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Atomic Force Microscopy to Study Interacting Forces in Phospholipid Bilayers Containing General Anesthetics

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Summary

Atomic force microscopy (AFM) can be used to reveal intimate details about the effect of anesthetics on phospholipid bilayers. In AFM, surfaces are probed using a tip revealing lateral structural features at 10–20-nm resolution and height features at 0.5-nm resolution. Additionally, information on the viscoelasticity of the surface can be gained by examining the forces of tip–surface interactions. This is also known as force spectroscopy. In this chapter, the use of AFM to observe and quantify anesthetic-induced changes in phospholipid bilayers is detailed. The procedures developed to create supported phospholipid bilayers are described and the techniques developed to generate the best AFM images and force spectroscopy results have been revealed.

Key Words: Atomic force microscopy; force spectroscopy; halothane; phospholipid bilayer; DPPC; phase transition.

1. Introduction

The changes in the physical and chemical properties of biological membranes owing to incorporation of anesthetics are of great interest for understanding the mechanism of anesthetic action. The molecular theory of anesthetic action includes the hypothesis that anesthetics alter the lipid membrane structure, and therefore, its biophysical properties. Changes in lateral pressure within bilayers ***(1)***, lipid packing in membranes ***(2–5)***, polarization of the membrane, and the degree of motional disorder in lipid chains ***(5,6)***, which leads to bilayer thinning ***(6–8)*** could all change the ability of cells to communicate with each other. Atomic force microscopy (AFM) is a valuable technique to address the structural changes in lipid bilayer membranes. AFM has proven to be advantageous not only for imaging biological samples in liquid media ***(9,10)***, but also for allowing the study of sample physical properties by measuring force of interactions between the AFM tip and the sample surface ***(11–13)***.

Previously, it was demonstrated that incorporation of general anesthetics into the bilayer produces structural alterations in a supported phospholipid bilayer ***(8)***, such as bilayer area and height (i.e., domain formation), each of which depends on the time of incubation and concentration of anesthetics. Similar domain formation was observed when the membrane underwent a melting transition ***(7)***. In spite of the visual similarity between an anesthetic-induced domain formation and the heat induced gel–liquid phase transition, when observed by AFM imaging, the mechanism of anesthetic action is likely to be different from the effect of membrane melting. It is assumed that the physical properties of the domains produced in lipid bilayers by halothane and by temperature are also different. To address these physical properties, the atomic force microscope can be used as a force apparatus; it has proven to be

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an advantageous tool for the measurements of interaction forces between AFM probe and the surface, giving information about the physical properties of the sample surface ***(14–16)***. In this chapter, the procedures used to generate planar phospholipid bilayers supported on a mica substrate are described in detail. Also the sample conditions, which provide AFM images of the highest quality are detailed. Finally, the approach to force spectroscopy is conveyed.

2. Materials

2.1. Chemicals and Substrates

1,2-Dipalmitoyl phosphatidylcholine (DPPC) (Avanti Polar-lipids Inc., Alabaster, AL) is used without further purification. Pure halothane (Sigma Chemical Co., Saint Louis, MO) is used throughout. Freshly cleaved ASTMV-2 quality, scratch-free ruby mica (Asheville-Schoonmaker Mica Co., Newport News, VA) is used throughout the study as a substrate. Micro Cleanning Solution from ArrayIt® (Sunnyvale, CA 94089 USA) is used. Distilled, deionized, nanopure quality water is used in the generation of all vesicles.

2.2. AFM Accessories

Magnetically, (Au–Cr-coated) type I Maclevers® (Agilent Technologies AFM [Tempe, Arizona] are used for magnetic A/C (MAC) mode imaging and for force measurements. The nominal spring constants of Au–Cr-coated Maclevers used are 0.6 N/m. The tip radius of curvature is quoted as being typically 25 nm. Colloidal gold spheres of 5 and 14 nm in diameter from Ted Pella Inc. (Redding, CA) ***(17)*** are used for calibration of the AFM scanner's height resolution. A calibration grid from Molecular Imaging is used for calibration in the lateral direction.

