Electron Microscopy Analysis of Multilamellar Vesicles Prepared from Synthetic Lipids: A Model System for Studying Membrane Structure in the Molecular Cell Biology Classroom and Laboratory

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Introduction

Why study cell membranes?

The cell is the fundamental unit of all living organisms, ranging from the unicellular *archaea* and bacteria (*prokaryotes*) to higher multicellular plants and animals (*eukaryotes*). All cells are bounded by a complex and dynamic plasma membrane, which functions principally to maintain cellular and organismal steady state by performing complex energy transformations and regulating the flow of information for the cell. The cell membrane also performs a number of vital housekeeping functions, which include control of the transport of substances between extracellular and intracellular environments, participation in cell signaling cascades by hosting receptors of extracellular ligands, and facilitating critical cell-to-cell communications in multicellular organisms (Karp, 2005).

Considerable research over the last fifty years has significantly increased our understanding of cell membranes and their structural organization. Every membrane is fundamentally comprised of a dynamic lipid bilayer that supports a variety of transmembrane and membrane-associated proteins. The specificity of cell function is in part attributed to variations in the composition and molecular ratios of lipids and proteins in the membrane bilayer as well as surface carbohydrates associated with these components (Alberts, 2004).

Since membranes play such an important role in the life of a cell, it is important to discuss the structure/function aspects of membranes early on in our molecular cell biology course. Concomitantly, we require "hands-on exposure" to the operation of the transmission electron microscope (TEM) in the laboratory. Students are reminded that the TEM has played a critical role in our understanding of cell membrane structure and function.

Where does one go to find a sample for introductory TEM membrane analysis? Conventional processing, sectioning and mounting of kidney, liver, intestine, red blood cells, *etc.*, for analysis of membranes in the TEM, is time consuming and quite challenging to students as a first exposure to membrane study. However, we have discovered an easy, rapid, and reliable method of artificial membrane preparation that captures student interest and enables the class to gain an appreciation of membrane structure. This method involves the preparation of multilamellar vesicles (MLVs or liposomes) (Chatterjee and Agarwal, 1988) composed of a purified phospholipid (with cholesterol), addition of phosphotungstic acid as a negative stain (Perrett *et.al.*, 1991), and application of the stained MLV suspension to formvar-carbon coated copper grids for TEM analysis (Bangham and Horne, 1964).

The entire protocol can be completed in a three hour lab period enabling the students to efficiently prepare and analyze the concentric bilayer membrane profiles of MLVs. With this successful and exciting first exposure to membranes, it becomes an easy task to introduce the students to more complex methods of sample preparation to study membrane structure and other cellular structures of selected tissue types. In this report, we describe the methods and results of a feasibility study using MLVs as a model system for examining membrane bilayer structure.

