Protein over-expression induces the elongation of cell membrane nanodomains

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In cell membranes, lipids and proteins are organized into sub-micrometric nanodomains of varying size, shape and composition, performing specific functions. Despite their biological importance, the detailed morphology of these nanodomains remains unknown. Not only can they hardly be observed by conventional microscopy due to their small size, but there is also a lack of models to describe their structuring. Here, we use a combination of analytical calculations and Monte Carlo simulations to show that increasing protein concentration leads to an elongation of membrane nanodomains. The results are corroborated by Single Particle Tracking measurements on HIV receptors, whose level of expression in the membrane of living cells can be tuned. These findings highlight that protein abundance modulates nanodomain shape and potentially their biological function. Beyond biomembranes, this meso-patterning mechanism is of relevance in several soft-matter systems because it relies on generic physical arguments.

The plasma membrane forms a hydrophobic barrier to separate the interior from the exterior of cells and a two-dimensional fluid matrix for proteins. Its constituents are unevenly distributed and form numerous domains whose composition and physical properties are different from the average membrane properties. A number of studies have reported the existence of nanodomains involved in biological functions such as signal transduction1, regulation of membrane trafficking2 or infectious processes3,4. As a result, the entire membrane might be considered as an assembly of nanodomains5,6. Over the past twenty years, super-resolution microscopy techniques among which Single Particle Tracking (SPT) have revealed that these nanodomains are structures with dimensions ranging from a few to several hundred nanometers7. SPT allows to follow the dynamics of individual molecules in living cells with unique accuracy over dozens of seconds (for review see9,11). While the behavior of a single trajectory might be stochastic, the statistical behavior of many long trajectories provides insight on properties such as membrane receptor activation, assembly or dissociation of signalling clusters, or protein reorganization due to virus interactions12–16. One point that caught our attention is that some nanodomains show more elongated contours when membrane proteins are over-expressed: Merklingler et al. revealed by super-resolution microscopy that increasing the expression level of syntaxin induces clustering and elongation of the nanodomains in which it is confined17 (compare Fig. 5 and 5S therein). In addition, in 2003 we had observed by SPT the existence of crescent-shaped nanodomains containing the µ-opioid receptor (unpublished data from12, see Fig. S4) that remained unexplained until now.

To analyze the impact of protein expression level on nanodomain elongation, we adopt in parallel theoretical and experimental approaches. We performed mesoscale numerical simulations, as in Ref.18, where a vesicle made of two species having different spontaneous curvatures is simulated with a Monte Carlo (MC) algorithm coupling the composition and the membrane elasticity. Below the critical temperature, it leads to the formation of nanodomains, instead of a macrophase separation. As a function of the parameter values, we observe the formation of elongated nanodomains. These simulations are guided by analytical calculations indicating the range of physical parameters entering the numerical model for which the elongation mechanism occurs. In parallel, we performed SPT experiments (Fig. 1) on three trans-membrane proteins involved in HIV infection: CD4, CCR5 and CXCR43,19. It has been recently shown that their distribution at the surface of the target cells is heterogeneous2024, and proposed that membrane nanodomains, by concentrating the receptors, would favor virus entry into the target cells3,2526. We have used the 293-Affinofile cell line to record the receptor trajectories at different expression levels. These cells, in which CD4 and CCR5 expression can be independently induced27, were further transduced to stably express low or high level of CXCR4. We have taken ad-
vant of these cell lines to record the trajectory of these proteins at different expression levels. Our observations reveal that an elongation of membrane nanodomains occurs upon protein over-expression.

