signal for undergoing rapid fusion with the PM within seconds. The excess membrane added to the PM is retrieved via compensatory endocytosis on a longer time-scale (minutes). We have been using endocrine BON cells, a cell line derived from a human carcinoid tumor, which are capable of calcium-regulated secretion. Secretion is stimulated, almost instantly during tether extrusion, by UV-uncaging of a photolabile calcium chelator loaded into the cytosol. Thus, a significant transient membrane addition to the PM can be turned on at will and the consequences for extrusion dynamics can be studied.
We observe mainly two types of response upon stimulation: a sudden jump in tether length L of ~10 μm followed by an increased extrusion velocity, or a sudden increase of slope. Both responses are consistent with exocytotic addition of area to the PM. However, only a fraction of such responses are well-correlated with UV-uncaging of intracellular calcium. This suggests exocytosis, or other mechanisms of membrane addition to the PM may independently be triggered by other means, possibly by tether pulling itself.

Membrane Fusion

1831-Pos Board B675
Molecular Mechanisms of Vesicle Fusion
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We have performed large scale molecular dynamics simulations using coarse-grained lipid models to study the fusion of vesicles. Our earlier work showed that lipid tail splay played an important role in the first lipids to cross from one vesicle to another. In this work we study the effect of different lipid types on the molecular mechanism of the initial fusion events. Lipid types studied include phosphatidylcholine and phosphatidylethanolamine lipids. In order to examine the importance of lipid splay, lipids with symmetric and asymmetric tail lengths have been studied. We calculate the free energy barrier to fusion by using the umbrella sampling method to determine the potential of mean force as a function of the distance between the center of mass of the two vesicles. We will discuss the comparison of the different curves for the different lipid types and the role of molecular mechanism(s) in vesicle fusion. We will also discuss how the interactions between lipid molecules influence fusion.

1832-Pos Board B676
Imaging Single Virus Fusion Reveals the HIV-1 Entry Pathway
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Viruses whose fusion proteins are activated by interactions with cellular receptors at neutral pH, including HIV-1, are assumed to fuse directly with the plasma membrane. However, direct virus fusion at the cell surface has not been explicitly demonstrated. To differentiate between HIV-1 fusion with the plasma membrane (PM) and with endosomal membrane (EM), we performed time-resolved single virus imaging. Pseudoviruses bearing HIV-1 Env glycoprotein were generated and co-labeled with a content marker (Gag-GFP) and a red lipophilic membrane dye. Upon virus maturation, the Gag-GFP is cleaved by viral protease yielding a smaller GFP-tagged fragment that is readily released from virions permeabilized with saponin. This marker thus provided a convenient means to detect small pore formation during virus-cell fusion. Double-labeled viruses were adhered in the cold to HeLa-derived cells expressing CD4 and coreceptors. Fusion was triggered by shifting to 37°C and monitored by laser scanning confocal microscopy. Imaging of single HIV-cell fusion revealed the occurrence of both PM and EM fusion events. Fluorescent viruses undergoing PM fusion transferred their lipid marker (hemifusion), but not content marker (full fusion). By contrast, full fusion with an endosome was consistently observed. These EM fusion events were manifested in disappearance of a content marker, but not the lipid dye due to its limited dilution in an endosomal membrane. These results demonstrate that, contrary to a common perception, HIV-1 enters HeLa-derived target cells by receptor/coreceptor-mediated endocytosis followed by fusion with endosomes and delivery of viral nucleocapsid into the cytosol.

