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Theoretical study of vesicle shapes driven by coupling curved proteins and active cytoskeletal forces[†]

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Eukaryote cells have a flexible shape, which dynamically changes according to the function performed by the cell. One mechanism for deforming the cell membrane into the desired shape is through the expression of curved membrane proteins. Furthermore, these curved membrane proteins are often associated with the recruitment of the cytoskeleton, which then applies active forces that deform the membrane. This coupling between curvature and activity was previously explored theoretically in the linear limit of small deformations, and low dimensionality. Here we explore the unrestricted shapes of vesicles that contain active curved membrane proteins, in three-dimensions, using Monte-Carlo numerical simulations. The activity of the proteins is in the form of protrusive forces that push the membrane outwards, as may arise from the cytoskeleton of the cell due to actin or microtubule polymerization occurring near the membrane. For proteins that have an isotropic convex shape, the additional protrusive force enhances their tendency to aggregate and form membrane protrusions (buds). In addition, we find another transition from deformed spheres with necklace type aggregates, to flat pancake-shaped vesicles, where the curved proteins line the outer rim. This second transition is driven by the active forces, coupled to the spontaneous curvature, and the resulting configurations may shed light on the formation of sheet-like protrusions and lamellipodia of adhered and motile cells.

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Curved membrane proteins,¹ for example membrane embedded proteins with non-zero intrinsic curvature,²⁻⁸ flexible nanodomains^{7,9} or curved membrane-attached proteins,^{1,7,10-12} have been identified to play an important role in driving the formation of various membrane shapes.^{4,7,13–17} Coupling between non-homogeneous lateral distribution of membrane components and membrane shapes may be a general mechanism for the generation and stabilization of highly curved membrane structures,¹⁸ such as spherical buds, membrane necks, thin tubular or undulated membrane protrusions.^{13,14,17,19-29} In addition, it was found in many cellular processes that the curved proteins, or complexes containing the curved proteins, are able to recruit the cytoskeleton of the cell to produce additional protrusive forces, for example due to actin polymerization.^{30,31} Such protrusive forces are active, meaning that they consume energy (ATP) and maintain the system out of thermal equilibrium.³² The resulting

^c Department of Chemical and Biological Physics, Weizmann Institute of Science, Rehovot 7610001, Israel. E-mail: nir.gov@weizmann.ac.il steady-state configurations of the system may therefore differ from those in thermal equilibrium. Note that in cellular membranes such active forces can originate also from other sources, such as ion pumps.^{33,34}

Curved membrane proteins with a convex shape, such that they induce outwards bending of the membrane, that also recruit the cytoskeletal forces which push the membrane outwards, can serve as efficient initiators of membrane protrusions. This mechanism was first suggested theoretically,³⁵ and has since been found in experiments.³⁶⁻⁴³ This coupling of convex curvature and recruitment of actin polymerization is therefore emerging as an efficient cellular mechanism for the production of actin-based protrusions.44 It also appears to be exploited by certain viruses during their budding from the infected cell.^{45,46} Previous studies of the coupling between curved membrane proteins and the cytoskeletal forces were mostly limited to the linear regime³⁵ or to simplified geometries,47 and indicated that convex proteins can undergo phase separation and aggregation at lower concentrations (or higher temperatures) when the protrusive forces are present.⁴⁸ We study here the membrane shapes and the aggregation properties of such systems using numerical simulations, which allow us to go beyond the linear deformations limit. We find that the presence of the active protrusive forces affects the

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phase-separation (budding) transition, as well as induces transitions into new classes of shapes that are not accessible in the equilibrium (passive) systems. The ability of active processes, associated with curved proteins, to lead to global shape transitions was previously found in numerical simulations,⁴⁹ where activity was in the form of proteins with fluctuating spontaneous curvature.

1 Theoretical model

The energy of the membrane is expressed as the sum of contributions of membrane bending, direct interactions between membrane proteins and outward protrusive cytoskeletal forces,

$$W = W_{\rm b} + W_{\rm d} + W_{\rm F},\tag{1}$$

respectively.

For membrane bending energy the standard Helfrich expression⁵⁰ is used,

$$W_{\rm b} = \frac{\kappa}{2} \int_{A} (C_1 + C_2 - C_0)^2 \mathrm{d}A, \qquad (2)$$

where the integral runs over the whole area *A* of the membrane with bending stiffness κ , C_1 and C_2 are principal curvatures and C_0 is the spontaneous curvature of the membrane. The proteins on the membrane are modeled as patches of the membrane with given spontaneous curvature c_0 .^{6,9,10} On the patches occupied by the curved proteins we therefore set $C_0 = c_0$ and elsewhere we assume a symmetric membrane $C_0 = 0$.

For direct interactions between neighboring proteins we assume the step potential,

$$W_{\rm d} = -w \sum_{i < j} \mathscr{H} \big(r_0 - r_{ij} \big), \tag{3}$$

where *w* is a direct interaction constant, the sum runs over all protein–protein pairs, r_{ij} are their mutual in-plane distances, $\mathscr{H}(r)$ is the Heaviside step function and r_0 is the range of the direct interaction. We consider here attractive interactions w > 0, that induce phase-separation of the proteins.⁴⁸

Finally, the energy contribution of the local protrusive forces due to the cytoskeleton is

$$W_{\rm F} = -F \sum_{i} \hat{n}_i \cdot \vec{x}_i, \tag{4}$$

where *F* is the size of the force, the sum runs over all proteins, \hat{n}_i is the outwards facing normal to the membrane at the location of the protein *i* and \vec{x}_i is the position vector of the protein *i*.

The activity of the proteins appears as a force pointing at the outwards normal of each vertex that is occupied by a protein. The normal force term in the energy (eqn (4)) acts like a timevarying external potential, which therefore manifestly makes the system out-of-equilibrium. In other words, unlike the terms that are due to local interactions in the membrane due to curvature or protein–protein binding, the active force term has the form of a fictitious external potential pulling on each protein in the direction of the instantaneous outwards normal. As the membrane changes its shape, this fictitious external potential changes. A system with time-varying external potentials is not in thermal equilibrium.

2 Monte-Carlo simulations

The membrane is represented by a set of N vertices that are linked by tethers of variable length *l* to form a closed, dynamically triangulated, self-avoiding two-dimensional network^{51,52} of approximately 2N triangles and with the topology of a sphere. The lengths of the tethers can vary between a minimal value, l_{\min} , and a maximal value, l_{\max} . Self-avoidance of the network is ensured by choosing the appropriate values for l_{max} and the maximal displacement of the vertex s in a single updating step. In this work we used s/l_{min} = 0.15 and l_{max}/l_{min} = 1.7. The dynamically triangulated network acquires its lateral fluidity from a bond flip mechanism. A single bond flip involves the four vertices of two neighboring triangles. The tether connecting the two vertices in diagonal direction is cut and reestablished between the other two, previously unconnected, vertices. The treatment is within the Rouse description, as it ignores the effects of hydrodynamics.

The microstates of the membrane are sampled according to the Metropolis algorithm. The probability of accepting the change of the microstate due to vertex move or bond flip is min[1,exp $(-\Delta E/kT)$], where ΔE is the energy change, k is the Boltzmann constant and T is absolute temperature. The energy for a given microstate is specified in eqn (1). The bending energy is discretized as described by Gompper and Kroll.^{51–53} For each set of parameters, the system is initially thermalized. Ensemble averaging is done over 200 statistically independent microstates.

In this work we set N_c of the total N = 3127 vertices to represent proteins, which have spontaneous curvature in the range between $c_0 = 0$ (for flat proteins) and $c_0 = 1/l_{min}$ for the most highly curved proteins that can be described well by the discrete mesh. All other vertices represent symmetric membrane and have zero spontaneous curvature. The positive sign of c_0 for curved proteins means that the proteins have the tendency to curve the membrane outwards. If the two vertices representing proteins are nearest neighbors, there is an addition energy term -w assigned to their bond (direct protein-protein interaction). Direct interaction constant w is assumed to be of the order of the thermal energy kT_0 , where $T_0 \approx 300 \text{ K}$ is "room temperature", and membrane bending stiffness κ is of the order of $20kT_0$. In the following we fix the ratio $\kappa/w = 20$, unless stated otherwise. For the size of the protrusive force F, the natural choice in terms of the energy and length-scale of the problem, is of the order of the thermal energy kT_0 per minimal bond length l_{\min} .

Note that since the volume of the vesicle is not conserved, it can adjust to accommodate any shape of the membrane, and therefore the membrane is in a tensionless regime for the passive system. The active forces, pointing outwards, induce a finite membrane stretch and tension. In addition, we can introduce a non-zero pressure difference across the membrane, which can act to inflate the vesicle and induce a finite tension (Fig. S8–S15, ESI†).

