Atomic force microscopy of supported lipid bilayers

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Supported lipid bilayers (SLBs) are widely used in biophysical research to investigate the properties of biological membranes and offer exciting prospects in nanobiotechnology. Atomic force microscopy (AFM) has become a well-established technique for imaging SLBs at nanometer resolution. A unique feature of AFM is its ability to monitor dynamic processes, such as the interaction of bilayers with proteins and drugs. Here, we present protocols for preparing dioleoylphosphatidylcholine/dipalmitoylphosphatidylcholine (DOPC/DPPC) bilayers supported on mica using small unilamellar vesicles and for imaging their nanoscale interaction with the antibiotic azithromycin using AFM. The entire protocol can be completed in 10 h.

INTRODUCTION

Biological membranes fulfill several important functions, such as acting as permeable barriers that allow the inside environment of the cell or organelle to differ from that outside and mediating cell communication and cell interactions. Over the past decade, evidence has accumulated for the existence of lipid rafts, that is, small (10–200 nm) membrane domains that are enriched in sphingolipids and cholesterol and which are believed to play important biological roles[1–4]. A variety of proteins partition into lipid rafts, including glycosylphosphatidyl inositol (GPI)-anchored proteins and transmembrane proteins[5]. Sphingolipid clusters rich in both cholesterol and GPI-anchored proteins tend to be insoluble in Triton X-100 at 4 °C, forming detergent-resistant membranes[6]. These preparations have become the ruling method for assigning lipid and protein raft affinity, even though they do not reflect membrane organization at steady state[6].

The structure and properties of biological membranes can be strongly affected by their interaction with drugs; in turns, this interaction modulates the drug activity and toxicity[7–9]. The conformation of acyl groups, the membrane thickness, the phase transition temperature, the membrane potential and hydration of head groups and the membrane fusion properties are just a few examples of traits that can be modified upon interaction with drugs. Membrane domains such as lipid rafts may have a number of important consequences on this interaction, including enhancement of penetration and insertion of the molecules at the domain boundary. Hence, there is currently much interest in understanding drug–membrane interactions at the molecular level.

Supported lipid bilayers (SLBs) are valuable model systems to study the properties of biological membranes and processes such as molecular recognition and membrane fusion[10,11]. SLBs also offer exciting prospects in nanobiotechnology, for example, for the design of patterned biosurfaces with given functionalities[10–13]. In addition, these structures offer the possibility to apply a range of surface analytical techniques that would be difficult to apply on real membranes, such as time-of-flight secondary ion mass spectrometry (SIMS)[13,14] and atomic force microscopy (AFM; **Fig. 1**). With its ability to image biosurfaces at high resolution and in buffer solutions[15,16], AFM offers exciting new opportunities for exploring the nanoscale properties of membranes, either on living cells[16–18] or on model SLB systems[19–22]. Of particular interest in SLB research is the possibility to observe time-dependent processes such as the interaction of lipid membranes with proteins[23], peptides[24] and drugs[25], and the growth of single lipid domains[26].

Preparation of SLBs

Mica is the most commonly used material for preparing SLBs. Muscovite mica is a nonconducting layered mineral composed of multiple 1-nm thick layers, which can be easily cleaved with the help of adhesive tape to produce clean, atomically flat surfaces. Glass and silicon oxide wafers are other suitable materials for forming SLBs, but organic contaminants and particles should be carefully removed before use, for example, by washing in concentrated acidic solution followed by ultrasonication in water solutions. Note that gold surfaces may also be functionalized with self-assembled monolayers of organic alkanethiols for preparing so-called hybrid alkanethiol/lipid bilayers[27].