To understand for which parameter values this elongation occurs, we first study analytically the shape of one membrane nanodomain in thermodynamical equilibrium. Generically, it is now understood that its stability comes from the action of two competitive interactions \[28\]. On the one hand, a short-range attraction between components of the same nature and/or repulsion between components of different nature \[29, 30\] gives rise to a line tension at the boundary between two phases. On the other hand, a mid-range repulsive interaction due to the coupling between membrane composition and membrane elasticity and fluctuations prevents macrophase separation \[31\]. We study for which range of the physical parameters an elongated shape is more stable than a circular one. The model considers generically an elliptic nanodomain of semi-axes \(r_0 a\) and \(r_0 a\), with \(a \geq 1\), so that the ellipse has area \(A = \pi r_0^2 a\). Its aspect ratio \(AR\), defined as the ratio of major to minor axes, is \(AR = a^2\). The total energy of the system is \(E_{tot}(a) = E_{bulk} + E_{rep} + E_{line}\) where \(E_{bulk} \propto r_0^2\) is the cohesive energy of the nanodomain due to inter-molecular short-range forces, and is simply proportional to its area. \(E_{bulk}\) does not depend on \(a\) and will be skipped in the following calculations. The repulsion energy between the membrane components inside the nanodomain is supposed to be pairwise and to have a finite range \(\xi\):

\[
E_{rep} = E_0 \int_{A \times A} \rho(r) \varphi \left( \frac{|r - r'|}{\xi} \right) \rho(r') d\mathbf{r} d\mathbf{r}'
\]

where \(\varphi\) is the interaction potential, and \(\rho(r)\) the particle density inside the nanodomain. The parameter \(E_0\) sets the strength of the repulsion. In the case where this repulsion energy comes from the coupling with the membrane elasticity, the molecules forming the nanodomain induce a spontaneous curvature \(C_0\) different from the average one (assumed to be 0 for simplicity sake). It has been shown that the screening length is \(\xi = \sqrt{\kappa/\sigma}\) and that \(E_0 \propto \sigma C_0^2\) in the low tension limit \[18, 32\]. Here \(\kappa\) and \(\sigma\) are respectively the membrane bending modulus and surface tension. In principle, the function \(\varphi\) decays exponentially at long distances, being for example a Bessel function. In order to get an analytically tractable model, we assume it to be the Gaussian \(\varphi(|r - r'|/\xi) = \exp[-(|r - r'|^2/(2\xi^2)], which is sufficient at the scaling level. We also suppose that \(\rho(r) = \frac{1}{2} \exp \left[ - \frac{1}{2} \left( \frac{r_x^2}{r_0} + \frac{r_y^2}{r_0} \right)^2 \right] \) is a Gaussian density with \(r = (x, y)\). The prefactor 1/2 ensures \(\int_{R^2} \rho(r) d^2r = \pi r_0^2\). Introducing the dimensionless repulsion length \(\ell = \xi/r_0\), one gets \(E_{rep}(a) = \pi^2 E_0 r_0^2 f(a)\) where

\[
f(a) = \frac{\ell^2}{\sqrt{\ell^2 + 2a^2} \sqrt{\ell^2 + 2/a^2}}
\]

The line energy reads \(E_{line} = \lambda P\) where \(\lambda\) is the line tension. The circumference \(P\) of an ellipse of semi-axes \(\alpha\) and \(\beta\) is given by the elliptic function. However, a very good approximation by Ramanujan is \(P \approx \pi \left[ 3(\alpha + \beta) - \sqrt{(3\alpha + \beta)(\alpha + 3\beta)} \right]\), thus \(E_{line}(a) = \pi \lambda r_0 g(a)\) with

\[
g(a) = \left[ 3 \left( \frac{a + 1}{a} \right) - \sqrt{\left( \frac{3a + 1}{a} \right) \left( \frac{3a + 3}{a} \right) \left( \frac{3a + 3}{a} \right) \left( \frac{3a + 1}{a} \right) \left( \frac{3a + 3}{a} \right) \left( \frac{3a + 1}{a} \right) \right] \right.
\]

Fig. 2 (left) shows how \(E_{tot}/(\pi r_0 \lambda) = \pi \varepsilon f(a) + g(a)\) behaves in function of \(a\) for various values of \(\ell\), where we have introduced the new dimensionless parameter

\[
\varepsilon = \frac{E_0 r_0^3}{\lambda}
\]

measuring the relative strengths of repulsion and line energies. One observes that there is a range of values of \(\ell\) for which \(a = 1\) is not an energy minimum. It implies that there exist values of the repulsion length \(\xi\) for which the most stable nanodomain shape is an ellipse \((a > 1)\), and not a disc \((a = 1)\). Their relative stability can be addressed by examining the behavior of \(E_{tot}\) close to \(a = 1\). Expanding \(f\) and \(g\) at order 2 \[31\]

\[
f(a) \approx f(1) - \frac{4\ell^2}{(2 + \ell^2)^2} (a - 1)^2 + g(a) \approx g(1) + \frac{3}{2} (a - 1)^2.
\]

