

The Effects of Tension, Curvature, and Lipid Diffusion on the Enrichment of Ras Proteins in Model Membranes

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An intriguing aspect of the cell membrane that provokes study is lipid-anchor enrichment of lipidated proteins on the cell membrane. Specifically, recent *in vitro* studies involving the enrichment of Ras proteins were done to see how enrichment was affected by changes in membrane curvature. These studies demonstrated the Ras protein's inclination to the expanded, or outer, leaflet of highly curved membranes. Other research done *in vivo*, however, provoked further inquiry into Ras protein behavior, since when highly curved protrusions were created in living cells, Ras proteins showed affinity for the inner leaflet. This inspired research to determine the exact mechanism (e.g. tension, curvature, lipid diffusion tendencies) affecting Ras protein spatial localization. Since mutated Ras can initiate uncontrolled cell division (cancer), this research would allow for deeper understanding of the mechanisms of the Ras protein, in order to inhibit uncontrolled signaling and binding to the cell membrane. A molecular level mean field density functional theory, discretized in space and encoded in Fortran, was used to create multiple types of model membranes and calculate the relative densities of the N-Ras protein as a function of membrane curvature, tension and concentration of lipids in the outer leaflet. The hypothesized belief was that Ras proteins adsorb to the inner leaflet more with increased tension and concentration of lipids in the outer leaflet *in vivo* since these factors would significantly increase the concentration of lipids in the outer leaflet; results instead proved that it is more so the diffusion of lipids from the inner to the expanded leaflet of curved membranes that could drive a shift in the lateral pressure fields to allow more Ras protein enrichment in the inner leaflet, as seen in the *in vivo* experimental studies.

Introduction

Despite rapid advances in technology and methodology to further observe the mechanisms of the cell membrane, much is left to be discovered of protein spatial localization to the cell membrane (1). The lipid bilayer performs diverse functional processes, many of which are facilitated by proteins that are integrated within the bilayer or that reside on its surface. The kinds of proteins that are recruited to the membrane, and whether or not they are activated is determined by the local environment of the membrane, which can be determined by the types of lipids in a given region of the membrane, the local pH and pressure, or forces exerted on the membrane by the underlying cytoskeleton. One group of proteins--Ras proteins--must interact with the bilayer in order to function and are known for their role in cell proliferation, differentiation, and cell survival (2). An intriguing sector of membrane research is the study of the mechanisms that drive the interactions between the membrane and lipidated proteins like those of the Ras family. Lipidated proteins like those found in the Ras family are proteins that are attached to a hydrocarbon chain, allowing them to easily adsorb into the cell membrane (1,3). These hydrocarbon chains (also known as lipid anchors) have been proven to exhibit certain attractions to certain traits of the membrane.

Lipid anchored proteins in the Ras family are known for their role in signaling cascades such as the MAPK/ERK pathway that aids in transcription, cell growth, and proliferation. They are usually found in the inner membrane of living cells and are activated by the binding of GTP, which in turn signals other proteins to initiate transcription (4,5). Research pertaining to the attractive mechanisms of the Ras proteins is significant because of their capacity to cause cancer when mutated. Ras protein mutations are found in 20% to 25% of all human tumors and even 90% of pancreatic cancers, so the correlation between mutated Ras proteins and cancer cannot be ignored, provoking research to help inhibit overproliferation (6). There are three types of Ras genes in humans, N-Ras, H-Ras, and K-Ras, which differ in the types of lipid anchors they attach to. In this study, we focused our analysis on the N-Ras protein, which features the palmitoyl and farnesyl lipid anchors. Since mutated Ras proteins are not inhibited by a negative feedback loop in their proliferation, this can lead to cancer since there is no inhibition of transcription and, therefore, cell over proliferation. Research in relation to the mechanism of Ras/membrane interactions could lead to the discovery of methods for preventing such mutations in the protein.