3. Methods

3.1. Vesicle Solution Preparation

Multilamellar vesicles are prepared by ultrasonication of phospholipid solutions. First, a controlled volume of phospholipid dissolved in chloroform is added to a 10-mL round bottom flask. The chloroform is then removed using a gentle stream of dry nitrogen. Following this, the phospholipid is suspended in low-p*I* buffer or pure water, as appropriate, and stirred for 30 min at room temperature. This produces a cloudy suspension. The suspension is then subjected to 10 rounds of 10 min ultrasonication, followed by 15 min of stirring in a cold water bath. After this regimen, the DPPC solution is found to become clear, suggesting the dissolution of large particles.

3.2. Supported Bilayer Formation

The liquid sample cell is washed thoroughly. First, it is subjected to a 10-min sonication in a diluted microcleaning solution (MCS). The MCS is diluted by mixing 50 mL of 20X MCS and 950 mL ultrapure H_2O to make 1 L of 1X MCS. After sonication, the cell is rinsed with ultrapure water, and then sonicated 10 min further in ultrapure water. This procedure removes all debris from the cell.

To create the supported planar bilayer, mica is freshly cleaved with double-sided adhesive tape before each experiment. Mica consists of negatively charged layers that are bound together by large, positively charged interlayer cations (K^+ in the case of muscovite mica). Each stratum consists of two hexagonal layers of SiO_4, which are cross-linked by aluminum atoms having OH_3 groups incorporated. The electrostatic bonds between K^+ and O atoms

from the layer are weak and easily broken. This layer is disrupted after a cleavage procedure, exposing a basal plane covered by K^+. Thus, freshly cleaved mica in water carries a slightly negative charge, with a density of 0.57 ions/nm ***(18)***. It is possible to adsorb a DPPC lipid bilayer on mica without any further modification of the mica surface. Supported planar bilayers are prepared for AFM imaging by the vesicle fusion method ***(8)*** as follows. Aliquots of 20 μL (lipid concentration: 0.5 mg/mL; incubation time: 10 min) liposome solutions are deposited on freshly cleaved mica. After a well-defined period of time (usually 10 min) the mica is gently rinsed with ultrapure water and the cell is filled with ultrapure water. After this, the samples of supported bilayers are ready for imaging. Then, the sample chamber is mounted onto the AFM scanner. Supported bilayers are always kept in water, never dried or exposed to air.

To prepare bilayer samples with halothane, two methods are put forward. First, addition of halothane into the vesicle solution, followed by an incubation of 1.5 h. A supported bilayer could then be formed from the halothane-loaded vesicles method of vesicle fusion as described earlier for pure vesicles. Second, addition of small volumes (60 μL) of pure anesthetic into the AFM liquid cell containing the established supported bilayer, which is covered with 1 mL of water. The sample is incubated for 1.5 h after the addition of halothane. Then, the nonpartitioned halothane is carefully removed by pipeting with all the water filling the cell. The cell is then gently rinsed by slowly adding water by pipet and removing it again. Following this, the cell is filled with nanopure water. Fluorescence experiments show that an incubation time of 1.5 h is optimal for halothane partitioning between water and bilayer and reach equilibrium, whether one method is used or the other ***(19)***. Both ways of adding halothane give similar results. For the sake of simplicity and reproducibility, the second method is used for all subsequent experiments.

3.3. AFM Imaging

AFM is a surface imaging technique with nanometer-scale lateral resolution and 0.1 nm resolution normal to the sample, which operates by monitoring the forces of interaction acting between a probe and a sample, while the sharp probe scans the sample surface. For imaging, MAC mode AFM is used; this is a tapping mode available through Molecular Imaging AFM in which a magnetically coated probe oscillates near its resonant frequency driven by an alternating magnetic field. All images are taken using a Pico SPM microscope with AFMS-165 scanner (Molecular Imaging Inc.) in a liquid cell. All samples are imaged in ultrapure water because degradation was observed in image quality with increasing p*I* ***(8)***. The highest contrast images are always obtained by imaging in ultrapure water ***(21)***.