There are essentially two methods for forming SLBs, that is, the Langmuir–Blodgett (LB) technique and the fusion of lipid vesicles (**Fig. 2**). In the LB method (**Fig. 2a**)[28], a Langmuir trough

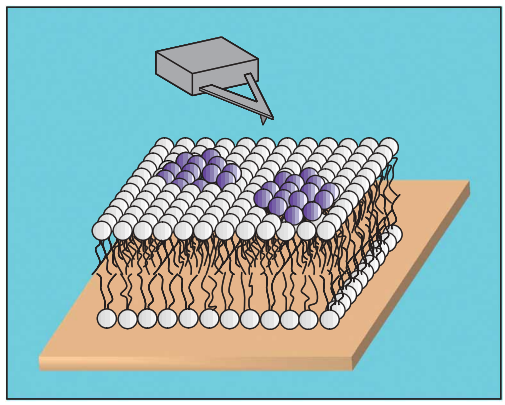

Figure 1 | Atomic force microscopy (AFM) of supported lipid bilayers (SLBs). SLBs are valuable model systems to study biological membranes and offer exciting prospects in nanobiotechnology. SLBs are particularly well suited for dynamic, real-time imaging studies using AFM.

consisting of a rectangular Teflon bath equipped with moveable barriers is used to compress the lipid molecules at the air–water interface. Lipids are usually spread at the air–water interface in hexane/ethanol or chloroform/methanol mixtures and then compressed after letting the solvent to evaporate for 15 min (**Fig. 2a**, left panel). A sensor records the surface pressure at the interface, which can be expressed as a function of the interfacial area. The obtained surface pressure versus area isotherms can provide useful information on the packing and organization of the lipid molecules[28]. In the LB technique, the monolayer of amphiphilic molecules is transferred at constant surface pressure and constant speed onto a solid support, usually mica (**Fig. 2a**, center panel). Careful control of surface pressure and lifting speed is essential to avoid artifacts such as defect formation or feature alignment of the deposited structures. Lipid monolayers interact with mica through the polar heads, thus exposing the hydrophobic tails to the environment. Supported monolayers are stable in air, not in water, and should therefore be examined in air. Transferring a second lipid layer onto a mica-supported lipid monolayer yields a supported bilayer which best mimics cellular membranes (**Fig. 2a**, right panel). Supported bilayers should always be kept and analyzed in aqueous solution because they are not stable in air.

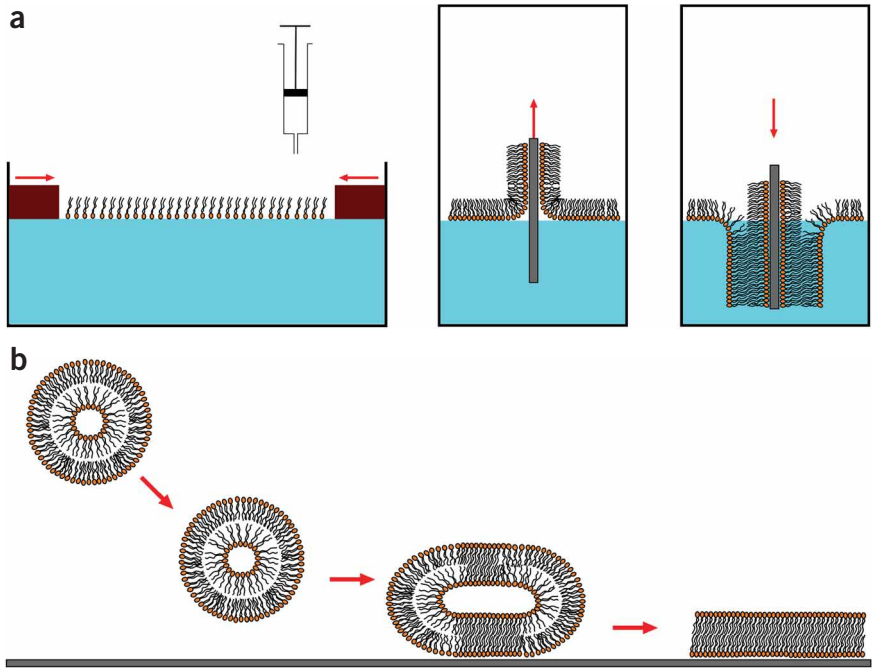

Figure 2 | Preparation of supported lipid bilayers (SLBs). Two methods commonly used for preparing SLBs for atomic force microscopy analysis: (**a**) Langmuir–Blodgett technique and (**b**) fusion of lipid vesicles.

