CHAPTER

RESOLVING THE THICKNESS AND MICROMECHANICAL PROPERTIES OF LIPID BILAYERS AND VESICLES USING AFM

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PART I INTRODUCTION

Lipid bilayers and vesicles serve as models for biomembranes and cells (1) and show increasing applications in medical and non-medical fields (2,3). A number of methods have been established to make 2-D lipid bilayers (4): 1) supported lipid bilayers by vesicle fusion or Langmuir-Blodgett (LB) dip coating, 2) tethered bilayer membranes by hydrophilic linkers or biotinylated receptors, 3) polymer-supported lipid layers, 4) micro-arrayed lipid layers, and 5) black lipid membranes spanning the holes of porous supports. Small unilamellar vesicles can be prepared from multilamellar vesicle dispersions either by sonication (5) or extrusion (6). The study of structure, morphology, and stability of lipid bilayers and vesicles is important to the understanding of membrane fusion as well as in drug delivery, gene therapy, and biosensor design.

AFM images a surface by scanning a sharp tip attached to a cantilever at a close distance to the surface (7). AFM is one of the newest and most important tools for biomembrane analysis because it provides unrivaled molecular-level understanding of structure, stability, and layer interactions. AFM provides surface topographical images with spatial resolution close to 1 Å and force-versus-distance curves with detection limit close to $10^{-12}$ N. AFM has become the preferred method for imaging soft materials such as molecular crystals, proteins, and living cells (8). The ideal way to image lipid layers and biomembranes is to conduct scanning in the natural solution environment of the constituents. Minimizing image force to below a threshold breakthrough force further ensures minimum disturbance of the surface structure. Force measurement has become an indispensable part of AFM analysis of biomembranes and biomaterials. AFM force curves provide information on the structure and surface charges, stability, and surface interactions of lipid layers in addition to the bilayer thickness. AFM imaging and force measurement are combined to provide a micromechanical map of the biomembrane or cell surface in force mapping or force-volume imaging (9).
This chapter describes various experimental methods used to determine the structure of lipid layers, specifically the bilayer thickness and elastic constants using EggPC as an example.

PART II SUPPORTED LIPID BILAYER (SLB) THICKNESS

The structure and stability of SLBs with or without biological or synthetic additives have been studied extensively by AFM (10). The ideal way to image fluid-like lipid bilayers with the least disturbance is the soft contact or tapping imaging mode, which uses the standard silicon nitride (Si$_3$N$_4$) tips in the natural solution environment. After a solid substrate, such as mica, graphite, glass, silicon wafer with or without surface modification, is brought to a close distance to the AFM tip, the lipid vesicle solution is injected directly into an AFM liquid cell through inlet tubing. The liquid cell is often sealed by an o-ring. Sometimes an open-cell configuration is preferred. SLBs are often formed in situ as a result of vesicle rupture and fusion after sufficient incubation time. Excess vesicles can be removed by flushing the liquid cell with buffer solutions. A different pH or salt solution can be introduced during measurement by solution exchange through the inlet/outlet tubing.

Bilayer thickness can be determined by the step height at the bilayer domain edges. An example of bilayer thickness determination is given in Figure 1 of egg yolk phosphatidylcholine or EggPC (11). Multilamellar EggPC vesicle solution is extruded through a polycarbonate membrane with an average pore size of 200 nm using a LiposoFast extruder from Avestin. The extrusion method produces larger vesicles that rupture into bilayers upon adsorption to produce the EggPC SLB. AFM imaging and force measurement are conducted using a Nanoscope IIIa AFM (Digital Instruments) and
an E scanner (maximum scan area = 16 × 16 μm²). AFM sectional height analysis of the
SLB step edges in water yields a value of 6.27 ± 0.57 nm. The bilayer film thickness
agrees with the hydrated bilayer thickness measured by x-ray diffraction (12). The value
is larger than the headgroup-to-headgroup bilayer distance (~ 4 nm) due to hydration of
the headgroup. Sackmann points out that freely supported lipid-protein bilayers are
separated from the substrate by a 1-nm thick water layer (13). 1H-NMR estimates that
the average thickness of the water layer between the single bilayer and the glass bead
surface is 1.7 ± 0.5 nm (14).

Figure 1. The AFM amplitude image and sectional height analysis of the corresponding
height image of EggPC SLB patches on mica in water. The thickness of the EggPC bilayer
patches is 6.27 ± 0.57 nm, which agrees with 6.58 ± 0.37 nm from force curve analysis.

