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Fluctuations and conformational stability of a membrane patch with curvature inducing inclusions

Weria Pezeshkian ^a and John H. Ipsen ^{*b}

Membranes with curvature inducing inclusions display a range of cooperative phenomena, which can be linked to biomembrane function, *e.g.* membrane tubulation, vesiculation, softening and spontaneous tension. We investigate how these phenomena are related for a fluctuating, framed membrane through analysis of a discretized membrane model by Monte Carlo simulation techniques. The membrane model is based on a dynamically triangulated surface equipped with non-interacting, up-down symmetry breaking inclusions where only terms coupled linearly to mean-curvature are maintained. We show that the lateral configurational entropy plays a key role for the mechanical properties of the semi-flexible membrane, *e.g.* a pronounced softening at intermediate inclusion coverages of the membrane and generation of membrane tension. Tensionless framed membranes will remain quasi-flat up to some threshold coverage, where a shape instability occurs with formation of pearling or tubular membranes, which below full coverage is associated with segregation of inclusions between the curved and flat membrane geometries. For inclusions with preference for highly curved membranes the instability appears at dilute inclusion coverages and is accompanied by strong configurational fluctuations.

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1 Introduction

The interplay between membrane inclusions and membrane shape deformations has been subject to renewed focus during the recent years since it has become clear that many cellular mechanisms such as signaling and endocytosis involve the organisation in the biological membranes of curvature active membrane inclusions with accompanying vesicular budding or tubulation phenomena.^{1–3} However, there is currently no theoretical consensus about which physical mechanisms are dominating these processes. This has lead to a plethora of descriptive nomenclature adopted from cell biology, *e.g.* curvature sensing, curvature generating and membrane scaffolding proteins.³ A major focus has been put on the clustering capacity of membrane inclusions which can facilitate budding or tubular invaginations.^{1,4–6} Numerous studies have demonstrated that besides the direct interactions between membrane inclusions, perturbations of the flexible lipid membrane induced by inclusions can give rise to interactions that can facilitate clustering of the

inclusions.⁷ Such interactions can *e.g.* originate from maximising the lipid molecule configurational entropy⁸ (depletion forces), minimising the lipid domain interface generated by membrane inclusions⁹ (capillary forces) or as a results of changes in the membrane geometrical shape or shape fluctuations by inclusions such as or curvature forces or thermal Casimir forces between inclusions.^{6,10–14} In the present work we only consider the latter class of membrane perturbations by inclusions. Our study suggests that in addition to these established membrane mediated interactions, a new clustering mechanism by curvature inducing inclusions can cause a cooperative transition from semi-flat membrane conformations with dispersed inclusions to tubular or bud domains enriched with the inclusions. We analyse the simplest possible membrane systems involving curvature inducing proteins with no direct interactions described by Monte Carlo simulation of a dynamically triangulated surface model.

In absence of membrane inclusions the energy of a fluid lipid membrane conformations is well described by the Helfrich Hamiltonian^{15,16}

$$\mathcal{H}_0 = \int dA \left(\frac{\kappa}{2} (2H - \bar{C}_0)^2 + \frac{\bar{\kappa}}{2} K \right) \quad (1)$$

where H and K are the local mean and Gaussian curvatures of the surface. κ and $\bar{\kappa}$ are the associated bending and Gaussian rigidities. $\bar{C}_0$ is the spontaneous curvature representing the preferred mean curvature due to some global asymmetry (up-down)

