DESTRUCTIVE NANOINDENTATION TECHNIQUES TO STUDY BIOLOGICAL MEMBRANES

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https://doi.org/10.37904/nanocon.2023.4764

Abstract

Atomic Force Microscopy (AFM) belongs to the nanoimaging methods employing the fine driving of the probe movement, where its interaction with the sample gives a detailed view of the surface structure. The ability to control the motion of the sample and thus its force interaction with the surface provides the possibility to map the sample's mechanical properties at the nanoscale, but also, for example, to influence the sample, even destructively, which can provide further interesting information. AFM ability to study the structure of biological molecules is here represented by the supported lipid bilayer (SLB), a synthetic model for cellular membranes. The traditional approach of SLB structural study can be extended by the destructive use of AFM probe, penetrating through the solid structure of the double layer, leaving a typical pattern in the measured curves. The characteristics of this mark then help in the detailed characterisation of the mechanical properties of this synthetic membrane in particular.

Keywords: Phospholipid bilayers, Atomic Force Microscopy, Force-Distance Curves, Smoothing splines, Local extrema

1. INTRODUCTION

Phospholipid bilayers (PLB) [1] are a fundamental structural component of biological membranes that surround living cells and organelles within cells. PLBs can be prepared in-vitro, from liposome particles. Liposomes are artificial vesicles that mimic the structure of biological membranes, consisting of one or more phospholipid bilayers surrounding an aqueous compartment. They are widely used in research, drug delivery, and other applications.

Atomic Force Microscopy presents an interesting approach to studying PLBs in-situ [2], under semi-physiological conditions without marking or other modifications. However, it is usually used for imaging topography, which can be affected by various effects, such as oxidative stress. However, in this presentation, we would like to present a different way of using AFM microscopy to study the properties of PLB, which is destructive nanoindentation. In this process, a force is applied to the probe, which penetrates through the bilayer structure **(Figure 1**), and a typical pattern, called a rupture event (RE), is created in the measured force-distance curve. This approach can also be applied to membranes of living organisms such as bacteria and tissue cultures. Compared to standard approaches in force-distance curve processing [3], which are relatively well-automated, special mathematical approaches need to be developed here.

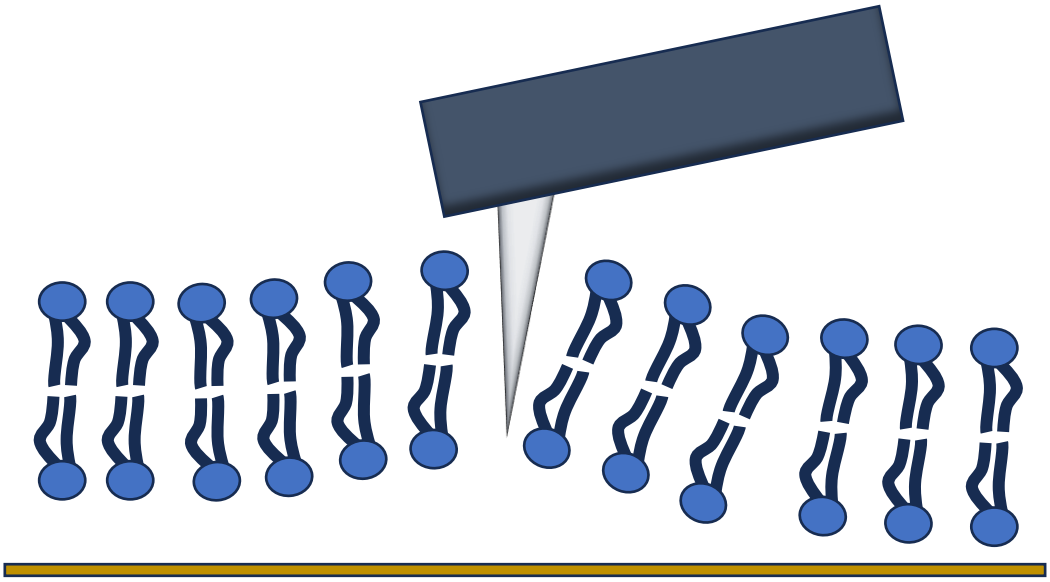

Figure 1 Penetration of the phospholipidic bilayer with a sharp tip of an AFM probe, a schematic view

The processing of the observed force-distance data used by us is based on smoothing splines and estimation of the first derivative of the curve. This allows us to identify the rupture event and to detect corresponding local extrema with sufficient accuracy.