In addition to 3D topographical imaging, AFM enables direct force measurements
between the tip and surface by moving an AFM tip up and down at one point on the
sample surface. The force-versus-distance curves, or in short force curves, yield not only
bilayer thickness but also its micromechanical properties. It has been shown that film
thickness from force curves matches precisely the value from sectional height analysis of
bilayer step edges if similar image force is used in both cases. AFM force calibration
plots are converted to force curves by defining the point of zero force and the point of
zero separation (15). Zero force is determined by identifying the region at a large
separation, where the deflection is constant. Zero separation is determined from the constant compliance region at high force where deflection is linear with the expansion of the piezoelectric crystal or by the end of the jump-in process. Discontinuity, called the jump-in point, has been a typical feature in force curves measured on lipid bilayers and adsorbed surfactant films above a threshold force (16). This threshold force has been used as the upper limit of the image force in the soft-contact AFM imaging mode most useful for organic, polymeric, and biological samples (17). The jump-in process during tip approach is due to spring instability. The process corresponds to the rupture and removal of the bilayer portion from the tip/substrate gap, most probably by a lateral push-out mechanism. The threshold force (or maximum steric barrier) is reported to be proportional to the surface excess of the film, and can be used to compare packing density within similar adsorption class (18).

Figure 2 is a representative force curve measured on the EggPC SLB. Only force values obtained with the same AFM tip are compared. The radius of the contact tip (= 33.2 ± 6.6 nm) is calibrated by imaging the TGT01 gratings (Mikro-Masch) (19). The
spring constant of the cantilever is calibrated using the deflection method against a reference cantilever (Park Scientific Instruments) of known spring constant (0.157 N/m) (20). The calibrated spring constant (0.17 ± 0.05 N/m) is used. Force curves are obtained in liquid contact mode only. Multiple force curves are obtained on the same SLB. In Figure 2, the repulsion starts at 6.58 ± 0.37 nm and the jump-in occurs at 4.57 ± 0.27 nm with a maximum force = 1.6 ± 0.3 nN. The repulsion up to the jump-in point can be described as a combination of hydration, steric force, and mechanical deformation for bilayer films. The force curves measured on the lower, flat background in both extruded and sonicated vesicle adsorption cases show characteristics of tip/mica interactions in water. It can be concluded that mica is not fully covered by the SLB in this case.

<table>
<thead>
<tr>
<th>Pluronic®</th>
<th>Composition</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>L81</td>
<td>(PEO)<em>{20}(PPO)</em>{40}(PEO)_{20}</td>
<td>~2866</td>
</tr>
<tr>
<td>L121</td>
<td>(PEO)<em>{10}(PPO)</em>{60}(PEO)_{10}</td>
<td>~4000</td>
</tr>
<tr>
<td>P85</td>
<td>(PEO)<em>{26}(PPO)</em>{39.5}(PEO)_{26}</td>
<td>4600</td>
</tr>
<tr>
<td>F87</td>
<td>(PEO)<em>{61.1}(PPO)</em>{39.7}(PEO)_{61.1}</td>
<td>7700</td>
</tr>
</tbody>
</table>

Next let’s look at the SLB film thickness variation due to the incorporation of a macromolecular additive as studied by AFM force measurement (21). Four Pluronic copolymers (L81, L121, P85, F87) (BASF) are used to prepare Pluronics-modified EggPC SLBs. The Pluronics are incorporated into EggPC vesicles according to literature procedures (22). The concentration of Pluronics is kept at 0.02 %w/w well below the critical micelle concentration (CMC). The nominal structures and the number of EO (hydrophilic) and PO (hydrophobic) units are listed in Table 1. Pluronic copolymers are known to enhance the stability of lipid vesicles (23).
Fused bilayers are observed for Pluronics with short PEO chain length. Figure 3 compares the force curve of EggPC SLB with those treated with Pluronics (EggPC/L81, EggPC/L121, EggPC/P85, and EggPC/F87). The approaching force curves show typical features of SLBs including the steric repulsion up to a threshold followed by the jump-in to contact. The distances of the repulsive force onset and jump-in points are plotted in Figure 4. The force onset distance corresponds to an unperturbed bilayer thickness at a small contact force (~ 0.2 nN). Figure 4 shows that the onset distance increases with increasing PEO chain length, which suggests that Pluronic copolymers have been integrated into the SLB with PEO chain protruding into the solution. Similar chain length dependence has been found by Wang et al. of adsorbed Pluronic copolymer layers on a self-assembled monolayer (SAM) of n-octadecyltrichlorosilane (24). A likely structure of the Pluronics-modified SLB is drawn in Figure 5. On the other hand, the jump-in
distances remain unaffected by the PEO chain length. This observation is consistent with

the interpretation of the force gap as the tip jumping across the interior of the bilayer.

