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SORTING OF AMPHIPHILE MEMBRANE COMPONENTS IN CURVATURE AND COMPOSITION GRADIENTS

Aiwei Tian
Upenn, aiwei@seas.upenn.edu

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SORTING OF AMPHIPHILE MEMBRANE COMPONENTS IN CURVATURE AND COMPOSITION GRADIENTS

Abstract
Phase and shape heterogeneities in biomembranes are of functional importance. However, it is difficult to elucidate the roles membrane heterogeneities play in maintaining cellular function due to the complexity of biomembranes. Therefore, investigations of phase behavior and composition/curvature coupling in lipid and polymer model membranes offer some advantages.

In this thesis, phase properties in lipid and polymer giant vesicles were studied. Line tension at the fluid/fluid phase boundary of giant lipid unilamellar vesicles was determined directly by micropipette aspiration, and found to be composition-dependent. Dynamics of calcium-induced domains within polyanionic vesicles subject to chemical stimuli were investigated, which revealed the strength of molecular interaction and suggested applications in triggered delivery.

In addition, curvature sorting of lipids and proteins was examined. Lipid membrane tethers were pulled from giant unilamellar vesicles using two micropipettes and a bead. Tether radius can be controlled and measured in this system. By examining fluorescence intensity of labeled molecules as a function of curvature, we found that Dil dyes (lipid analogues with spontaneous curvatures) had no curvature preference down to radii of 10 nm. Theoretical calculation predicted that the distribution of small lipids was dominated by entropy instead of bending energy. However protein Cholera toxin subunit B was efficiently sorted away from the high positive curvature due to its negative spontaneous curvature. Bending stiffness was determined to decrease as curvature increased in homogeneous membranes with ternary lipid mixtures near a critical consolute point, revealing the strong preferential intermolecular interactions of such mixtures. In addition, diffusion controlled domain growth was observed in tethers pulled from phase-separated vesicles, which provides a new dynamic sorting principle for lipids and proteins in curvature gradients.

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Dennis E. Discher

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Tobias Baumgart

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SORTING OF AMPHIPHILE MEMBRANE COMPONENTS IN CURVATURE AND

COMPOSITION GRADIENTS

Aiwei Tian

A DISSERTATION

in

Chemical and Biomolecular Engineering

Presented to the Faculties of the University of Pennsylvania

in

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Supervisor of Dissertation
Signature____________________
Tobias Baumgart

Co-Supervisor
Signature____________________
Dennis E. Discher

Graduate Group Chairperson
Signature____________________

Dissertation Committee:
Tobias Baumgart
John C. Crocker
Dennis E. Discher
Paul A. Janmey
for Yongfa Tian and Shuping Li
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suggested applications in triggered delivery.

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CHAPTER 1 INTRODUCTION TO THE PHASE AND CURVATURE SORTING OF LIPIDS AND MACROMOLECULES

Membrane domains and curvatures are important in maintaining cellular functions, such as cell signaling and vesicle trafficking. However, the complexity of biomembranes in composition makes it difficult to elucidate the functional importance of membrane heterogeneities. Therefore, modeling cell membranes as simple bilayer mixtures provides advantages. In this thesis, the phase properties and curvature/composition coupling in lipid and polymer model membranes are investigated.

This introduction includes two parts. The first part is about phase segregation in vesicle membranes, which mainly discusses the basic principles governing phase separation in lipid vesicles from summarizing different types of mixtures and the phase separation achieved recently in polymer vesicles. The second part focuses on the mechanism and examination of curvature/composition coupling in membrane tubes.

1.1 Phase Heterogeneity in Lipid and Polymer Vesicles

1.1.1 Phase separation in lipid vesicles

The study of model membranes with phase heterogeneity is important to the
understanding of biological membranes and cellular function. The membrane raft hypothesis has been intensely debated since Simons formulated it in the late 20-century (Simons 1997). The raft hypothesis states that cell membranes contain cholesterol/sphingolipid-rich domains, which provide platforms for protein associated cell signaling. Correspondingly, properties of laterally phase-separated membranes that could model raft formation are becoming increasingly important. Their investigation will test hypotheses and develop models that could help to elucidate biophysical phenomena, such as membrane trafficking (Ryan 2003; Bonifacino 2004); viral infection processes; and, cell signaling. In parallel, research in model membranes with phase heterogeneity will advance the fabrication of functional and programmable materials through an understanding of natural materials.