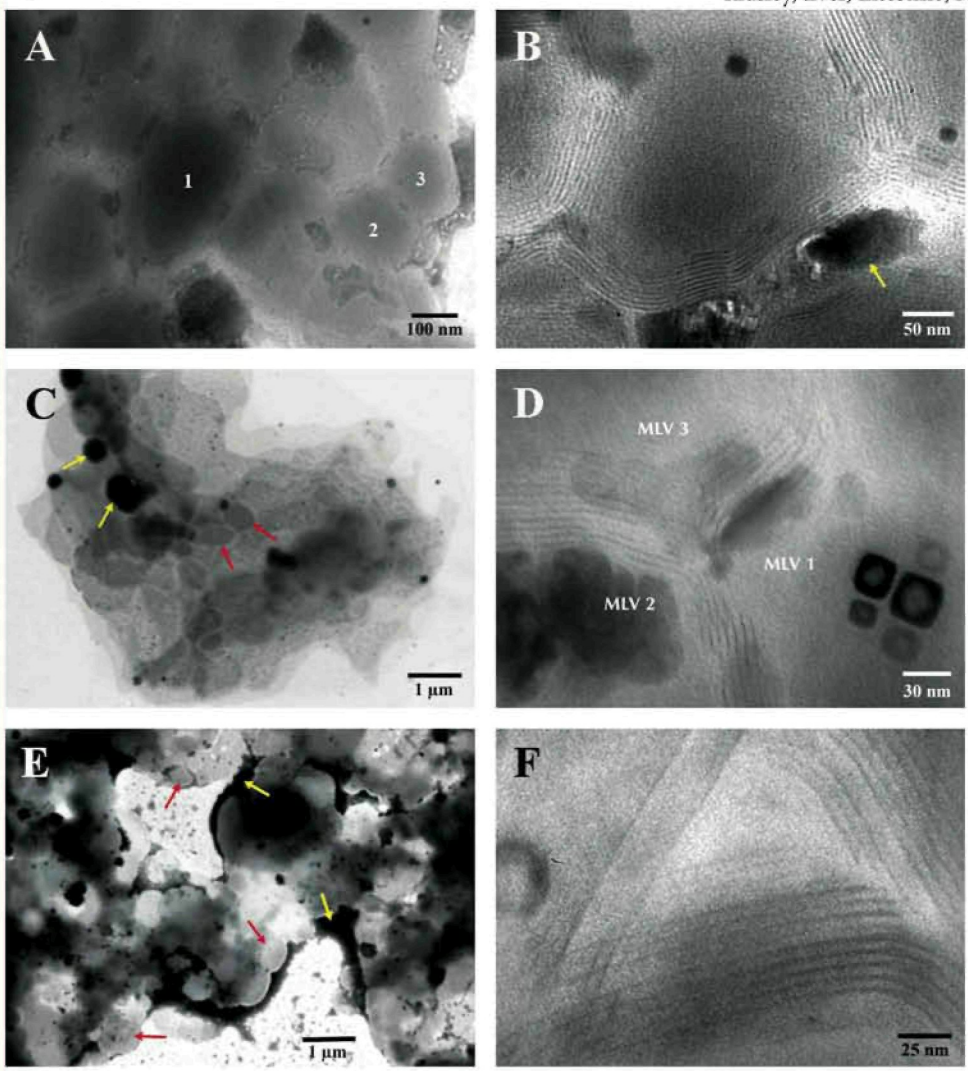

Figure 1. Comparison of three types of MLVs shown at low (A,C,E) and high magnification (B,D,F). MLVs within each type vary in size and shape and show peripheral concentric membrane profiles. Red arrows identify specific MLVs. Yellow arrows identify PTA deposits. A) DLPC MLV cluster. Select MLVs are numbered. B) DLPC MLV membrane profiles. PTA deposit shown at right. C) Egg PC MLV cluster. D) Egg PC membrane profiles of three MLVs. Salt crystals shown at right. E) SPM MLV cluster. PTA shows strong affinity for surface of MLVs. F) SPM MLV concentric membrane profiles.

Preparation, Staining and Mounting of MLVs for TEM Analysis...

Materials

Several purified phospholipids were used to prepare MLVs in this study: 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC); egg PC (ovalecithin or OVPC), and porcine brain sphingomyelin (SPM). The above lipids were obtained from Avanti Polar Lipids (Alabaster, AL) solubilized in chloroform at concentrations of 10 mg/ml (Egg PC and SPM), and 20 mg/ml (DLPC). Cholesterol was also obtained

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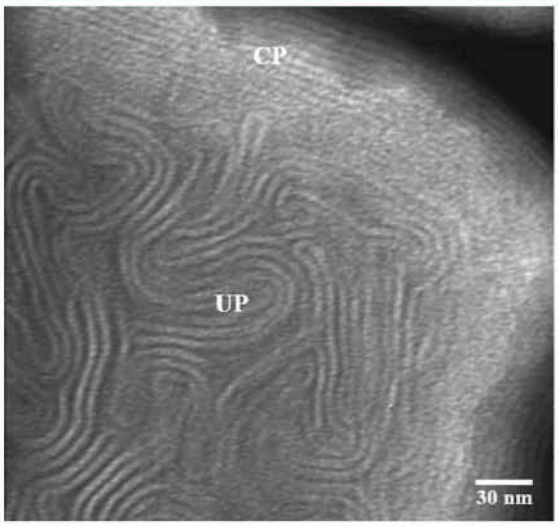