The PEO chains sticking out of the EggPC lipid bilayer can be either random coils or extended chains. Figure 6 compares our experimental data of the force onset distances with calculations based on random coil and fully extended chain configurations, respectively. The following equations are used. Fully extended chain length is

\[ L_f = a \times N \]

Random coil chain length is

\[ L_r = a \times N^{3/5} \]

\( a \) is the EO monomer chain length (= 0.35 nm) and \( N \) is the number of EO units. Pluronics-modified EggPC bilayer thickness:

\[ L_{f_{\text{bilayer}}} = L_0 + 2L_f \text{ (nm)} \]

if the chain is fully extended and

\[ L_{r_{\text{bilayer}}} = L_0 + 2L_r \text{ (nm)} \]

if the chain is a random coil. \( L_0 \) is the undisturbed EggPC bilayer
Figure 6 shows that experimental data are in good agreements with calculated values based on the random coil chain.

**Figure 5.** Scheme of onset point of repulsive force on bilayer (undisturbed bilayer thickness); c) Scheme of jump-in point (bilayer thickness). The onset point of the repulsive force increased with increasing PEO chain length and the jump-in point is around 4 ~ 5 nm.

**Figure 6.** Comparison of Pluronics-modified SLB thickness based on AFM force curves and theoretical calculations. The number in the parenthesis in X-axis is the PEO chain length.
PART III SONICATED UNILAMELLAR VESICLE (SUV) BILAYER THICKNESS AND MORPHOLOGY

Compared to other micromechanical methods such as shape fluctuation method (25), magnetic-field-induced orientation (26), and micropipette aspiration method (27), AFM can provide information on: 1) bilayer thickness; 2) adsorbed liposomes of small sizes between 20 and 100 nm; 3) mechanical property variation at nanoscale; 4) local deformation and rupture events induced by the tip; and 5) surface and adhesive forces. AFM measurement focuses on the properties of vesicle population with the smallest sizes because large vesicles rupture into bilayers upon adsorption. Small vesicles with size between 50 and 150 nm have been found to passively target several different tumors because this size range is a compromise between loading efficiency (which increases with increasing size), stability (which decreases with increasing size), and ability to extravasate in tissues with enhanced permeability (which decreases with increasing size) (3). Size control may be a simple way for targeted drug delivery because vesicle distribution among different organs and tissues is related to its size.

Compared to studies on SLBs, much fewer articles address the structure of adsorbed yet unruptured vesicles. Shape instability, size, and softness of vesicles often prevent unambiguous AFM imaging. Perhaps the earliest images of intact liposomes are obtained by Shibata-Seki et al. from dipalmitoyl phosphatidylcholine (DPPC) and cholesterol mixture (28). Others subsequently confirm the characteristic features of adsorbed vesicles as first reported. The features include: 1) flattened spheres with length to width ratio less than 1 (about 0.4 in their case); and 2) image quality dependence on contact pressure either by changing image force or using tips of different curvature.

Egawa and Furusawa reported a conical relief image of sonicated phosphatidylethanolamine (PE) vesicles with diameter around 100 nm adsorbed on its own bilayer (29). The conical image has been attributed to unruptured yet flattened
vesicle with height to diameter ratio of 0.175. Thomson et al. imaged liposomes 70 nm in diameter made from pethyldimyristoyl phosphatidylcholine (EDMPC) and cholesterol mixture on aminopropylsilane modified mica (30). Closely packed liposomes have been imaged but can be easily disturbed by the scanning process. The paper by Raviakine and Brisson provides many details about vesicle adsorption and fusion by AFM imaging (31). In the paper, supported vesicular layer (SVL) is used to differentiate adsorbed and intact vesicles from adsorbed and single-bilayer disks as in SLBs. It is found that vesicles of all sizes adsorb on mica, but only vesicles with sizes below a critical rupture radius remain intact. The bilayer disks exhibit constant height of 5 nm while the intact vesicles exhibit height variation from 10 to 40 nm. The shape of liposomes are found to change both with applied image force and adhesion between biotin and streptavidin that are incorporated into vesicle and substrate layer respectively (32). Kumar and Hoh reported intact vesicles of phospholipid and cholesterol mixture that exhibited saucerlike structure in addition to the usual rounded protrusion (domelike structure) (33). The saucerlike structure is attributed to wetting and fusion near the edge of the vesicle. A force curve is obtained on the intact vesicle that shows monotonic repulsion with onset at 15 nm. The flattening from the outer edges toward the center has been studied as a function of time by Jass et al., and is described as the first step in a multistep processes leading toward the formation of SLBs (34).

Here we describe an AFM study of EggPC SUVs with diameter less than 50 nm, adsorbed on mica (11). AFM tip is moved on top of individual vesicles so that mechanical properties of the smallest vesicles can be measured at the nanoscale. The EggPC vesicle undergoes reversible shape changes from convex to flattened and concave shape with increasing image force. In addition to the monotonic repulsion due to vesicle resistance to the AFM tip advancement and compression, there exist several characteristic breaks in the force versus distance curves. Hertzian analysis of the slope
of the repulsion gives a measure of the vesicle elastic properties. The breaks in the force curve are interpreted as the tip jumping across the bilayer and allow the determination of bilayer thickness.