3 Results and discussion

Using our simulations we aim to improve the understanding of clustering of curved and active proteins on the membrane and how this process of demixing is coupled with membrane shape changes. Especially we are interested in the budding of curved protein clusters. We expect the demixing and budding to be enhanced by attractive direct interaction between the proteins and the additional membrane deformation induced by the protrusive (cytoskeletal) forces recruited by the proteins.

In our Monte-Carlo simulations the whole membrane is the triangulated surface. To quantitatively analyse the demixing of the proteins in our system, we define the ensemble averaged mean cluster size as

$$\langle \bar{N}_{\rm vc} \rangle = \left\langle \frac{\sum\limits_{i} N_{\rm vc}^{(i)} N_{\rm cl}^{(i)}}{\sum\limits_{i} N_{\rm cl}^{(i)}} \right\rangle,\tag{5}$$

where the angle brackets denote the canonical ensemble average. Inside the brackets, *i.e.*, for a given microstate, \bar{N}_{vc} is the mean cluster size and the sums run over all clusters of vertices representing proteins. In the sums, $N_{vc}^{(i)}$ is the number of vertices in cluster *i* and $N_{cl}^{(i)}$ is the number of clusters of size $N_{vc}^{(i)}$

3.1 Phase transition in thermal equilibrium

In Fig. 1 we plot the cluster size distribution and snapshots of typical microstates of vesicles with curved proteins, in the absence of active protrusive forces. The system is in thermal equilibrium‡ at different temperatures and densities (area coverage fraction, $\rho = N_c/N$) of curved proteins. The cluster size distributions are given from averaging over convergent MC realizations.

At low average protein densities the equilibrium vesicle shapes remain quasi-spherical, with clusters that increase in size with decreasing temperature (in the far left column of Fig. 1, the largest clusters are composed of 5 proteins at $T/T_0 = 1.33$ and of 8 proteins at $T/T_0 = 0.63$). At higher average protein densities, cluster sizes increase and curved protein buds appear on the membrane.

At even larger average protein densities, the vesicle shapes deviate drastically from quasi-spherical and large necklace-like protein clusters often form. The size of these necklace-like clusters and the number of "beads" they contain increase with decreasing temperature. These necklace-like structures form since the isotropically curved proteins can not form flat aggregates, due to their spontaneous curvature, and resemble aggregates calculated for membrane-adsorbed spherical particles.⁵⁴ The theory of self-assembly of curved proteins can approximately explain observed necklace-like structures (see S1, ESI[†]),

while anisotropic curved proteins may form aggregates with other geometries on vesicles.^{55,56} Necklace-like membrane protrusions have been observed in cellular membranes, under different conditions,⁵⁷ and in many *in vitro* experiments.^{58,59}

We compare these simulation results with the prediction of the linear stability analysis (corresponding to the spinodal) of Gladnikoff *et al.* (eqn (S8) in its ESI†).⁴⁵ The linear stability analysis yields the critical thermal energy, $kT^{(c)}$, below which the instability occurs and buds start to form:§

$$kT^{(c)} = 12w(1-\rho)\rho\left(1+\sqrt{\frac{l_{\min}^2 F c_0}{12w}\left(1-\frac{1}{\rho R_0}\right)}\right),$$
 (6)

where $1/R_0$ is mean membrane curvature at the site of a curved membrane protein. In the following we approximate R_0 with the radius of a spherical vesicle with the same membrane area $A.\P$. The expression in eqn (6) is in the limit of vanishing membrane tension, which is valid for our system, while eqn (S8) in the ESI† of Gladnikoff *et al.*⁴⁵ gives the expression in the presence of tension.

In thermal equilibrium, in the absence of the protrusive forces F = 0, this simplifies to: $T^{(c)} = 12w(1 - \rho)\rho/k$, which is plotted in Fig. 1. It can be seen that the prediction of the linear stability analysis qualitatively agrees with our simulation results: above the critical temperature line the protein budding is weak and the cluster size distribution is highly peaked at the size of isolated proteins, while below it buds are larger (together with the corresponding membrane deformation) and the size distribution exhibits a secondary peak at aggregates containing 8 or more proteins (which is the number required to form the smallest closed spherical cluster of proteins). For a more quantitative description, $T^{(c)}$ is defined from the simulations as the temperature where $\langle \bar{N}_{vc} \rangle = 2$ (eqn (5)),||

§ We express the binding interaction between viral proteins I introduced in Gladnikoff et al.45 (see the forth term in the expression for the membrane free energy, eqn (S1), in its ESI^{\dagger}) with our direct interaction constant *w* (see eqn (2)). We get that the aggregation interaction energy contribution per viral protein is $J\phi(1-\phi)/2$, where ϕ is the local concentration of viral proteins. Change of ϕ from 0 to 50% gives an energy change of J/8. On the other hand in our simulations the energy contribution due to direct interaction between curved proteins is defined on bonds between vertices, where each bond carries energy -w, if the vertices connected by the bond both represent curved proteins. In a hexagonal mesh the concentration $\phi = 1/2$ for a given vertex corresponds the case where 3 out of 6 bonds carry the energy -w and the energy per vertex is therefore -3w/2. Comparing the energy changes per protein when ϕ changes from 0 to 50% in both models, we get J = -12w. We also identify ϕ_0 in ref. 45 with our normalized average densities of curved proteins $\rho = N_c/N$, *H* with one half of our spontaneous curvature c_0 and f_c with our F per a^2 , where a is protein lateral size. We use $a = l_{min}$ and take into account that in our simulations we assume tensionless membrane (σ = 0). In our model the membrane is not flat. For a membrane with curvature $1/R_0$ at the site of the curved protein, the spontaneous curvature c_0 (or 2H in eqn (S8) in the ESI of ref. 45) effectively changes to $c_0(1 - 1/\rho R_0)$. We can then express from eqn (S8) in the ESI of ref. 45 the critical temperature $T^{(c)}$. ¶ To estimate R_0 we assume a network of equilateral triangles with sides of

lengths $l = (l_{\min} + l_{\max})/2$. This gives $R_0 \approx \sqrt{\sqrt{3}l^2 N/8\pi} \approx 0.35 l_{\min}\sqrt{N}$.

|| The points $\langle \bar{N}_{vc} \rangle$ as a function of temperature (see Fig. 2b) where fitted using a function $y(x) = a_1/x^{a_2} + a_3$, where a_1 , a_2 and a_3 are free parameters and then y(2) was taken as the estimate for $T^{(c)}$. For non-zero *F* only points at temperatures above the transition into a pancake-like shapes are taken into account when fitting. The error bars in Fig. 2 were obtained by fitting points $\langle \bar{N}_{vc} \rangle \pm \varepsilon$, where ε 's are standard deviations of $\langle \bar{N}_{vc} \rangle$.

[‡] Note that in some cases, for example when large necklace-like clusters form, thermal equilibrium is not always easily obtainable. However, after monitoring different measures for membrane shape change and demixing, we expect that the presented results correspond to the correct phase behaviour.



Fig. 1 Microstates of the vesicles in thermal equilibrium (absence of protrusive forces), for different average densities of curved proteins ρ and relative temperatures T/T_0 . The blue vertices represent the protein-free bilayer and have zero spontaneous curvature; red vertices denote the curved proteins and have spontaneous curvature c_0 . In the corresponding cluster-size distributions, the *y*-axis is the ensemble averaged number of protein clusters of each size and the *x*-axis is the protein cluster size. Black solid curve denotes the prediction of the critical temperature by the linear stability analysis (eqn (6)), while gray points connected by dashed line denote the line where $\langle \bar{N}_{vc} \rangle = 2$. Red solid curve marks the critical boundary of the phase space below which the self-assembly theory predicts aggregate growth (for $r = 1.35l_{min}$, $R_0 = 10r$; eqn (8)).

which agrees quite well with the predicted budding transition line, as plotted in Fig. 1 and 2a, b. We find that the mean aggregate size collapses to a universal curve when the temperature is scaled by $T^{(c)}$ (Fig. 2c). The agreement with eqn (6) is also found as function of protein interaction strength (Fig. 2d), and as function of vesicle radius (Fig. S7, ESI†). We conclude that the critical temperature for budding in the passive system agrees very well with the prediction of the linear stability model⁴⁵ (eqn (6)), at least for the low density regime where the linear stability analysis holds.