Introducing \(A_\ell = 4\ell^2/(2 + \ell^2)^3\), it follows that

\[
\frac{1}{2} \frac{d^2 E_{tot}}{da^2} = -\pi^2 E_0 r_0^2 A_\ell + \frac{3}{2} \pi \lambda r_0
\]

Ellipses are stable when \(\frac{d^2 E_{tot}}{da^2} < 0\), i.e. \(A_\ell > \frac{3}{2 \ell^4}\); \(A_\ell\) has a maximum \(A^* = \frac{8}{27\pi}\) at \(\ell^* = 2\). There exists a region of stability of ellipses if \(A^* > 3/(2 \pi \varepsilon)\), i.e. \(\varepsilon > \frac{81}{16\pi^2}\).

Since the numerical prefactors in our expressions come from the choices of repulsive potential Gaussian shape \(\varphi\) and Gaussian density profiles in the nanodomain, we simplify the principal results as follows: (i) Elliptic domains are stable for \(E_0 r_0^3 > \lambda\), i.e. for strong enough repulsion strength \(E_0\), large enough domain radius \(r_0\) or weak enough line tension \(\lambda\). (ii) If this condition is satisfied, \(\ell = \xi/r_0\) must belong to an interval \([\ell^*, \ell^*]\) (see Fig. S9) distributed around the maximum abscissa \(\ell^*\) of order unity to stabilize ellipses with respect to discs. If the repulsion range is too short as compared to the domain radius, repulsion is not strong enough to destabilize domains. If it is too long, the gain in elongating the domain cannot compensate the line energy cost. (iii) The stability of ellipses requires that the repulsion range \(\xi\) and the cluster radius \(r_0\) are on the same order of magnitude.

In the context of protein nanodomains, \(E_0 \approx \sigma C_0^2\) and we use the realistic values \(\sigma = 10^{-4} J/m^2\), \(C_0 = 0.05 \text{ nm}^{-1}\), \(\lambda = 1 \text{ pN} \ [28]\), and \(r_0 = 200 \text{ nm}\) (see below). Then \(\varepsilon = E_0 r_0^3/\lambda > 1\) and \(\pi r_0 \lambda \approx 150 \text{ kBT} \ [24]\). Hence our scaling law shows that for realistic parameter values, circular domains can become unstable even at the sub-micrometric scale in the cell membrane context.
To confirm these results, we perform MC simulations, where we also observe elongated nanodomains, in particular when increasing concentration of the component forming the domains. We use a vesicle model, the discretized version of a continuous biphasic membrane model, developed in Ref. [18]: a lattice-gas model, with Ising interaction parameter $J_I$, describes the binary mixture of two species A and B. It is coupled to a discretized Helfrich model accounting for the membrane elasticity, where the local spontaneous curvature $C$ depends linearly on the composition. In these simulations, the species A can be considered as a phase containing particular lipids and/or some membrane proteins. It has a spontaneous curvature $C_A$ different from the majority phase (species B with $C_B = 2/R < C_A$ where $R$ is the average radius of the vesicle). We chose $c_1 = R(C_A - C_B) = 10$ [33]. Besides, we observe in the experimental domains as shown in Fig. 1 small boundary fluctuations. This implies that the line tension $\lambda$ of the domain boundary has to be high enough and thus that the interaction parameter $J_I \equiv J_I/\left(\kappa_B T\right)$ is significantly larger than its critical value $J_{I,c} = \ln(3)/4 \simeq 0.27$ for an hexagonal lattice. Therefore we focus on the value $J_I = 0.5$. We then run simulations with a typical value $\kappa = 20 \kappa_B T$ and a dimensionless surface tension $\tilde{\sigma} = \sigma R^2/\left(\kappa_B T\right) = 300$, which corresponds to quasi-spherical vesicles. [33], and study a rather low A-species concentration $\tilde{\phi} = 0.20$ versus a higher one $\tilde{\phi} = 0.35$. We run long simulations, up to $3 \times 10^{10}$ MC steps on 2562 vertices to have good enough statistical sampling [18]. To measure the AR of domains lying on a quasi-spherical surface, we project each of them onto the plane tangent to the average sphere at the domain center of mass and to compute domain covariance matrix with its in-plane coordinates $(x, y)$. The AR is then simply the ratio of the square roots of its two eigenvalues (see SM).