A well-established recipe is used to prepare EggPC SUVs (5). Multilamellar vesicle (MLV) solution is obtained by dissolving appropriate amounts of EggPC lipids in chloroform/methanol (2:1 \( v/v \)) and evaporating the solvent with nitrogen. After drying in a desiccator connected to a rotary vacuum pump for thirty minutes, the lipids are re-suspended by stirring them in an aqueous buffer solution (20 mM NaCl) at a concentration of 0.5 mg/ml. SUVs are produced from the MLV suspension by sonication to clarity (about one hour) in a sonicator bath (Branson 2200). The suspension is kept in an ice bath during the sonication process. Sonicated samples are centrifuged for 1 hour at 16,000 rpm to remove large lipid fragments by Sorvall OTD70B Ultraspeed Centrifuge.

Images of EggPC vesicles are obtained in both liquid contact mode and liquid tapping mode. Figure 7 shows the amplitude and deflection images for tapping and contact respectively and the cross-sectional height profiles from the corresponding height images. The images consist of spherical objects on a flat background. The lateral width or diameter of the spheres is measured to be 48.6 ± 11.4 nm and 69.3 ± 12.8 nm on the sectional height profiles of tapping and contact respectively. The diameter is taken from the width of the peak at the baseline in sectional height profiles. The lateral width values are higher than the vesicle diameter in solution 37.0 ± 7.9 nm by dynamic light scattering. The discrepancy is generally attributed to the flattening of vesicles on surface and tip casting its shadow over the object, so-called tip convolution effect. The height of vesicles from sectional height profiles in Tapping and Contact Mode is determined to be 13.9 ± 2.2 nm and 3.9 ± 0.4 nm respectively. The height value from tapping mode is about 40% to 50% of the vesicle diameter in solution. Causes for the unreasonably low height values from contact mode include vesicle movement during
contact mode imaging and the vesicles being severely compressed by the tip. The intermittent contact motion of the tapping tip is known to reduce frictional and adhesive forces on the sample surface. By minimizing image force, it is possible to obtain stable images of intact vesicles in either tapping or contact mode. Tapping causes less deformation of the vesicle, and it can maintain a smaller image force than contact mode.

With increasing image force, EggPC vesicles exhibit three distinctive morphologies: convex-shaped vesicles where the highest point is at the center as shown in Figure 8(a), disk-shaped vesicles with uniform height across much of the vesicle as shown in Figure 8(b), and concave-shaped vesicles where the center is depressed as shown in Figure 8(c). The lateral diameter of the vesicle increases from $48.6 \pm 11.4$ nm for convex shape to $73.7 \pm 11.5$ nm for disk shape and to $89.8 \pm 6.6$ nm for concave shape. The
morphological change is reversible under varying image force. The convex-shaped vesicles are similar to the conical relief and dome-like structure in other studies (29,33). The disk-shaped vesicles are similar to the saucer-like vesicles (33,34). While the saucer-like vesicles are cited as an intermediate state of vesicle fusion due to adhesion between vesicle and substrate, the morphological changes observed here can only be attributed to the compressive tip. The small size of the SUVs means that the adhesion alone is not enough to collapse the vesicle edge to form saucer-like vesicles (35). The morphological change due to tip compression is schematically illustrated in Figure 9. A convex shape is obtained in the lowest indentation region of the force curve; a planar shape is obtained at an intermediate indentation, and a concave shape is obtained at the highest indentation. For the concave shape, when the tip moves across the vesicle with an applied force held more or less constant, the amount of indentation is largest at the center of the vesicle. Several factors may contribute to maximum depression at the center.
1) It is known that the mechanical response of thin films couples to the substrate, which results in an increase (50%) of the apparent modulus (36). The coupling increases with decreasing film thickness. Therefore, the tip indents more in the central region of a sessile droplet.