Note that in the tensionless limit, as we have here, the spontaneous curvature only affects the budding (phase-separation) transition if there are active forces (eqn (6), compare Fig. 1 and Fig. S17, ESI†). The spontaneous curvature alone (in the presence of finite tension), without direct protein–protein adhesion, does not lead to a budding transition in the limit of linear stability of a flat uniform membrane.⁶⁰

The phase separation of the passive system was additionally approximated with a two-dimensional model of self-assembly of curved proteins (see S1 for details, ESI[†]). The total free energy of the model reads

$$F = M[\tilde{x}_{1}\tilde{\mu}_{1} + kT\tilde{x}_{1}(\ln\tilde{x}_{1} - 1)] + M\sum_{i=1}^{\infty} \left[x_{i}\mu_{i} + kT\frac{x_{i}}{i}\left(\ln\frac{x_{i}}{i} - 1\right)\right] - \mu M\left(\tilde{x}_{1} + \sum_{i=1}^{\infty} x_{i}\right).$$
(7)

Here, \tilde{x}_1 and x_i are the number densities of nanodomains in the weakly curved region and highly curved aggregates, respectively. The energy contributions come not only from the free energies per nanodomain (μ_i and $\tilde{\mu}_1$), but also from configurational entropy, while the Lagrange multiplier μ assures a constant number of nanodomains in the system through a conservation relation. We minimize *F* with respect to the number densities, arriving at the equilibrium distributions for aggregate size (eqn (S10), ESI†). This model predicts that the critical density beyond which the growth of aggregates is energetically favourable is given by (eqn (S11), ESI†)

$$\tilde{x}_{\rm c} \approx \exp\left(\frac{\Delta f - w}{kT}\right),$$
(8)

where $\Delta f = f_c - f_{sp}$ is the difference between the energy of a single protein nanodomain on the highly curved necklace-like aggregates (f_c) and on the weakly curved membrane region (f_{sp}). Comparison between the model phase transition and MC simulations of aggregate distributions shows good overall agreement, with large necklace-like aggregates appearing below the calculated transition line (Fig. 1).

The distribution of cluster sizes develops a secondary peak below the transition line, indicating the formation of large aggregates. For low temperature and large protein densities, the distribution of lengths of necklace-like aggregates is



Fig. 2 Results for the equilibrium system with curved proteins ($c_0 = 1/l_{min}$) without active forces F = 0. (a) Contour plot of the ensemble averaged mean cluster size $\langle \bar{N}_{vc} \rangle$ (eqn (5)) as a function of protein density ρ and relative temperature T/T_0 . The prediction of the critical temperature $T^{(c)}$ by the linear stability analysis (eqn (6)) is shown (solid yellow line), as well as the points where $\langle \bar{N}_{vc} \rangle = 2$ (green). (b) Critical temperatures as predicted by the linear stability analysis (solid line) and from simulations ($\langle \bar{N}_{vc} \rangle$ = 2, green points). Representative snapshots are added for the mixed phase (for ρ = 0.05, T/T_0 = 4/3) and the budded phase (for ho = 0.08, T/T_0 = 0.625). (c) Mean cluster size $\langle \bar{N}_{\rm vc} \rangle$ as function of the temperature normalized by the critical temperature $T/T^{(c)}$, for $\rho =$ 5, 7.5, 8, 8.5, 9, 9.5, 10.5, 11, 11.5, 12, 12.5, 14, 15, 15.5, 17, 20 and 25%. The horizontal dashed line indicates where $\langle \bar{N}_{vc} \rangle$ = 1. Inset: $\langle \bar{N}_{vc} \rangle$ as a function of T/T_0 for three values of ρ . (d) $\langle \bar{N}_{\rm vc} \rangle$ as a function of $T/T^{(c)}$ for two different values of the direct interaction constant w. The average protein density is $\rho = 9.5\%$ (inset: the same but as a function of T/T_0).

predicted to be exponential (eqn (S9), Fig. S1, S2 (ESI[†]) and ref. 61). However, in our small vesicles and due to the simulations getting "stuck" in particular aggregate geometries, this feature of the distribution is not observed.

3.2 Phase transitions in the presence of active protrusive forces

Next, we consider the effects of active protrusive forces on the system. In Fig. 3 we plot the typical shapes and aggregate size distribution as in Fig. 1, but with $F = 1kT_0/l_{min}$. We can see that the active protrusive forces promote demixing and budding of the convex curved proteins, such that the transition temperature $T^{(c)}$ is shifted to higher temperatures and lower densities, as expected and predicted by the linear stability analysis⁴⁵ (eqn (6)).

A more dramatic effect of the active forces is seen in Fig. 3 at low temperatures, where below the budding transition there is now a second transition to a new class of shapes that was not seen in the equilibrium system (Fig. 1). Namely, below the red dashed curve we find that the vesicles change from deformedspherical to flattened pancake-like shapes, where all or nearly



Fig. 3 Same as on Fig. 1 but for the system with active protrusive forces $F = 1kT_0/l_{min}$. Approximate temperatures below which a transition into a pancake-like shapes is observed are indicated with red dots connected with dashed lines.

all the proteins aggregate at the rim, forming one large cluster in the form of a closed ring. We locate the transition into the pancake-like shapes regime from the sharp change of the slope of the mean cluster size $\langle \bar{N}_{vc} \rangle$ as function of *T* (see Fig. 4b and d),



Fig. 4 Results for the system with active curved proteins ($c_0 = 1/l_{min}$, $F = 1kT_0/l_{min}$). (a) Contour plot of the ensemble averaged mean cluster size $\langle \bar{N}_{VC} \rangle$ (eqn (5)) as a function of protein density ρ and relative temperature T/T_0 . The prediction of the critical temperature $T^{(c)}$ by the linear stability analysis (eqn (6)) is show (solid yellow curve), as well as the points where $\langle \bar{N}_{vc} \rangle$ = 2 (green) and approximate temperatures below which a transition into a pancake-like shapes is observed (red). (b) $\langle \bar{N}_{vc} \rangle$ as a function of temperature normalized by the critical temperature $T/T^{(c)}$, for $\rho = 5, 7.5, 8, 8.5, 9, 9.5, 10.5, 11, 11.5, 12, 12.5, 14, 15, 15.5, 17, 20$ and 25%. The horizontal dashed line indicates where $\langle \bar{N}_{vc} \rangle = 1$. Inset: $\langle \bar{N}_{vc} \rangle$ as a function of T/T_0 for three values of ρ . (c) Critical temperatures as predicted by the linear stability analysis (solid line) and from simulations (green points). Approximate temperatures below which a transition into a pancake shapes is observed in the simulations are shown in red. Representative snapshots are added for the mixed phase (for $\rho = 0.05$, $T/T_0 = 4/3$), the budded phase (for $\rho = 0.14$, $T/T_0 = 4/3$) and the pancake phase (for $\rho = 0.105$, $T/T_0 = 0.625$). (d) $\langle \bar{N}_{vc} \rangle$ as a function of temperature for three different values of the actin protrusive force (legend). The average protein density is ρ = 9.5%. For F = $1.5kT_0/l_{min}$, the snapshots of steady-state microstates are show for $T/T_0 = 0.625$ (pancake), 1 (prolate) and 1.33 (quasi-spherical).

as shown in Fig. 3, 4a and c.** The transition is sharp (see Fig. 5a), which suggests similarity to a first-order phase transition (although the system is out-of-equilibrium). However, we did not find any significant hysteresis (Fig. S5, ESI[†]).

Note that in Fig. 4b we plot $\langle \bar{N}_{vc} \rangle$ as a function of $T/T^{(c)}$, where $T^{(c)}$ is the critical temperature from eqn (6). In the presence of the actin protrusive force the curves collapse well at temperatures above the pancake-like shape transition, as in the passive case (Fig. 2b and c). However, at lower temperatures, where this shape transition dominates the system behavior, there is no such collapse as function of $T/T^{(c)}$. This is expected, since the linear analysis of Gladnikof *et al.*⁴⁵ does not take into account large-scale global shape changes, which are highly non-linear. A similar



Fig. 5 (a) Zoom-in of the lower left corner of Fig. 3, showing system snapshots at low protein densities. Approximate temperatures below which a transition into pancake-like shapes is observed are also indicated (red). (b) Zoom-in on the edge of the pancake shapes for $\rho = 10.5\%$, $T/T_0 = 0.625$ (left) and $\rho = 15.5\%$, $T/T_0 = 0.625$ (right), to highlight their convoluted shape. The circle in the middle has radius $l_{min} = 1/c_0$ and indicates the length-scale of a single spherical bud of proteins.

behavior is found for different protein–protein interaction strengths (Fig. S4, ESI[†]). As expected, a stronger force promotes the transition to pancake-like shapes at higher temperatures (Fig. 4d).