In an attempt to minimize the osmotic shock to vesicles during rinsing, the original solutions are thus prepared with a low-p*I*. This rinsing process occurs before the AFM tip encounters the solution. Therefore, the tip is never exposed to a liposome solution, and should remain free of lipid contamination. Magnetically, (Au–Cr-coated) type I Maclevers (Molecular Imaging Inc.) are used for MAC mode imaging and force measurements. The scan rate is fixed to 20 mm/s. The standard MAC mode fluid cell (Molecular Imaging) is used throughout.

A supported DPPC bilayer is formed on mica from vesicle solution, rinsed with water, and covered with 1 mL of water in the AFM liquid cell; pure halothane (60 μL) is added into the liquid cell and the cell is incubated for 1.5 h. After this, nonpartitioned halothane is removed by gently rinsing and refilling the cell with water. The cell is mounted into the microscope and after an equilibration of 10–15 min, the sample is imaged in water in tapping mode (MAC mode). The amplitude set point for imaging is chosen at the near maximum of the resonance frequency (80–90% of the maximum amplitude).

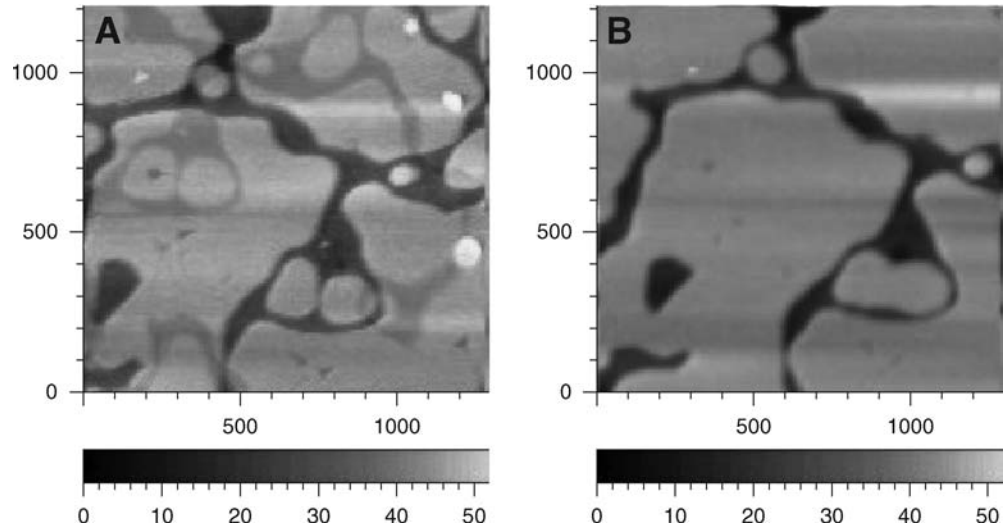

Fig. 1. A typical AFM image of DPPC bilayer with halothane incorporated. Supported bilayer was formed on mica from vesicle solution, rinsed with water and covered with 1 mL of water in AFM liquid cell, excess of halothane (60 μL) was added into the liquid cell, and the cell was incubated for 1.5 h. After this, nonpartitioned halothane was removed with water, AFM cell was gently rinsed and filled with water, Bilayer was imaged in taping mode (MAC mode). **(A)** imaged immediately after sample preparation and **(B)** after 30 min of imaging bilayer converts into thin domains.

A typical image of the DPPC bilayer with halothane incorporated is shown in **Fig. 1**. Halothane incorporation results in domain formation (*see* **Fig. 1**) ***(20)***. The thickness of the higher and lower domains is measured as 3.5 nm.

3.4. AFM Force Measurements

In AFM force measurements, the probe is moved toward the sample, and the cantilever deflection is measured as a function of the extent of the piezo electric tube. During approach to the surface the attractive and repulsive forces being measured are characterized by van der Waals and electrostatic interactions, as well as solvation, hydration, and compression-related steric forces ***(11)***. The probe is then retracted from the surface. The retraction force curves often show a hysteresis referred to as an adhesion pull-off event, which can be used to determine adhesion forces between the probe and the sample.