1833-Pos Board B677
The Gaussian Curvature Elastic Energy of Membrane Fusion Intermediates, and a Possible Mode of Action of Fusion-Mediating Proteins
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Calculating the Gaussian curvature elastic energy of fusion intermediates requires knowledge of the Gaussian curvature elastic modulus, κ. κ can be measured for lipids that form Q_{II} phases. It is shown that one can estimate κ for non-Q_{II} phase lipids by studying phase behavior of lipid mixtures. κ is between -5 and -10 k_BT for biological lipid compositions. The Gaussian curvature elastic energy of fusion intermediates is positive and $\geq$ the total bending energy calculated previously: it increases the total energy of fusion intermediates by 100 k_BT or more. This large contribution makes the predicted intermediate energies compatible with observed lipid phase behavior in excess water. An order-of-magnitude fusion rate equation is used to show that a current theory now predicts stalk energies that are slightly too large, by about 30 k_BT, to rationalize the observed rates of stalk-mediated processes in pure lipids. Despite this discrepancy, when the effect of κ is included, current models of fusion intermediate energy can make semi-quantitative predictions about how proteins mediate biomembrane fusion. Fusion-mediating proteins must lower the stalk energy by several tens of k_BT relative to lipidic stalks. One way proteins could do this is by altering the elastic constants of the patches of monolayer that fuse, by inserting peptides. Preliminary data using fusion peptides and membrane-spanning peptides is compatible with this role for peptides. The energies of stalks, fusion pores, rhombohedral (R) phase and Q_{II} phase relative to L_α phase all depend on approximately the same assembly of monolayer elastic constants; ($2k_m\delta$ J_s-κ). Thus the influence of peptides on stalk and fusion pore energy can be studied by measuring the effects of peptides on R and Q_{II} phase stability (Siegel, Biophys. J.; Dec. 2008).

1834-Pos Board B678
Determination of Free Energy Barriers to Initial Fusion of Vesicles
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The fusion of two vesicles is studied by means of molecular dynamics simulations using coarse-grained lipid models. We use the umbrella sampling method to determine the potential of mean force as a function of the distance between the center of mass of two vesicles. The free energy barrier to the initial fusion step between two vesicles is determined from the peak in the PMF curve calculated at a center of mass distance just prior to the initial fusion step. The two CG lipid models used were the original model by Marrink, de Vries, and Mark (J. Phys. Chem. B 2004, 108,750) and its recently improved and extended version, the MARTINI model. We find that the free energy barrier for the initial fusion event varies by more than an order of magnitude between the two models. The source of the difference is found in the greater repulsive character of the interaction between the hydrophobic tail particles and the charged headgroup particles in the MARTINI model compared to the older model. This interaction results in the lipid tails being confined to the hydrophobic region of the vesicle to a greater extent and the splay of the lipid tails being limited. These factors reduce the probability of triggering an initial fusion event.

1835-Pos Board B679
Insights Into The Energetics Of Neuronal SNARE Complex Formation
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Eukaryotic cells transport material between intracellular compartments by vesicles that bud from a donor and afterwards fuse with a target organelle. Best-studied is the molecular machinery that drives the Ca2+-dependent release of neurotransmitters from synaptic vesicles. Key players in the exocytotic fusion are three proteins synaptobrevin 2, syntaxin 1a, and SNAP-25. Synaptobrevin resides in synaptic vesicles, whereas syntaxin and SNAP-25 are anchored in the plasma membrane. They belong to the so-called SNARE protein family, which members are involved in all other vesicle fusion steps. SNARE proteins are tail-anchored membrane proteins that assemble into a stable membrane-bridging complex. As SNARE assembly is accompanied by extensive structural rearrangements from mostly unstructured monomers into a tightly packed parallel four-helix bundle, it is thought that zipper-like formation of the SNARE bundle between opposing membranes provides the energy that drives fusion. It is unclear, however, whether the assembly energy indeed suffices for membrane merger. Unfortunately, a marked hysteresis in the folding and unfolding transition of the SNARE complex prevents the direct determination of the free energy of assembly. We have now investigated the assembly process by isothermal titration calorimetry. We found that the structural changes upon assembly are reflected in extremely large favorable enthalpy changes, counterbalanced by a large positive entropy change. Moreover, as SNARE complex is essentially irreversible, we made use of the fact that assembly in vitro proceeds in discrete steps. This allowed us to assess the energetics of each assembly step individually.