To further gain understanding of the mechanisms of membrane enrichment of Ras proteins, the effect of membrane curvature on Ras spatial localization was studied. This study was done experimentally using liposomes of varying diameters and fluoresced Ras proteins. Confocal fluorescence microscopy was used to visualize the intensity of Ras protein enrichment in liposomes of varying diameter, *in vitro*. The results supported the hypothesis that the lipid anchors of Ras proteins are more inclined to adsorb to more highly-curved membranes (7,8). Once the same scenario was modeled theoretically, it was discovered that when the cell bilayer is curved, the lateral pressure of the expanded leaflet (in this case the outer leaflet of the liposomes) decreases and that of the compressed leaflet (in this case the inner leaflet of the liposomes) increases, as modeled in Figure 1. Because of this, more Ras proteins were seen to bind to the outer leaflet where there was lower lateral pressure. This lower lateral pressure allowed for greater Ras enrichment to the outer leaflet since the work of insertion of the lipid anchors of Ras proteins to the outer leaflet decreased.

However, other research done *in vivo* studying the effect of curvature had different results than the previous *in vitro* experiment. This was done by taking living cells (specifically cells of the PC12 family) and using optical tweezers to create long protrusions in the cell, as illustrated in Figure 2 (9). Fluoresced Ras proteins attached to the membrane and the intensity of enrichment was measured using confocal fluorescence microscopy. Experimental results indicated that Ras proteins showed affinity for the inner leaflet, though it would have been intuitively assumed that there would be greater Ras enrichment to the outer leaflet because of its lower lateral pressure when curved, according to previous *in vitro* research (10, 7, 11). This provoked the hypothesis that other factors, such as tension or concentration of lipids in the outer leaflet of the cell, affect the affinity of enrichment of Ras proteins *in vivo*. Possible stimuli in living cells like the presence of the cytoskeleton, which provides mechanical resistance to deformation (tension) to the cell membrane, or the enzyme flippase, which transports lipids between the bilayer leaflets, are relevant factors in such *in vivo* experiments, and could provide the explanation for the differences between the *in vivo* and *in vitro* observations. Specifically, it was thought that as both tension and concentration of lipids in the outer leaflet increases, there would be a shift in the lateral pressure *in vivo* that would allow more Ras binding to the inner leaflet.

To study the effect of tension and concentration of lipids in the outer leaflet on Ras protein enrichment, multiple model liposomes of the same diameter were represented using a mean field density functional, and the relative density of Ras protein enrichment was calculated for

manipulations of each. This theoretical approach, in contrast with the experimental approaches discussed previously, allows the thermodynamics and physicochemical properties of the system to be studied and explained. Any set of parameters and variables could be changed computationally to see its effect instead of having to obtain extensive materials. This approach is also more controlled in preventing sources of error.

Methods

This theoretical approach involves the use of a mean field density functional. The frame of the mean field density functional, as shown in Equation 1, allows for the energetic and entropic interactions of the lipids to be accurately represented theoretically.

Through this functional, the coupling and unique interactions of the multiple different forces in the cell membrane are accurately accounted for. The packing constraint, for example, accounts for that fact that there is no water or free space in the lipid bilayer, so the total density of all components is constant. This ensures that the system is incompressible at every position (12). The attractive energy term accounts for the Van der Waals forces between lipid tails within the lipid bilayer by calculating the interaction energy between each monomer on the lipid tails. Essentially, this term puts a weighting factor on certain configurations of the monomers of the lipids so the attractions with the most weight are perpendicular to the bilayer, as this is the configuration that is most thermodynamically favorable.

This mean field density functional was discretized in space so it could be read by Fortran. This partition function was used to calculate the relative density of Ras proteins associated with the curved bilayer in comparison with a planar bilayer. This was done by accounting for the fact that both the lipid anchors of N-Ras adsorbed to a planar membrane, and those adsorbed to a curved membrane, must have chemical potentials that are equivalent to the N-Ras anchors in bulk solution, at equilibrium. Because of this, the lipid anchor chemical potentials within a planar and curved bilayer were set equal to each other to obtain the relative density of N-Ras enrichment, by means of Equation 2-4.