Figure 2. Egg PC MLV. Internal undulating membrane profiles (UP) contrast with uniform concentric peripheral membrane profiles (CP).

from Avanti Polar Lipids and was solubilized in chloroform at 10 mg/ml. MLVs were prepared in aqueous buffer (diffraction buffer) containing 0.5 mM HEPES and 150 mM NaCl at pH 7.3. Samples were stained with 2% phosphotungstic acid (PTA) prepared from PTA crystals (Electron Microscopy Sciences, Fort Washington, PA) in deionized water at pH 7.3. Formvar, carbon-coated, 300 mesh copper grids (Electron Microscopy Sciences) were used to whole-mount MLV suspensions.

Methods

Preparation of DLPC, egg PC, and SPM MLVs MLVs were prepared by first combining 0.75 mg of purified DLPC, Egg PC or SPM (from stock solutions) together with aliquots of cholesterol stock to achieve a 0.6:1 cholesterol-to-phospholipid mole ratio (xx µl, xx µl, and xx µl of 10 mg/ml stock for DLPC, Egg PC and SPM, respectively) in separate 13 × 100 mm glass test tubes.

Each sample was dried under a steady stream of nitrogen gas while vortexing to evaporate chloroform solvent; nitrogen gas was used to inhibit oxidation of sample lipids. This drying approach results in the deposition of a thin film of lipid on the lower inside walls of the test tube. Each tube was wrapped in aluminum foil (to shield from excessive exposure to light) with the open end left uncovered and dried in a desiccator chamber for 1 hr. After drying, lipid samples were resuspended in 300 µl of diffraction buffer and vortexed for 3 min to create a suspension of MLVs. 100 µl of MLV suspension was transferred to a 1.5 ml microfuge tube to which was added 100 µl of 2% phosphotungstic acid. The PTA was mixed with the MLV suspension by gentle aspiration. A 10 µl aliquot of this sample was applied to a formvar, carbon-coated 300 mesh copper grid previously placed on a parafilm strip and anchored to the edge of a strip of Scotch tape applied through the center of the parafilm strip. The MLV suspension was incubated on the formvar grid for 1 hr. The sample was maintained in a humid environment by placing alongside a damp paper towel and covering with a petri dish. After incubation the residual MLV suspension was wicked away by touching a small wedge of filter paper to the edge of the grid. The sample grid was placed in a TEM pre-pump vacuum film chamber until microscopic examination.

Electron Microscopy Technique

MLV samples were examined using a JEOL 100S TEM equipped with an AMT digital imaging system. Samples were examined at 80 KV acceleration voltage and images were captured between 10,000 and 500,000 × magnification.

Determination of MLV membrane bilayer widths

25 transects were acquired for a specific MLV sample. Each transect was performed in the peripheral region of an MLV where concentric bilayer membranes were easily observed and of consistent width. A transect extended at right angles to the membrane from the middle of one dark line (a dark line represents the phosphate head groups of two opposing membranes with an intervening water space) to the middle of the fourth nearest dark line. The four internal white lines captured by a transect represent the opposing hydrophobic acyl chains of the bilayer phospholipid molecules. The average length and standard deviation of the 25 transects was determined and this value was divided by four to determine the average width of one bilayer membrane. When possible, all measurements for a specific sample were made on MLVs in one grid hole to assure a greater uniformity of membrane width measurements.

Protocols for Testing MLV Sample Stability

To determine the stability of MLV membranes during extended drying of negatively stained sample in vacuum, egg PC MLVs were stained, mounted and examined at 3, 16, 26 and 38 hr for changes in membrane structure.