1836-Pos Board B680
Clustering of Syntaxin-1A in Model Membranes is Modulated by Phosphatidylinositol-4,5-bisphosphate and Cholesterol
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Syntaxin-1A is part of the SNARE complex that forms in membrane fusion during neuronal exocytosis of synaptic vesicles. Together with SNAP-25, the single-span transmembrane protein syntaxin-1A forms the receptor complex on

the plasma membrane of neuroendocrine cells. Previous studies have shown that syntaxin-1A is found in clusters that are different from lipid rafts in neuroendocrine plasma membranes. However, the interactions that promote these clusters have been largely unexplored. Here, we have reconstituted syntaxin-1A into lipid model membranes and show that cluster formation of syntaxin depends on cholesterol in a lipid system that lacks sphingomyelin and therefore does not form typical liquid-ordered phases that are commonly believed to represent lipid rafts in cell membranes. The cholesterol-induced clustering of syntaxin is found to be reversed by as little as 1 mol % of the regulatory lipid phosphatidylinositol-4,5-bisphosphate (PIP2), and PIP2 is shown to bind specifically and electrostatically to syntaxin, presumably mediated by the positively charged juxtamembrane domain of syntaxin. Possible consequences of these results to the regulation of SNARE mediated membrane fusion are discussed.

1837-Pos Board B681

Lipid Mixing and Content Release in Single-Vesicle, SNARE-driven Fusion Assay with 5 ms Time Resolution

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A single-vesicle, fluorescence based SNARE-driven fusion assay enables simultaneous measurement of lipid mixing and content release with 5 ms/frame, or even 1 ms/frame, time resolution. The v-SNARE vesicles, labeled with lipid and content markers of different color, dock and fuse with a planar t-SNARE bilayer supported on glass. A narrow (< 5 ms duration), intense spike of calcein fluorescence due to content release and dequenching coincides with inner-leaflet lipid mixing within 10 ms. The spike provides much more sensitive detection of productive hemifusion events than do lipid labels alone. Consequently, many fast events that were previously thought to be prompt, full fusion events are now re-classified as productive hemifusion events. Both full fusion and hemifusion now occur with a time constant of 5-10 ms. At 60% DOPE lipid composition, productive and dead-end hemifusion account for 65% of all fusion events. However, quantitative analysis shows that calcein is released into three-dimensional space above the bilayer (vesicle bursting), rather than the thin aqueous space between bilayer and glass. Evidently at the instant of inner-leaflet mixing, flattening of the vesicle increases the internal pressure beyond the bursting point. In the future, additional height of the aqueous space may enable proper content release. To achieve this goal, single vesicle-vesicle fusion will be tested by tethering v-SNARE vesicles onto supported lipid bilayers. Also GUVs with t-SNARE proteins will be ruptured onto polymer cushion to create a floating target membrane for v-SNARE vesicles to fuse with.

1838-Pos Board B682

Visualizing Viral Fusion At The Single-particle Level

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Specific fusion of biological membranes is a central requirement of many cellular processes and is the key event in the entry of enveloped viruses into cells. Though many biochemical and biophysical studies have contributed to an understanding of the mechanisms underlying fusion, important questions remain about the sequence and orchestration of events underlying the process. Conventional fusion assays are generally limited to observation of ensembles of multiple fusion events, making more detailed analysis difficult. We have developed an in vitro two-color fluorescence assay that enables us to monitor the kinetics of individual fusion events. The resulting 'molecular movies' allow us to dissect the reaction kinetics at a level of detail previously inaccessible. Analysis of lipid and content mixing trajectories of single viral particles provides further evidence of a hemifusion intermediate preceding pore formation. Distributions of the lag times of events reveal multiple long-lived kinetic intermediates leading to hemifusion followed by a single rate-limiting step to pore-formation. We interpret the series of intermediates preceding hemifusion as the result of multiple copies of the trimeric hemagglutinin fusion protein participating in a single fusion event.