The most popular method for preparing SLBs is the fusion of lipid vesicles on solid supports (**Fig. 2b**)[29–32]. As detailed in the PROCEDURE, lipids are first solubilized in organic solvent. After solvent evaporation under nitrogen and subsequent desiccation under vacuum, the dried lipid film is resuspended in aqueous buffer solution yielding a multilamellar vesicle (MLV) suspension. From this suspension, small unilamellar vesicles (SUVs) can be obtained using various approaches, sonication being the most popular one. The suspension is sonicated to clarity using a titanium probe sonicator while keeping the suspension in an ice bath, after which the suspension is filtered on nylon filters to eliminate titanium particles. Then, fusion is achieved by heating the SUV suspension in contact with freshly cleaved mica for 45–60 min at a temperature between 45 and 60 °C. The supported bilayers are finally gently cooled to room temperature (~20 °C) and rinsed abundantly with the appropriate image buffer. Although the exact mechanism of bilayer formation from SUVs is not fully understood yet, the process involves adsorption of the vesicles on the surface, deformation, flattening and rupture to form a continuous SLB (**Fig. 2b**)[31,32]. Compared to LB deposition, the drawbacks of the fusion method are the impossibility to prepare asymmetric bilayers composed of two layers of different nature and the lack of control of the lateral pressure in the lipid layers. However, the fusion approach is simpler and permits lipid diffusion as in free-standing bilayers.

Currently, there is much interest in developing methods for preparing more biologically relevant membranes that incorporate membrane proteins, because these play essential roles in cellular processes and are highly relevant to human physiology and disease[22]. Membrane proteins can be reconstituted at high density into a lipid bilayer to form 2D crystals that are ideally suited for AFM analysis[33]. An alternative approach is to incorporate membrane proteins into preformed SLBs destabilized by detergents used in membrane biochemistry (dodecyl-β-maltoside or dodecyl-β-thiomaltoside)[34]. Here, a major advantage is the extremely small amount (~1 pmol) of protein needed to obtain a high protein density in the lipid bilayer. Also, the versatility and simplicity of the technique should make it very useful for the conception of biosensors and nanobio devices involving membrane proteins.

In this procedure, we present protocols that we have used to prepare phase-separated dioleoylphosphatidylcholine/dipalmitoyl-phosphatidylcholine (DOPC/DPPC) bilayers supported on mica, and to image their nanoscale interaction with the antibiotic azithromycin using AFM (**Fig. 3**)[35]. The procedure is applicable to SLBs of various compositions (mixtures of phospholipids, sphingolipids, cholesterol and proteins) and may be used to investigate a variety of membrane-interacting compounds such as drugs, proteins and peptides (**Fig. 4**)[36,37]. Regarding reproducibility and statistics, we recommend recording images on different SLBs prepared independently, using different tips. Also, it is important to demonstrate that the observed membrane morphological changes are due to the action of the drug (or membrane-interacting compound) rather than to sample damage by the scanning tip. A first control experiment is to record an image of a new SLB zone at the end of the time-dependent experiment to confirm that similar morphological changes are observed. Another control consists in recording successive images of the SLB in the absence of any drug to show that, in these conditions, the SLB morphology is not altered. Clearly, the full potential of AFM in SLB research will be best exploited when combined with other advanced microscopy and spectroscopy techniques, such as SIMS and fluorescence techniques[4].

MATERIALS

REAGENTS

- L-α-DOPC and L-α-DPPC (Sigma-Aldrich)
- Azithromycin, 94% purity (Pfizer)
- Mica (Agar Scientific)
- Analytical grade ethanol, methanol, HCl and $CHCl_3$ (VWR)
- $CaCl_2$ (VWR)
- Milli-Q water (Millipore)

EQUIPMENT

- Nanoscope IIIa MultiMode AFM equipped with a liquid cell (Veeco Metrology Group)
- Oxide-sharpened microfabricated Si_3N_4 cantilevers with spring constants of 0.01 N m^{-1} (Microlevers; Veeco Metrology Group)
- Steel sample pucks (Veeco Metrology Group)
- Glue: EPO-TEK 377 (Gentec Benelux)
- 500 W Probe sonicator (Fisher Bioblock Scientific)
- 0.2-μm Nylon filters (Whatman)

REAGENT SETUP

Solutions Dissolve azithromycin in 0.1 N HCl. Prepare the following aqueous solutions for forming and imaging the SLBs: Tris/NaCl/$CaCl_2$ (10 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, pH 7.4), Tris/NaCl (10 mM Tris, 100 mM NaCl, pH 7.4) and Tris/NaCl/azithromycin (10 mM Tris, 100 mM NaCl, 1 mM azithromycin, pH 7.4).