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between the two faces of the membrane. In this study $\bar{C}_0 = 0$. For an inclusion free membrane without topology changes the second term of eqn (1) is invariant and can thus be left out of consideration. Eqn (1) must be extended when the membrane is decorated with inclusions. For simple circular, up-down symmetry breaking inclusions the most relevant term with regard to power counting is

$$\mathcal{H}_1 = \lambda \int dA \rho H, \quad (2)$$

suggested by Leibler in his seminal work on membrane inclusions.¹⁷ ρ is the local density field for membrane inclusions while the parameter λ represents the local coupling to curvature. In mean-field theory eqn (1) and (2) gives a local preferred mean curvature $-\frac{\lambda}{\kappa}$, while the local rigidity is unchanged. The next relevant terms do not break the up-down symmetry¹⁸

$$\mathcal{H}_2 = \frac{1}{2} \Delta \kappa a_p \int dA \rho (2H)^2 + \frac{1}{2} \Delta \bar{\kappa} a_p \int dA \rho K, \quad (3)$$

where $\Delta \kappa$ and $\Delta \bar{\kappa}$ represents the local change in the bending and Gaussian rigidities due to the inclusions and a_p is the cross-sectional area of the inclusion. A perturbative analysis (rigid membrane) of the terms in eqn (3) gives rise to membrane mediated forces between the inclusions which resembles the forces predicted by Goulian *et al.*¹⁰ and exact agreement is found in the strong coupling limit ($\Delta \kappa = -\Delta \bar{\kappa} = \infty$).¹⁸ It is also in agreement with computer simulations on semi-flat membranes which showed that such couplings to local rigidity results in inclusion clustering.^{19,20} A similar perturbative analysis of eqn (2) shows that it does not give rise to membrane mediated forces.^{12,18} It has been demonstrated experimentally that for some inclusions, like Shiga-toxin, aggregation is a prerequisite for invaginations of the membrane and a soft membrane-inclusion interface abolish the aggregation.^{6,7} This suggests that both the terms eqn (3) (aggregation) and eqn (2) (symmetry breaking and tubulation) are in play.²¹ However, in this work we show that even in the absence of eqn (3), *i.e.* $\Delta \kappa = \Delta \bar{\kappa} = 0$, such phenomena of concomitant aggregation and tubulation (or budding) can take place at system conditions where strong fluctuations impede the perturbative arguments.

One approach to access the non-perturbative properties of flexible membranes with inclusions is to implement a dynamically triangulated surface model (DTS)^{22–25} with curvature inducing proteins with no direct interactions but excluded volume effect. Monte Carlo simulation of the DTS model demonstrate that compositional and conformational fluctuations of the membrane with curvature inducing inclusions play an essential role for physical phenomena such as membrane softening, tension generation, and cooperative phase separation involving both composition and membrane curvature. We show by use of this approach that at very high coverage and very low coverage the conformational properties of the quasi-flat membrane is well described by the mechanical properties of Helfrich's Hamiltonian eqn (1). At intermediate coverages

fluctuation effects due to lateral entropy become significant and the membrane is softened with reduced effective bending rigidity depending on the local curvature preference of the inclusions. The semi-flat membrane patch is conformationally unstable at high membrane coverage and form pearling membrane structures. For increasing membrane curvature preference of the inclusions this instability move into the dilute regime accompanied with vigorous conformational fluctuations. Recently, a DTS-study of membranes governed by the eqn (1), eqn (2) and aspects of eqn (3) have been performed in context of binding of curvature active inclusions to vesicles.²⁶

Our results are relevant for numerous experimental studies showing spontaneous formation of high-curvature tubules at membranes in presence of local curvature inducing inclusions, *e.g.* pathogen toxins,¹ antimicrobial peptides,^{27,28} Ca^{2+} ,²⁹ or the ganglioside GM1,³⁰ involving shape instability of a membrane patch. Related phenomena like the large-scale shape deformations of free standing lipid vesicles or distribution of inclusions on membranes with fixed prescribed curvature are not covered in this study.