In the experiments, first the supported bilayer in tapping MAC mode is imaged. After the image is completed and saved, the regime is switched into the contact mode and then, using the same cantilever, the interacting forces are measured by positioning the AFM tip on different locations using the image obtained in tapping mode. Force curves are collected at 10 locations on the bilayer and then averaged. The force spectroscopy experiment consists of monitoring the interaction between the AFM tip and the substrate by sensing the cantilever deflection (Z_c) as a function of the piezo elongation (Z_p) and as the tip is moved toward and away from the substrate (i.e., raw data). The raw data averaged for each sample are shown on **Fig. 2**.

Force measurements were performed on DPPC bilayers formed from water solutions with and without halothane. It is a well-known fact that the force interactions depend on the velocity of the surface approach ***(11)***, especially for soft, viscoelastic materials. The frequency sweep has been varied over about four decades, from 0.01 to 10 Hz. The *Z*-range is 50 nm. Note that

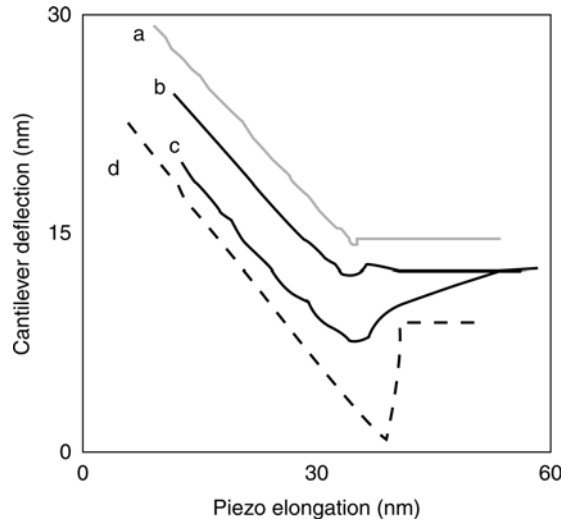

Fig. 2. Cantilever deflection dZ_c as a function of the piezotube elongation when the tip is retracted from the sample. DPPC bilayers deposited on mica were measured under three conditions: (b) the DPPC bilayer at $T = 20°C$ (c) the DPPC bilayer at $T = 50°C$ (d) the DPPC bilayer with halothane incorporated. The adhesion curve on bare mica (a) is shown for comparison.

varying the scan frequency affects simultaneously the velocity at which the tip is withdrawn from the bilayer and the contact time between the tip and the sample. At a fixed applied load, increasing the frequency sweep rate increases the tip velocity yet decreases the contact time.

Statistical analysis reveals that 10 measurements at different locations of the same area are required to determine parameters such as the adhesion and the long-range forces with 10% accuracy. Data are collected over a time period of 2 h, and a total of 100 force curves are analyzed for each sample. Force curves are saved as plots and then converted into text files for analysis. After the raw data are collected, they are converted into the "force vs separation plots," averaged and analyzed.

3.5. AFM Force Analysis

During AFM force measurements, the probe is moved toward the sample and the cantilever deflection is measured as a function of the extent of the piezo electric tube. Force measurements using the atomic force microscope (described in **Subheading 3.4.**) give the raw data of cantilever deflection (δV) vs piezo movement (z_{piezo}). Raw data are transformed numerically under a Matlab environment. As the tip–sample separation cannot be independently measured, a systematic procedure for calculating the sensitivity of the apparatus is developed *(7)*. This measurement assumes that tip and the mica surface are brought into a nondeformable contact in the higher loading region. Raw data (Z_c vs Z_p) are then converted into force (F) vs surface-tip separation (D) using Hooke's law: $F = k\,Z_c$ where k is the spring constant of the cantilever, with the geometric relationship $D = Z_c - Z_p$ for incremental changes.