The organization of the proteins into a circular cluster around the rim of a flat vesicle is highly effective in stretching out the flat membrane parts. We indeed find that these regions are almost devoid of proteins (Fig. 3 and 5a), since these regions are energetically unfavorable for the curved proteins. The stretching of the membrane in these regions also acts to suppress aggregation of the curved proteins,⁴⁵ and the rim aggregate is highly stable (see Movie S1, ESI†).

Below a critical density, we find that there are simply not enough proteins to form a continuous cluster around the rim of the flattened vesicle, and the system changes to a different class of shapes (Fig. 5a). In this regime the curved proteins form arc-like clusters that line the flattened ends of an elongated vesicle, and remain highly dynamic (see Movie S2, ESI†). Since the curved proteins are isotropic, the curvature of the rim of the flattened vesicles along circumferential direction is much smaller compared to c_0 . The curved protein therefore tend to bend the rim and cause it to undulate along this direction (Fig. 5b). At large protein densities we find that the excess proteins crowd the rim and cause it to undergo

^{**} The error bars on the pancake-transition curve in Fig. 4c mark the region in which the transition occurs, *i.e.*, the region between observed no-pancake states with lowest *T* and pancake states with largest *T* (Fig. 3).

buckling and curling, so as to be able to accommodate more curved proteins (Fig. 3). At the highest densities, the excess proteins extend from the rim cluster as spherical and necklace-like clusters (Fig. 3).

We can propose the following mechanism that drives the transition from deformed spherical vesicles with small buds to pancake-shapes in the presence of the active protrusive forces. When the proteins are isolated, or in very small clusters, the membrane is rather flat and the protrusive force promotes the aggregation of small clusters,⁴⁵ since the active force is directed at the outwards normal at each protein, thereby enhancing the outwards deformation that the curved protein induces. However, for larger clusters that are highly curved, the active forces point in different directions which acts to inflate and deform the clusters (Fig. 6a). We can give a rough estimate of the critical cluster size at which the in-plane projection of the active forces at the cluster edge are large enough to compete with the direct attraction between the proteins (w), and can destabilize spherical aggregates. The cluster size for an angle θ is: $N_{\text{cluster}} =$ $2\pi c_0^{-2}(1 - \cos(\theta))/a$, where $a \sim 0.8 \times l_{\min}^2$ is the area per protein. The critical angle can be estimated by the following force balance: $F\sin(\theta) \simeq w/l_{\min}$, where the projection of the active force in the plane of the membrane at the edge of a cohesive cluster competes with the binding energy of an additional protein at the cluster rim. From this estimate we expect that the active forces will destabilize spherical aggregates above a critical cluster size. This estimate does not provide us with an accurate expression that can be compared quantitatively to the simulations, beyond noting that the shape of the pancake transition line in the phase diagram should follow the contours of the mean cluster size. The observed transition line is indeed found to follow a critical cluster size contour (Fig. 4a). As the temperature decreases and the mean cluster size increases beyond the critical size, the system transitions to another global configuration where the proteins form a rim cluster that is highly stable and contains almost all the proteins. In this configurations there are no side-ways active forces that act to destabilize the



Fig. 6 (a) Schematic illustration of the side-ways active protrusive forces (black arrows) that act to destabilize spherical aggregates (red) of curved proteins, on a flat membrane (blue). (b) Schematic illustration of the forces when a flat protein aggregate (red) drives the growth of a tubular protrusion from a flat membrane.



Fig. 7 (a) Same as Fig. 3, but for flat active proteins ($c_0 = 0$, $F = 1 kT_0/l_{min}$). Transition curve for hydra-like shapes is shown in dashed red line, the $\langle N_{cl} \rangle = 2$ line in dash green line and the critical temperature $T^{(c)}$ (eqn (6)) in solid black line. (b) Contour plot of the ensemble averaged mean cluster size $\langle \bar{N}_{vc} \rangle$ as a function of protein density ρ and temperature *T*. Transition lines as in (a), only $T^{(c)}$ in yellow. (c) Asphericity (eqn (10)) as a function of temperature. Red vertical dashed line indicates the transition from quasi-sphere to tube-like shapes. (d) $\langle \bar{N}_{vc} \rangle$ as a function of spontaneous curvature for interaction constant $w = 0kT_0$ (blue), $1kT_0$ (green) and $1.5kT_0$ (red); for $\rho = 11\%$ and $T/T_0 = 0.7$. Error bars denote standard deviations. Some corresponding snapshots are shown, where colors of the arrows match the color of the corresponding data points.

protein aggregate, but rather the active forces now act to stretch the whole vesicle in the same direction as the deformation due to the proteins' curvature, and thereby stabilize the pancake configuration.

The transition into the pancake shape can be arrested by applying an osmotic pressure difference, which acts to maintain the vesicle in a spherical shape of maximal internal volume. In the S7 (ESI⁺) we derive the critical pressure at which the pancake phase disappears from the phase diagram, which scales as: $p_c \sim (F/a) \sim 1kT_0/l_{\min}^3$, where $d \sim 1/c_0$ is the thickness of the pancake shape. However, already at much smaller pressures the critical temperature shifts to much lower values (Fig. S9-S13, ESI⁺). This arises from the following effect: the applied pressure inflates the pancake shape, increasing the radius of curvature at the rim such that it becomes sufficiently different from the value preferred by the proteins $(1/c_0)$. By calculating the pancake width as function of pressure, as a balance between the pressure and the bending energy of the proteins along the pancake rim, we can estimate the value of the pressure at which the pancake width increases significantly such that the bending energy is increased by an order of $\delta E \sim kT$ (Fig. S15, ESI[†]). This pressure scales as: $p_{\rm c}' \sim \sqrt{\delta E \kappa c_0^3 / R^3}$, which for our vesicle gives $p_{\rm c}' \sim 0.04 k T_0 / l_{\rm min}^3$.

Note that even in the absence of any osmotic pressure there is an entropic pressure due to the collisions between the membranes on the two opposing flat surfaces of the pancake shape. This pressure, which is well known from membrane stacks, ^{62,63} turns out to be negligible in our case.

One striking feature of the pancake transition line shown in Fig. 3 (see also Fig. S8–S13, ESI[†]) is that the critical temperature has a maximum at $\rho \sim 9\%$, and decreases for higher densities. We can qualitatively understand this, as arising from surplus proteins that can not fit within the circular cluster along the pancake rim. The surplus proteins attach to the rim aggregate from the sides, inducing sideways forces that bend the rim. The net effect of these sideways active forces is similar to the effect of the isotropic osmotic pressure, making the pancake shape thicker and reducing the bending energy gain for having all the curved active proteins along the pancake rim (see also Fig. S6, ESI[†]). The critical temperature needed to break-up the rim cluster is therefore lower for these higher densities.

3.3 Dependence on the spontaneous curvature of the active proteins

We next explore the role of the spontaneous curvature of the active proteins in driving the shape transition discussed above. We start by calculating the phase diagram for flat active proteins, *i.e.*, with $c_0 = 0$. The result is shown in Fig. 7a. We find that the budding transition line is still well described by the linear stability expression (eqn (6)). At low temperatures we find a global shape transition from quasi-spherical vesicles to shapes with highly elongated protrusions, which are driven by the protrusive force provided by a cluster of proteins at the protrusion's tip. An approximate transition curve, which marks the transition into such hydra-like shapes regime (Fig. 7a and b) can be obtained

from locating the sharp change of the slope of the asphericity's^{††} dependence on *T* (Fig. 7c).

This shape transition can be understood by calculating the conditions that allow a tube-like protrusion to start growing from the vesicle, driven by the protrusive force induced by a circular protein aggregate (Fig. 6b). This occurs when there is a force balance between the protrusive force provided by the protein cluster at the tip and the elastic restoring force due to membrane bending (as in tether pulling): $N_{\rm cl}F \simeq 2\pi\kappa/\sqrt{N_{\rm cl}}$. From this force balance we derive the radius of the protrusions

(details in the ESI,† eqn (S17)): $R_{\rm c} = \left(\frac{2\kappa a}{F}\right)^{1/3}$ (where *a* is the average area per protein), which is in very good agreement with the simulated widths of the protrusions (Fig. S3, ESI†). As the temperature decreases, the mean cluster size increases until it is larger than the threshold size (Fig. 6b) for the elongation of tube-like protrusions (Fig. 7b). In this phase the shapes are highly dynamic and unstable, with protrusions merging and growing (see Movie S3, ESI†).