To determine the stability of MLV membranes during extended incubation at 4°C prior to negative staining, an egg PC MLV suspension was stored at 4°C for 72 hr followed by staining, mounting and examination in the TEM.

The structural effects of washing the MLV membranes to remove preparative diffraction buffer were examined as follows. An unstained 150 µl aliquot of a specific MLV suspension was placed in a 1.5ml microfuge tube and centrifuged for 5 min at 7000 rpm to pellet the MLVs. The supernatant (diffraction buffer) was removed without disturbing the pellet; 300 µl of water was added and the pellet was resuspended by gentle aspiration. The MLV sample was centrifuged and washed two more times, re-suspended in 150 µl dH_2O, stained, mounted and examined by TEM as previously described.

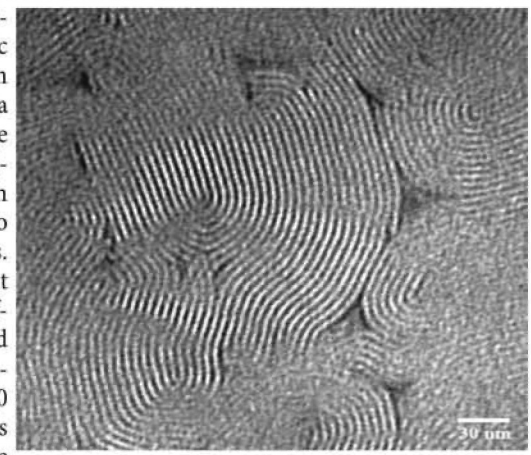

Figure 3. Egg PC fingerprint-like membrane profiles from a water-washed MLV sample.

Results and Discussion

Artificial MLV membranes of DLPC, egg PC and SPM were prepared by a rapid and simple protocol. Under low magnification (10,000 ~100,000 ×), clusters of MLVs were observed (Fig. 1A, C, E). Negatively stained concentric membrane bilayer stacks of each MLV type were easily observed at higher TEM magnification (100,000 ~ 500,000 ×; Fig. 1B, D, F). These concentric lamellar profiles were observed at the periphery of each MLV type where the membranes were thin and flattened but were usually not seen in the thicker interior region (Bangham and Horne, 1964; Olson *et.al.*, 1979). In order to determine the nature of the MLV interior, we processed MLVs by a conventional method of 1% OsO_4 fixation, dehydration, and embedding in Spurr's resin, followed by thin-sectioning, treatment with uranyl acetate and lead citrate staining. The MLV interior may contain additional concentric membranes toward the center or may contain vacuous spaces of varying width. Freeze fracture studies of liposomes confirm our structural observations of whole mount negatively-stained and conventionally processed MLVs (Zasadzinski, 1986; Handjani-Vila *et.al*, 1993).

However, undulating lamellar profiles were seen occasionally in the interior of egg PC MLVs (Fig. 2) and resembled profiles reported by Bangham and Horn (1964) in MLVs containing egg PC and cholesterol.

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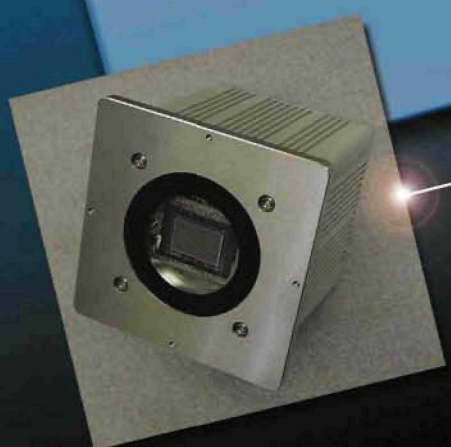