2) A high volume percentage of material is bound by the surface near the edge of a droplet and spreads less upon compression of the tip. Contrarily, at the center of the vesicle, less resistance exists against squeezing of the trapped liquid portion.
Figure 10 represents a typical force curve captured on EggPC vesicles. While it is impossible to place the tip exactly at the center of the vesicle, the force curve with the largest onset repulsion distance was selected from a set of force curves obtained in the vicinity of a vesicle, and is used to represent the interaction between the apexes of AFM tip and vesicle. The onset force distance = 32.2 nm in Figure 10 falls in the range of measured vesicles size between 30 and 40 nm. The repulsion is not continuous, but displays characteristic breaks. During approach, one or two breaks are observed, around 19 and 7 nm respectively. While the exact locations of the breaks vary somewhat, the gap distance between the beginning and the end point of the jump-in is constant at 4.8 ± 0.4 nm. During retraction, similar jump-out gap is also observed though apparently not as well defined. This unique gap distance coincides with lipid bilayer thickness.
We hypothesize that during approach, the bilayer portion at the top of vesicle gives away to the pressing tip and slides aside, followed by continuous compression of the tip on the vesicle, until tip approaches and breaks through the bottom portion of the bilayer enclosure. It is surprising that vesicle maintains its enclosed shape while the tip bridges across different parts of the bilayer shell. The stepwise deformation during approach is shown in Figure 11: 1) compression of vesicle; 2) breakthrough of top portion; 3) further compression of vesicle with tip bridging top portion; and 4) breakthrough of bottom portion with tip spanning the whole vesicle. The process is reversed during retraction except that the vesicle is stretched to 150% of its intrinsic diameter before the final detachment of tip from vesicle. This adhesive interaction may also contribute to the shape stability of vesicle bridged by the AFM tip. Vesicles are known to self-heal after perforation due to high line tension.

**PART IV SUV MICROMECHANICAL PROPERTIES**

The mechanical properties of phospholipid membranes can be related to many vesicle behaviors, such as their formation, stability, size, shape, fusion, and budding processes. Bending rigidity is a fundamental and characteristic mechanical property of vesicles and is related to the stability and strength of bilayer (37).
Force curves can be used to extract quantitative micromechanical constants of SLBs and SUVs. The force curves can be fitted to the Hertzian model \( \delta = AF^b \) (\( \delta \) is the indentation on the vesicle and \( F \) is the load force) by assuming a spherical shape for the tip. The indentation, \( d \), from the difference between the cantilever distance \( z - z_0 \) and cantilever deflection \( d - d_0 \) is described in the following Equation:

\[
[z-z_0] = [d-d_0] = \delta = A(d-d_0)^{2/3} = 0.825 \frac{k^2 (R_{tip} + R_{ves}) (1-\nu_{ves}^2)^2}{E_{ves}^2 R_{tip} R_{ves}} (d-d_0)^{2/3}
\] (1)

\( E_{ves} \) is the Young’s modulus of the vesicle, \( R_{tip} \) and \( R_{ves} \) are the radii of the tip and vesicle, respectively, \( \nu_{ves} \) is the Poisson’s ratio of the vesicle, and \( k \) is the cantilever spring constant. \( \nu_{ves} \) is assumed to be 0.5 (38). The spring constant and tip radius are calibrated to be 0.17 N/m and 33 nm, respectively. \( R_{ves} \) is taken to be equal to \( z_0/2 \).

Experimental data can be fitted to the above Equation with two fitting parameters \( A \) and \( z_0 \). In general, the fitted \( z_0 \) value is found to be consistent with the visually examined contact point. Thus, \( z_0 \) in our experiment is determined by the onset point of the repulsive force. \( E_{ves} \) is then computed from \( A \) by least square fitting.

Bending modulus \( k_c \) is deduced from Young’s modulus based on the following Equation (39):

\[
k_c = \frac{E_{ves} h^3}{12(1-\nu_{ves}^2)}
\] (2)

\( h \) is the bilayer thickness.

Laney et al. (38) has calculated elastic properties from averaged data of approach and retraction force curves and they discard the force plots with discontinuities. The
approach part of the force curves is suitable for the indentation calculation because significant adhesive forces in retraction can affect the measurement of indentation (40). Generally, the force curves on vesicles are characterized by two repulsive regimes.

Figure 12a shows a typical deflection versus z position plot on a vesicle containing cholesterol (EggPC 80:cholesterol 20) (41). Figure 12b is a force curve converted from 12a by defining points of zero force and zero separation. The force curve can be divided into four regions as labeled. In region I, the non-contact region, the tip is far away from vesicle and the force between the tip and the vesicle is zero. Region II illustrates the elastic deformation of the vesicle under tip compression and therefore can be used to calculate the Young’s modulus. Region III corresponds to further tip compression after the tip penetrates the vesicle’s top bilayer. Region IV reflects the cantilever deflection when it is in contact with the hard mica substrate after penetrating through the vesicle’s bottom bilayer. Theoretically, the slope of region IV in Figure 12a should be 1.0 because the deflection of the cantilever is identical to the z direction movement of sample on hard surface (42). Based on the fitted data, a slope of $0.9967 \pm 0.0036$ is obtained.