By fixing a low temperature and large enough density, we explore the dependence of the global vesicle shape transitions on the spontaneous curvature of the active proteins. This is shown in Fig. 7d, where we change the spontaneous curvature of the active proteins from $c_0 = 0$ (flat proteins) to $c_0 = 1/l_{min}$ (the spontaneous curvature used in the previous section). We find that as c_0 is increased, the multi-tube-like shapes transform continuously into shapes that have a single tubelike part and flattened arc-like clusters at the two tips. Above a critical spontaneous curvature there is a sharp transition into the flattened shapes with continuous rim cluster. From our simple estimates (Fig. 6a and b) we predict that the critical cluster size that enables the pancake shape transition decreases with increasing c_0 , while the tube-like shape transition does not depend on this parameter. We therefore expect that above a critical value of c_0 the pancake shape will dominate, as we observe (Fig. 7d).

†† Deviation of the vesicle from quasi-spherical shapes can be characterized conveniently in terms of the asphericity. To this end, we make use of the gyration tensor, whose components are defined for a discrete object through

$$S_{ij} = \frac{1}{N} \sum_{k=1}^{N} r_{k,i} r_{k,j} \qquad (i,j=1,2,3).$$
(9)

Here, $r_{k,i}$ is the *i*-th Cartesian coordinate of the position vector \vec{r}_k of the *k*-th particle. The origin of the coordinate system is located at the center of mass and the sum runs over all particles of the object. From the principal moments (*i.e.*, the eigenvalues) $\lambda_1 \geq \lambda_2 \geq \lambda_3$ of S_{ij} (calculated using the algorithm outlined by Smith⁶⁴), we obtain the asphericity of the object⁶⁵

$$Asph = \frac{\left\langle (\lambda_1 - \lambda_3)^2 + (\lambda_2 - \lambda_3)^2 + (\lambda_1 - \lambda_2)^2 \right\rangle}{2\left\langle (\lambda_1 + \lambda_2 + \lambda_3)^2 \right\rangle}$$
(10)

with $\langle \cdots \rangle$ denoting ensemble averages. We point out that a one-dimensional object (where $\lambda_2 = \lambda_3 = 0$) leads to A = 1, a two-dimensional axisymmetric disk (where $\lambda_1 = \lambda_2$ and $\lambda_3 = 0$) entails A = 1/4, and a sphere (where $\lambda_1 = \lambda_2 = \lambda_3$) gives rise to A = 0. The asphericity A has frequently been used in the past to characterize polymers and membranes.^{66–68}

4 Conclusions

We have explored here the coupling of convex proteins (such as complexes that contain IRSp53) that recruit the protrusive force of the cytoskeleton (most commonly due to actin polymerization), using Monte-Carlo computer simulations. We found that the presence of the protrusive forces gives rise to the formation of protein aggregates and budding at a higher temperature and lower average protein density, compared to the passive system that is in thermal equilibrium (no active forces). This is in an agreement with analytic linear-stability analysis.⁴⁵ The more robust aggregation and budding due to the recruited active forces of the cytoskeleton has important consequences for a variety of biological processes, such as budding of viruses^{45,46,69} and initiation of cellular protrusions (such as filopodia) during development and cell motility.

Beyond the budding transition, we found a new and unexpected global shape transition that occurs only in the presence of the active forces. In this transition the spherical vesicle is transformed into a flat, pancake-like shape, with all the curved proteins and associated cytoskeleton forces along the circular rim. This structure resembles the lamellipodia and ruffles of spreading and motile cells⁷⁰ (see Movies S1 and S2, ESI[†]), where the actin polymerization is localized to the highly curved leading edge. Our results show that lamellipodia-like structures spontaneously form when convex proteins recruit the protrusive force of the cytoskeleton, on a closed membrane. This mechanism is in agreement with recent experiments providing evidence for the role of such curved protein complexes, involving I-BAR proteins, at the leading edge of lamellipodia.⁷¹

While we do not take into account many physical processes that occur in the lamellipodia of cells, such as adhesion and accounting for the complex orientations of the branched actin network, we do believe that our results may shed light on the formation of such structures in living cells: adhesion is not required for cells to form sheet-like membrane protrusions.^{70,72} The orientations of the actin filaments play a dominant role for structures such as filopodia that have a core of highly oriented actin filaments. In the sheet-like structures, where actin is branched, the role of oriented fibers may be less dominant. Including the complexity of the actin network is beyond the scope of the models that we are exploring at present. Future studies could test our predictions, by exploring the spontaneous curvature properties of the actin nucleators at the leading edges of these cellular structures.

In our simulations, the forces exerted by the active proteins on the membrane do not maintain global force balance, since the proteins are not symmetrically distributed on the surface of the vesicle in general, while in a real cell these forces are balanced of course. Most commonly, in an adhered living cell the forces balance by adhesion to an external substrate. Despite the lack of adhesion, and therefore lack of global force balance, we do not expect the nature of our conclusions to qualitatively change: (1) In the "pancake" class of shapes the active forces nearly balance, even in our free vesicle system. (2) In the case of flat active proteins that induce long protrusions, the basic

tendency of protein clusters to pull tether-like protrusions should not change if the protrusions are being drawn from a vesicle that has some part of it stuck to a substrate. In Fig. S16 (ESI[†]) we plot the mean total active force (per protein) as function of the spontaneous curvature of the proteins. Indeed, in the pancake phase the configuration of proteins gives rise to near force balance, while in the phase with long protrusions it is less well maintained. However, the discrepancy is not very large, indicating that it is not likely to dominate the overall class of shapes that the system forms, which should not be strongly affected by the small force imbalance. Since we are interested in shape changes, we disregard any center-of-mass motion. While the role of adhesion on the shape of the vesicle is a subject that we plan to study in the future, and is outside the scope of the present paper, we simulated the shapes of vesicles in the pancake and protrusions regimes where a small patch is permanently anchored to a fixed flat substrate (Movies S7 and S6, respectively, ESI⁺). We find that the shapes maintain essentially the same dynamics as was observed for the free vesicles, even though there is now an imposed global force balance.

Another class of active proteins are those that exert forces that maintain a local force balance, such as a local force dipole due to the activity of membrane pumps⁷³ or due to curvaturechanging proteins.³⁴ These were not explored here, as we focused on the direct local forces that can be exerted on the membrane by the cytoskeleton. Due to the anchoring of the actin cytoskeleton to the membrane, the application of protrusive force at the sites of polymerization nucleation (represented by the proteins in our model), may be balanced by a net inwards pull exerted by the cytoskeleton on the membrane away from these sites. This inwards effective pull can be spread laterally over a larger membrane area compared to the localized protrusive forces. Such a delocalized network applies an overall dipolar force profile on the membrane, which maintains force balance. Since our model does not include the details of the actin network structure, and its anchoring to the membrane, the description of this more realistic application of the forces by the cytoskeleton is beyond the current study.

Let us note that flat oblate membrane shapes can be obtained without the presence of active protrusive forces, for vesicles with low volume to area ratios.^{10,16,74,75} However, in our simulations the volume of the vesicle is free to relax, and under such conditions we find that the active forces are essential for the curved proteins to drive the global flattened vesicle shape transition.

In addition, a variety of tubular and flattened shapes may be stabilized in the absence of active forces, due to the presence of anisotropic curved membrane proteins (or protein complexes).⁷⁶ Tubular protrusions can occur due to accumulation of anisotropic membrane components, as was shown theoretically^{21,28,29,77-80} and supported by experiments.^{9,21,24} Vesicles with flattened edges can be stabilized by anisotropic (arc-like) proteins that form a cluster at the edge of the flattened regions.⁸⁰ We also note that the edge of the disc-shaped vesicles that we obtained here are highly convoluted (Fig. 5b), due to the isotropic curvature of the proteins.

Lamellipodia in cells may avoid this and maintain a smooth edge by using anisotropic proteins that recruit the actin polymerization. All of these considerations motivate the exploration of the vesicle shapes induced by coupling active forces with anisotropic membrane constituents in our future studies.

To conclude, our study highlights the rich variety of membrane shapes that may be induced by curved membrane proteins that recruit the active forces of the cytoskeleton.³⁰ These include steady-state shapes that are not possible for the passive, equilibrium system. Future computer simulations could explore further the space of these non-equilibrium, and dynamic membrane shapes.

Author contribution

MF and SP provided Monte-Carlo simulations; NG provided the model for active proteins and linear stability analysis; AI, VKI and MD provided the model of self-assembly in equilibrium.

Conflicts of interest

There are no conflicts to declare.