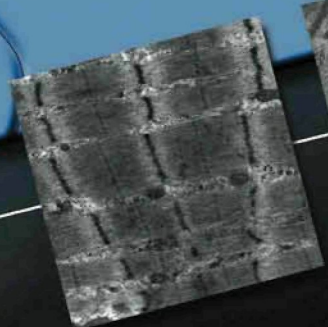

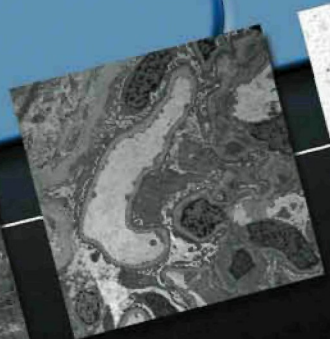

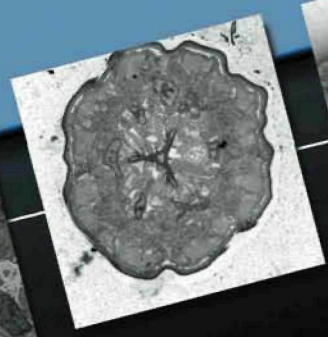

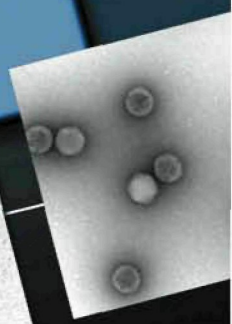

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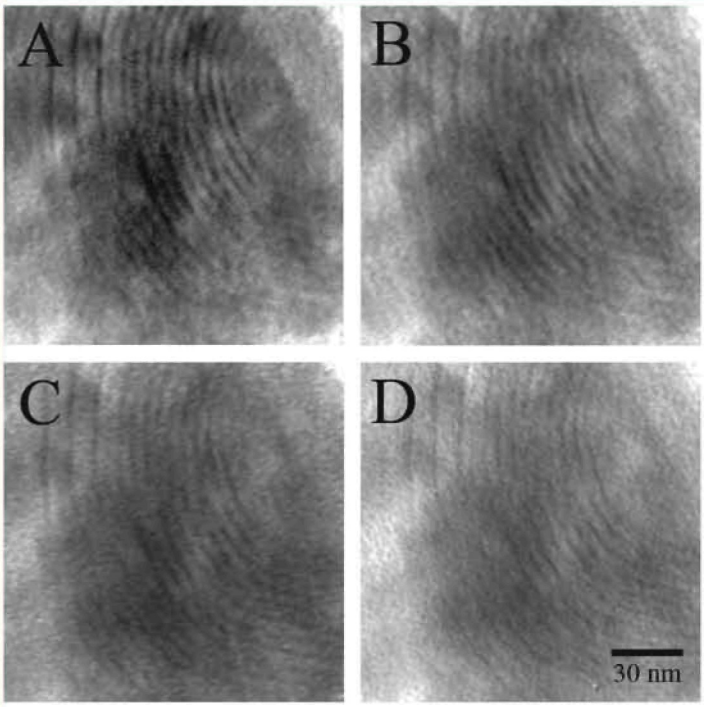

Figure 4. Egg PC MLV section imaged at A) 3 hr B) 16 hr C) 26 hr, and D) 38 hr. Stacked MLV lamellar structures remained unchanged throughout the incubation period. Sample remained in the microscope column during the sequential imaging period.

Bangham and Horn interpreted these structures as elongated rod-like micelles, not lipid bilayers. Thus, conclusive structural characterization of the undulating profiles remains uncertain.

Dramatic "fingerprint-like" lamellar profiles were occasionally seen in water-washed egg PC MLV samples (Fig. 3). Similar fingerprint-like profiles were reported by Chapman *et.al.* (1968) in MLV samples prepared from egg PC. Bangham and Horne (1964) also reported similar profiles in MLV samples prepared from a mixture of egg PC and progesterone.

