Supplementary Note

Membrane bending by protein-protein crowding

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Analytical model of spontaneous curvature generation by crowding pressure. We propose a simple thermodynamic model to explain how high protein coverage can drive an increase in membrane spontaneous curvature. Our results suggest that highly curved membranes can be formed and stabilized by the gain in entropy within a layer of adhered proteins as the membrane surface curves.

The free energy of the protein-coated membrane is modeled as:

$$ F(A, R, N) = F_{\text{membrane tube}} + F_{\text{prot}} = \kappa \frac{1}{2R^2} A + \gamma A + F_{\text{prot}}(\bar{A}) $$

where $A$ is the surface area of the membrane, $R$ is the radius of the tubule, $\gamma$ is the surface tension, and $N$ is the number of bound proteins. $\bar{A}$ is the area that the proteins can diffuse within, which is slightly larger than the area of the membrane tube. Specifically, $\bar{A} = 2\pi R^2 + 2\pi L(R + \sigma/2) = A(R + \sigma/2)$ where $R$ is the distance from the center of the tube to the center of a protein with height $\sigma$, which is not equal to $R$, the distance from the center of the tube to the surface of the membrane. In the second equality we have introduced the standard Canham-Helfrich form of the elastic bending energy of a membrane $^1$.

Then we take the derivative of this equation and set it to 0 to find a free energy minimum:

$$ \frac{\partial F}{\partial R} = 0 = \frac{\partial}{\partial R} \left( \kappa \frac{1}{2R^2} A \right) + \frac{\partial}{\partial R} (\gamma A) + \frac{\partial F_{\text{prot}}}{\partial A} \cdot \frac{\partial \bar{A}}{\partial R} $$

The second term on the right is zero, since $\gamma \bar{A}$ does not vary with $R$. Then we have:

$$ 0 = -\frac{2\kappa}{2R^2} A + (-p) \left( -\frac{A \sigma}{2R^2} \right) $$

Here we have used the thermodynamic definition of pressure ($p$) in two dimensions as the free energy per change in area.

This expression simplifies to:

$$ R = \frac{2\kappa}{\sigma p} $$

Therefore, the sigma appearing in this equation represents the protein height. Increasing sigma decreases the predicted tubule radius.
This relation also predicts that as pressure increases (i.e., the concentration of adhered proteins increases), the membrane spontaneous curvature will increase to provide more space for the proteins on the membrane surface. Therefore, the energetic cost of bending is accommodated by an increase of entropy in the protein layer. To estimate the pressure produced in the crowded protein layer, we approximate the proteins as non-attracting disks. We apply the Carnahan-Starling equation of state for hard disks $^{2,3}$ to calculate pressure as a function of density. In two dimensions the Carnahan-Starling equation has the following form, where $\eta$ is the fraction of membrane area covered by proteins (modeled as non-interacting disks), $k_B$ is the Boltzmann’s constant, and $T$ is temperature.

$$p = \frac{4\eta}{\sigma^2 \pi} \left[ 1 + 2\eta - \frac{0.44\eta}{(1-\eta)^2} \right] k_B T$$