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Theoretical study of vesicle shapes driven by coupling curved proteins and active cytoskeletal forces (Supplementary Information)

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S1: A theoretical model of self-assembly of curved nanodomains in a two-component membrane

We use the theory of self-assembly to describe the accumulation of curved membrane nanodomains composed of lipids and proteins into spherical or necklace membrane protrusions. The curved nanodomains (of total number *N*) are initially distributed in the weakly curved spherical membrane surface of constant mean curvature $H=1/R_0$. We assume that the nanodomains are laterally mobile over the membrane surface. For isotropic curved membrane protrusion of constant high mean curvature H=1/r. Here, *r* is the radius of curvature everywhere on the membrane protrusion which may be a sphere or necklace formation (see Fig. S1) and assume $R_0 > r$.



Figure S1 Growth of necklace-like protrusions is energetically favorable when critical concentration \tilde{x}_c is surpassed.

For the sake of simplicity we assume that the free energy of a single flexible membrane nanodomain can be written in the form [V. Kralj-Iglič et al. Deviatoric elasticity as a possible physical mechanism explaining collapse of inorganic micro and nanotubes, Physics letters A, 2002]:

$$f = \frac{\xi}{2} (H - H_0)^2 a_o \,. \tag{S1}$$

where H_0 is the intrinsic mean curvature of an isotropic membrane nanodomain, ξ is the elastic constant and a_0 is the area per single nanodomain. In aggregates of curved flexible membrane nanodomains the local membrane bending constant is $k_c = \xi/4$ and the membrane spontaneous curvature $c_0 = 2H_0$.

Curved flexible membrane nanodomains in aggregates interact with neighbouring membrane nanodomains. We denote the corresponding interaction energy per curved flexible membrane nanodomain (monomer) in an aggregate composed of *i* nanodomains as w(i) where we assume that the energy w(i) depends on the size of the aggregate composed of *i* nanodomains. The mean free energy per nanodomain in a curved aggregate (where H = D = 1/r) composed of *i* nanodomains can be written as:

$$\mu_i = f_c - w(i) \,, \tag{S2}$$

where $f_c = f(H=1/r)$ and w(i) > 0. We assume that in the weakly curved spherical regions of the membrane (having $H=1/R_0$) the concentration of nanodomains is always below the critical aggregation concentration and therefore nanodomains cannot form twodimensional flat aggregates. The mean energy per nanodomain in the weakly curved membrane regions is $\tilde{\mu}_1 = f_{sp}$, where $f_{sp} = f(H=1/R_o)$. The number density of curved proteins in the weakly curved membrane regions is

$$\tilde{x}_1 = \frac{\tilde{N}_1}{M},\tag{S3}$$

where \tilde{N}_1 is the number of monomeric curved nanodomains in the weakly curved membrane regions and *M* is the number of lattice sites in the whole system. The distribution of highly curved aggregates in the membrane protrusions on the scale of number density is expressed as

$$x_i = \frac{iN_i}{M},\tag{S4}$$

where N_i denotes the number of aggregates with aggregation number *i*. The number densities \tilde{x}_1 and x_i must fulfil the conservation condition for the total number of flexible nanodomains in or on the membrane:

$$\tilde{x}_1 + \sum_{i=1}^{\infty} x_i = N/M.$$
(S5)

The free energy \mathscr{F} of all nanodomains in or on the membrane can be written as:

$$\mathscr{F} = M[\tilde{x}_1 \ \tilde{\mu}_1 + kT\tilde{x}_1(\ln \tilde{x}_1 - 1)] + M\sum_{i=1}^{\infty} \left[x_i \ \mu_i + kT\frac{x_i}{i} \left(\ln \frac{x_i}{i} - 1 \right) \right] - \mu M(\tilde{x}_1 + \sum_{i=1}^{\infty} x_i)$$
(S6)

where μ is the Lagrange parameter assuring conservation of protein concentrations. The above expression for the free energy also involves the contributions of configurational entropy. We minimize \mathscr{F} with respect to \tilde{x}_1 and x_i :

$$\frac{\partial \mathscr{F}}{\partial \tilde{x}_i} = 0, \frac{\partial \mathscr{F}}{\partial x_i} = 0, i = 1, 2, 3, \dots,$$
(S7)

which leads to equilibrium distributions:

$$\tilde{x}_1 = \exp\left(-\frac{f_{sp} - \mu}{kT}\right),\tag{S8}$$

$$x_i = i \, \exp\left(-\frac{i}{kT} \left[f_c - w - \mu\right]\right),\tag{S9}$$

where we assumed for simplicity that w(i) = w is independent of aggregate size. The quantity μ can be expressed from Eq. S8 and substituted in Eq. S9 to get:

$$x_i = i \left[\tilde{x}_1 \cdot \exp\left(\frac{f_{sp} + w - f_c}{kT}\right) \right]^i.$$
(S10)

We see that if the concentration \tilde{x}_1 is small, aggregate growth will not be favorable, since $x_1 > x_2 > x_3 \dots$ Furthermore, x_i can never exceed unity, leading to the maximal possible value of the number density of monomeric curved flexible nanodomains in the weakly curved parts of the membrane when \tilde{x}_1 approaches $\exp[(f_c - f_{sp} - w)/kT]$. The critical concentration is therefore

$$\tilde{x}_c \approx \exp\left(\frac{\Delta f - w}{kT}\right),$$
(S11)

where $\Delta f = f_c - f_{sp}$ is the difference between the energy of a single nanodomain on the highly curved membrane protrusion and the energy of the single nanodomain in the weakly curved membrane region with:

$$\Delta f = \frac{\xi a_o}{2r} \left(\frac{1}{r} - 2H_0\right) - \frac{\xi a_o}{2R_0} \left(\frac{1}{R_0} - 2H_0\right).$$
(S12)

If \tilde{x}_1 is above \tilde{x}_c , the formation of a very long necklace membrane protrusions composed of curved membrane proteins is energetically favourable. It can be seen from Eq. S11 that longitudinal growth of the necklace membrane protrusions is dependent on the energy difference Δf (Eq. S12) and the strength of the direct interaction between nanodomains *w*. The critical concentration \tilde{x}_c strongly depends on H_0 .

In the approximation limit $R_0 \gg r$ we can rewrite Eq. S12 as:

$$\Delta f \simeq \frac{\xi}{2r} \left(\frac{1}{r} - 2H_0\right) = \frac{2k_c}{r} \left(\frac{1}{r} - c_0\right),\tag{S13}$$

where k_c and c_0 are the local bending constant and spontaneous curvature of aggregates of nanodomains, respectively. We may rewrite Eq. S11:

$$\tilde{x}_c \approx \exp\left(2\frac{k_c}{kT}\frac{a_o}{r^2}\left(1-c_0r\right) - \frac{w}{kT}\right).$$
(S14)

For $1 < c_0 r$ the value of Δf is always negative. The theoretically predicted existence of necklace membrane protrusions (without application of the local forces) within the self-assembly theory is in line with our MC predictions.

Since the density of nanodomains in or on the membrane is defined with the conservation condition (Eq. S5), this also gives us the relation between normalized temperature T/T_0 and total curved nanodomains concentrations $\rho = N/M$. Using the parameters from the MC simulations, we may graph dependencies $x_i(i)$, as seen in Fig. S2. Above small concentrations and especially above \tilde{x}_c , aggregates start to form, where the peaks of the distributions are strongly dependent on the total protein concentration in the lattice. We see that the critical line beyond which aggregate growth is favourable agrees well with the results of MC simulations.



Figure S2 Aggregate concentrations in dependence on number of nanodomains in the aggregate for different number of flexible nanodomains on the membrane.

S2: A theoretical analysis of the critical cluster size that enables tubular shapes for flat active proteins

The conditions that trigger the transition into the tubular-shapes (Fig.7) are given by the following force balance: The force applied at the tip of the cylindrical protrusion by the cluster of active proteins is

$$F_a = F \frac{\pi R^2}{a},\tag{S15}$$

where F is the force per active protein, R is the radius of the cylinder, and a is the area of a protein on the membrane. This is balanced by the restoring force of the membrane bending energy

$$F_b = \kappa \frac{2\pi}{R},\tag{S16}$$

with κ the bending modulus. The force balance gives the radius of the cylindrical protrusions in this phase of the vesicle shapes

$$R_c = \left(\frac{2\kappa a}{F}\right)^{1/3}.$$
(S17)

The prediction of Eq. S17 is in good agreement with simulations (see Fig. S3), where we took for *a* the area that corresponds to one vertex in a hexagonal mesh, $a = \sqrt{3} l_0^2/2$, where $l_0 = (l_{min} + l_{max})/2$.