![Figure 12](image-url)

**Figure 12** (a) Deflection versus z position approaching curve. (b) Force curve converted from (a).
By fitting our experimental data (region II and region III) to the Hertzian model we obtain exponent $b$ of 0.6632 (region II) and 0.9227 (region III), respectively. The poor fit on the high loading force (region III) ($b = 0.9227$) suggests that the Hertzian model severely fails in region III. The exponent $b = 0.6632$ which is close to the 2/3 in region II is remarkable. Although the Hertzian model describes the contact between two solid bodies on the basis of continuum elasticity theory without adhesion force (43), the good fit in region II suggests that Hertzian model may also be applicable to describing the elastic deformation between the tip and the vesicle within the limit of small indentation.

Figure 12c. Force curve data fit with Hertzian model for region II in Figure 12a. The squares are experimental data. Experimental data can be described by power equation $\delta = 9.9291F^{0.6632}$. The solid line is the Hertzian model $\delta = AF^{2/3}$ with $A = 9.9291$. The measured compression (indentation) versus loading force agrees with the Hertzian model in the case of the first repulsive force region.

Region II illustrates the elastic deformation of the vesicle under tip compression, and is used in subsequent calculations for determining the micromechanical properties of the vesicle. Figure 12c is the indentation versus load force converted from data in Figure 12a region II. It shows that in the beginning of the compression (indentation), the Hertz model can simulate the experimental findings very well. A deviation from the model is
observed at high load force (larger indentation, the second repulsive regime) illustrating the limitation of the model.

Young’s modulus is calculated using \( R_{\text{tip}} = 33 \text{ nm}, R_{\text{ves}} = z_0/2 \) (the onset point of repulsive force is regarded as the size of the vesicle), \( \gamma = 0.5 \), and \( k = 0.17 \text{ N/m} \). Bending modulus is a characteristic property of the vesicles, which is closely related to the activities of liposomes and the gel-liquid phase transition of liposome’s bilayer membrane. According to solid-state mechanics, Young’s modulus is related to bending modulus as \( E_{\text{ves}} = k_c/I \) where \( I \) is the cross-sectional moment. The value \( I \) for a three-dimensional, isotropic planar surface is \( h^3/[12(1 - \nu^2)] \) where \( h \) is the thickness of the bilayer and \( \nu \) is Poisson’s ratio (38,39). The bending modulus \( k_c \) is calculated using Equation (2) with \( h = 4.57 \times 10^{-9} \text{ m} \), and \( \nu = 0.5 \). The Young’s modulus and the bending modulus for pure EggPC are found to be \( 1.97 \pm 0.75 \text{ MPa} \) and \( (0.21 \pm 0.08) \times 10^{-19} \text{ J} \), respectively. The calculated values are compared with literature values in Tables 2 and 3. The Young’s modulus of the EggPC vesicle from force curves is one order of magnitude smaller than the reported value (\( \sim 10^7 \text{ MPa} \)) (44). The discrepancy is probably due to different measurement environment (11).

### Table 2. Young’s modulus of biological samples

<table>
<thead>
<tr>
<th>Material</th>
<th>Young’s Modulus (E MPa)</th>
<th>Method</th>
<th>Remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptic vesicles</td>
<td>0.2 ~ 1.3</td>
<td>Force mapping</td>
<td>Size: 90 ~ 150 nm adsorbed on mica</td>
<td>38a</td>
</tr>
<tr>
<td>DMPC vesicles</td>
<td>15</td>
<td>Osmotic swelling</td>
<td>Size: 160 ~ 180 nm in solution</td>
<td>44</td>
</tr>
<tr>
<td>DOPC vesicles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EggPC vesicles</td>
<td>1.97 ± 0.75</td>
<td>Force plot</td>
<td>Size: &lt; 60 nm adsorbed on mica</td>
<td>11a,b</td>
</tr>
</tbody>
</table>

* DMPC: dimyristoylphosphatidylcholine; DOPC: dioleoylphosphatidylcholine

The calculated bending modulus is in the same range as that reported in literature between \( 10^{-19} \) and \( 10^{-20} \text{ J} \) (Table 3). Variations in bending modulus measured have been reported and the reason for the discrepancy still needs further analysis (45).
Table 3. Comparison of bending modulus of egg yolk phosphatidylcholine