2 Methods

The large-scale conformational properties of framed, fluid membranes are modeled by a dynamically triangulated, self-avoiding surface subject to periodic boundary conditions in the X and Y directions of the rectangular frame. This setup is thus not suitable for analysis of global shape changes of vesicles but applies to a membrane patch and is directly comparable with analytic modeling of membranes in the Monge representation. Since the Euler characteristics of the surface is vanishing the number of vertices N_v , triangles N_t and links between vertices N_l are fixed by the relation $6N_v = 3N_t = 2N_l$. The self-avoidance is ensured by a spherical bead of diameter d at each vertex and a maximum tether length between neighboring vertices $\sqrt{3}d$.²³ d is chosen as the unit length. There are several different methods to calculate the elastic energy for a triangulated surface,^{22–25,31} but here we follow the procedure described by Ramakrishnan *et al.*²³ since it allows for great flexibility in the calculation of geometrical surface quantifiers. This method involves a set of discretised geometrical operations to calculate vertex based quantities like the normal vector $\mathbf{N}_\nu$, the surface area $A(\nu)$ (one third of the area of its neighbouring triangles), and the shape operator $S(\nu)$ which produces the local principal curvatures $c_1(\nu)$, $c_2(\nu)$ and the associated principal directions $\mathbf{X}_1(\nu)$, $\mathbf{X}_2(\nu)$.

For the description of a framed membrane with curvature inducing inclusions the simplest possible model energy expression takes the form

$$E = \frac{\kappa}{2} \sum_{\nu}^{N_v} A(\nu) (c_1(\nu) + c_2(\nu) - \eta(\nu) C_0)^2 \quad (4)$$

which is an extension of Helfrich's mean-curvature elastic energy including local variations in the spontaneous curvature, corresponding to eqn (1) and (2), formulated on a random triangular lattice. C_0 is the up-down symmetry breaking local spontaneous

curvature representing the curvature imprint by an inclusion, which is equivalent to $\bar{C}_0$ at full coverage. The N_ν inclusions are represented by occupation variables $\eta(\nu) = 0, 1$. In this model there is no direct interaction between the inclusions while the configurational entropy is affected by the excluded volume effect provided by the lattice and the vertex beads.³²

The equilibrium properties of the framed membrane is analysed by Monte Carlo simulation techniques with the three standard Monte Carlo moves for DTS: link flip, vertex moves and translational inclusion moves (Kawasaki moves).²³ For systems with constant frame tension (τ) there is an additional move to control the membrane frame area A_p . Consider a framed membrane with a projected area (A_p) on XY plane in a rectangular box ($L_x \times L_y \times L_z$) with periodic boundary conditions in the X , Y and Z directions. To each Monte Carlo Sweep (MCS) with a probability of $P_A = 1/(N_\nu \tau_A)$ the bilayer frame area is updated ($\tau_A = 5$ MCS in all simulations) and with probability of $1 - P_A$, trial moves corresponding to N_ν vertex moves, N_l link updates, N_i inclusion moves are performed. The procedure to update the projected area is as follows: first, the box sides will be updated to $L_x \rightarrow L_x + \Delta L_x$ and $L_y \rightarrow L_y + \Delta L_y$ in which ΔL_x is chosen randomly in the interval $[-0.04d, 0.04d]$ and $\Delta L_y = \Delta L_x(L_y/L_x)$ so L_x/L_y remains constant. The X , Y coordinates at each vertex is then updated to $X \rightarrow (1 + \Delta L_x/L_x)X$ and $Y \rightarrow (1 + \Delta L_y/L_y)Y$. Note that the L_z and Z coordinates of the vertices remain unaffected. To accept or reject the move, Metropolis algorithm with the acceptance probability (P_{acc}) at temperature T is performed, where

$$P_{acc} = \min \left[1, \left(\frac{A_{p2}}{A_{p1}} \right)^{N_\nu} \exp \left(\frac{-\Delta E + \tau \Delta A}{k_B T} \right) \right] \quad (5)$$

guarantee a correct τNT ensemble. A_{p1} and A_{p2} are the current and the trial projected areas and $\Delta A = A_{p2} - A_{p1}$. ΔE is the corresponding change in the system energy eqn (4). $k_B T$ is the thermal energy.