1839-Pos Board B683

Functional and Structural Measurements of HIV gp41 Fusion Protein Constructs

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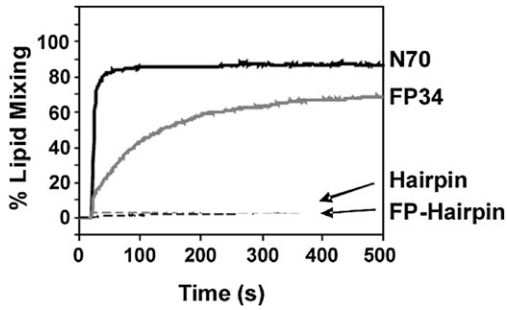
The initial step of HIV infection is fusion between the viral and target cell membranes. Fusion is mediated by the HIV gp41 protein and its N-terminal "fusion peptide" (FP) which binds to target cell membranes. Shorter constructs of gp41 that contain the FP usually catalyze vesicle fusion and such fusion at physiological pH was measured for three different gp41 constructs which differed in their numbers of N-terminal gp41 residues. "FP34" and "N70" were respectively models of the FP and "pre-hairpin intermediate" gp41 conformation while "FP-hairpin" was a model of the final "six-helix-bundle" gp41 structure. N70 induced rapid fusion, FP34 induced moderate fusion, and FP-hairpin induced no fusion and even arrested fusion induced by FP34. The data therefore suggest that the six-helix bundle conformation stops membrane fusion. In related work, solid-state nuclear magnetic resonance measurements probed the membrane locations of three different FP constructs with very different fusion rates. There was a positive correlation between fusion rate and depth of membrane insertion for the FP in either helical or β strand conformation. The key determinant of fusion rate may therefore be FP membrane location rather than conformation.



1840-Pos Board B684

SNARE-Mediated Adhesion Kinetics in Giant Membrane Systems

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Giant membrane *in vitro* systems are powerful and relatively unexplored model systems to probe mechanisms of SNARE-mediated adhesion and fusion. We present results of a joint experimental-modeling study of SNARE-mediated adhesion kinetics of giant unilamellar vesicles (GUVs) with supported bilayers (SBLs). The large size of GUVs allows control of physical parameters such as tension and direct observation of adhesion kinetics unavailable in small unilamellar vesicle (SUV) systems. In the gravity-imposed contact zone between v-SNARE GUVs and t-SNARE SBLs, reflection interference contrast microscopy (RICM) revealed initial membrane separation ~50 nm, consistent with Helfrich theory predicting intermembrane repulsion from thermal undulations. Tight adhesion in several discrete patches within the contact zone then develops over ~10 min, with total patch area growing linearly in time. We mathematically modeled the adhesion kinetics, which comprises two stages. (a) Patch nucleation kinetics. Nucleation is limited by the mean membrane separation which exceeds the reach of cognate SNAREs in apposing membranes. Complexation requires fluctuations to bring cognate SNAREs into proximity and the nucleation rate depends on tension and SNARE density. (b) Patch growth. Once nucleated a patch encourages further complexation because the membrane separation is within SNARE reach. We find two classes of patch growth kinetics. (i) Mobile SNARE complexes. Complex osmotic pressure then drives patch growth and patch area grows quadratically in time. (ii) Immobile SNARE complexes. Patches of tightly clustered SNARE complexes are predicted: patch growth, limited by SNARE diffusion, is linear in time. The experimental data are consistent with model (ii). The SNARE complex density in the patch inferred from the areal growth data suggests that complexes were shoulder-to-shoulder as expected in clusters generated by SNARE-SNARE interactions.

1841-Pos Board B685

Relative Rearrangements Of Synaptotagmin3 C2A And C2B Domains Are Influenced ay Calcium, Lipids And SNARE Proteins

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Synaptotagmins are able to bind negatively charged lipids in Ca2+ dependent manner and act as Ca2+ sensors. Together with SNARE and additional auxiliary proteins synaptotagmins coordinate Ca2+ triggered vesicle exocytosis, although the exact mechanism remains unclear. Synaptotagmins are transmembrane proteins comprised of a short intralumenal/extracellular sequence,