The presented publication gives an insight into the possibilities of preparing phospholipid bilayers for their study by AFM. Advanced processing of force-distance curves allows for better characterization of these synthetic membranes.

2. MATERIALS AND METHODS

2.1. PREPARATION OF SUPPORTED LIPID BILAYER

SLB monolayers were prepared via the commonly employed method of unilamellar vesicle fusion. Small unilamellar vesicles (SUVs) can be prepared as described elsewhere [4], from variety of phospholipid derivatives, such as 1-palmitoyl-oleoyl-sn-glycero-phosphocholine (POPC)[5]. Deposition of bilayer SLB monolayer is a spontaneous procedure if the transition temperature of the bilayer is lower than the temperature of the environment. Briefly, 0.5 mM solution of SUVs in TRIS buffer pH=7.2 is incubated on the surface of freshly cleaved mica sheet (2Spi, West Chester, PA, USA) for 60 minutes under laboratory temperate in a wet chamber preventing the solution drying out. The mica surface was then washed carefully with fresh TRIS buffer by adding and removing with a pipette, the sample was considered to be thoroughly washed after ten buffer exchanges. The sample thus prepared was immediately subjected to AFM examination.

2.2. AFM EXPERIMENTS ON SUPPORTED LIPID BILAYER

Before performing AFM experiments on the prepared SLBs, the AFM was equilibrated and calibrated. Bruker Dimension FastScan Bio atomic force microscope (Bruker Nano Surfaces, Santa Barbara, CA, USA) was employed in all experiments. Soft silicon nitride cantilever with super sharp probe (tip radius 1-2 nm) PEAKFORCE-HIRS-F-B (Bruker Nano Surfaces) were equilibrated prior calibration for 45 minutes in the TRIS buffer. The calibration of stiffness and sensitivity was performed using the built-in calibration manager in Bruker NanoScope software ver. 9.4 (Bruker Nano Surfaces), giving parameters of sensitivity in the range 21-23 nm/V and cantilever stiffness in thew range between 0.09 to 0.12 N/m for individual probes.

Each experiment started by imaging the surface in Peak Force QNM mode, where a low force was applied, 50 pN. The displayed area size was 5 µm and the resolution was 1000x1000 pixels^2. The other parameters were set automatically using the ScanAsyst algorithm.

Force-distance curves (FDC) were measured at specific points using the "Point and Shoot" module with typical settings - setpoint 5 nN, sampling frequency 1000 points, sampling rate 0.5 Hz, Z-axis range was shortened to 1 µm.

2.3. DATA PROCESSING

The images of the surface were processed in the Gwyddion software[6], where theraw data were flattened by the aligning rows function, scars were removed and low-pass filter was applied to remove the noise captured in a data file.

FDCs were visualized in the Bruker NanoScope Analysis software ver 3.0 (Bruker Nano Surfaces) as force vs. separation curves, where the appropriate X and Y positions of the local extremes on the curves can be evaluated – thus giving parameters of bilayer thickness and strength.

3. RESULTS AND DISCUSSION

Phospholipidic bilayers creating incomplete monolayers are visualized as islands on the background of the supporting substrate (**Figure 2**, left). The thickness of this layer can be measured as Z-scale profile of the visualized sample (**Figure 2**, right), thus providing precise information about the SLB structure.