3 Results and discussion

Monte Carlo simulations of the triangulated surface model were performed with fixed κ , C_0 , T , θ , N_ν and either fixed τ or A_p . Furthermore, the model parameters were fixed at $\kappa = 20k_B T$ or $30k_B T$ with $N_\nu = 1840$, $C_0 = 0.2, 0.4, 0.6, 0.8, 1[1/d]$ and inclusion coverages $\theta = N_i/N_\nu$ in the range from 0 to 1. Thermalisation were checked by initially putting together the inclusions as shown in Fig. 1A top at vanishing frame tension. After $\sim 10^6$ MCS the inclusions were separated and randomly distributed on the membrane at low θ values (Fig. 1A bottom) indicating that the lateral configurational entropy is dominating their organisation. Fig. 1B shows snapshots after equilibration for $\theta = 0.22, 0.35, 0.2$ and $C_0 = 0.4, 0.4, 1[1/d]$ respectively. As depicted, for higher inclusion coverage, the membrane may change its morphology to include highly curved, tubular or budded surface configurations accompanied by elevated concentrations of inclusions in the curved domains of the membrane. This suggests that the inclusions participate in a compositional phase separation phenomena involving geometrical transformations of the membrane. Simulations performed with random initial distribution of the inclusions give the same results. To get some quantitative characterisation of this phenomenon we define an inclusion–inclusion contact measure n_c as the average number of nearest neighbour inclusions of a given inclusion. Fig. 2 shows n_c as a function of C_0 for 6 different inclusion coverages (θ) at $\tau = 0$. For all the coverages at $C_0 = 0.2, 0.3[1/d]$ n_c is indistinguishable from the $C_0 = 0$ case. This reflects the fact that the membrane remains semi-flat with homogeneous distribution of inclusions. For $C_0 \geq 0.4[1/d]$ n_c is significantly increased at high coverage, which indicates that some clustering or compositional phase separation takes place. Furthermore, the behaviour shown in Fig. 1B suggests that it is associated with the shape transition to tubular configurations. A simple mean-field analysis of eqn (4) shows that mean-curvature elasticity frustration becomes important with increasing concentrations of

Fig. 1 Snapshots from simulations with zero fixed tension ($\tau = 0$). The blue vertices represents the membrane surface and the red vertices are membrane with inclusions. (A) (i) The inclusions are placed in a patch as the starting configuration of the simulation. (ii) The final configuration after 12 million Monte Carlo steps. (B) At intermediate concentration and high C_0 flat configuration of the membrane becomes unstable (iii) more invaginations can form.

Fig. 2 (A) Average inclusion-inclusion contacts n_c as a function of C_0 for different coverages at $\kappa = 20k_B T$. (B) Schematic phase diagram showing upon crossing certain concentrations, the shape configuration of the membrane changes. For θ and C_0 values within the striped region, membrane remain in quasi-flat metastable configurations. A small perturbative force brings the systems to a highly curved configuration (see Fig. 5).

inclusions which can drive such changes of the membrane shape. However, a detailed description is more complex, which *e.g.* is revealed by analysis the configurational fluctuations of quasi-flat membranes.

3.1 Quasi-flat membranes

Although, the Hamiltonian eqn (4) is fully symmetric w.r.t. Euclidean symmetry, the simulation setup with periodic boundary conditions break this symmetry. For quasi-flat configurations it is natural to parameterize the deviations of the membrane from the $z = 0$ plane by Monge representation: $(\vec{x}, z(\vec{x}))$ with $\vec{x} = (x, y)$. To lowest orders in $\vec{\nabla} z(\vec{x}) = (\partial_x, \partial_y)z(x, y)$ an effective free energy difference for the conformations of the semi-flat framed membrane takes the form:

$$\Gamma^{(2)}(z(\vec{x}), \delta\hat{\rho}(\vec{x})) = \int dA_p \left(\frac{\tau}{2} (\vec{\nabla} z(\vec{x}))^2 + \frac{\hat{\kappa}}{2} (\vec{\nabla}^2 z(\vec{x}))^2 - \hat{\lambda} \nabla^2 z(\vec{x}) \delta\hat{\rho}(\vec{x}) + \frac{1}{2} f^{(2)}(\bar{\rho}) (\delta\hat{\rho}(\vec{x}))^2 \right) \quad (6)$$

