The molecular mechanism of Nystatin action is dependent on membrane biophysical properties and lipid composition †


Phase behavior of POPC/DPPC and POPC/ESM lipid systems
To understand the factors guiding Nys partition towards and/or aggregation within the membrane in relation to the presence of ordered domains, it is necessary to know with quantitative detail the relative amount and composition of the lipid phases for the lipid mixtures under the selected conditions, i.e. the partial phase diagram 1,2. Partial phase diagrams for POPC/N-palmitoyl-SM 3 and POPC/DPPC 4,5 mixtures have been previously determined. In the present study, we further determined the phase diagram for POPC/ESM mixtures and confirmed the properties of POPC/DPPC mixtures under our experimental conditions. Figure S1 shows the variation of the anisotropy of t-PnA as a function of temperature for POPC/ESM and POPC/DPPC (Figure S1.A) mixtures. At low temperatures the anisotropy is very elevated and representative of the gel phase. Upon increasing the temperature, a steep decrease in the fluorescence anisotropy of t-PnA is observed towards values typical of the fluid phase. The temperatures at which these transitions occur can be used to plot the solidus and the liquidus boundaries of the partial binary phase diagrams of POPC/ESM and POPC/DPPC mixtures (Figure S1.B,C) 3.

The POPC/DPPC phase diagram determined in this study is in agreement with the previously published diagram for this system 5, and the POPC/ESM phase diagram resembles the one previously determined for POPC/N-palmitoyl-SM mixtures 3. The phase diagrams show a broad fluid/gel phase coexistence, which at 25°C occurs from 45 to 70 mol% of ESM (Figure S1.B) and from 38 to 91 mol% of DPPC (Figure S1.C).

Figure S1: Partial binary phase diagrams of POPC/ESM and POPC/DPPC mixtures.
(A) t-PnA fluorescence anisotropy as a function of temperature for POPC/ESM (●) and POPC/DPPC (●) 60:40 mixtures (the lines represent a non-linear fitting of a sigmoidal growth function). These data were used to determine the solidus and liquidus boundaries of the binary phase diagram for (B) POPC/ESM 3 and (C) POPC/DPPC 4,5 using experimental data (●) and complemented with literature data (○). Experimental data were corrected for the width of the transition of the pure lipids according to Mabrey and Sturtevant.6 The dotted lines are hypothetical. It is considered that there are one fluid phase (f) and two gel phases (s1, rich in POPC and s2, rich in ESM or DPPC) 3.
Determination of Nys partition towards membranes

Nys partition towards lipid membranes can be determined using the observed concomitant increase of its fluorescence emission intensity. In this way, the coefficient of Nys partition towards the membrane was calculated by fitting Eq. 1 to a plot of the intensity of fluorescence emission of Nys at a fixed concentration (ΔFI) for increasing concentration of lipid:

\[ \Delta I = \frac{\Delta I_{\text{max}} K_p [L]}{[W] + K_p [L]} \]  
(Eq. 1)

Figure S2 presents two representative curves and corresponding non-linear fit of Eq. 1 obtained for lipid vesicles composed of a mix of POPC:ESM containing 20 mol% or 50 mol% of ESM.

**Figure S2:** Nystatin partition (K_p) towards POPC/ESM membranes.

Variation of the fluorescence intensity of Nys as a function of accessible lipid concentration in POPC/ESM mixtures containing (x%) 20 mol% and (ca) 50 mol% of ESM. The data presented are from a representative experiment. The final K_p values are given in Table 1 in the main text. The dotted lines correspond to the non-linear regression fit of Eq.1 to the experimental data points and was used for obtaining K_p.

Control assays of Nys pore formation

Control experiments where only valinomycin was added to the mixtures were performed in order to assess the fluorescence emission values obtained for maximal gradient dissipation without any other gradient disturbing molecules, i.e. Nystatin.

**Figure S3:** Valinomycin-induced membrane permeabilization.

(A,C) The fluorescence intensity of entrapped Pyranine after dilution in K+ free buffer was evaluated overtime through ratiometric measurements of pyranine excited at 450 and 405 nm (450/405). Valinomycin was added at ap. 50 minutes to (A) POPC/ESM and (C) POPC/DPPC mixtures. In (A) 20, 50 and 70 mol% of ESM are represented by black, light grey and dark grey lines, respectively. In (C) 20, 50 and 90 mol% of DPPC are represented by black, light grey and dark grey lines, respectively. After (A) ap. 70 and (C) 80 minutes, triton X100 (0.1% v/v) was added to samples to recover the initial fluorescence intensity ratio. These experiments were repeated at least three independent times and the values are median representative curves of those experiments. (B,D) The percentage of gradient dissipation was calculated at different time points after the addition of valinomycin to POPC vesicles containing (B) 20, 50 and 70 mol% (black, light grey and dark grey bars, respectively) of ESM and (D) 20, 50 and 90 mol% (black, light grey and dark grey bars, respectively) of DPPC. Data are the mean ± standard deviation of at least 3 independent experiments. In all these experiments 20 and 50 mol% of ESM or DPPC correspond to 0 and 20% of gel phase, respectively. Vesicles with 98% of gel phase are represented by 70 mol% of ESM and 90 mol% DPPC.
AFM imaging of Nys-induced effects on ESM-containing membranes

Figure S4: AFM phase imaging of Nys-induced ESM-enriched expanded gel phase.
Image of a POPC/ESM 50:50 SLB obtained ~1h15 after the addition of 0.7 μM Nys, where it is possible to distinguish the gel (1), expanded gel (2) and ld (3) phases through their different viscoelastic response.

Notes and references