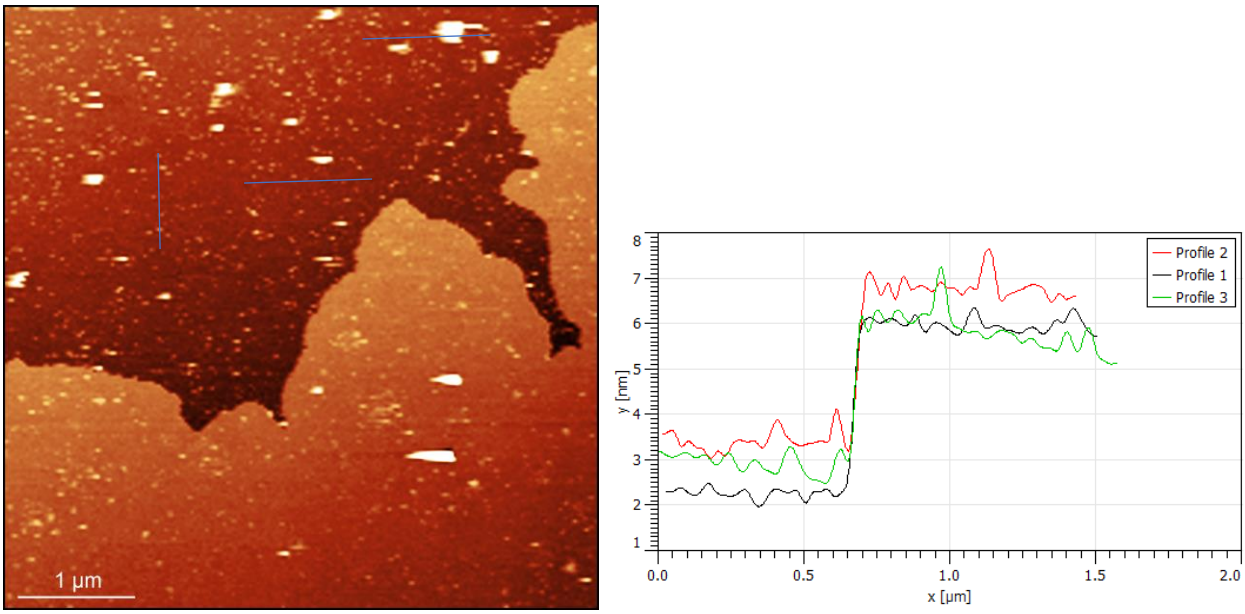

Figure 2 Islands of phospholipidic bilayer present white maps on darker background (left), bilayer thickness measured in three different places shown on the graphs of Z-scale profiles (right), position of the profiles is indicated with blue lines in the AFM image.

Atomic Force Microscopy (AFM) is a powerful tool used in the study of phospholipid bilayers, which are often used to mimic biological membranes [7]. The AFM technique allows for the probing of surfaces using a tip, revealing lateral structural features at 10–20-nm resolution and height features at 0.5-nm resolution1. This high resolution enables the study of intimate details about the effect of various factors on phospholipid bilayers1.

Phospholipid bilayers are a key component of biological membranes, and their study is crucial for understanding the physical and chemical properties of these membranes [8]. The bilayers are arranged in two layers, each consisting of a head and two tails. Changes in these bilayers, such as changes in lateral pressure, lipid packing, polarization of the membrane, and the degree of motional disorder in lipid chains, can all affect the ability of cells to communicate with each other1.

In the context of AFM, the use of mica as a substrate is common due to its atomically flat surface, which allows for the formation of uniform and stable phospholipid bilayers. These bilayers can then be studied under various conditions to observe changes in their structure and properties.

For instance, one study used AFM to observe and quantify anesthetic-induced changes in phospholipid bilayers. It was found that the incorporation of general anesthetics into the bilayer produced structural alterations, such as changes in bilayer area and height1. These changes were dependent on the time of incubation and concentration of anesthetics [9], [10].

In conclusion, the use of AFM in the characterization of phospholipid bilayers formed on mica provides valuable insights into the structure and behavior of biological membranes. This technique allows for the detailed study of these bilayers under various conditions, contributing to our understanding of biological processes and the development of new therapeutic strategies [11].

If the SLB lamellae form a compact layer (**Figure 3**) on the surface and therefore the above-mentioned islands are not present, the thickness of the double layer cannot be determined simply from the Z-profile readout.