After this, the lateral pressure fields in the inner and outer monolayers were observed to find possible correlation with N-Ras recruitment tendencies.

Results

Results indicated that, although tension does have an effect on Ras protein enrichment, its effect was not substantial enough to cause more recruitment of Ras proteins to the inner leaflet when highly curved, as seen in the previously referred *in vivo* experiment. Although the presence of the cytoskeleton in living cells does impose tension, its presence cannot alter the spatial localization of Ras proteins enough to redirect adsorption of Ras from the outer leaflet to the inner leaflet. As seen in Figure 3, as membrane diameter decreases (increased curvature) the density of N-Ras proteins in the outer leaflet generally increases. When tension is imposed, there is a very similar trend with only a small decrease in N-Ras enrichment to the outer leaflet. Attention was therefore turned to the influence of the diffusion tendencies of the membrane's lipid constituents. By manipulating the value of, what has been termed as the *relaxation ratio* (*RR*), the amount of lipids that migrate to the outer leaflet upon curvature of the membrane can be controlled, and in effect, the concentration of lipids in each membrane leaflet can be modulated.

It was theorized that when the membrane is curved, there is a disruption in equilibrium, causing lipids to passively diffuse from the compressed inner leaflet to the expanded outer leaflet. Diffusion progresses until a new equilibrium, for the new geometry of the system, is achieved. However, lipids can continue to flip from the inner leaflet to the outer leaflet, past the system's equilibrium point, because of the active transport of the flippase enzyme. It was theorized that the activity of the lipid flippase enzyme, which is present only in live cells, was the factor causing the disparity in the experimental results between the *in vitro* and *in vivo* studies. Therefore, the concentration of lipids in the outer leaflet was manipulated to simulate the effect that the enzyme flippase has in living cells, which could alter Ras protein spatial localization *in vivo*. Results of this specific study substantiated the original hypothesis that as concentration of lipids in the outer leaflet increases, so does N-Ras enrichment to the inner leaflet. As seen in Figure 4, Ras protein enrichment to the inner leaflet is highest when there is the highest concentration of lipids in the outer leaflet (large *RR*) and lowest when there is a low concentration of lipids in the outer leaflet (small *RR*).

The hypothesis is also substantiated through Figure 5. As vesicle diameter decreases, there is consistently higher N-Ras enrichment to the outer leaflet when there are lower concentrations of lipids flipped to the outer leaflet. This would make sense since the outer leaflet experiences a decrease in lateral pressure since the membrane is curved, due to its expansion--however, curving the membrane also induces lipid diffusion to the outer leaflet, which would contribute to an *increase* in outer leaflet lateral pressure. With low relaxation ratios, lipid diffusion is not substantial enough to counteract the pressure relief in the outer leaflet with curvature, which promotes the adsorption of the N-Ras lipid anchors to the outer leaf. However, for high relaxation ratios the amount of lipids that flip to the outer leaf is large enough to cause a shift in the pressure profiles, such that lateral pressure actually accumulates in the outer leaf with the influx of lipids, despite the leaflet's expansion when curved. In turn, this also causes a reduction in lateral pressure within the inner leaflet, thereby promoting N-Ras adsorption to the inner, rather than the outer, leaflet under these conditions. It was found that the value of the relaxation ratio at which this shift in behavior occurred (i.e. the value at which equilibrium is recovered in the membrane via lipid trans-bilayer diffusion) is around $RR = 0.56$. As indicated in figures 4 and 5, at relaxation ratios above this value, N-Ras preferentially adsorbs to the inner leaflet, while at values below this equilibrium value, there is increased adsorption in the outer membrane leaflet.