Mica supports Cut mica squares (25 mm^2) and glue them onto steel sample pucks. After drying, cleave the mounted mica with the help of adhesive tape.

AFM liquid cell Clean the AFM liquid cell with detergent and rinse abundantly with Milli-Q water, further rinse with ethanol and then dry with a gentle nitrogen flow.

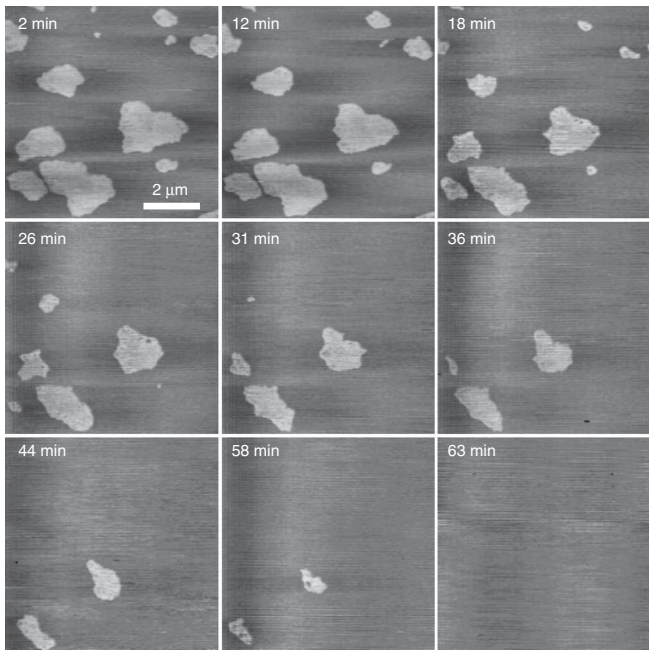

Figure 3 | Real-time imaging of bilayer–drug interactions. Atomic force microscopy height images (*z*-scale: 10 nm) of a mixed dioleoylphosphatidylcholine/dipalmitoylphosphatidylcholine (1/1, mol/mol) bilayer supported on mica, recorded following incubation with the antibiotic azithromycin (1 mM) at increasing incubation times. Reprinted with permission from ref. 35.

PROCEDURE

Formation of SLBs ● TIMING ~4 h

1| Dissolve DOPC and DPPC in 10 ml $CHCl_3$/methanol 2/1 (vol/vol) in two glass tubes at 1 mM final concentration.

2| Evaporate an equimolar mixture of the two lipids under nitrogen and dry in a desiccator under vacuum for 2 h.

3| Resuspend the dried lipid film in Tris/NaCl/$CaCl_2$ buffer at 1 mM final lipid concentration to obtain an MLV suspension. ▲ CRITICAL STEP Suspend the lipids in a calcium-containing buffer because this ion favors vesicle adsorption and SLB formation[32].

4| To obtain SUVs, sonicate the suspension to clarity (five cycles of 2 min) using a probe sonicator (500 W; 35% of the maximal power; 13-mm probe diameter) while keeping the suspension in an ice bath.

5| Filter the suspension on a 0.2-μm nylon filter to eliminate titanium particles from the probe sonicator.

6| Place 2 ml of the SUV suspension in contact with freshly cleaved mica supports and heat for 45 min at 60 °C.

7| After slowly cooling down the system to room temperature, carefully rinse the samples with Tris/NaCl to remove the SUV excess.

8| Mount the wet samples onto the AFM scanner while avoiding dewetting of the SLBs. Gently add 100 μl of the Tris/NaCl imaging buffer on the sample surface.

9| Place microfabricated cantilevers with ~0.01 N m^{-1} spring constants and oxide-sharpened tips in the liquid cell. Fill the cell with imaging buffer and mount it on top of the sample.