Statistical data are extracted from a large set of measurements. The authors use the Gaussian distribution in the following form:

$$P(x) = C\frac{1}{\sigma\sqrt{2\pi}}e^{-(x-\mu)^2/(2\pi^2)} \quad (1)$$

where $P(x)$ is the number of samples at the particular x point in the histogram, μ is the mean, σ is the standard deviation, and C is the normalization constant. The bin size of the histogram is determined statistically by dividing the range of measurements by the square root of the number of measurements. The average of the adhesion force and the standard deviation are obtained from the analysis of the whole set of the force curves, which are represented in histogram form ($P[x]$) and then adjusted using a Gaussian law.

3.5.1. Adhesion Forces

Surface force profiles are measured among the silicon nitride tip, mica, and a pure DPPC bilayer vs the profiles in a DPPC bilayer with halothane partitioned into it. To extract information about adhesive forces, the approach part of the force curve was analyzed. Raw data of the cantilever deflection corresponding to the retraction of the tip from the surface (after contact with the tip), are shown in **Fig. 2**, plots a–d. Data corresponding to the mica surface immersed in water are presented for comparison. Mica is characterized by an abrupt jump out of contact (plot a in **Fig. 2**).

To analyze adhesive forces, the same statistical analysis was conducted that was used in the previous work *(7)*. It has been demonstrated that the force of a unit interaction between an AFM tip and a surface can be determined from statistical analysis of a series of detachment force measurements. For a statistical analysis based on adhesive forces originating from a discrete number *n,* individual interactions or bonds have been used. The total force distribution follows Poisson statistics, where both the adhesion force (F_{adh}) and the variance (σ) originates from a number of individual bonds n:

$$F_{adh} = nF_s$$

and

$$\sigma^2 = nF_s^2$$

The force of one bond (F_s) is therefore given by the square of the variance of the force divided by F_{adh}. The value n is the ratio between F_{adh} and F_s. This analysis has the advantage that the knowledge of the mean radius of curvature is not required, and gives information on the nature of the bilayer. **Table 1** presents the standard deviation of the adhesion force in the gel phase DPPC bilayer and bilayer with halothane incorporated, as measured by the authors. Data for gel phase DPPC bilayer at room temperature and fluid phase DPPC bilayer at elevated temperature from the previous paper *(7)* are shown for comparison.

This analysis allows to correlate microscale changes in the bilayer physical properties with molecular structure and mobility of individual lipid molecules. Tail mobility can be seen as the number of molecules n in contact with the AFM probe: the larger the n, the higher the mobility. A change in mobility increases the number of bonding (n) and, thus, F_{adh} and σ.

3.5.2. Repulsive Forces

Repulsive forces are extracted by analyzing the approach part of experimental force curves. **Figure 3A** shows four patterns of repulsive forces, measured through the AFM tip when approaching the sample surface of pure mica (plot a), DPPC bilayer at room temperature (plot b), DPPC bilayer at 50°C (plot c) (*see also* **ref.** *7*), and DPPC bilayer with an excess of halothane (plot d). The zero force intercept obtained from **Fig. 3B** is used to determine the "effective bilayer thickness" corresponding to the related tip–sample distance.

Table 1
Experimental Adhesion Force (F_{adh}) and Its Standard Error Sigma on DPPC Bilayer and DPPC With Halothane, Measured at Room Temperature; Calculation of the Number of Bindings *n* and the Mean Force of a Single Bond F_s

	DPPC[a] 22°C	DPPC	DPPC + halothane
F_{adh}	1 nN	1.5 nN	7 nN
σ	0.6 nN	0.8 nN	2 nN
F_s	0.3 nN	0.4 nN	0.5 nN
n	3	4	14

[a]Data on DPPC bilayer at room and elevated temperature, measured earlier during melting transition ***(11)*** are shown for comparison.