Numerous physical and chemical properties of lipid vesicles with phase heterogeneity have been investigated involving interfacial phenomena, critical phase behavior, as well as the coupling of phase behavior and membrane topography. For example, phase boundary line tension has been characterized in giant unilamellar vesicles (Baumgart 2003; Peter I. Kuzmin 2005; Esposito 2007; Tian 2007). Line tension at the domain boundary of phase segregated membranes, an important interfacial phenomena of 2-D phase behavior, is found to regulate global membrane shape (Baumgart 2003), domain size, domain growth dynamics (Kohyama 2003) and vesicle budding in cells (Vind-Kezunovic 2008). In
addition, critical phenomena including phase boundary fluctuations of model membranes have received increasing attention (Esposito 2007; Honerkamp-Smith 2008). The existence of critical fluctuations in the plasma membrane at physiological temperatures suggests the presence of lipid rafts in cell membranes (Veatch 2008). The coupling of phase separation with membrane curvature is of great interest (Baumgart 2003; Parthasarathy 2006; Yanagisawa 2008), as it provides insight into membrane sorting and trafficking.

In the following sections, phase separated vesicles with different lipid mixtures will be discussed. To understand the basic principle of phase separation, I will begin with simple binary systems.

1.1.1.1 Phase separation in binary mixtures

In binary mixtures, intensive studies were carried out experimentally and theoretically using two types of lipids with different chain lengths (Findlay 1978; Jorgensen 1995; Xiang 1998; Shi 2005; Longo 2009). For example, numerous temperature-composition phase diagrams of DC_nPC/DC_{n-x}PC (x ≥ 2) mixtures were investigated, such as mixtures of dipalmitoylphosphatidylcholine (DPPC)/dimyristoylphosphatidylcholine (DMPC) (Mabrey 1976; Jacobs 1977; Xiang 1998), dilauroylphosphatidylcholine (DLPC)/distearoylphosphatidylcholine (DSPC) (Mabrey 1976; Jorgensen 1995), and
DMPC/DSPC (Mabrey 1976; Jorgensen 1995). Here $n$ denotes the number of carbon atoms in the chains, and $x$ is the difference in the chain length between the components of the binary mixtures. Comparing phase diagrams of DC$_n$PC/DC$_{n-x}$PC ($x \geq 2$) mixtures, one sees that the temperature and composition range of the phase coexisting region becomes wider when $x$ increases. Also, as seen from images of GUVs observed via fluorescence microscopy, the characteristic shapes of the gel domains change from line, quasicircular, to dendritic shapes as $x$ increases (Bagatolli 2000).

Phase separation in binary mixtures can also be achieved using two types of lipids with different numbers of double bonds. Temperature-composition phase diagrams of mixtures with chain difference of two double-bonds (e.g. dioleoylglycerophosphocholine (DOPC)/DPPC) and one double-bond (e.g. palmitoyloleoylglycerophosphocholine (POPC)/DMPC, POPC/DPPC and POPC/DSPC) were studied (Curatolo 1985; Schmidt 2009). Comparing the phase diagram of DOPC/DPPC and POPC/DPPC, the phase coexisting region grows wider as the difference in number of double bonds between binary components increases (Curatolo 1985; Schmidt 2009).