<table>
<thead>
<tr>
<th>Method</th>
<th>Size / Shape</th>
<th>Bending modulus $k_c$ ($\times 10^{-19}$ J)</th>
<th>$T$ (°C)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase contrast microscopy</td>
<td>Long unilamellar tubular vesicle (11 µm &lt; L &lt; 34 µm, 17 &lt; L/r &lt; 83)</td>
<td>2.3 ± 0.3</td>
<td>22.0</td>
<td>25a</td>
</tr>
<tr>
<td></td>
<td>Cylindrical vesicle (&gt; 10 µm)</td>
<td>1 - 2</td>
<td>25</td>
<td>25b</td>
</tr>
<tr>
<td></td>
<td>Quasi-spherical vesicle (&gt; 10 µm)</td>
<td>1 - 2</td>
<td>25</td>
<td>25c</td>
</tr>
<tr>
<td></td>
<td>Spherical (&gt;10 µm)</td>
<td>0.4 - 0.5</td>
<td></td>
<td>46a,b</td>
</tr>
<tr>
<td>Magnetic-field-induced orientation</td>
<td>Cylindrical rods (5-30 µm diameter, &lt;200 µm long)</td>
<td>0.4</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>AC electric field</td>
<td>Spherical vesicle (Diameter &gt; 20 µm)</td>
<td>0.247</td>
<td></td>
<td>45a</td>
</tr>
<tr>
<td></td>
<td>Spherical vesicle (~15–70 µm diameter)</td>
<td>0.66 ± 0.06, 0.45 ± 0.05</td>
<td></td>
<td>45b</td>
</tr>
<tr>
<td>AFM force curve</td>
<td>Spherical vesicle (Diameter &lt; 60 nm) on mica substrate</td>
<td>0.21 ± 0.08</td>
<td>22 ± 1</td>
<td>11a,b</td>
</tr>
</tbody>
</table>

Table 4 lists the bending modulii of pure, cholesterol-modified, and Pluronics-modified EggPC vesicles. The data show that EggPC vesicles are stiffened by adding cholesterol and Pluronic copolymers.

Significant increase in bending modulus when cholesterol is incorporated into EggPC vesicles. It has been reported that the phospholipid bilayer packing geometrical structures are changed by cholesterol insertion and thus changing fluidity and intra-vesicle interaction (47). After the cholesterol is incorporated into phospholipid bilayers, the small hydrophilic 3β-hydroxyl headgroup of cholesterol is located in the vicinity of the lipid ester carbonyl groups, and the hydrophobic steroid ring orients itself parallel to the acyl chains of the lipid (48). Thus, the movement of the acyl chains of the phospholipid bilayer has been restricted. Below the gel phase transition temperature ($T_m$) of lipids, cholesterol addition increases the fluidity of lipids, while above $T_m$ the mobility and fluidity of the lipid chains are restricted ($T_m$ of EggPC = -15 °C) (49). Moreover, hydrogen bonding between cholesterol’s β-OH and the carbonyl groups of the lipid enhances the stability of the bilayer (50). The interaction between the cholesterol and phospholipid
bilayer results in an increase in membrane cohesion, as shown by increases in the mechanical stiffness of the membranes.

**Table 4. Bending moduli of pure, cholesterol-modified, and Pluronics-modified EggPC vesicles**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bending Modulus</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure EggPC</td>
<td>0.27 ± 0.10</td>
<td>11a,b</td>
</tr>
<tr>
<td>EggPC: Chol (85:15)</td>
<td>1.68 ± 0.21</td>
<td>41</td>
</tr>
<tr>
<td>EggPC: Chol (80:20)</td>
<td>1.49 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>EggPC: Chol (70:30)</td>
<td>1.44 ± 0.56</td>
<td></td>
</tr>
<tr>
<td>EggPC: Chol (50:50)</td>
<td>1.81 ± 0.41</td>
<td></td>
</tr>
<tr>
<td>EggPC/F88</td>
<td>1.50 ± 0.40 (103 EO, 40 PO)</td>
<td>21</td>
</tr>
<tr>
<td>EggPC/F127</td>
<td>1.64 ± 0.28 (100 EO, 65 PO)</td>
<td></td>
</tr>
<tr>
<td>EggPC/F108</td>
<td>2.06 ± 0.38 (133 EO, 50 PO)</td>
<td></td>
</tr>
</tbody>
</table>

The slope of the long-range repulsive force regime in Figure 3 can be used to calculate the elastic properties of the Pluronics-modified EggPC SUVs based on Eqn. (1) and Eqn. (2). The slopes of EggPC/F88 and EggPC/F127 are found to be lower than that of EggPC/F108. The slope of EggPC/F127 is close, but slightly higher than that of EggPC/F88. Both F127 and F88 have similar PEO size while PPO chain of F127 is longer. Table 3 lists the calculated bending modulus values. The bending modulus of Pluronics-modified EggPC SUVs increases by an order of magnitude from that of pure EggPC. The magnitude of the increase is comparable with cholesterol/EggPC vesicles. These data show that the bending moduli of Pluronics-modified EggPC SUVs are a function of PEO and PPO chain lengths. The dramatic increase can be attributed to PPO block incorporation and PEO chain on the surface. For Pluronics-modified SUVs, the physical stability is improved by the PEO steric repulsion (22b,23b). The bending modulus obtained on F127 vesicles is \((1.64 \pm 0.28) \times 10^{-19} \text{ J}\), slight larger than that of F88 \((1.50 \pm 0.40) \times 10^{-19} \text{ J}\). The increase can be attributed to longer PPO chain length of F127. While comparing bending moduli of F127 and F108, the circumstances are somewhat complicated since both have different PPO and PEO chain lengths. The bending modulus of F108 is higher than that of F127 although the PPO chain length of
F108 is smaller. However, F108 has longer PEO chain and stiffer shell-like “coating” on the vesicles seems to contribute to bending modulus increase.