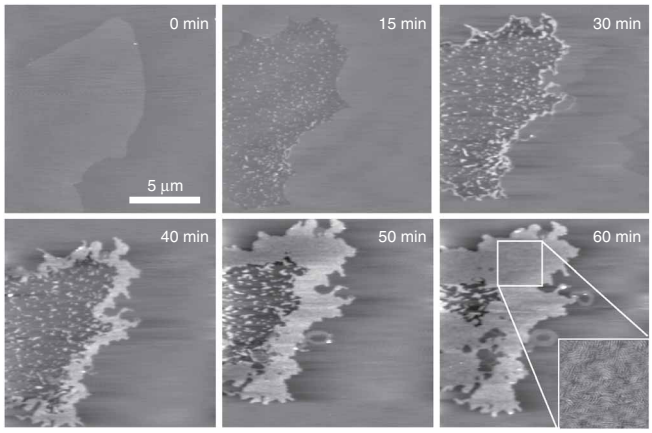

Figure 4 | Real-time imaging of bilayer–peptide interactions. Atomic force microscopy height images (*z*-scale: 10 nm) of a dioleoylphosphatidylcholine/dipalmitoylphosphatidylcholine/dioleoylphosphatidic acid (495:500:5) bilayer recorded before (0 min) and after (15, 30, 40, 50 and 60 min) addition of a simian immunodeficiency virus peptide solution (10 μM). The inset (60 min) is a higher magnification (3 × 3 $μm^2$; *z*-range: 2 nm) of the white box region that reveals nanostructures attributed to cylindrical reverse micelles. Reprinted with permission from ref. 37.

▲ CRITICAL STEP SLBs should always be kept hydrated because they are not stable in air. Thus, avoid any contact with air bubbles because these may cause reorganization of the bilayers. With the MultiMode liquid cell (Veeco Metrology Group), best results are sometimes obtained without using an O-ring to seal the sample and tip. However, protect the scanner (e.g., metallic cap or Parafilm) if you do not use the O-ring.

10| Wait for 10 min to minimize thermal drift. Focus the laser beam onto the cantilever end and adjust the photodiode signal. Make sure to keep the temperature of the setup constant (e.g., 20 °C); if necessary use a temperature-controlled liquid cell.
▲ CRITICAL STEP The packing and miscibility of lipids within SLBs strongly depend on temperature[26], meaning this parameter should be carefully controlled. Depending on the lipids used, a temperature increase of only few degrees may lead to bilayer domain remodeling.

Imaging mixed DPPC/DOPC bilayers ● TIMING ~2 h

11| Engage the AFM tip and start scanning the DPPC/DOPC bilayer surface in contact mode at low resolution (e.g., image size of 50 × 50 μm^2), while adjusting the feedback parameters.
▲ CRITICAL STEP For SLBs, good results are generally obtained in contact mode, in which the AFM tip is raster scanned over the sample while the cantilever deflection, thus the force applied to the tip, is kept constant using feedback control (constant-force mode). However, for fragile samples, it may be useful to switch to tapping mode, which uses an oscillating tip to scan the surface. Because lateral forces during imaging are greatly reduced, this dynamic mode is less destructive than contact mode.

12| Maintain the imaging force at small values, that is, <250 pN.
▲ CRITICAL STEP The image resolution dramatically depends on the imaging force[38]. In buffered solutions, it is easy to maintain an applied force in the range of 100–500 pN. Due to thermal drift, readjust the applied force between each recording.

13| Record high-resolution images (e.g., image size of 5 × 5 μm^2) to resolve the DPPC and DOPC phases (**Fig. 3**, upper left panel).
▲ CRITICAL STEP DPPC/DOPC (1:1) bilayers should always show phase separation at room temperature, in the form of elevated gel domains of DPPC, surrounded by a continuous fluid matrix of DOPC. However, note that the geometry of the DPPC domains can greatly vary from one preparation to another, with a size ranging from 100 nm to several microns.

14| Take vertical cross-sections across representative images to measure the relative height of the two lipid phases.
▲ CRITICAL STEP AFM can measure vertical dimensions with Å resolution, an option which is very useful for measuring the relative height of bilayer domains and for validating the quality of the SLBs. For DPPC/DOPC bilayers, check whether the step height between the two lipid phases is in the order of 1.1 nm (ref. 39).
? TROUBLESHOOTING

Imaging of bilayer–drug interactions ● TIMING ~4 h

15| Record an image of a region of interest, that is, a region which is representative of the entire SLB and which shows well-defined domains (**Fig. 3**, upper left panel).