In the research discussed so far, phase separation was achieved due to a difference of chain melting temperatures in the binary components. Phase heterogeneity can be also induced by hydrophilic head group interactions. Lipids with titratable negatively charged head groups such as phosphatidic acid (PA), phosphatidylserine (PS), or phosphatidylglycerols
(PG) were found to phase separate when mixed with zwitterionic lipids such as phosphatidylcholine (PC). The phase separations were induced by changing pH and/or adding calcium. Heterogeneity in lipid membranes of PC/PA was found at physiological temperature (Garidel 1997), and was enhanced by lowering pH from 7 to 4 as determined from the temperature-composition phase diagrams (Bhagat 2010). As pH is lowered, the decrease in the ionization of PA can reduce the electrostatic repulsion between charged head groups and increase the attractive hydrogen bonding interactions, and thus give rise to phase separation (Garidel 1997). In addition, the cross-bridging of negatively charged lipids by bivalent ions Ca$^{2+}$ also induces phase separation. Heterogeneous membrane was observed to be induced by calcium addition in PC/PA and PC/PS binary mixtures (Jacobson 1975; Hinderliter 1994). Although the observation of large scale phase separation in giant unilamellar vesicles (GUV) composed of PC/PA or PC/PS using fluorescence probes is still under debate (Haverstick 1987; Shoemaker 2003), coexisting domains in GUVs made with the highly anionic lipid dihexanoylglycerophospho- myoinositolbisphosphate (PIP2) and PC have been observed from the dye phase-preference induced by calcium cross-bridging (Christian 2009).

In addition to lipids with charged and neutral headgroups, those with different zwitterionic headgroups and same chains were found to have phase separation. For example, dihexadecanoylglycerophosphoethanolamine (DPPE)/DPPC mixtures have been found to
form membranes with coexisting domains; however, the temperature range for phase coexistence is still controversial (Caffrey 1987; Shi 2005). Phase separation was observed experimentally at temperatures between the melting temperatures of pure DPPE and DPPC (315-333K) (Caffrey 1987), but no aggregation or phase heterogeneity was seen in computer simulations in the above temperature range (Shi 2005; Leekumjorn 2006), instead occurring at a lower temperature (Shi 2005; Wong 2007). The discrepancy between experiment and simulations was claimed to be caused by the inaccuracy of the coarse-grained model used in simulation (Shi 2005). The phase heterogeneity stems from the stronger interaction between the PE headgroups in contrast to PC headgroups.

Furthermore, phase behavior in binary membranes is also studied in mixtures of cholesterol (Chol) and saturated chain lipids, such as mixtures of Chol and DPPC. The temperature-composition phase diagram of DPPC/Chol was intensively investigated (Vist 1990; Huang 1993; McMullen 1995; Xiang 1998). However, due to the complicated nature of PC/Chol interaction, there exist numerous phase states and the phase diagram is still under debate (Vist 1990; Huang 1993; McMullen 1995; Xiang 1998). Several favoring interaction states between lipids and Chol were postulated in terms of number of lipids per Chol (McMullen 1995; Tamai 2008). The phase separation of saturated chain PC and Chol could be based on the different types of interactions. In vesicle membranes with mixtures of unsaturated lipid and cholesterol, however, coexisting domains are not
observed.

Two to three decades ago, membrane phase behavior investigations were focused on binary mixtures. The coexisting phases are typically gel/fluid detected by the irregular domain shape as well as the diffusion coefficients of lipids in different phases (Korlach 1999).

1.1.1.2 Phase separation in ternary mixtures

In recent years, since the postulation of membrane rafts, fluid/fluid coexisting membranes containing Chol have received increasing attention. With the investigation of dye phase preference and the improvement of GUV formation, the examination of phase behavior is no longer confined to small unilamellar vesicles (SUV) and traditional methods, such as differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR), X-ray diffraction, and differential thermal analysis (DTA). The investigation of fluid/fluid phase behavior in GUVs with ternary lipid mixtures using fluorescence microscopy offers some advantages (Baumgart 2003; Veatch 2003). For example, micron-sized fluid domains are visible via fluorescence microscopy and can be manipulated such that physical properties at the domain boundary can be determined (Tian 2007; Yoon and Cicuta 2010), and domain dynamic behavior such as coarsening and diffusion can be examined (Jensen 2007; Yanagisawa 2007).
Membranes consisting of ternary lipid mixtures of high melting temperature lipid, low melting temperature lipid and Chol can segregate into liquid-ordered (Lo) and liquid-disordered (Ld) coexisting domains with circular phase boundaries. The Lo phase is rich in Chol and high melting temperature lipid. It has a higher packing density and smaller molecular area than Ld phase, which is rich in the low melting temperature lipid. The role of Chol in the phase behavior of ternary lipid mixtures is important. It regulates physical and chemical properties of lipids. For instance, it fluidizes the high melting temperature lipids (Tierney 2005), and increases the bending stiffness of low melting temperature lipids (Henriksen 2006). The ternary mixture membranes with two coexisting fluid phases are more biologically relevant than membranes with binary mixtures in terms of membrane components and fluidity.