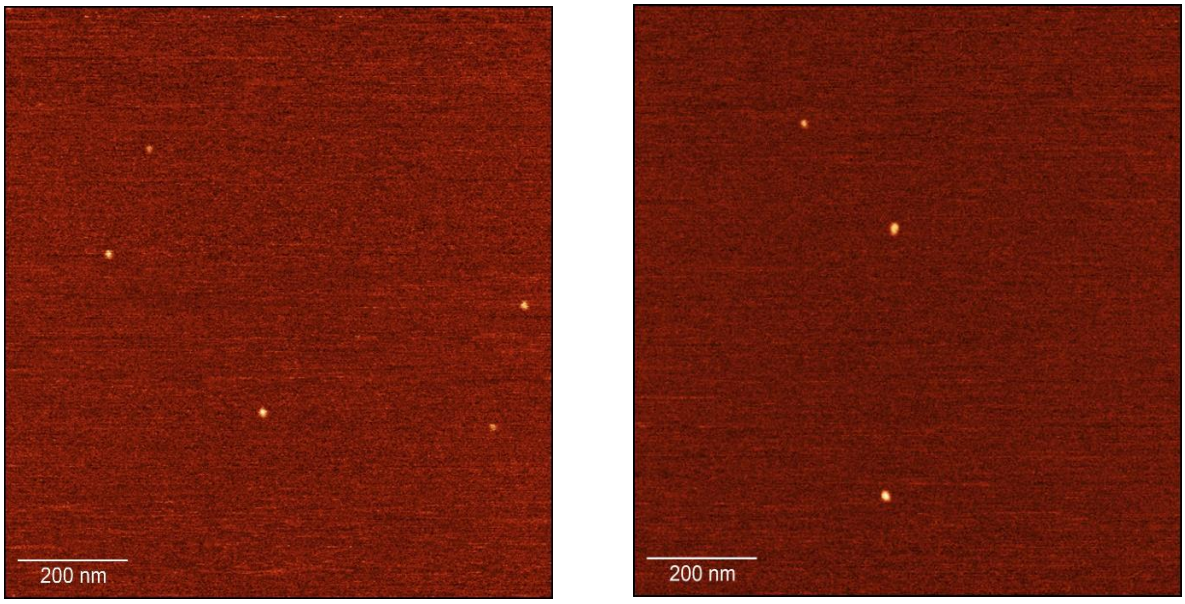

Figure 3 Homogeneous SLB structure randomly displayed at two sample locations. The sample shows the capture of several liposomal particles (SUVs) as white objects, but the thickness of the bilayer cannot be determined.

Thus, if it is not possible to determine the thickness of the SLB by reading its profile on the surface of the underlying mica, as in the case of island formation (**Figure 2**), it is possible to determine this thickness by penetrating the probe through the bilayer when a relatively high force is applied - units of nanonewtons. During the deformation of the SLB, typical patterns (so called rupture events, RE) in the force-distance curves are recorded, as shown in **Figure 4**. The distance between local minimium and maximum in on x-axis is equal to the bilayer thickness, the force in the point of local maxima corresponds to the density and compactness of the studied layer.