Here $\sigma$ is used to calculate the area that each protein occupies on the membrane surface (i.e., the protein diameter), whereas above we used $\sigma$ to denote protein height. Thus we are implicitly assuming that these dimensions are roughly equal. The first term of this equation represents the pressure generated by non-interacting disks colliding with boundaries. This term increases linearly with coverage and is equivalent to the ideal gas equation of state. The second term represents the pressure generated by protein-protein interactions, and increases nonlinearly with coverage. Calculations of membrane spontaneous curvature, $1/R$, as a function of $\eta$, $\sigma$, were performed using GNU Octave software, where a $\kappa$ of $10k_B T$ $^4$ was used. This value for bending rigidity was chosen on the basis of data from multiple measurements on highly fluid membranes resembling the composition used in our experiments $^5$. The resulting relationship between spontaneous curvature and coverage (Fig. 3C) is highly non-linear over the range of coverage observed for tubulated membranes, indicating that surface pressure is dominated by protein-protein interactions, rather than protein-boundary interactions. These predictions illustrate how protein-protein crowding could create large surface pressures as membrane coverage by proteins increases, and is thus capable of playing an important role in bending membranes to high curvatures.
**FLIM-FRET data analysis to determine protein densities.** The determination of fluorophore densities in random distributions from Förster energy transfer has been investigated in a number of previous studies\(^6\)\(^-\)\(^8\). In two dimensions, appropriate for these membrane studies, simple analytical solutions have been derived for the case where the fluorophore labeled molecules can be considered to be points. However, in our experiments on protein crowding, the protein diameter is a substantial fraction of \(R_0\), the distance of half energy transfer, and the radial distribution of acceptors around a donor fluorophore is significantly dependent upon the protein diameter and hence surface coverage. For these reasons, the simple analytical expressions are not applicable. Therefore, we used computer simulations to determine the protein density corresponding to a measured fluorescence decay. In the computer simulations, random configurations of proteins with diameter of 4.3 nm (corresponding to the ENTH domain) were created around a central donor labeled molecule at a series of protein densities. The protein configurations were relaxed by Monte Carlo diffusion until the radial distribution function converged. For each configuration the decay was calculated by computing the total energy transfer rate to all acceptors, and from that rate, the probability, \(p(t)\), of donor excitation as a function of time.

\[
p(t) = \exp \left[ -\frac{t}{\tau} \left( 1 + \sum_n \left( \frac{R_0}{R_n} \right)^6 \right) \right]
\]

Here \(t\) is the donor fluorescence lifetime in the absence of acceptors and \(R_n\) is the distance from the central donor to an individual acceptor. We used values of \(R_0\) and \(\tau\), as provided by the dye manufacturer. To generate a decay characteristic of a particular density, decays from 3000 individual configurations were averaged. Dye-labeling efficiencies less than unity were accommodated by randomly excluding a fraction of the proteins in a configuration from the energy transfer rate calculations. The calculated decay at each density was convoluted with a Gaussian representation of our instrument resolution function to produce a list of simulated decays as a function of total protein density. Experimentally obtained donor fluorescence decay curves were fit to obtain the protein density by scaling the simulated decays to the area of the experimental curve and finding the minimum sum of squared error from the list of simulated
decays. This procedure produces excellent fits to the experimental data; representative examples are shown in Figure S6.

**Fluorescence intensity fluctuation analysis to calibrate protein densities measured using FLIM-FRET.** In order to test the accuracy of FLIM-FRET measurements of protein density on membrane surfaces, we applied an intensity based method, using fluorescence fluctuation spectroscopy. The method is based on statistical analysis of fluorescence intensity (photon counts per unit time). Because it is extremely sensitive to the position of the sample surface with respect to the focal plane of the objective it is not a viable option for measuring the density of fluorescent proteins on the rapidly fluctuating surfaces of giant vesicles. However, we were able to directly compare it to our FRET-FLIM method by performing both measurements on the surface of stationary supported lipid bilayers (flat membrane surfaces). These measurements are conceptually fairly simple. We take a fluorescence lifetime image of a supported bilayer containing DOGS-NTA-Ni lipids saturated with our FRET labeled mixture of hisENTH. From this image the density is determined using our FRET analysis as described above. On an identical supported bilayer we take a confocal fluorescence image of a very dilute (nearly resolving single molecules) sample of singly labeled hisENTH. From this image the sample density can be determined using fluorescence fluctuation analysis. We then take an image of another identical bilayer saturated with the singly labeled hisENTH. The protein density on this saturated sample can be determined by comparing its intensity to the dilute sample, accounting for self-quenching on the dense sample. The saturated sample density measured in this way can be compared directly to the FRET based measurement.