In the phase of tubular shapes, there are several protrusions (typically 2-3) that pull in opposite directions to provide an approximate overall force balance, and maintain the relative stability of this shape. Some fusions of protrusions do occur, especially for cases with a larger number of thinner protrusions, so that their number fluctuates.

An alternative to the derivation of the estimate of the protrusion's width given above can be obtained as follows: the total work done by the active forces that pull and extend a protrusion of length *L*, combined with the curvature energy, is given by

$$W = \frac{\pi R^2}{a} FL + 2\pi RL \frac{\kappa}{2} \frac{1}{R^2},\tag{S18}$$

where we assume that the cylinder is very long compared to its radius, so that its surface area is given by: $A \simeq 2\pi RL$. For a fixed area constraint, such that we can substitute $L = A/(2\pi R)$, we can rewrite this work function as

$$W = \frac{RF}{2a}A + A\frac{\kappa}{2}\frac{1}{R^2}.$$
(S19)

Differentiating this work with respect to *R* we find that the minimum is given by the radius of Eq. S17.



Figure S3 Radius of cylindrical protrusions as a function of the κ to *F* ratio for the system with almost flat active proteins with parameters $c_0 = 1/(0.9l_{\min})$, $\rho = 11\%$, $w = 1kT_0$ and $T/T_0 = 0.7$ (see top-left hydra-like snapshot on Fig. 7d). Black solid curve is the prediction of Eq. S17, while red dots are the results of the simulations with error bars indicating standard errors.

SI3: Cluster size dependence on the strength of the direct interaction for active system See Fig. S4.



Figure S4 Mean cluster size $\langle \bar{N}_{vc} \rangle$ as a function of $T/T^{(c)}$ for two different values of the direct interaction constant (legend), for an active system with $F = 1 kT_0/l_{min}$. The average protein density is $\rho = 9.5\%$. The graphs do not collapse, unlike in the passive system (Fig. 2d).

SI4: Testing for hysteresis of the pancake transition

See Fig. S5.



Figure S5 Hysteresis test for the transition into the pancake shape (corresponding to the system shown in Figs. 3,4), showing ensemble averaged mean cluster size for active curved proteins as a function of temperature for two different initial states – above (blue) and below (red) pancake transition. Average protein density is $\rho = 11\%$. Error bars denote standard deviations.

SI5: Cluster size dependence on the density

The activity-driven transition is clearly seen in Fig. 4b of the main text – in the mean cluster size $\langle \bar{N}_{vc} \rangle$ as a function of temperature T/T_0 for different average densities of proteins ρ . Without the active protrusive force, $\langle \bar{N}_{vc} \rangle$ monotonically increases with ρ and decreases with T, while the protrusive force gives rise to the sharp transition into pancake-like shapes. The lower stability of the rim aggregate at high protein densities, that we already noticed in Fig.3, is manifested in the non-monotonic dependencies of $\langle \bar{N}_{vc} \rangle$ on ρ and T (Fig. S6).



Figure S6 Ensemble averaged mean cluster size as a function of the average density of curved proteins with $c_0 = 1/l_{min}$. Results with active protrusive force $F = 1 kT_0/l_{min}$ are shown for $T/T_0 = 0.625$ (solid) and without it for $T/T_0 = 0.4$ (dashed).

SI6: Vesicle size dependence of the budding and pancake transition

The dependence of the pancake transition on the vesicle radius mirrors the effect on the overall cluster size distribution: a smaller vesicle has smaller protein clusters and a lower transition temperature (Fig. S7).



Figure S7 Dependence of the budding (green) and pancake (red) transition curves as functions of the number of vertices composing the vesicle with $F = 1 kT_0/l_{\min}, c_0 = 1/l_{\min}, \rho = 9.5\%$. Spherical vesicle with the same membrane area *A* has the radius $R_0 \approx 0.35 \sqrt{N}$ (in units of l_{\min}). Black solid curve is the prediction for the budding transition line from the linear stability analysis.

SI7: Osmotic pressure dependence of the pancake transition

We studied the effects of adding an isotropic osmotic pressure, which adds a term of the form $-p \cdot V$ to the energy of the vesicle.

We begin by estimating the pressure that balances the active forces of the active proteins along the circular rim of the pancake shape. The work done by the active proteins and the osmotic pressure is

$$W \simeq -p\pi R^2 d + \frac{\pi R d}{a} FR, \tag{S20}$$

where we treat the pancake as very thin compared to its radius $(d \sim 1/c_0 \ll R)$, so that its volume $V \simeq \pi R^2 d$. We keep the membrane area constant, so maintain: $A = 2\pi R(d+R)$. Substituting this constraint into Eq. S20, we find a critical pressure that balances the protein forces, when $p_c \sim F/a \sim kT_0/l_{min}^3$. At higher pressures, the isotropic pressure overwhelms the protein active forces, and prevents the pancake shapes.

However, as we can see from the phase diagram on Figs. S8 to S13 (details of the simulations are descibed below), we find that there is significant shrinkage of the pancake phase already at much lower pressures. We can explore the interplay between the osmotic pressure, the pancake shape and the bending energy that keeps the circular protein cluster along the highly curved rim. By considering only the bending energy and work done by the osmotic pressure, we write an energy functional

$$W \simeq -p\pi R^2 d + \frac{\pi R d}{2} \kappa \left(\frac{1}{2} \left(\frac{1}{R} + \frac{2}{d}\right) - c_0\right)^2.$$
(S21)

Since we are interested in a regime of low pressures, where the pancake becomes thicker but still maintains $d \ll R$, we can simplify the mean curvature

$$W \simeq -p\pi R^2 d + \frac{\pi R d}{2} \kappa \left(\frac{1}{d} - c_0\right)^2.$$
(S22)

Minimizing this functional, while maintaining the constant surface condition, provides the steady-state width and radius, for a given surface area A. It turns out that we can approximate R as constant, since it changes very little, and the steady-state width can be approximated as

$$d \simeq \sqrt{\frac{\kappa}{c_0^2 \kappa - pR}}.$$
(S23)

Plugging this width into the bending energy of the proteins at the rim (second term in Eq.S22), and equating this bending energy to some threshold value δE (of order kT) at which the proteins can be thermally activated to leave the highly curved rim, we get for the critical pressure the expression

$$p_c' \simeq 2\sqrt{\frac{\kappa}{R^3}}\sqrt{c_0^3 \delta E}.$$
(S24)

Using the values of our simulations in Fig. S14, and noting that the change in the bending energy of proteins at the transition is of the order $\delta E \sim 0.07 kT$ (see Fig. S15), this critical pressure is: $p'_c \sim 0.01 kT_0/l_{min}^3$, which is close to the values that we found to affect the pancake transition temperature.

In the simulations in the main text the membrane is (almost) tension free. However by including the osmotic pressure, we can expect the membrane tension to increase. To evaluate for membrane tension, we added to the hamiltonian for the membrane energy, besides the -pV energy term, also the term for tension energy:

$$W_A = \frac{k_A}{2} \sum_{i=1}^{N_i} \left(\frac{a_i}{a_0} - 1\right)^2,$$
(S25)

where k_A is the elastic constant of the membrane and the sum runs over all of N_t triangles of the network, a_i is area of triangle *i*, and a_0 is area of a tensionless triangle. For a_0 we choose area of the equilateral triangle, $a_0 = \sqrt{3} l_0^2/4$, with side lengths $l_0 = (l_{min} + l_{max})/2$. We define membrane tension as the average tension energy per membrane area,

$$\sigma = \left\langle \frac{W_A}{A} \right\rangle,\tag{S26}$$

where A is area of the membrane for a given microstate and bra-ket denote canonical ensemble average.

From Fig. S14 we see that for no osmotic pressure, p = 0, membrane tension is around $0.0212kT_0/l_{min}^2$. As can be seen by comparing the phase diagram in Fig. 3 with Fig. S8, we can see that, as expected, the tension term (Eq. S25) does not change the behavior of the system for p = 0. When we introduce the osmotic pressure, the pancake protein rim disassembles (on Fig. S14 at $p \approx 0.0065 kT/l_{min}^3$) while the membrane tension is still close to the value at p = 0. Near the border of the pancake phase, the behavior is quite dynamic, the protein aggregate at the rim can disassemble and reassemble, and with that the vesicle shape looses and gains again the pancake-like shape (see Movies S4 and S5). At larger osmotic pressures (on Fig. S14 for $p > 0.01kT/l_{min}$), the pressure difference starts to dominate the behavior, the vesicle swells and membrane tension starts to increase (see Fig. S14).