It is well known that the stability of polymer-coating liposome is improved in terms of steric repulsive force between the polymer chains dangling outside of the liposome. In earlier studies, the steric stabilization effect has been described by aggregation and fusion among vesicles in solution (22b,23b). Kostarelos et al. draw a similar conclusion by comparing prolonged vesicle durability against flocculation and ?potential change (23b). It has been reported that the bending and mechanical properties of vesicles from diblock polymer exceeds those of phospholipid liposomes by a factor of 5 or more (51). Thus, the significant bending modulus improvement can be attributed to PEO (incorporated) and PPO chains (dangling outside of EggPC vesicles). Figure 13 shows a schematic of the steric stabilization effect of copolymer on liposome. A block copolymer may aggregate to form micelles (core/shell structure) consisting of a swollen core of insoluble blocks surrounded by a flexible fringe of soluble block (52). As an analogy to the polymer micelle, the PEO chains dangling outside the vesicle surface may exhibit a shell-like effect to provide steric stabilization to the vesicle. The shell-like structure also serves as a kind of “net” or “coating” of vesicles (53). With longer PEO chain length (>19 units), a finite steric barrier is formed (24). The PPO chain incorporated into the lipid bilayer acts in a similar fashion as cholesterol to restrict the lipid molecular movement and fluidity. For copolymer with longer PEO chain length (F88, F108 and F127), shell-like wall structure is strong enough to keep all EggPC vesicles in an intact form upon adsorption on mica (21).
This chapter describes experimental methods to determine the thickness and micromechanical properties of supported bilayers and vesicles using AFM imaging and force measurement. Fused bilayers and sonicated unilamellar vesicles of EggPC are used as examples to illustrate the various approaches for film thickness determination. The AFM imaging of lipid layers is ideally conducted in natural biological solutions using tapping or contact mode at below a threshold force. The adsorbed vesicles of EggPC below 50 nm in size can be imaged in an intact state using the soft contact mechanism.

**PART V SUMMARY REMARKS**

Figure 13. Scheme of the Pluronics PEO (a) and PPO effect (b) on the stability of the EggPC vesicle. (a) SUVs with long attached PEO chains can form shell-like structure (black dotted circle) due to sufficient steric repulsion provided by the dangling PEO chains away from the vesicle surface. (b) The PPO chain incorporated inside the lipid bilayer restricts the bilayer fluidity and movement, and makes the lipid bilayer membrane more rigid.
The bilayer thickness can be accurately determined by the step height at bilayer domain edges. Alternatively, the jump-in distances can be used to obtain the bilayer thickness. The jump-in distances change when biological or polymeric additives are integrated into the lipid bilayer. The distance increase can be matched to the hydrophilic block chain length when nonionic Pluronic copolymers are adsorbed onto the EggPC bilayer. Jump-in points are also observed in supported intact vesicles, with one corresponding to the upper bilayer and another corresponding to the lower, surface-attached bilayer. The two gaps are identical in value, which is the lipid bilayer thickness. These gaps can be interpreted as abrupt jumps of the AFM tip across the top and bottom portion of the bilayer enclosure during approach and retraction. AFM can be also used to monitor the shape changes at different image forces. The indentation curve converted from the force curve can be fitted to the Hertz spherical contact model in order to extract the Young’s modulus of the vesicle. The calculated Young’s modulus and bending modulus of the EggPC SUVs are $(1.97 \pm 0.75) \times 10^6$ Pa and $(0.21 \pm 0.08) \times 10^{-19}$ J respectively. The elastic constants are compared to literature values from various micromechanical tests of liposomes. AFM methods are the most direct way to determine bilayer film thickness and offer new insights into adsorption, spreading, fusion, self-healing, and mechanical properties of biomembranes and liposomes at the nanoscale.


23. (a) Woodle, M. C.; Newman, M. S.; Working, P. K. In Stealth liposomes; Lasic D. Martin F., Eds; CRC Press: Boca Raton, 1995; p103-118 (b) Kostarelos, K.; Luckham, P. F.; Tadros, Th. F. *Steric Stabilization of Phospholipid Vesicles by Block Copolymers* -