Equilibrium within the membrane is defined by the the chemical potentials of the membrane's inner and outer leaflets. If the difference between the chemical potentials of the inner and outer leaflets is zero, the system is at equilibrium. As discussed above, curving the bilayer disrupts the system out of equilibrium. This is because the change in geometry shifts the chemical potentials out of balance--a balance that can be slowly reattained via the reorganization of lipids to a new optimal configurational state. The equilibrium relaxation ratio (which designates the number of lipids that must flip to the outer leaflet upon curvature to obtain the leaflet concentrations of lipids that would settle the membrane at equilibrium) is 0.56--hence, at this relaxation ratio, the difference in chemical potentials between the membrane leaflets is zero. The direction of lipid diffusion is governed by the direction of the chemical potential gradient. For this theoretical system, a positive difference in chemical potential between the inner and outer leaflets motivates lipid diffusion from the inner leaf, to the outer leaf (as would occur at the onset of bending the bilayer). This corresponds to values of the relaxation ratio below 0.56. Alternatively, a negative chemical potential difference is indicative of a system that would drive lipids to diffuse from the outer to the inner leaflet. Physically, this state could only be achieved via an external energy source (e.g. active transport provided by the lipid flippase enzyme) that forced the system "beyond" its equilibrium configuration by transporting more lipids from the inner leaflet to the outer leaflet than is energetically favorable. This situation applies to relaxation ratios above the equilibrium value of 0.56.

A graph of the change in chemical potential change between membrane leaflets as a function of membrane curvature for a variety of relaxation ratios also substantiates the hypothesis since, as seen in Figure 6, a positive chemical potential gradient, shown by the purple ($RR = 0$) and blue ($RR = 0.25$) curves, causes passive diffusion of lipids from the compressed inner leaflet to the outer leaflet as it curves. However, as the flipping of lipids to the outer leaflet increases past the point of equilibrium ($RR=0.56$), there is a negative change in chemical potential, indicated by the pink ($RR = 0.80$) and orange ($RR = 1.15$) curves, which can only be achieved through active transport that enzymes like the lipid flippase partake in. This is justified since in figures 4 and 5, it is revealed that as lipids continue to flip from the inner leaflet to the outer leaflet through active transport after

equilibrium has been reached, there is a further decrease in lateral pressure in the inner leaflet and a further increase in the lateral pressure in the outer leaflet that allows greater Ras enrichment to the inner leaflet as previously seen *in vivo*.

Lastly, the hypothesis was substantiated through lateral pressure profiles within the inner and outer leaflets, as seen in Figure 7. In the inner leaflet, there is a decrease in lateral pressure in curved membranes with a relaxation ratio above that of the equilibrium value and an increase in lateral pressure in the outer leaflet relative to a planar bilayer. This corresponds to the negative change in chemical potential for $RR > 0.56$ caused by the active transport of lipids from the inner leaflet to the outer leaflet, thereby further decreasing the lateral pressure in the inner leaflet and increasing the lateral pressure of the outer leaflet. The shift in lateral pressure allows more Ras proteins to bind to the inner leaflet as seen *in vivo* and demonstrated theoretically in Figures 4 and 5.

Discussion and Conclusion

As seen through results, the original hypothesis was partially substantiated. Although tension was proven to somewhat alter Ras protein recruitment to the membrane, it was more so the concentration of lipids in the outer leaflet that motivated a shift in lateral pressure, ultimately driving the adsorption of N-Ras to the inner leaflet. In summary, when the cell membrane is curved, its equilibrium is disturbed, and a chemical potential gradient is created. This allows for the passive diffusion of lipids from the compressed inner leaflet to the expanded outer leaflet until equilibrium is attained. However, the presence of the enzyme flippase in living cells allows for further active transport of lipids to the outer leaflet, against the chemical potential gradient. This increase in the concentration of lipids in the outer leaflet caused by the enzyme flippase further increases the lateral pressure of the outer leaflet and further decreases that of the inner leaflet, therefore allowing more Ras enrichment to the inner leaflet *in vivo* in highly curved membranes. These results differed from those of previous *in vitro* studies since the liposomes used in these experiments did not have factors such as the cytoskeleton (can impose tension on the cell) or the enzyme flippase (can actively transport lipids across leaflets). These results conclude that it has been consistently proven that, in both *in vitro* and *in vivo*, the lateral pressure is the driving force of Ras protein adsorption to the membrane when curved, but *in vivo*, more Ras proteins are seen to enrich to the inner leaflet because of the increased concentration of lipids in the outer leaflet by the active transport of flippase, which shifts the lateral pressure fields of the leaflets and reduces the work of insertion of the N-Ras lipid anchors into the inner leaflet. As previously discussed, these results further the understanding of the mechanism of Ras protein attraction to the membrane and promote efforts to inhibit mutated Ras over proliferation of cells by assessing where they are more likely to enrich.

