Cluster phases of membrane proteins

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A physical scenario accounting for the existence of size-limited submicrometric domains in cell membranes is proposed. It is based on the numerical investigation of the counterpart, in lipidic membranes where proteins are diffusing, of the recently discovered cluster phases in colloidal suspensions. I demonstrate that the interactions between proteins, namely, short-range attraction and longer-range repulsion, make possible the existence of stable small clusters. The consequences are explored in terms of membrane organization and diffusion properties. The connection with lipid rafts is discussed, and the apparent protein diffusion coefficient as a function of their concentration is analyzed.

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I. INTRODUCTION

The question of membrane functional organization is a key issue in modern cell biology [1–10]. The central problem is to establish the relationship between the (dynamical) organization and functions of the different constituents of the membranes. In this context, many microscopy techniques are implemented to have access, with the highest possible spatial and temporal resolutions, to the distribution and dynamics of membrane proteins and lipids, in particular to their diffusive properties in connection with their crowded environment.

There exist a large variety of situations, in live cells [1,3,8,10–13] or in model membranes [4,14], where membrane proteins have been found in oligomers or small clusters using these techniques. This colocalization is supposed to facilitate encounters of partners in different processes, such as complexes involved in signal transduction [1–3]. Lipid rafts [2,15,16] are usually invoked to account for colocalization. These membrane submicrometric domains enriched in certain lipids (e.g., cholesterol and sphingolipids) are supposed to recruit proteins having a higher affinity for their composition. They are believed to ensue from a lipidic microphase separation [2,15,16]. However, a consensus has not yet been reached to explain why the separation process stops and domains remain size limited [16], and attempting to understand the relationship between these structural patterns and diffusion of their constituents remains topical [1,2,5,7,12].

At the same time, there is an increasing interest in colloid science for systems presenting a cluster phase. It is the fruit of a competition between a short-range attraction (e.g., a depletion interaction) which favors clusterization, and a longer-range repulsion (e.g., electrostatic) which prevents a complete phase separation, because when clusters grow their repulsion also grows and the repulsion barrier can no longer be passed by thermal activation. The result is an equilibrium phase with small clusters of concentration-dependent size. It has been suggested [17] that the existence of cluster phases is not a singular behavior in a specific system. They indeed occur in different physical systems (e.g., colloids, star polymers, proteins) and for a large variety of interactions [17–25]. Such patterns have already been experimentally observed or simulated in two dimensions (2D) [14,23,25,26].

I propose, as an alternative paradigm, to reinterpret the aggregation of membrane proteins in terms of two-dimensional cluster phases, because membrane proteins, like colloids, interact with energies of the order of magnitude of the physiological thermal energy k_BT ($T \approx 310$ K) [2,27–30]. I discuss in this framework the mechanism driving the formation of so-called rafts. I propose that proteins spontaneously congregate in small clusters instead of constituting a "gas" of independently diffusing inclusions; they promote the formation of nanodomains in membranes. The submicrometric, limited size of these nanodomains now appears naturally in this scenario, as a result of the competition between attraction and repulsion. By contrast with the lipid raft scenario where domains result from a lipidic microphase separation and then recruit specific proteins, the present mechanism proposes that domain formation is mainly driven by protein interactions. Recent experiments in live cells support the point of view that protein-protein interactions are necessary to induce clustering, whereas lipids alone are not sufficient in the system studied [13]. I also relate the protein diffusive properties as a function of their concentration to the limited size of clusters, itself depending on concentration. I demonstrate that the mean long-term diffusion coefficient D of proteins decreases when the mean cluster size grows: $D \propto 1/\langle n \rangle$ where $\langle n \rangle$ is the mean cluster size (its number of proteins). I also anticipate that $\langle n \rangle$ grows with the protein concentration ϕ [14]. Thus D also depends on ϕ [12]. From this point of view, contact is made with prior experiments, where D was found to decrease significantly when ϕ increases [31] or that were interpreted by appealing to such a behavior [5]. I propose a simple scenario leading to the law $D \sim 1/\phi$, requiring that $\langle n \rangle \propto \phi$. Previous attempts to address this issue, based on low-ϕ expansions of D, are not adapted to catch the physical mechanisms capable of accounting for this behavior [32]. At the end of this paper, I propose experiments to validate this scenario definitely.