$\hat{\rho}(\vec{x})$ is the density w.r.t. the projection plane and $\delta\hat{\rho}(\vec{x})$ is its deviation from its equilibrium value $\bar{\rho}$. Eqn (6) is not a simple linearisation of eqn (4) around a plane, but it fully encompasses the full non-linear Hamiltonian.^{33–35} The equivalence between the physical frame tension τ and the fluctuation tension has previously been confirmed numerically for simple model membranes.^{36–38} $\hat{\kappa}$ is a renormalized bending rigidity, $\hat{\lambda}$ is a renormalized version of coupling to curvature $\lambda = \kappa C_0 d$, $f^{(2)}(\bar{\rho}) = \frac{\partial^2 f}{\partial \rho^2}$, where $f(\hat{\rho}(\vec{x}))$ describes the effective free energy for the inclusions in the plane to be considered below. Eqn (6) can be approached by standard Fourier analysis of the fluctuations:

$$\Gamma^{(2)}[\mathbf{u}(\vec{q})] = \frac{1}{2} \sum_{\vec{q}} \mathbf{u}(\vec{q}) \mathbf{M}(\vec{q}, \bar{\rho}) \mathbf{u}(\vec{q})^T. \quad (7)$$

Here, $\mathbf{M}(\vec{q}, \bar{\rho})$ is the compliance matrix

$$\mathbf{M}(\vec{q}, \bar{\rho}) = \begin{pmatrix} \hat{\kappa} q^4 + \tau q^2 & -\hat{\lambda} q^2 \\ -\hat{\lambda} q^2 & f^{(2)}(\bar{\rho}) \end{pmatrix}, \quad (8)$$

$\vec{q} = (q_x, q_y)$ are the elements of Fourier space and $\mathbf{u}(\vec{q}) = (z(\vec{q}), \delta\hat{\rho}(\vec{q}))$. A simple stability analysis of eqn (7) suggests that the quasi-flat system with nearly homogeneous distribution of inclusions will remain stable for $\det[\mathbf{M}(\vec{q}, \bar{\rho})] = q^2((\tau + q^2 \hat{\kappa}) f^{(2)}(\bar{\rho}) - q^2 \hat{\lambda}^2) \geq 0$, where the lower limit mark the condition for curvature instability as suggested by Leibler.¹⁷ This instability involves both the membrane shape and the distribution of inclusions, *e.g.* observed in the correlation functions

$$\langle \mathbf{u}(\vec{q}) \otimes \mathbf{u}(-\vec{q}) \rangle = k_B T \mathbf{M}^{-1}(\vec{q}, \bar{\rho}) \quad (9)$$

The l.h.s. of eqn (9) consist of correlation functions obtained in the framed geometry which are easily measurable from the simulation setup described in the previous section, while the r.h.s. involves expressions in terms of coefficients of eqn (6). In particular the high correlation function can be expressed as

$$\langle z(\vec{q}) z(-\vec{q}) \rangle = \frac{k_B T}{\left(\hat{\kappa} - \frac{\hat{\lambda}^2}{f^{(2)}(\bar{\rho})} \right) q^4 + \tau q^2}. \quad (10)$$

The form of eqn (10) gives the possibility to estimate τ and the effective bending modulus $\kappa_{\text{eff}}(\bar{\rho}) = \hat{\kappa} - \hat{\lambda}^2 / f^{(2)}(\bar{\rho})$ by fitting to the measured height correlation function from computer simulations of quasi-flat membranes in both fixed A_p and fixed τ ensembles. Prior to further analysis we tested and validated the equivalence between the physical frame tension and the fluctuation tension for simple membranes and membranes with inclusions given by model eqn (4) over a range of C_0 and θ values and frame tensions τ . A similar simple interpretation is not available for κ_{eff} . Eqn (10) also gives the possibility to

estimate the excess area $\frac{\delta A}{A_p} = \frac{A - A_p}{A_p}$:

$$\frac{\delta A}{A_p} = \int \frac{d^2 q}{(2\pi)^2} q^2 \langle z(\vec{q}) z(-\vec{q}) \rangle$$

$$= \frac{k_B T}{8\pi\kappa_{\text{eff}}} \ln \left(\frac{\kappa_{\text{eff}} N + \tau A_p}{\kappa_{\text{eff}} + \tau A_p} \right). \quad (11)$$