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Fig.1: The extended outer leaflet has lower lateral pressure and is favorable for lipid anchor binding

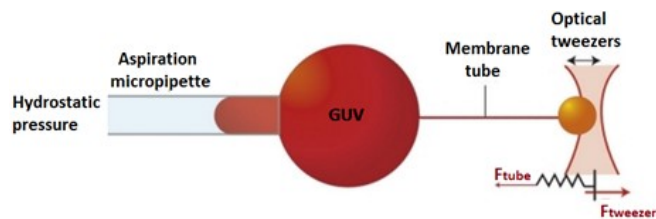


Fig. 2: Optimal tweezers creating an extended membrane tube

$$F = E_{attractions} + E_{configurational} + E_{headgroups} - T(S_{configurational} + S_{translational}) + E_{packing\ constraint}^{repulsive}$$

Equation 1: Frame of the Mean Field Density Functional

$$\beta\mu_{anchor}^{bulk} = \beta\mu_{anchor,E}(\vec{c}) = \beta\mu_{anchor,E}(\vec{c} = 0)$$

Equation 2: Equivalency of Chemical Potentials between Phases

$$\beta\mu_{bulk}^{anchor} = \beta\mu_E^{anchor}(\vec{c}) = \ln[\rho_E^{anchor}(\vec{c})\lambda_{anchor}^2] - n_{tails}^{anchor} \ln[q_E^{anchor}(\vec{c})]$$

Equation 3: Equation for the Chemical Potential with respect to Density and the Partition Function

$$\frac{\rho_{anchor,E}(\vec{c})}{\rho_{anchor,E}(\vec{c} = 0)} = \left(\frac{q_{palmitoyl,E}(\vec{c})q_{farnesyl,E}(\vec{c})}{q_{palmitoyl,E}(\vec{c} = 0)q_{farnesyl,E}(\vec{c} = 0)} \right)$$

Equation 4: Ratio of Anchor Densities is Proportional to the Ratio of Anchor Partition Function

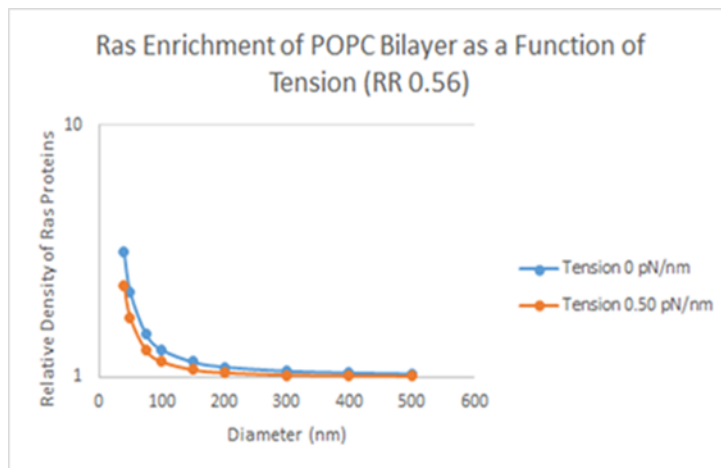


Fig 3: Effect on Tension of Ras Protein Enrichment to the Outer Leaflet* *Note: the “RR” (also known as relaxation ratio) value is a means by which the concentration of lipids that flip to the outer leaflet is measured.