Fig. 2 (middle) shows obtained AR distributions. We note that the curves intersect at $AR_0 \simeq 2$. Domains with an $AR \leq 2$ (respectively $AR > 2$) are thus called roundish (resp. elongated). The increase in concentration leads to an increase in the proportion of elongated domains from 28% to 40%. Additional values are provided in the SM. We also measure the typical cluster sizes. As expected [18], the domains for $\tilde{\phi} = 0.35$ have a larger typical size than the ones at $\tilde{\phi} = 0.2$ (Fig. S11). The average cluster size growing when $\tilde{\phi}$ is increased, more clusters fulfill the condition $r_0^2 > \lambda/E_0$ and therefore become elongated as predicted by the analytical model. Now we estimate the parameter $\varepsilon$. We get $\varepsilon \sim 0.01 \text{ pN}$ from the value of $J_I$ and a vesicle radius $R = 10 \mu m$ [54]. The surface tension has been estimated to be $\sigma \sim 10^{-8} \text{ J/m}^2$ with those parameters and the domain curvature is $C_A = (c_1 + 2)/R \sim 1 \text{ \mu m}^{-1}$ [34]. The observed domain radius $r_0$ is about 2 $\mu m$. It follows that $\varepsilon = E_0 r_0^2/\lambda \approx 10$, consistently larger than 1. In the SM, we are led to similar conclusions by exploring a second numerical model where proteins are represented as point-like objects as described in Ref. [35].

| Number of Proteins | Low Expression | High Expression |
|--------------------|---------------|----------------|
| CD4                | 2800 ± 300    | 100000 ± 13000 |
| CCR5               | 3600 ± 800    | 125000 ± 8000  |
| CXCR4              | 15000 ± 2000  | 1200000 ± 7000 |

Finally, we present our observations by SPT on HIV cells. Starting from the cell line described by Johnston and collaborators [27], we have generated stable cell lines presenting a high or a low number of copies of the receptor CXCR4. Since the expression of partner receptors CD4 and CCR5 can be simultaneously and independently controlled in these cell lines, we were able to generate cells presenting any possible combination of protein expression at their surface (Table I, Figs. S1 and S2). Note that this model is relevant regarding HIV infection since,
whatever the expression level of the three proteins, the cells can be infected by HIV-1 strains requiring CCR5 or CXCR4 (see Fig. S3) [36, 37]. We have performed SPT experiments to track these different proteins in different conditions. The collected data give the positions of the tracked proteins every 40 ms for ≥ 30 s. 482 individual trajectories have been acquired with different expression levels of each protein. For each, we have isolated the confined parts of the trajectories thanks to the confinement index of Ref. [38]. We have then measured the sizes and shapes of these confinement zones. If we again define the radius \( r_0 \) through the ellipse area \( A = \pi r_0^2 \) (see analytical model), we find the typical values \( r_0 \simeq 150 \text{ nm} \) (respectively 200 nm) in the low (resp. high) expression state. Details are given in Table S1.