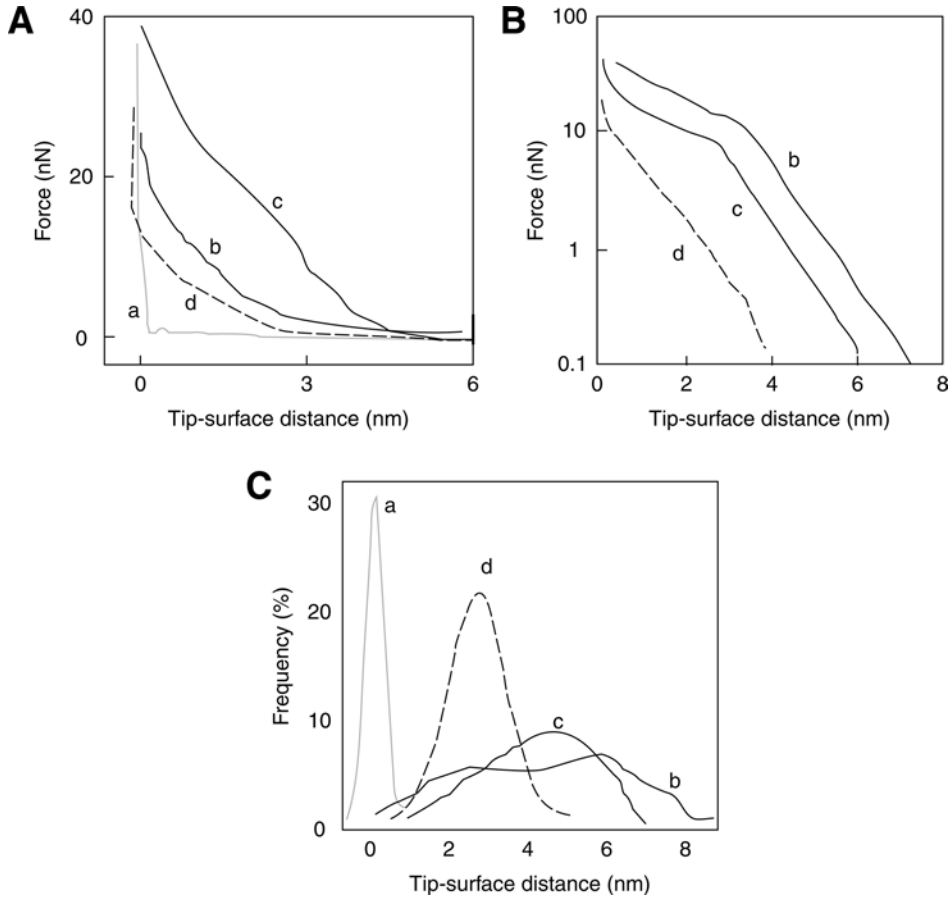

Fig. 3. **(A)** Surface force as a function of a tip–surface distance in liquid cell when the tip is approaching the sample; **(B)** logarithmic representation of data in panel **(A)**; **(C)** Distribution of the "effective thickness" determined from force analysis **(A)** on mica, DPPC and DPPC with halothane incorporated. Plots on **3A–C** are ascribed as: (a) bare mica (is shown for comparison), (b) the DPPC layer at $T = 22°C$, (c) the DPPC layer at $T = 50°C$, and (d) the DPPC layer with

4. Notes

1. All liposome solutions are sonicated at controlled temperature between 25° and 30°C, not to exceed the melting temperature of the DPPC bilayer (42°C) by addition of ice or cold water into the sonication bath.
2. When preparing supported bilayers on mica, the time of incubation and amount of solution added has to be adjusted, in order to obtain good coverage of mica by the bilayer. To form a supported DPPC bilayer on mica small aliquots (20–30 µL of 0.5 mg/mL) DPPC solution in water were used. The incubation time can vary from 5 to 30 min.
3. Sometimes it is necessary to produce holes in the bilayer in order to measure the bilayer thickness. One way to do so is using the AFM tip and scratch a hole in the bilayer by imaging a very small area at high scanning rate and high load force in contact mode. Although this method produces a hole in the bilayer, it may also produce mechanical defects in the tip, and contaminate the lipids adsorbing to the tip from the sample, which reduces the resolution and quality of the image. Another, less-invasive method is used to produce holes. First, one needs to scan a prepared, supported bilayer, because in general, holes are naturally present in the bilayer. If not, one can rinse the cell with supported bilayer with a stream of water from a plastic bottle; this procedure readily produces the required holes, and does not contaminate the cantilever.

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