To implement our approach to comparing sample density measurements we prepared supported lipid bilayers containing 4 or 7.5 mol% DOGS-NTA-Ni lipids mixed with DPhPC lipids were formed using existing protocols and a 8.5:1 ratio of acceptor (Atto 594) and donor (Atto 532) labeled hisENTH proteins. Next, FLIM-FRET measurements of saturated protein density were performed on these surfaces using the same protocol described above for giant vesicles. Then, another supported lipid bilayer of
identical composition was formed and dilute and saturated densities of singly labeled proteins were
determined. This procedure has several steps. (i) A low coverage of proteins on the membrane surface
was created by exposing the surface to a total protein concentration of 2.5 nM of Atto532 labeled
hisENTH. The average fluorescence lifetime was measured on these surfaces. (ii) On the same low
coverage membrane, a confocal fluorescence image of the sample surface was measured with photon
counting using a cooled GaAsP pmt (Hamamatsu). From this data, the mean, <k>, and variance, <\Delta k^2>,
of the pixel intensities (counts in a 2 ms dwell time) were computed. These values were used to calculate
the average number of proteins in the laser focus using the expression,

\[
\langle N \rangle = \frac{0.5 \langle k \rangle^2}{\langle \Delta k^2 \rangle - \langle k \rangle^{0.11}}
\]

The concentration of proteins on the surface is then

\[
C_{\text{dil}} = \frac{2\langle N \rangle}{\pi \omega_0^2}, \text{ where } \omega_0 \text{, the } 1/e^2 \text{ radius of the focused}
\]

laser beam was 200 nm based on a bead measurement calibration. (iii) Next, the protein concentration
was raised to 400 nM, saturating the binding sites on the supported membrane surface with bound
proteins. The resulting count rate was very high requiring an increase in attenuating filter OD in the
excitation beam. The mean of the pixel photon counts <k>_{sat} was recorded for these saturated surfaces.
(iv) The saturated protein concentration, uncorrected for fluorophore self-quenching, was calculated by
scaling the dilute protein concentration with the ratio of filter OD and photon count rates, using the
expression

\[
C_{\text{sat,uncorr}} = \left( \frac{OD_{\text{sat}}}{OD_{\text{dil}}} \right) \left( \frac{\langle k \rangle_{\text{sat}}}{\langle k \rangle_{\text{dil}}} \right) C_{\text{dil}}.
\]

(v) The fluorescence lifetime measured on the
saturated membrane surfaces was significantly lower than on the dilute surfaces due to self-quenching
(homo-FRET) between the closely spaced fluorophores. Therefore the protein concentration was
corrected for self-quenching using the expression

\[
C_{\text{sat}} = C_{\text{sat,uncorr}} \left( \frac{\tau_{\text{dil}}}{\tau_{\text{sat}}} \right).
\]

The resulting value was compared with the saturated concentration value measured by FLIM-FRET (Supplementary Figure S3b).
The values measured in this analysis were as follows. For membranes containing 4 mol% DOGS-NTA-
Ni lipids, OD_{sat}=5.5, OD_{dil}=3.1, \langle k \rangle_{dil}=19, \langle \Delta k^2 \rangle_{dil}=457, \langle k \rangle_{sat}=69, \tau_{dil}=2.15 \text{ ns, } \tau_{sat}=1.24 \text{ ns, } C_{\text{sat, intensity}}=0.0103 \text{ proteins/nm}^2 \text{ (16.5\% hisENTH coverage), } C_{\text{sat, FLIM-FRET}}=0.0125 \text{ proteins/nm}^2 \text{ (20\%}
hisENTH coverage). For membranes containing 7.5 mol% DOGS-NTA-Ni lipids, OD<sub>sat</sub>=5.5, OD<sub>dil</sub>=3.1, 
<k><dil>=19, <Δk><dil>=457, <k><sat>=71, τ<sub>dil</sub>=2.15 ns, τ<sub>sat</sub>=0.67 ns, C<sub>sat, intensity</sub>=0.0189 proteins/nm<sup>2</sup> (30.2% hisENTH coverage). C<sub>sat, FLIM-FRET</sub>=0.0180 proteins/nm<sup>2</sup> (28.8% hisENTH coverage). Based on the variations in densities calculated from different images and consideration of other potential sources of uncertainty in the measurements we estimate the overall uncertainty in the density determinations to be +/-20%.

References