II. SHORT- AND LONGER-RANGE INTERACTIONS

There exist several nonspecific short-range attractive forces between proteins embedded in membranes, each with a range of a few nanometers and a binding energy of order

k_BT. They first of all consist of a depletion interaction due to the 2D osmotic pressure of lipids on proteins, which tends to bring them closer when they are about a nanometer apart [27]. There also exist hydrophobic mismatch interactions between proteins, the hydrophobic core of which does not match the width of the membrane [2]. The energy cost of the subsequent membrane deformation increases with the distance between two identical proteins, thus resulting in an attractive force. The energy scale is of order k_BT [29]. In membranes with several lipid species, another scenario leads to attractive forces: proteins recruit in their neighborhood lipids which best match their hydrophobic core. The closer the proteins, the more energetically favorable the configuration. Binding energies are also of order k_BT or larger [2,30]. A protein-driven mechanism for domain formation invoking such forces has been proposed [2,30], but the limited domain size due to additional repulsive forces has not been discussed in this context.

Membrane inclusions are also affected by nonspecific longer-range repulsive forces. Electrostatic repulsion between like charged proteins is usually considered as negligible because it is screened beyond a few nanometers in physiological conditions: at physiological ionic strength $I_\varphi \sim 0.1M$, the Debye screening length is of the order of 1 nm [33]. Only proteins with (unreasonable) charges of several hundreds of elementary charges can give a repulsion of a fraction of k_BT at 10 nm. By contrast, there exist repulsions due to the elastic deformation that proteins impose on the membrane when they are not, strictly speaking, cylindrical inclusions but conical ones, or peripheral proteins [28]. For example, using the formulas of this reference for transmembrane proteins with a moderate contact angle of 10°, one finds that the repulsive energy barrier at 10 nm is 0.10 k_BT for a typical bending rigidity $\kappa=100\ k_BT$. For instance, rhodopsin has a contact angle larger than 10° [34].

Thus the ingredients for the existence of cluster phases are present in assemblies of membrane proteins, and cluster phases should generically exist in cell membranes. Below, I shall take a typical binding energy of $-4\ k_BT$ [18] and an energy barrier of 0.1 k_BT at about 10 nm.

III. MONTE CARLO SIMULATIONS

To test the relevance of this mechanism, I have performed Monte Carlo simulations of systems of $N=100$ particles ($N=200$ for the highest density considered $\phi=0.1$), interacting via pairwise potentials displaying a hard-core repulsion, a short-range attraction, and a longer-range repulsion [25] (see Fig. 1). I have chosen physically and biologically relevant parameters as justified above. I have observed a strong robustness of the cluster phase with respect to the potential shape. I have tested repulsive terms decaying linearly, algebraically (as $1/r^2$ or $1/r^4$ [28]), or exponentially [22] with the distance r between molecules, as well as attractive ones varying exponentially or linearly with r. In all cases, a cluster phase exists at equilibrium (i.e., after very long runs) for wide ranges of parameters. Clusters coexist with a gas of monomers, of density depending on the potential and (weakly) on the density ϕ (as observed experimentally in Ref. [14]). What is important is not the precise shape of the potential but the existence of a short-range attraction of a few k_BT and of a longer-range, weaker repulsion extending over a range larger than the typical cluster diameter. Therefore I have focused on a potential shape already studied in detail [22,23,25]:

