distribution of only one of the lipid components in the bilayer. Introducing asymmetry in the distribution of one of the bilayer lipids naturally results in the asymmetric distribution of other lipids, which have to make up for the generated area difference between the leaflets. If the bilayer is composed of only two types of lipids, the leaflet compositions are well defined. However, in the presence of multiple lipid species, different assays will have to be performed to quantify the leaflet-specific abundance of each membrane component and monitor its (potentially different) translocation kinetics.

The ability to prepare robust models of asymmetric proteoliposomes provides new opportunities for studying membrane protein function, including their interaction with lipids, small molecules, and other proteins, in a more physiologically relevant context. Tools like those developed by Markones et al. bring us closer to answering one of the major remaining open questions in membrane biophysics, namely, the functional significance of the ubiquitous but energetically expensive membrane asymmetry of the PM. Markones et al. remind us that even though a perfect model does not exist, great progress can be made by choosing the most appropriate one.

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