AFM-Based Force-Clamp Indentation

Force-Clamp Monitors the Lipid Bilayer Failure Kinetics

The lipid bilayer rupture was here explored by means of AFM-based force clamp. For the first time to our knowledge, this technique has been used to evaluate how lipid membranes respond when compressed under an external constant force in the range of nN. We were able to directly quantify the kinetics of the membrane rupture event and the associated energy barriers, in distinction to the classic studies performed at constant velocity.

Force Signature of the Membrane Mechanical Stability

Biological membranes are known to perform their function under a complex combination of forces. The micropipette aspiration technique has shown to be valuable to gain quantitative mechanical information [1]. Due to the chemical diversity of cell membranes, techniques with nanometric resolution, like optical tweezers [2], atomic force microscopy (AFM) [3] and AFM-based force spectroscopy (FS) [4], emerged as an excellent approach to probe the local properties of lipid bilayers. By means of FS, it has been well established that the vertical force applied to supported lipid bilayers (SLBs) is a direct measurement of the lateral interactions between phospholipid neighbor molecules [5]. Force-distance curves show a discontinuity in the approaching curve that marks the penetration of the AFM tip through the bilayer. The force at which this process occurs has been interpreted as the maximum force the bilayer is able to stand before breaking, the so-called breakthrough force, $F_B$. Subtle variations in the chemical structure of the phospholipid molecules [6] as well as in the physicochemical environment [7] give rise to differences in the $F_B$ value, which can therefore be considered as the fingerprint of the mechanical stability of a specific lipid membrane in a determined environment.

In a traditional FS experiment, the tip is driven towards to and away from the surface through vertical motion of the piezo positioner at a constant velocity while the resulting force is measured. In this kind of configuration, force is measured but not controlled and the determined $F_B$ is loading-rate dependent. Because force is the measurable and controllable magnitude of the mechanical rupture, it is appealing to study the bilayer rupture process under constant force conditions.
This force-clamp mode was implemented by the group of Fernandez to study the stepwise unfolding of proteins [8] and capture conformational changes in polysaccharides [9] at a constant pulling force. Therefore, force-clamp spectroscopy represents an ideal platform to study the lipid film rupture kinetics.

**Indenting at Constant Force**

Experimentally, the lipid bilayers of a model phospholipid (DPPC) are spread onto the planar substrate (mica). Then, a set of force curves in the constant velocity mode is performed in order to determine the corresponding $F_b$ distribution. The recorded events are represented in a histogram. Once the $F_b$ mean value is determined, force-clamp experiments can be performed setting the constant applied force ($F_c$) to be below the mean $F_b$. In a force-clamp experiment, the AFM tip approaches the surface at a constant velocity (fig. 2Aa) until the set $F_c$ is reached and kept constant (clamped) by continuously readjustments of the tip-surface distance (fig. 2Ab). A sudden decrease of the force (and the tip-surface separation) (fig. 2B) caused by the lipid failure (fig. 2Ac) triggers the movement of the piezo to reinstate the tip position, consequently restoring the force to the $F_c$ set value (fig. 2Ad). This is evidenced as a single-step in the separation vs. time trace (fig. 2B) that corresponds to the average height of the lipid bilayer observed in force-separation plots for constant velocity experiments (ca. 3 nm). The time the SLB withstands before failure is defined as the time to breakthrough (tb). After the rupture event, the tip keeps on compressing against the mica substrate and once the set dwell time is reached, the tip retracts at constant velocity (fig. 2Ae). Well-defined rupture or failure events observed in the force vs. time recording correspond well with a step in the separation vs. time plot (fig. 2B), evidencing the penetration of the AFM tip through the lipid film (fig. 2B inset).

As shown in figure 3a, the $F_b$ distribution obtained in constant velocity mode for the mica-DPPC SLBs gave an average value of 14.0 ± 1.3 nN. Accordingly, for the AFM-FC experiments, a range of $F_c$ below 13 nN was chosen. By fitting the corresponding histograms of tb to an exponential decay function, a mean lifetime ($t$) is obtained for each $F_c$ value studied. The inverse of $t$ is defined as the rate of the rupture process, $\alpha$. For the $F_c$ range studied, we found an exponential dependence of the rate of the rupture process on the applied $F_c$, as evidenced in the linear plot of $\ln \alpha$ vs. $F_c$ in figure 3b.

When the membranes are subjected to a constant compression force $F_c$, the energy barrier height is reduced by an amount that equals $\Delta x \cdot F_c$, for $\Delta x$ representing the distance from the native conformation to the transition state conformation along
the reaction coordinate, beyond which phospholipid lateral interactions fail [10]. It corresponds to the Arrhenius-Bell expression, where \( E_0 \) is the activation energy (height of the energy barrier) of the process in absence of external force and \( \alpha_0 \) is the rupture rate constant in the absence of external applied force.

\[
\ln \alpha(F) = \ln \alpha_0 + \frac{\Delta x \cdot F_c}{k_B \cdot T}
\]

The model implies a probabilistic behavior of the process with a single rate constant, which can be obtained from the single exponential fit to the average lipid failure trajectory. Fitting the experimental data to the equation gives rise to values of \( \alpha_0 = 1.9 \cdot 10^{-3} \text{ s}^{-1} \) and \( \Delta x = 3.74 \text{ pm} \) (fig. 4). We estimated the energy barrier for the DPPC bilayer rupture at zero external force, \( \alpha_0 = 14.97 \text{ kBT} \). The \( \alpha_0 \) value obtained by means of AFM-FC is comparable to the value obtained by the traditional constant-velocity AFM-FS method taking into account the dependence of the \( F_b \) value to the loading rate. For this system, a value of about 12 kBT was obtained by varying the loading rate in dynamic force spectroscopy experiments.

**Conclusion**

In brief, our results illustrate a well-defined approach to study kinetics of lipid membranes rupture at the nanoscale. By means of AFM-FC we followed the kinetics of bilayer failure of model SLBs for the first time to our knowledge. We were able to determine the parameters - \( \alpha_0 \) and \( \Delta x \)- characterizing the energy barrier that governs the lipid bilayer rupture when assumed as two-state process with a single energy barrier for SLBs (fig. 5). Although the activation energy has been previously determined by means of AFM-FS at various velocities, AFM-FC allowed for the determination of the activation energy (\( \alpha_0 \)) as well as the distance to the transition state along the reaction coordinate (\( \Delta x \)). Finally, we are confident that this kind of study on the kinetics of lipid bilayers rupture will be extended and applied to the study of other molecular thin films, including systems of higher complexity like models mimicking cell membranes.

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**References**

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