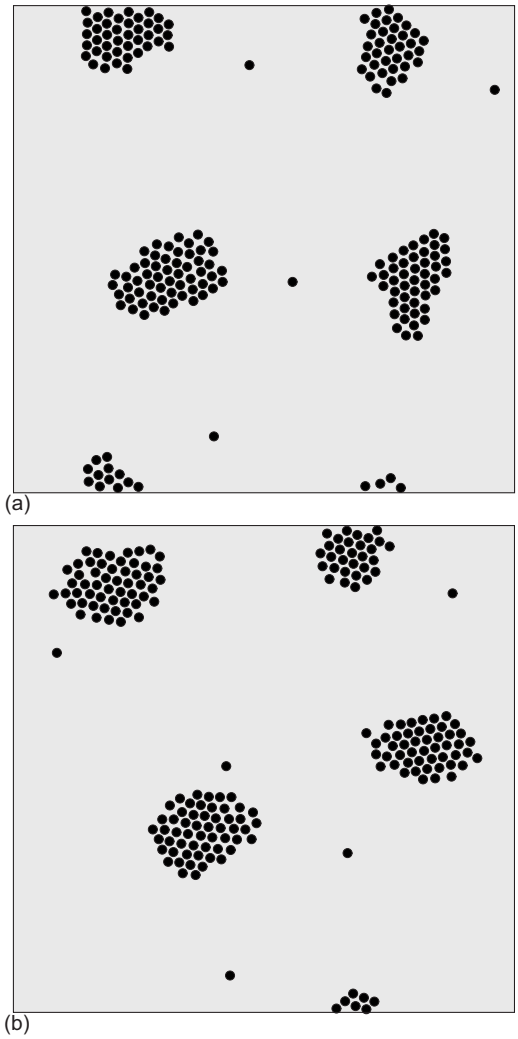

FIG. 1. Two snapshots of the cluster phase at $\phi=0.1$ ($N=200$ proteins, box side $a=0.25\ \mu$m, periodic boundary conditions). The time delay between the two snapshots is 0.5 ms [(a) $t=31.0$ ms, (b) $t=31.5$ ms]. Clusters diffuse slowly and appear nonrigid, deformable, and fluctuating.

$$U(r) = -\varepsilon_a \exp(-\gamma_a r) + \varepsilon_r \exp(-\gamma_r r). \quad (1)$$

The parameters are chosen so that, as required above, the binding energy between two proteins is $-4\ k_BT$ and the energy barrier is 0.1 k_BT. The following values satisfy this requirement: $\varepsilon_a=32\ k_BT$, $\varepsilon_r=0.3\ k_BT$, $1/\gamma_a=2$ nm, and $1/\gamma_r=16$ nm. In spite of the high value of ε_a, the binding energy is low because the attractive part is cut at $r=d_0$ due to the hard-core repulsion (inset of Fig. 2). This hard-core diameter is chosen as $d_0=4$ nm, the typical diameter of a protein of average molecular weight [14]. The proteins are given a "bare" diffusion coefficient $D_0=1\ \mu\text{m}^2/\text{s}$ (the diffusion co-

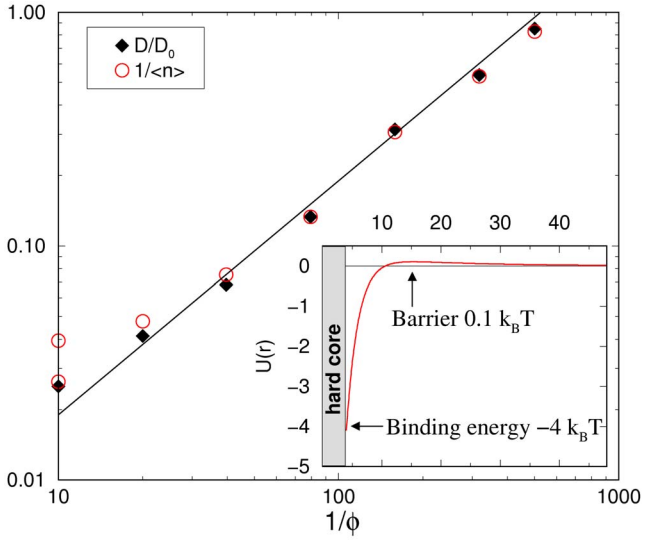

FIG. 2. (Color online) Diffusion coefficient D/D_0 (diamonds) and inverse mean cluster size $1/\langle n\rangle$ (circles) as functions of the inverse density $1/\phi$, in log-log coordinates, for the potential U discussed in the text (see [36] for $\phi=0.1$). The full line has slope 1, for comparison. Inset: the potential $U(r)$ (in units of k_BT; r in nanometers).