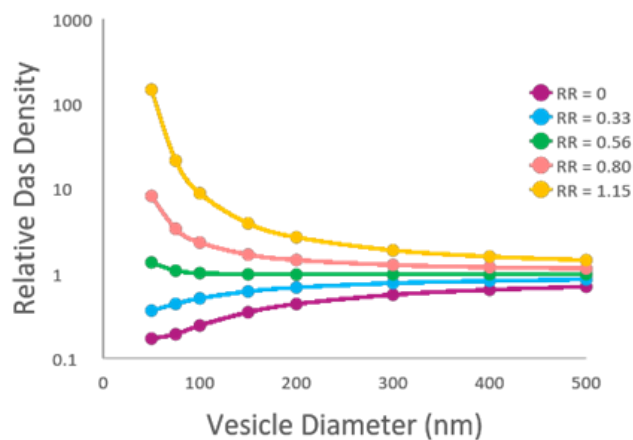


Fig 4: The Effect of Concentration of Lipids in the Outer Leaflet on Ras Protein Enrichment in the Inner Leaflet

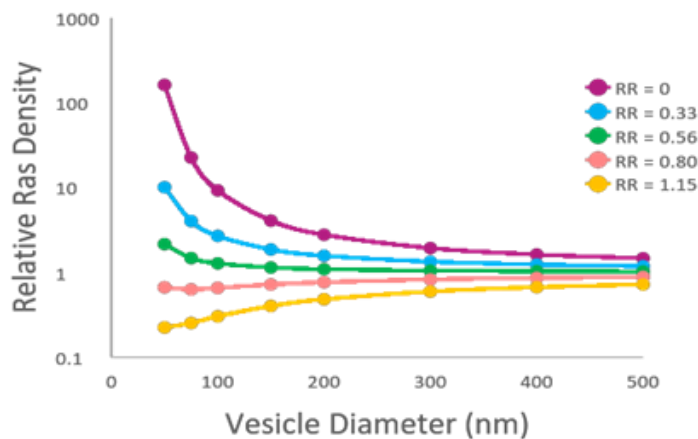


Fig 5: The Effect of Concentration of Lipids in the Outer Leaflet on Ras Protein Enrichment in the Outer Leaflet

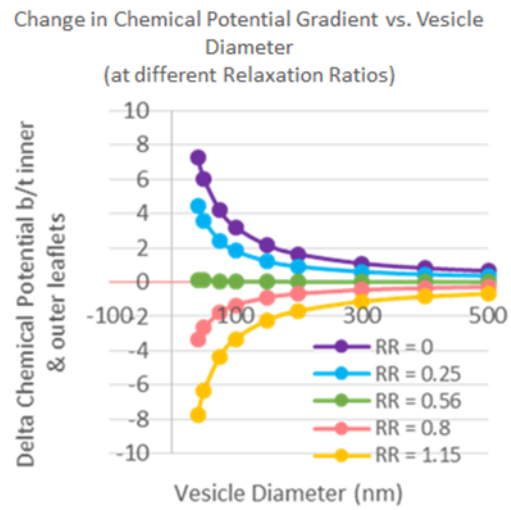


Fig 6: Change in Chemical Potential vs Vesicle Diameter

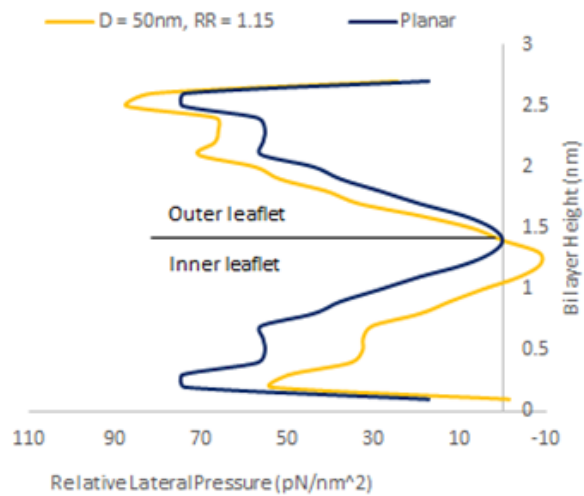


Fig 7: The Effects of Concentration of Lipids in the Outer Leaflet on Lateral Pressure Fields of the Membrane