Phase-separated membranes with ternary mixtures were explored in mainly three composition types. The most intensively studied mixtures consist of unsaturated chain lipid, saturated long chain lipid, and Chol (e.g. DOPC/DPPC/Chol (Veatch 2003) and DOPC/Sphingolipid/Chol (Baumgart 2003)). However, not all mixtures with unsaturated chain lipid, saturated long chain lipid and Chol can form coexisting liquid domains: such a list of mixtures has been examined by Veatch et. al. (Veatch 2003). In addition, ternary mixtures containing branched chain lipids were found to have coexisting fluid phases (Veatch 2006). Diphytanoyl PC (DphyPC) is a typical branched chain lipid. Due to its very
low melting temperature, coexisting liquid phases were observed in mixtures with DphyPC/SOPC/Chol and DphyPC/POPC/Chol, as well as at a large temperature and composition range of DphyPC/DPPC/Chol (Veatch 2006). The above two types of ternary mixtures can display fluid coexisting phases besides gel/fluid phases. The third type of ternary mixture, having only gel/fluid phase coexistence, consists of single monounsaturated chain lipid, saturated chain lipid and Chol (e.g. POPC/DPPC/Chol (Zhao 2007)), as well as short chain saturated lipid, long chain saturated lipid and Chol (e.g. DPPC/DLPC/Chol (Feigenson 2001)). However, small coexisting fluid domains may be present in the third type of ternary systems but are outside of the length scales observed directly via fluorescence microscopy (Filippov 2007).

Similarly to temperature-composition phase diagrams of binary mixtures of Chol and saturated lipids, phase diagrams of ternary mixtures containing Chol are complicated and still debated, especially in the composition region with high concentration of the high melting temperature lipids (Veatch 2003; de Almeida 2007).

To summarize the phase behavior in lipid membranes, generally, the majority area of the heterogeneous region in the phase diagram is occupied by gel/fluid or gel/gel phase coexistence for binary mixtures, and fluid/fluid or gel/fluid coexisting phases for ternary mixtures. The capability to form membranes with coexisting domains mainly depends on the difference in melting temperatures between lipids in the mixture.
In addition to spontaneous phase separation and domain formation triggered by varying pH and metal ions addition, similar heterogeneities can also be induced by peptide and protein crosslinking (Hammond 2005; Arouri 2008). Phase separation in vesicles composed of more than 3 components (e.g. quaternary mixtures (Tilcock 1988)) is not typically considered as a model system and therefore, is not discussed here.

1.1.2 Phase separation in polymer vesicles

Similar to lipids, diblock copolymers with hydrophilic and hydrophobic blocks can self-assemble into vesicles in water forming so-called polymersomes when the weight fraction of the hydrophilic block is about 35% (Discher 2006). Polymersomes have thicker and tougher membranes than liposomes due to the high molecular weight of polymers, which offer advantages in drug delivery.

The investigation of lateral phase heterogeneity in polymersomes could aid the understanding of molecular interactions, offer new insights in bilayer membrane phase behavior, expand the applications in controlled release, and stimulate the development of surface patterning. However, the exploration of laterally heterogeneous polymersomes is a rather new field. No report was found until recently (Christian 2009).