Membrane bilayer width of each MLV type was measured from captured images. The average SPM MLV membrane width (60 Å) was greater than DLPC (54 Å) and egg PC (54 Å). SPM's wider membrane width is correlated with its longer carbon chains. SPM has a heterogeneous array of acyl chains, each containing 18 to 24 carbons. In contrast, DLPC acyl chains have 18 carbons; egg PC has a heterogeneous mixture of acyl chains containing 16 and 18 carbons and a minor acyl chain species of 20 carbons.

The membrane width of egg PC reported by Bangham and Horne (1964) was 44 Å. Since our method of measurement included a portion of the water space, it was not surprising that our bilayer width (54Å) was slightly greater than Bangham's reported width. An attempt to compare membrane widths reported in the literature is imprecise since various factors can influence membrane width, such as ambient humidity (Jacob, 1999).

Egg PC MLV membrane structure is remarkably stable under various conditions such as extended incubation at 4°C in diffraction buffer prior to staining. This observation supports the assumption that liposomes maintained at 4°C are physically stable for months (Zimmer, 1999; Mozafari, 2002). MLVs also remained stable over a 38 hr incubation period, during which a negatively stained sample was kept in the TEM column for timed imaging at selected intervals (Fig. 4). A second stained sample, stored for 30 days in the TEM pre-pump chamber under vacuum, also showed excellent membrane detail. Similar results were observed following removal of diffraction buffer by washing with deionized water.

In contrast to the high stability of egg PC and SPM MLV membranes, DLPC MLV membrane integrity was clearly disrupted, especially by water washing to remove the MLV buffer (Fig. 5). This was expected since its 18 carbon acyl chains are di-unsaturated and subject to oxidation at the reactive double bonds. Although DLPC yielded excellent MLVs, samples had to be quickly mounted and fixed in order to minimize oxidation artifact before observation in the TEM.

Conclusion

This study reports a rapid, simple and reproducible method to prepare and observe MLV membrane structure in the TEM. MLV samples composed of phospholipids with saturated acyl chains exhibited long-term stability whereas MLV samples containing phospholipids with unsaturated acyl chains were less stable. The artificial membrane preparation protocol presented in this paper is easily incorporated into a cell biology classroom/laboratory module involving a study of the relationship of membrane structure to function. ■

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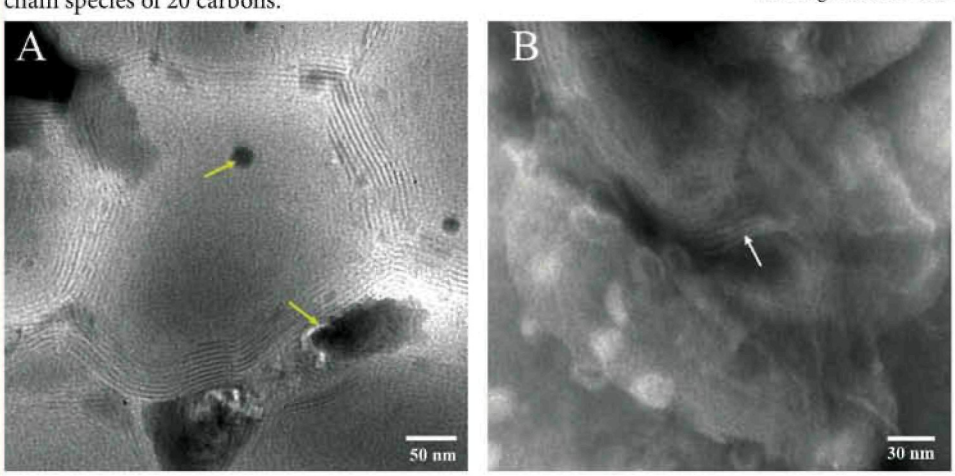

Figure 5. A) DLPC MLV cluster showing peripheral bilayer membrane profiles. Deposits of PTA are present (arrows). B) Water washed, distorted and folded DLPC MLV cluster showing scattered diffuse bilayer membrane profile (arrow).

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