efficient at vanishing concentration) [31,35]: at each Monte Carlo step (MCS), a randomly chosen protein attempts to move a distance δr forward in a randomly chosen direction; Here $\delta r=1$ Å $\ll d_0$. With this δr, the acceptation rate of MCSs is larger than 60%, even at the highest densities considered; The time step between two MCSteps is $\delta t=\delta r^2/(4D_0)=2.5$ ns. A Monte Carlo sweep corresponds to N MCSs. The simulation time is chosen so that error bars on D and $\langle n\rangle$ are smaller than 10% (more than 10^7 sweeps, i.e., 30 ms of real time). The protein average long-term diffusion coefficient D is measured at different concentrations $\phi=Nd_0^2/a^2$ (a is the size of the box with periodic conditions in which the proteins diffuse). By long term is meant processes occurring at time scales larger than the time needed to diffuse inside the clusters (typically 0.1 ms). The measures are performed after a long equilibration period. To be sure that equilibrium has indeed been reached, I simulate two systems with initial configuration chosen as (a) a random one where proteins later coalesce to form clusters; (b) a completely condensed state where all proteins belong to the same big cluster which later splits into smaller ones and gas. Equilibrium is considered to have been reached when both systems (a) and (b) are qualitatively identical (same number of multimeric clusters). The Monte Carlo time needed is generally shorter than 10^7 sweeps (however, see [36]). Note that the time needed to reach equilibrium in (a) is rather long because, after a transient period where small clusters appear via a binodal-like decomposition, larger clusters are formed by evaporation of the smaller ones. Evaporation is the result of escape of single proteins from the clusters, one after the other. The energy barrier to evaporate a single protein being of several k_BT, this is a slow process [36].

IV. RESULTS AND DISCUSSION

Once formed, clusters appear in simulations to be nonrigid, deformable, fluctuating (as illustrated on the two consecutive snapshots in Fig. 1, they can be seen as liquid droplets that reorganize rapidly; proteins diffuse inside the clusters with measured short-term diffusion coefficients larger than 0.01 $\mu m^2/s$), and long lived. By long lived, it is meant that clusters are stable at the time scale of the simulations (about 30 ms). However, some proteins constantly leave the clusters (via the evaporation process discussed above), diffuse freely in the gas phase, and are captured later by another cluster. Rare events of clusters being disintegrated or nucleating spontaneously in the gas have been observed. Therefore clusters are certainly not stable at long time scales (seconds or minutes) and are only transitory.

I computed the mean cluster size $\langle n\rangle$, counting monomers as clusters of size $n=1$. One can see in Fig. 2 that $\langle n\rangle$ was found to be proportional to ϕ over a wide range of concentration. Indeed, in 3D, it has been shown analytically in specific situations [19,22,24], and measured experimentally [17,18,20], that $\langle n\rangle$ is proportional to the particle concentration ϕ. My purpose here is not to demonstrate such a relation in 2D, but simply to note its validity in a wide range of situations in 3D and to observe numerically its equivalent in 2D. In addition to the numerical evidence presented here, further calculations, appealing, for example, to the theory of micellization [37], will be necessary to confirm this last point. They go beyond the scope of the present numerical paper.

Furthermore, the cluster distributions obtained in simulations are bimodal in all the range of concentrations where $\langle n\rangle \propto \phi$. A gas of monomers ($n=1$) coexists with large multimers ($n>1$), the distribution of which is Gaussian around a typical size n^*. With the parameters chosen here, there are virtually no small multimers (dimers, trimers, etc.). These distributions are illustrated in Fig. 3 at different concentrations ϕ. The relation $\langle n\rangle \propto \phi$ then comes from a subtle balance between monomers and multimers: as ϕ increases, the density of monomers is essentially constant while large clusters capture additional proteins (I recall that monomers are counted as clusters of size $n=1$). This behavior is reminiscent of experimental observations (Fig. 4 of Ref. [14]).