Surprisingly, the strong meso-scale lateral segregation in polymersomes is achieved with neutral and charged diblock copolymers, as little evidence of large-scale phase separation
in giant vesicles composed of charged and neutral lipids was observed. The phase separation is induced by calcium cross-bridging. The calcium-binding phase is solid-like and highly rich in negatively charged diblock copolymers poly(acrylic acid)-poly(butadiene) (PAA-PBD), whereas the other phase is fluid-like and rich in neutral poly(ethylene oxide)-poly(butadiene) (PEO-PBD). The phase heterogeneity of this system is sensitive to pH and calcium conditions, and thus the pH-calcium phase diagram was studied for weight fraction of 25% PAA-PBD. A very narrow pH and calcium concentration range was found to have phase coexisting membranes. Theoretical work was carried out to interpret the phase diagram (Christian 2009). Calcium concentration is critical for the formation of domains, and pH value can regulate the calcium condensation in PAA chains. At low calcium concentrations, crosslinking of PAA chains is not sufficient to overcome polymer entropy changes associated with demixing. Whereas at high calcium concentrations, the counterion entropy is so high that calcium is unable to be confined among PAA chains (Christian 2009). The formation of phase separated polymer vesicles reveals the interaction of polyanionic amphiphiles and ions.

1.2 Shape Heterogeneity in Lipid Membranes and Curvature/Composition Coupling
1.2.1 Biomembrane curvature

Bent membrane morphologies are observed in many fundamental cell-biology processes and structures. At molecular length scales, examples include the budding or fusion of transport vesicles in intracellular trafficking pathways (Rustom 2004) and the tubular shape of endoplasmic reticulum (ER) and Golgi membrane. Examples at length scales of cells include: dendrites and axons in neurons; a dividing cell; concave disk shape of red blood cells; and, filopodia on the cell surface (Parthasarathy 2007). These membrane curvatures are found to be of functional importance. Curvature is expected to couple to membrane composition and phase (Baumgart 2003; Roux 2005; Parthasarathy 2006; Derganc 2007; Sorre 2009; Tian 2009; Capraro 2010). Also, protein/lipid interactions can be affected by curvature (Machida 1980), and vice-versa (Blood 2006; Tristram-Nagle 2007). In addition, large curvature is found to mediate membrane fusion (McMahon 2010). However, the mechanisms of curvature coupling to composition and lipid/protein interaction are not clear. The quantitative understanding of the functional importance of shape heterogeneity is of increasing interest.

1.2.2 Methods of curvature generation

Several methods were developed to generate membrane curvature experimentally. High curvatures can be obtained in the form of cylindrical tethers with ten to hundreds of nm in
radii. Tethers could be pulled from giant vesicles (Bo 1989; Benoit 2009; Tian 2009) or cell membranes (Dai 1999; Oh 2009) using micropipettes, optical traps, magnetic tweezers, atomic force microscopy (AFM), hydrodynamic flow (Oh 2009) or even gravity of the membrane adhering bead (Bo 1989). The methods involving micropipette aspiration are described here. Briefly, the giant vesicle is aspirated using a micropipette, while a bead either held by another micropipette or an optical trap pulls a tether from the giant vesicle via streptavidin/biotin conjugation. This method has the advantage that the radii of the tether can be controlled by varying the aspiration pressure. The system is similar to tubular networks connecting to a large flat membrane patch within and between cellular organelles, such as Golgi and ER.

Membrane curvatures can also be realized by the spherical curvatures of different sizes (15nm ~ 800nm) of SUV (Hatzakis 2009; Kamal 2009). SUVs are made by extruding large vesicles in buffer through filters of different pore sizes. The formation of SUVs is less challenging from an experimental point of view than the tether pulling. However, the physical properties of SUV (i.e. membrane tension, radius) are difficult to probe and control.

In addition to methods mentioned above, supported bilayer membranes can be deformed on either specific curvature-patterned or rough surfaces (Parthasarathy 2006; Goksu 2009). In brief, SUVs are deposited on the microfabricated solid surface. Spontaneous rupture of
the SUVs leads to formation of a bilayer membrane on top of the curved surface with a thin water layer in between. Membrane curvatures generated in this fashion are rather stable compared to membrane tether. However, because the support surface and the membrane is rather close (only ~5nm water layer in between), membrane properties are easily affected by the surface support (e.g. lipid diffusion coefficient is slower on supported bilayer than in GUV).