Eqn (11) has been derived numerous times in the literature,^{39,40} but generally the areas, the tension and the bending rigidity have different connotations. $\frac{\delta A}{A_p} \ll 1$ gives a minimal criterion for the validity of the approximation eqn (6) and certainly it will be violated for $\tau = 0$ and $\kappa_{\text{eff}} \simeq 0$.

3.2 Constant A_p

Fig. 3B-i shows the obtained κ_{eff} values as a function of inclusion coverage at fixed A_p and a range of C_0 values. The corresponding estimates of τ appear in Fig. 3A. The fixed frame area $A_p = 2797[d^2]$ is chosen so that $\tau(\bar{\rho} = 0) = 0$. It can be noted that the obtained $\hat{\kappa} = \kappa_{\text{eff}}(\bar{\rho} = 0)$ is a little less than the model parameter κ given in eqn (4), which underline that the discretised model is an approximation of a continuum model and the fact that $\hat{\kappa}$ is a renormalised quantity.³⁵ Both κ_{eff} and τ are strongly dependent on the composition. κ_{eff} shows a minimum at intermediate compositions and returns to $\hat{\kappa}$ at full coverage, while τ is steadily increasing with inclusion coverage. The behavior of κ_{eff} can in absence of any direct interactions between membrane inclusions largely be explained as a consequence of $\hat{\kappa}$ and the lattice gas entropy which gives an approximate compositional free energy density

$$f(\hat{\rho}(\vec{x})) \simeq k_B T (\hat{\rho}(\vec{x}) \ln(a_p \hat{\rho}(\vec{x})) + (a_p^{-1} - \hat{\rho}(\vec{x})) \ln(1 - a_p \hat{\rho}(\vec{x}))), \quad (12)$$

where a_p is the projected vertex area and $a_p \bar{\rho}$ approximates the coverage θ . Since $f^{(2)}(\bar{\rho}) = \frac{k_B T}{\bar{\rho}(1 - a_p \bar{\rho})}$ the corresponding

effective bending rigidity obtained from eqn (10) becomes $\kappa_{\text{eff}} = \hat{\kappa} - \frac{\lambda^2}{k_B T} \bar{\rho}(1 - a_p \bar{\rho})$. A similar expression has been very useful for concomitant experimental estimation of $\hat{\kappa}$ and λ at low inclusion coverages.^{41,42} As indicated in Fig. 3B-i this expression for κ_{eff} describes the simulation results well for small C_0 with dependences of $\bar{\rho}$ nearly symmetric around half coverage. For higher C_0 values κ_{eff} become highly asymmetric with pronounced membrane softening at high coverage with re-entrance of the rigid membrane at full coverage. The measured frame tensions shown in Fig. 3A are increasing with ρ , indicating that the fluctuations cross over from bending excitations to capillary wave-like excitations. For small C_0 values the relationship is linear and well approximated by the spontaneous tension $\frac{1}{2}\kappa C_0^2 \theta$ suggested by Lipowsky,⁴³ while the induced frame tension becomes non-linear in $\bar{\rho}$ at $C_0 \geq 0.5[1/d]$. At the chosen system conditions the quasi-flat and homogeneous membrane remain stable, where κ_{eff} and τ are measurable from the fluctuations at all compositions.