Now I study the diffusive properties of particles in cluster phases. I first consider an isolated cluster of n proteins, modeled as an assembly of interacting Langevin particles with bare diffusion coefficient D_0. The center of mass of the assembly diffuses with a coefficient D_0/n, because the clusters considered are not rigid entities but loosely bound, fluctuating ones in which the proteins diffuse, as discussed previously [5]. If clusters interact weakly because they are sufficiently far away (Fig. 1), the long-term diffusion coefficient of each protein of the cluster is also equal to D_0/n [5]. If clusters contain $\langle n\rangle$ proteins on average (still counting a monomer as a cluster with $n=1$), then the mean long-term diffusion coefficient $D=D_0/\langle n\rangle$: if the system contains N proteins

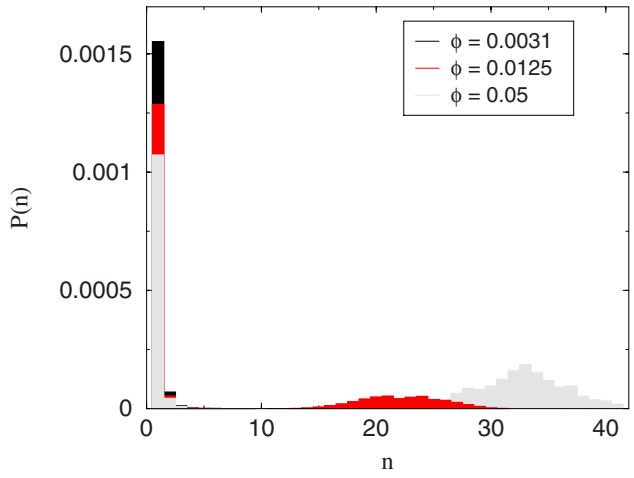

FIG. 3. (Color online) Numerical cluster-size distributions, for three different concentrations: ϕ=0.0031 (black), 0.0125 [red (dark gray)], and 0.05 (light gray). For ϕ=0.0031, multimers are not visible with the scale used because they are very scarce. The fractions $P(n)$ of clusters of size n are in the same units as ϕ: number of clusters per unit surface d_0^2.

$$D \equiv \frac{1}{N}\sum_{c=1}^{N_c} n(c)\frac{D_0}{n(c)}, \tag{2}$$

where $N_c = N/\langle n\rangle$ denotes the number of clusters, because a cluster c contains $n(c)$ proteins that diffuse, each with a diffusion coefficient $D_0/n(c)$. Thus $D = D_0 N_c/N = D_0/\langle n\rangle$. If R is the average cluster radius, then $D \propto 1/R^2$. Such an experimental behavior of D with cluster size has already been observed [4], but has been left unexplained. If in addition $\langle n\rangle \propto \phi$, then the effective diffusion coefficient of proteins in a cluster phase is inversely proportional to their concentration:

$$D = \text{const}/\phi. \tag{3}$$

As ϕ was increased, D was indeed observed in simulations to decrease dramatically, as expected (Fig. 2). One observes that $D \approx \text{const}/\phi$ over nearly two decades, thus confirming the hypothesis that cluster phases can account for this behavior. In addition, this relation holds even at moderate concentrations, $\phi \sim 0.1$. This demonstrates that the interactions between clusters are negligible and that the clusters diffuse independently at the time scale considered. Even though it was not identified as such, experimental evidence of this law was observed 25 years ago [31], without receiving a full explanation, apart from arguments invoking "crowding effects" or "aggregation" reminiscent of the clustering mechanism discussed here, but unable to predict quantitatively the dependence of D on ϕ [31]. The diffusion coefficient at typical cell membrane protein concentration ($\phi \sim 0.1$) appears to be reduced by a factor larger than 10, which is the decrease of D observed in cell membranes as compared to the same diffusion coefficient D_0 in model membranes at low ϕ [7,31].