1.2.3 Methods of curvature/composition coupling examination

The most common and straightforward way to examine curvature sorting is to fluorescently label the molecule, and examine the fluorescence intensity as a function of curvature (Parthasarathy 2006; Bhatia 2009; Sorre 2009; Tian 2009; Capraro 2010). Fluorescence intensity is proportional to the number of dyes in the absence of self-quenching, and can be easily measured through fluorescence microscopy. Degree of curvature can be measured and calculated based on known tether mechanical relations (Hochmuth 1982; Greenwood 2002), simply known as in designed surface patterning for supported bilayer (Parthasarathy 2006), or directly measured via electron microscopy (EM) (Bhatia 2009). The correlation between fluorescence intensity and curvature reveals the changes in molecules as a function of curvature.

Curvature sorting can also be examined through measuring material properties of the
highly curved membrane, which is relatively complex in both operation and conception compared to the former one. Material properties of the curved membrane (e.g. Bending stiffness) can be indirectly obtained through the correlation of measured physical properties (i.e. force, membrane tension) and membrane geometry (i.e. curvature) (Song 1990). A typical material property calculated to examine curvature sorting is bending stiffness. Bending stiffness reflects the energy required to bend the membrane. It is dependent on membrane compositions. Therefore, by examining the bending stiffness of the curved membrane in contact with a flat membrane reservoir as a function of curvature, curvature/composition coupling could be determined.

1.2.4 Curvature/composition coupling mechanism

Curvature/composition coupling could be achieved through two means: by molecular spontaneous curvature and by composition dependence of bending stiffness difference.

1.2.4.1 Curvature sorting by spontaneous curvature

The definition of the molecular spontaneous curvature here is the local degree of curvature at which the molecule deforms the membrane. The spontaneous curvature of the lipid is closely related to its intrinsic shape. Lipids with cylindrical shape have zero spontaneous curvatures; cone and inverted cone shape lipids have negative and positive spontaneous
curvatures, respectively (see Fig. 1). Spontaneous curvature can be measured from the inverted hexagonal phases by X-Ray (Kozlov 2007). However, unlike lipid spontaneous curvatures, protein spontaneous curvature depends on many factors, such as how the protein interacts with membrane and the intrinsic shape of the protein. For example, for membrane insertion proteins, various insertion depths could affect the spontaneous curvature generated by the protein regardless of its intrinsic shape (Campelo 2008). The membrane binding surface arc of the peripheral protein determines its spontaneous curvature. At present, there is no direct measurement of protein spontaneous curvatures.

![Diagram showing lipid shapes and spontaneous curvatures]

**Figure 1** Demonstration of lipid shape and spontaneous curvature

However, the measurement of the degree of protein partitioning in tubes connecting to a flat membrane reservoir provides a method to determine protein spontaneous curvatures (Tian 2009; Capraro 2010). Proteins and peptides that have been found to prefer positive curvatures due to their spontaneous curvatures are mostly those associated with endocytosis, such as dynamin (Roux 2010), amphiphsin N-BAR domains (Bhatia 2009), Epsin N-Terminal Homology (ENTH) domain (Capraro 2010), and cytoplasmic coat
proteins (COPI) (Manneville 2008). Proteins found to have negative or low curvature preference are mostly cargo proteins such as Cholera Toxin (Sorre 2009; Tian 2009), Shiga Toxin (Roemer 2007), and the cell penetrating peptide penetratin (Lamaziere 2009). Such proteins are able to deform membranes as observed by EM of tubulated liposomes (Peter and Butler 2004).

Large proteins can be efficiently curvature sorted by their spontaneous curvatures, whereas lipids even with a rather high spontaneous curvature have weak curvature couplings (Kamal 2009; Tian 2009). Whether a molecule can be curvature sorted by its spontaneous curvature mainly depends on the competition between entropy and the bending energy. If bending energy dominates, molecules can be efficiently sorted.