3.3 Tension free patch

At $\theta = 0$ the framed membrane patch can sustain semi-flat equilibrium configurations at vanishing tension and a range of negative tensions.^{44,45} At low inclusion coverage this is still the case, which makes it possible to perform the fluctuation analysis as above at $\tau = 0$. Fig. 3B-ii shows the results for κ_{eff} at $\tau = 0$ (varying A_p) as a function of the inclusion coverage for different C_0 values. It can be seen that κ_{eff} follows closely the behaviour found for the fixed A_p ensemble. In addition to the characterisation of the configurational correlations, the stability of the configurations are tested by performing simulations with both semi-flat and tubular initial conditions. At coverages above some threshold value $\theta^*(C_0)$ the semi-flat configurations are unstable and characteristic high curvature extensions of the membrane are formed, which is indicated by

Fig. 3 Simulation results for $\kappa = 20k_B T$: (A) frame tension as a function of protein coverage. The dashed lines: spontaneous tension $\tau_s = \frac{1}{2}\kappa C_0^2 \theta$. (B) Effective bending rigidity is a function of inclusion concentration, dashed line: theoretical prediction assuming that inclusions form an ideal mixture $\kappa_{\text{eff}} = \hat{\kappa} - \frac{\lambda^2}{k_B T} \bar{\rho}(1 - a_p \bar{\rho})$ where $(\theta = a_p \bar{\rho})$, λ was extracted using fitting (i) NTA_p ensemble (ii) $NT\tau$. The data points under the striped regions (filled circle points) are obtained from systems in quasi-flat metastable configurations.

Fig. 4 $\frac{\delta A}{A_p}$ for different θ at $\kappa = 20k_B T$, $\tau = 0$ and $N_\nu = 1840$. For $C_0 \geq 0.4[1/d]$ configurational fluctuations increases with inclusion coverage θ and at $\frac{\delta A}{A_p} \simeq 0.05$ the quasi-flat membranes becomes unstable.

the striped region in Fig. 3B-ii and Fig. 2B. For $C_0 = 0.2[1/d]$ and $C_0 = 0.3[1/d]$ the semi-flat configurations form long living meta-stable configurations even for $\theta > \theta^*(C_0)$, while the stable phase is dominated by characteristic pearling tube structures (see Fig. 5). At high coverage this can be interpreted as vesiculation,^{46,47} a mechanical instability found in vesicles with homogeneous spontaneous curvature. This phenomena is also well known from shape analysis of Helfrichs Hamiltonian eqn (1) with $\bar{C}_0 \neq 0$ where so called ‘‘Helfrich spheres’’ with $2H = \bar{C}_0$ are shed off until volume or area constraints may stop the process.^{47,48}

In general the formation of pearling membrane structures (strings of Helfrich spheres) are energetically favored over tubes or many buds.^{43,46} A minimal requirement for such mechanical instability in the membrane patch with periodic boundary condition is that a Helfrich sphere fits within the boundary: $\theta C_0 > \theta^*(C_0)C_0 \simeq \frac{4\sqrt{\pi+1}}{L}$. For $C_0 = 0.2, 0.3[1/d]$ this criterion is approximatively fulfilled. While this picture based on

membrane mechanics gives a good description at nearly full coverages the vesiculation is accompanied with phase separation at lower coverage $\theta \geq \theta^*(C_0)$, where the overall compositional constraint will stop the vesiculation. This phenomena is distinct from domain-induced budding or vesiculation where the interfacial line tension helps the formation.⁴⁹ Here, there is no preformed domains *e.g.* induced by in-plane intermolecular interactions. Note, that linear coupling between inclusion coverage and mean curvature does not promote interactions between the inclusions.³³ Rather, it is the mechanical instability which promotes the phase separation. A phenomenological phase equilibrium between inclusions in a flat membrane geometry and the Helfrich spheres can easily be established based on the model eqn (4). For $C_0 \geq 0.4[1/d]$, κ_{eff} is also following the behaviour found in the previous section. However $\theta^*(C_0)$ is dramatically reduced and no meta-stable semi-flat phase is detected. Thus, as C_0 is increased the membrane becomes unstable already in the dilute regime, and the averaged mean curvature $\theta^*(C_0)C_0$ at the threshold composition $\theta^*(C_0)$