I have also observed in simulations that small modulations of the parameter ε or γ in $U(r)$ can lead to segregation [1,13,27]: if two (or more) groups of proteins (A's and B's, which are not necessarily identical in the same group) are present in the simulation and if A-A and B-B interactions are slightly favored as compared to A-B ones, then the proteins segregate. There are A-rich and B-rich clusters because, even though it is entropically unfavorable, it is energetically favorable. For instance, an A-B binding energy 10% smaller than the equal A-A and B-B ones suffices to ensure segregation. At the biological level, this implies that groups of proteins that show a slight tendency to associate, because of their specific physicochemical properties, would segregate in distinct clusters (see [13]). This mechanism might play an important role in sorting together proteins that must congregate to perform biological functions [1,3]. This point will be quantified in future investigations.

The existence of cluster phases in live cells would mean that proteins spontaneously congregate in small clusters of a few entities or a few tens of entities in the plasma membrane instead of constituting a gas of independently diffusing inclusions, thus promoting nanodomains in plasmic membranes. By contrast to the lipid raft scenario where domains result from a lipidic microphase separation and then recruit specific proteins, the present mechanism proposes that domain formation is mainly driven by protein interactions (even though lipids *do* play an important role in the effective forces). Note that this scenario does not exclude a concomitant recruitment by protein clusters of specific lipids having a higher affinity for those proteins (and which participate in the effective attractions [30]), thus reconciling my hypothesis with the observation of detergent-resistant membrane fractions [15]. This mechanism, by constraining an increasing fraction of lipids to diffuse slowly (with clusters) as ϕ increases, could also explain why the diffusion constant of *lipids* decreases significantly when the concentration of *proteins* increases [31].

In spite of the evidence provided above, the existence of protein clusters has to be confirmed definitively at the experimental level. Even though such clusters have already been observed by freeze-fracture electron microscopy [4,14], their existence must be explored by different techniques in a wider range of situations, in cell and model membranes. Near-field scanning microscopy is an ideal tool because it is able to identify individual proteins after immobilizing the membrane onto an adequate substrate. Counting numbers of proteins in clusters is then in principle possible [9]. Such experiments would be able to investigate the dependency of cluster numbers $\langle n\rangle$ on protein concentration, as well as the correlation between $\langle n\rangle$ and D. Recently, high-frequency single-particle tracking has demonstrated that proteins are confined in nanodomains in the plasma membrane of live cells [7], of typical diameter a few tens of nanometers. An appealing hypothesis is that these nanodomains are the clusters under consideration. For proteins several nanometers apart, the previous size would correspond to clusters containing a few tens of proteins, in agreement with the previous simulations. The confirmation of this hypothesis would provide additional evidence of cluster phases in live cells.

I have proposed in this paper a paradigm leading to the formation of size-limited nanodomains in cell and model membranes. This scenario, based on reasonable hypotheses

about the energy and length scales in biological membranes, sheds light on several so far open issues in cell biology: (i) it provides a mechanism for the limited size of nanodomains; (ii) it gives a qualitative interpretation of previous experiments in model membranes [14,31]; (iii) it proposes a simple explanation leading to the proportionality law $D \propto 1/\phi$. If cluster phases were to be experimentally confirmed in model membranes and in live cells, it would mean that, by physical mechanisms, proteins generically gather in small assemblies in biological membranes, thus shedding new light on membrane functional processes.

Note added. A paper mixing experiments and numerical simulations has been published going in the same direction as the present work [38]. This paper also discusses the existence of membrane protein clusters due to short-range attraction and longer-range repulsion, in the case of syntaxin. As compared to the present work, the repulsion has a steric origin. In addition, the authors do not propose a similar quantitative study of the system properties as functions of the protein concentration.

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[35] This particular value of D_0 is chosen for the sake of simplicity and because it has the correct order of magnitude. However, all diffusion constants below will be given in units of D_0 and its precise value is irrelevant. Furthermore, fixing D_0 and the space scale via d_0 imposes the time scale. Thus all times calculated will be rough approximates.
[36] However, for ϕ=0.1, systems (a) and (b) are not qualitatively identical after 2×10^7 sweeps (seven big clusters vs four big ones). Thus, in Fig. 2, two values for $1/\langle n\rangle$ are plotted, the higher one corresponding to system (a). For D/D_0, both values are identical within 10% error bars.
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