We compare in Fig. 2 (right) the cumulated data of all the studied trajectories when the 3 proteins have a low expression level vs. high expression level. In the Supplemental Material (SM), we explain that some confinement zones with AR \( \leq 2 \) are however classified as elongated because they are in fact coiled elongated nanodomains. They appear as peaks in Fig. 2 (right), to be compared to Fig. S5. In over-expressed conditions, the AR distribution is significantly shifted towards higher values and the proportion of elongated nanodomains raises from 47% to 61%. Then, we have compared the behavior of each protein individually in a low or high expression context. Fig. S7 also shows that there is a neat increase of the proportion of elongated nanodomains regardless of the protein being considered. To go further, we have focused on the single-spanning CD4 and seven-spanning CCR5 receptors that have been abundantly studied in the literature [25, 39, 41]. We have analyzed changes of the shape of the confinement nanodomains of each of these proteins when it is the only one to be over-expressed. As shown in Fig. S8, we have observed that over-expression of the sole CCR5 protein is accompanied by a strong increase of the proportion of elongated nanodomains (33% to 76%) while for the CD4 protein, we have observed a slight increase of the proportion of elongated nanodomains (46% to 52%) that is however statistically significant.

This work thus combines theoretical and experimental approaches to show that increasing the concentration of the minority phase can lead to a noticeable elongation of nanodomains in membranes, as already observed experimentally [17] or in numerical simulations [42]. However, to our knowledge, this effect had never been quantified so far. A simple physical mechanism in thermodynamic equilibrium can be put forward to explain why elongated nanodomains are more stable than roundish ones under favorable circumstances. When \( \phi \) grows, nanodomains become more and more numerous with a growing typical size \( r_0 \) [18, 33]. However, too large domains are intrinsically unstable, because of the effective long-range repulsion due, for example, to membrane deformation induced by the spontaneous curvature of the domain constituents.

One way of dealing with this instability is to generate more elongated nanodomains above a critical size, in which the repulsive energy (of magnitude \( E_0 \)) is lower, at the price, however, of a higher line energy, proportional to the line tension \( \lambda \). More quantitatively, we propose a scaling argument, which writes \( E_0 r_0^3 > \lambda \), predicting when circular domains become unstable at the benefit of elongated ones, provided that the typical domain size \( r_0 \) must be comparable to the range \( \xi \) of the repulsion. In our experiments, we have measured \( r_0 \approx 150 \text{ nm} \) when receptors are over-expressed which indeed corresponds to the realistic value \( \xi = \sqrt{\kappa/\sigma} \approx 100 \text{ nm} \) in cells [28].

Pointing out the analogy between experimental and numerical nanodomain morphologies implicitly assumes that a curvature-composition coupling mechanism is at play in the case of HIV receptors nanodomains, at least for CCR5 and CXCR4, two class-A G-Protein Coupled Receptors (GPCRs). The spontaneous curvature induced by class-A GPCRs has very recently been investigated in detail in live cells [44]. A spontaneous curvature of about 0.04 nm\(^{-1}\) is deduced, presumably related to the crystal structures of those GPCRs that reveal their transmembrane part to be up-down asymmetric across the bilayer. This spontaneous curvature is typically in the range of values that can promote sub-micrometric nanodomains, as expected [28]. To our knowledge, no such measurements have been performed on CCR5 or CXCR4. But they belong to the same class A [19] and thus share structural similarities with those of Ref. [44]. The effect of over-expression is less marked for CD4. CD4 has only one transmembrane segment and the impact of over-expression of this protein on the local membrane curvature should indeed be less important than for CCR5 which has seven transmembrane segments. The present work then shows that the accumulation of proteins into nanodomains can conduct to a change of the morphology of these nanodomains. This could probably be extrapolated to other membrane proteins since such “untypical” nanodomain shape has already been observed with other proteins in different cell types without being explained.

Domain elongation thus reveals local accumulation of specific proteins in the cell membrane. Such an effect, influencing biological processes as HIV entry [45, 46], can be revealed by SPT thanks to its unique performances. From a soft-matter physics viewpoint [47], we conjecture further that elongation of nanodomains is the signature of the transition between 2D hexagonal and lamellar phases, where the minority phase transforms into parallel stripes [48]. Indeed, in phase diagrams ensuing from approximate calculations, a region of coexistence between these two phases was identified [49] that might contain the elongated nanodomain stability region, as numerical simulations suggest it [42, 50]. Refined calculations will be necessary to get a full understanding of meso-patterns in the future.
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On the other hand, if $c_1$ is too large the domains get as small as the lattice spacing.

[54] In Ref. [54], it is explained how renormalization group methods allow one to relate $\lambda$ to $R$ and $J_I$. 