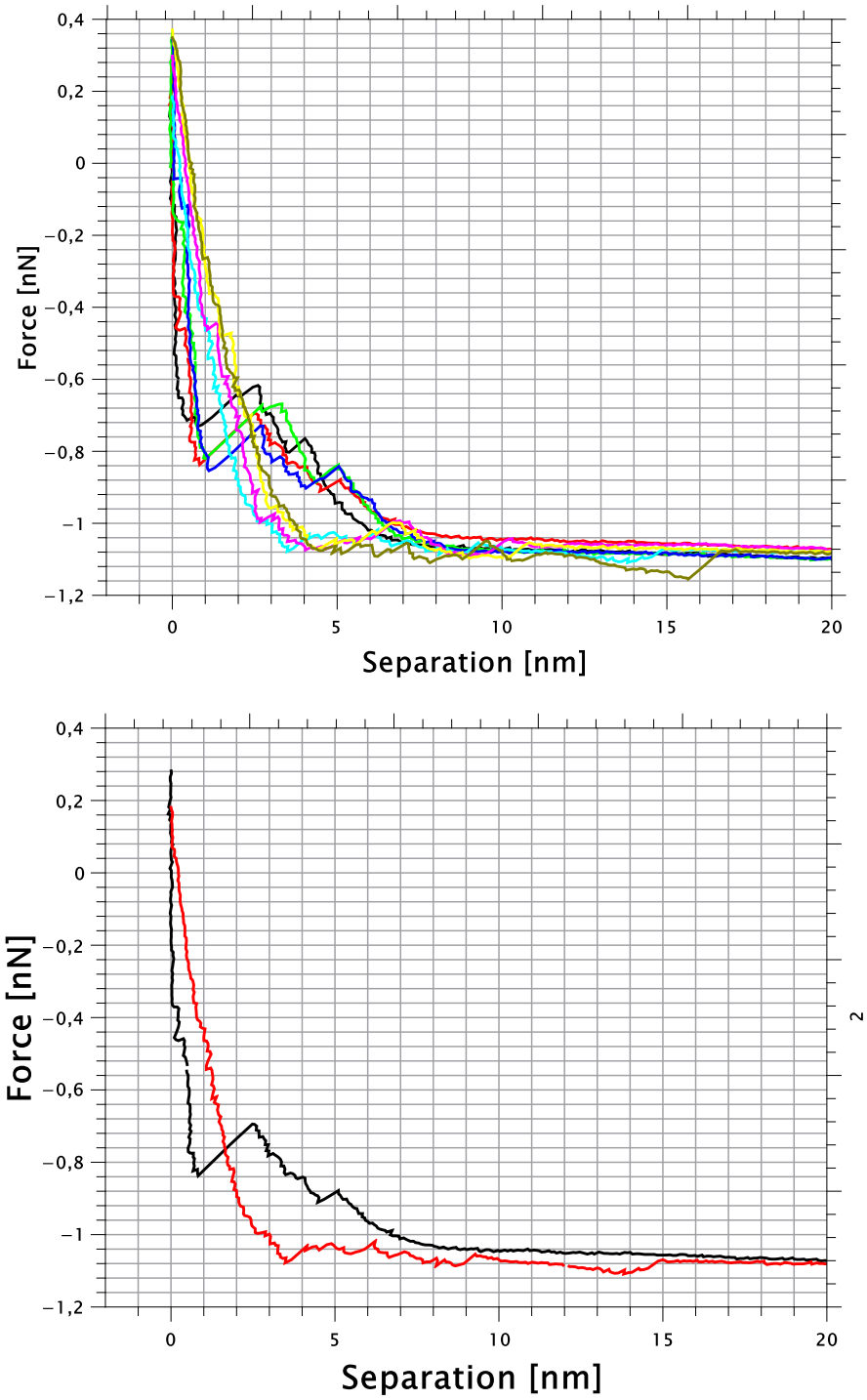

Figure 4 Force-separation curves showing the specific penetration through the structure of the SLB. The upper picture shows examples of different curves, the lower one shows many curves recorded at different places.

4. CONCLUSION

In conclusion, this work presents a novel approach to the preparation and characterization of phospholipid bilayers using Atomic Force Microscopy (AFM). The study demonstrates the successful formation of these bilayers on the atomically flat substrate of mica through the fusion of Small Unilamellar Vesicles (SUVs). This

method provides a reliable and reproducible means of creating synthetic membranes that closely mimic biological ones.The application of advanced processing of force-distance curves has significantly enhanced the characterization of these synthetic membranes. This technique allows for a more detailed understanding of the membrane's properties, including its mechanical and structural characteristics. The potential applications of this work are vast. The ability to accurately mimic and study biological membranes opens up possibilities in various fields, from understanding cellular processes to the development of new therapeutic strategies. Furthermore, the insights gained from this study could be instrumental in the design of biomimetic systems and devices. The bilayer thickness can be measured either by Z-scale profile of the bilayer islands or by penetration of AFM probe through the compact bilayer structure. Both experimental approaches lead to similar results for the thickness of the studied layers, and the measurement of penetration through these structures leads to additional parameters that characterize SLBs, such as their stability and density.

ACKNOWLEDGEMENTS

We acknowledge Masaryk University Interdisciplinary Grant MUNI/G/1125/2022 and CF Nanobiotechnology of CIISB, Instruct-CZ Centre, supported by MEYS CR (LM2023042) and European Regional Development Fund-Project "UP CIISB "(No. CZ.02.1.01/0.0/0.0/18_pl046/0015974).

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