becomes small (compared to $\frac{4\sqrt{\pi+1}}{\theta C_0 L}$). Therefore, the simple mechanical vesiculation cannot be the driving force for the instability in this regime. Furthermore, instead of the strings of Helfrich vesicles found at high coverage the irregular tubes with elevated concentrations of inclusions are formed at low coverage $\theta > \theta^*(C_0)$. The instability point $\theta^*(C_0)$ correlates well with the significant softening of the membrane as shown in Fig. 3B-ii, but it takes place for κ_{eff} well above the instability criterion of Leibler ($\kappa_{\text{eff}} = 0$) and apparently also above the crumpling transition point $\kappa_c \simeq k_B T$ of a simple framed, fluid membrane.⁴⁵ However, the instability at $\theta^*(C_0)$ is accompanied by strong fluctuations as suggested by eqn (11) at $\tau = 0$: $\frac{\delta A}{A_p} = \frac{k_B T}{8\pi\kappa_{\text{eff}}} \ln(N_\nu)$, which is confirmed by the measured $\frac{\delta A}{A_p}$ shown in Fig. 4. We can therefore conclude that the corrections to the quadratic approximation of eqn (6) becomes important. The first terms of such a perturbative correction to eqn (2) are precisely of the form of eqn (3) formulated in Monge

Fig. 5 Snapshots of fully covered membranes for 3 different C_0 values. For $C_0 = 0.2, 0.4[1/d]$, 5×10^6 MC moves was not enough to pass through the energy barrier between quasi-flat and highly curved configurations (a perturbative force was needed). For $C_0 = 0.6[1/d]$, periodic images of the simulation box are shown to illustrate long tubular structure.

representation, which can give rise to interactions between the inclusions and subsequently clustering. These results may thus suggest that the mechanisms promoting tubulation and segregation of inclusions are changing over the different (θ, C_0) parameter regions. More detailed simulation studies of the membrane with high C_0 inclusions in dilute regime and corresponding analytical studies beyond the level of linearized Helfich Hamiltonian are desired to clarify this.

3.4 Conclusion

In the present study it has been demonstrated that the compositional and configurational fluctuations can play a crucial role for the mechanical properties and stability of membranes with curvature inducing inclusions. In particular it is shown that the lateral compositional fluctuations of membranes with local curvature inducing inclusions at intermediate coverages can give rise to significant softening of the membrane. Such softening has been observed experimentally and in computer simulations for a many membrane systems with symmetric distributions of peripheral inclusions, *e.g.* for antibacterial peptides⁴¹ and GM1⁵⁰ or mixed lipid bilayer systems.^{42,51} However, for fixed framed membranes the conformational fluctuations are limited by the build-up of a spontaneous tension induced by the inclusions, which is an experimentally measurable quantity.³⁰ For tension less framed membranes the inclusions tend to increase the conformational fluctuations and at coverages above a threshold value a shape instability is triggered. At low local spontaneous curvature of the inclusions the instability is governed by the mechanical vesiculation transition at high coverage accompanied by geometric segregation of inclusions into spherical and flat configurations. For high local spontaneous curvatures the onset of the shape instability moves to dilute concentrations, where strong conformational fluctuations assist the segregation of inclusions into tubular membrane structures. In this regime the clustering is facilitated by a complex interplay between compositional and conformational fluctuations, where the lateral mixing entropy drives membrane softening causing strong conformational fluctuations and segregation. Our results thus point at another membrane inclusion clustering mechanism where the coupling between membrane geometry and inclusion facilitate membrane mediated interactions. This class of interactions may contribute to the lateral organization of membrane inclusions without reliance on lipid cooperative phenomena such as partitioning into lipid raft domains.⁵²

Conflicts of interest

There are no conflicts to declare.

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