Figure S8 Representative snapshots of the vesicle at protein densities $\rho = 5, 7.5, 10, 12.5$ and 15% and temperatures $T/T_0 = 0.6, 0.7, 0.8, 0.9$ and 1.0, for p = 0 (with $k_A = 1 kT_0$, $w = 1 kT_0$ and $F = 1 kT_0/I_{min}$). Approximate temperatures below which a transition into a pancake-like shapes is observed are indicated with red dots connected with dashed lines.



Figure S9 Representative snapshots of the vesicle at protein densities $\rho = 5, 7.5, 10, 12.5$ and 15% and temperatures $T/T_0 = 0.6, 0.7, 0.8, 0.9$ and 1.0, for $p = 0.005 kT/l_{min}^3$ (with $k_A = 1 kT_0$, $w = 1 kT_0$ and $F = 1 kT_0/l_{min}$). Approximate temperatures below which a transition into a pancake-like shapes is observed are indicated with red dots connected with dashed lines.



Figure S10 Representative snapshots of the vesicle at protein densities $\rho = 5$, 7.5, 10, 12.5 and 15% and temperatures $T/T_0 = 0.6$, 0.7, 0.8, 0.9 and 1.0, for $p = 0.01 kT/l_{min}^3$ (with $k_A = 1 kT_0$, $w = 1 kT_0$ and $F = 1 kT_0/l_{min}$). Approximate temperatures below which a transition into a pancake-like shapes is observed are indicated with red dots connected with dashed lines.



Figure S11 Representative snapshots of the vesicle at protein densities $\rho = 5$, 7.5, 10, 12.5 and 15% and temperatures $T/T_0 = 0.6$, 0.7, 0.8, 0.9 and 1.0, for $p = 0.015 kT/l_{\min}^3$ (with $k_A = 1 kT_0$, $w = 1 kT_0$ and $F = 1 kT_0/l_{\min}$). Approximate temperatures below which a transition into a pancake-like shapes is observed are indicated with red dots connected with dashed lines.



Figure S12 Representative snapshots of the vesicle at protein densities $\rho = 5$, 7.5, 10, 12.5 and 15% and temperatures $T/T_0 = 0.6$, 0.7, 0.8, 0.9 and 1.0, for $p = 0.02 kT/l_{min}^3$ (with $k_A = 1 kT_0$, $w = 1 kT_0$ and $F = 1 kT_0/l_{min}$). Approximate temperatures below which a transition into a pancake-like shapes is observed are indicated with red dots connected with dashed lines.



Figure S13 Representative snapshots of the vesicle at protein densities $\rho = 5, 7.5, 10, 12.5$ and 15% and temperatures $T/T_0 = 0.6, 0.7, 0.8, 0.9$ and 1.0, for $p = 0.025 kT/l_{min}^3$ (with $k_A = 1 kT_0$, $w = 1 kT_0$ and $F = 1 kT_0/l_{min}$).



Figure S14 Membrane tension σ (Eq. S26) as a function of osmotic pressure *p* for a membrane with elastic constant (Eq. S25) $k_A = 1 kT_0$, at temperature $T/T_0 = 0.7$, with $\rho = 11$ % of active proteins with direct interaction constant $w = 1 kT_0$ and protrusive force $F = 1 kT_0/l_{min}$. Averaging is done over 200 statistically independent microstates in steady state and error bars indicate standard deviations. Vertical dashed line indicates border of the pancake phase. Representative snapshots are shown for $p = 0.006 kT/l_{min}^3$ (pancake), $0.007 kT/l_{min}^3$ (protein rim disassembles and pancake shape is lost) and $0.08 kT/l_{min}^3$ (quasi-spherical shape).



Figure S15 Ensemble averaged bending energy of proteins W_{bp} as a function of osmotic pressure near the border of the pancake phase (indicated by dashed vertical line), for a system used also in Fig. S14. Averaging is done over 400 statistically independent microstates in steady state.



Figure S16 Ensemble average over 500 statistically uncorrelated microstates of the size of the normalized resultant of the protrusive forces (Eq. S27) as a function of the spontaneous curvature of active proteins, for $F = 1 kT_0$, $w = 1 kT_0$, $\rho = 11\%$ and $T/T_0 = 0.7$. As c_0 increases, the configurations go from hydra-like to pancake-like (see Fig. 7d in the main text). Error bars indicate standard deviations.

SI8: Normalized resultant of the protrusive forces

In our work we defined the local protrusive force due to the cytoskeleton at the active protein *i* as $\vec{F}_i = F \hat{n}_i$, where *F* is the size of the force and \hat{n}_i is the outward facing normal to the membrane at the location of protein *i* (see Eq. 4 in the main text). Here we define the normalized resultant of the protrusive forces,

$$\vec{r} = \frac{\sum_i \hat{n}_i}{\sum_i |\hat{n}_i|} \tag{S27}$$

where the sums run over all proteins. Note that size of vector \vec{r} is $r = |\vec{r}| = 0$ when the protrusive forces cancel out and the net protrusive force on the vesicle is zero, and r = 1 when all protrusive forces show in the same direction.

Fig. S16 shows the ensemble averaged r for different scenarios - pancake and hydra shapes. As we expected, pancake shapes have lower r than hydra shapes.



Figure S17 Representative snapshots for flat passive proteins with $w = 1 kT_0$ for protein with densities $\rho = 0.05, 0.11, 0.15$ at temperatures $T/T_0 = 0.7, 0.9, 1.1$. Black solid curve denotes the prediction of the critical temperature by the linear stability analysis (Eq. 6). Gray point with dashed line denotes where $\langle \bar{N}_{vc} \rangle = 2$ (for $\rho = 0.11$ and $0.15, \langle \bar{N}_{vc} \rangle > 2$ for all three temperatures shown).

SI9: Simulations with flat passive proteins

We also simulated membrane with flat passive proteins, where the only difference between vertices representing the proteins and the rest of the membrane is that the proteins feel the attractive direct interaction (Eq. 3).

In Fig. S17 we plot representative snapshots for different values of protein densities and temperatures. As expected, all shapes are quasi-spherical. There is only phase-separation due to direct protein-protein interactions.

MOVIES

Movie S1: Animation of snapshots in steady-state of the system with $\rho = 7\%$ of curved active proteins with $F = 1 kT_0/l_{min}$, $c_0 = 1/l_{min}$, $w = 1 kT_0$ at $T/T_0 = 0.6$ (see the last snapshot in the second line from below on Fig. 5a).

Movie S2: Animation of snapshots in steady-state of the system with $\rho = 5\%$ of curved active proteins with $F = 1 kT_0/l_{min}$, $c_0 = 1/l_{min}$, $w = 1 kT_0$ at $T/T_0 = 0.6$ (see the second snapshot in the second line from below on Fig. 5a).

Movie S3: Animation of snapshots in steady-state of the system with $\rho = 11\%$ of almost flat active proteins with $F = 1kT_0/l_{min}$, $c_0 = 1/(9l_{min})$, $w = 1kT_0$ at $T/T_0 = 0.7$ (see the top-left shape on Fig. 7d).

Movie S4: Animation of snapshots in steady-state of the system for osmotic pressure $p = 0.006 kT/l_{min}^3$ with $k_A = 1 kT_0$, at temperature $T/T_0 = 0.7$, with $\rho = 11$ % of active proteins with direct interaction constant $w = 1 kT_0$ and protrusive force $F = 1 kT_0/l_{min}$ (see Fig. S14).

Movie S5: Animation of snapshots in steady-state of the system for osmotic pressure $p = 0.007 kT/l_{min}^3$ with $k_A = 1 kT_0$, at temperature $T/T_0 = 0.7$, with $\rho = 11$ % of active proteins with direct interaction constant $w = 1 kT_0$ and protrusive force $F = 1 kT_0/l_{min}$ (see Fig. S14).

Movie S6: Animation of snapshots in steady-state of the system with $\kappa = 20kT_0$, $T/T_0 = 0.7$, $\rho = 11\%$, $w = 1kT_0$ (see Fig. 7d, green dots) and $c_0 = 1/(9l_{min})$ (top left shape on Fig. 7d, and movie S3), but with a patch of seven vertices fixed in space (denoted with green boxes).

Movie S7: Animation of snapshots in steady-state of the system with $\kappa = 20kT_0$, $T/T_0 = 0.7$, $\rho = 11\%$, $w = 1kT_0$ (see Fig. 7d, green dots) and $c_0 = 1/l_{min}$, but with a patch of seven vertices fixed in space (denoted with green boxes).