16| Exchange the imaging buffer with the 1 mM azithromycin solution. To this end, disengage the tip and remove the liquid cell, partially remove the Tris/NaCl buffer by contacting the border of the sample puck with precision wipes, while keeping the SLB surface fully hydrated. Gently add 100 µl of the Tris/NaCl/azithromycin solution, remove liquid in excess with precision wipes, and repeat this procedure two times. Finally, exchange the Tris/NaCl solution in the liquid cell with the Tris/NaCl/azithromycin solution, mount the cell onto the sample and engage the tip to find the bilayer region chosen in Step 15.
▲ CRITICAL STEP Exchanging fluids while imaging SLBs is a tricky step which may dramatically alter bilayer integrity.
? TROUBLESHOOTING

17| Record successive images of the bilayer region chosen in Step 15, while minimizing the applied force between each recording, to visualize the time-dependent remodeling of the SLB (**Fig. 3**).
▲ CRITICAL STEP Withdraw the tip from the sample surface between each recording to minimize sample damage by the scanning tip.

18| Stop the experiment after 4 h.

? TROUBLESHOOTING

No lipid domains or relative heights much larger than expected (Step 14)
The step height between the DPPC and DOPC phases should be in the order of 1.1 nm, reflecting differences in the film thickness and mechanical properties[39]. A completely smooth surface could reflect the presence of a bilayer of homogeneous

composition resulting from poor lipid mixing during the preparation. Alternatively, a smooth surface could reflect the bare mica surface due to poor preparation (e.g., contact with air).

Sample is altered upon injection of the drug solution (Step 16)
Exchanging fluids while imaging SLBs must be done carefully. The method presented in Step 16 presents some drawbacks, such as misalignment of the laser spot on the cantilever, loss of the region of interest, and time consumption. If these problems occur, it is best to work with an O-ring and to pump fluids into the liquid cell using syringes, gravity flow systems or computer-controlled fluid changes.

ANTICIPATED RESULTS

Newcomers will need to practice for a month or two to prepare and image SLBs in an accurate, reliable way. Undoubtedly, the trickiest part is to preserve the overall integrity of the fragile SLB during the various steps of the preparation and imaging procedure. **Figure 3** shows how the above protocols have been used to image SLBs and to monitor their dynamic remodeling upon interaction with drugs[35]. We found that incubation of DPPC/DOPC bilayers with the dicationic antibiotic azithromycin induces progressive erosion and disappearance of DPPC gel domains within 60 min. This effect was attributed to the disruption of the tight molecular packing of the DPPC molecules by the drug, in agreement with earlier biophysical experiments. In contrast, sphingomyelin and sphingomyelin/cholesterol domains mimicking lipid rafts were not modified by azithromycin. This higher membrane stability was suggested to reflect stronger intermolecular interactions between sphingomyelin molecules.

Using a similar approach, we also investigated the interaction of SLBs with tilted peptides, a class of short peptides found in many membrane-interacting proteins such as viral fusion proteins and neurotoxic proteins, and which are known to promote membrane fusion (**Fig. 4**)[36,37]. We found that incubation of SLBs with the simian immunodeficiency virus peptide leads to major bilayer remodeling. At short incubation time, a $\sim$2 nm thickness reduction of the DPPC domains was observed, reflecting either interdigitation or fluidization of lipids. After longer incubation times, these depressed DPPC domains evolved into elevated domains, composed of nanorod structures protruding several nanometers above the bilayer surface and attributed to cylindrical reverse micelles.

These studies demonstrate the power of using AFM imaging in lipid membrane research. Yet, we must emphasize that adsorbing membranes onto a solid support for analysis means the very central notion of a free-standing biomembrane separating two aqueous compartments is not preserved. As a result, membrane properties such as elasticity, fluidity and diffusion properties may be altered, thereby limiting the biological relevance of the measurements. This problem can be solved by adsorbing the membranes on supports that have well-defined pores[40,41] or holes[42]. These elegant approaches provide the required free aqueous volume between support and membrane, as well as a second compartment accessible to analysis. As already emphasized, another challenge for future research will be to explore SLBs that incorporate membrane proteins[22].

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