1.2.4.2 Curvature sorting by bending stiffness composition dependence

In addition to curvature sorting by spontaneous curvature, molecules can sense curvatures by bending stiffness composition dependence. Sorting by bending stiffness is a collective sorting behavior. From the definitions of bending stiffness and spontaneous curvature of mixtures according to Kozlov (Kozlov 2000), average bending stiffness of a mixture (in the absence of molecular interaction) is dominated by the component with the smallest stiffness unlike the overall spontaneous curvature which is determined by all the components. Therefore, even if a molecule has large bending stiffness, it could still
partition into a high curvature region as long as low bending stiffness molecules are well mixing with it. However, in the case of non-ideal mixing (optically homogeneous, but unmixed at molecular scale), sorting by bending stiffness is the governing mechanism (Sorre 2009; Tian 2009).

As described in section 1.2.4.1, large proteins instead of small lipids can be efficiently curvature sorted by spontaneous curvature. Entropy and bending energy were compared to evaluate the sorting efficiency. But in the situation considered here, lipids instead of proteins show curvature/composition coupling by bending stiffness. The difference is that we are not only comparing bending energy with entropy, but that another energy contribution - molecular interaction - needs to be considered; this third contribution works against entropy (or mixing). Curvature sorting of non-ideally mixing lipids (Sorre 2009; Tian 2009) and phases (Baumgart 2003; Roux 2005; Parthasarathy 2006; Jiang 2008; Heinrich 2010) all reflect curvature sorting by bending stiffness. Phase coexisting membranes are more easily to be sorted by curvature than microscopically homogeneous membranes with non-ideal mixing; ideally mixed membranes undergo no curvature sorting in absence of extreme spontaneous curvatures. The sorting efficiency here therefore reflects the strength of the preferential molecular interaction.

1.2.4.3 Curvature sorting mechanism conclusion
Generally, a protein is likely to be curvature sorted by its spontaneous curvature whereas lipids are sorted through the composition dependence of bending stiffness. Exceptions may arise when proteins aggregate or when lipids have extremely strong spontaneous curvatures. Bending energy, entropic energy and lateral intermolecular interactions are three important energy terms to determine the curvature sorting efficiency of molecules.
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2.1 Line Tension Measurement at Domain Boundary of GUV

Aiwei Tian, Corinne Johnson, Wendy Wang and Tobias Baumgart

2.1.1 Introduction

It has often been suggested that microdomains in biomembrane often termed raft play important roles in cellular function, including cargo transportation and signal transduction (Simons 1997; Edidin 2003). Fluid biological membrane phase coexistence can be experimentally modeled via liquid disordered (Ld) and liquid ordered (Lo) phases in membranes self assembled from synthetic or natural purified lipids. Line tension at the domain phase boundary, controls the kinetics of phase separation and domain sizes. Line tension could be an essential control parameter in-vivo for regulation of lateral compartmentalized membrane localized signaling complexes. In order to better understand line tension and its relationship to lipid components and composition, we studied this parameter at the phase boundary of giant unilamellar vesicle (GUV) model membranes by a micropipette aspiration technique.

The concept of line tension was introduced by Gibbs in his theory of capillarity (Gibbs
1878). Most experimental and theoretical studies of line tension have examined three-phase contact systems (Rowlinson and Widom 1982) where it was first introduced. Line tension is furthermore found in surface phase contact regions, where it is also termed “boundary tension” (Perkovic 1995). However, unlike the line tension in three-phase contact systems, the one occurring at surface phase contacts has been studied experimentally in a few material layers only, including Langmuir layers of polymers (Mann 1992; Roberts 1997) and surfactants (Muller 1991), as well as lipids.

Line tension at lipid monolayer gas and liquid-expanded phase domain boundaries was measured by Knobler’s group (Stine 1990; Muller 1991). McConnell and co-workers determined line tensions at fluid / fluid phase boundaries in a cholesterol / DMPC monolayer by measuring of the recovery rate of distorted to equilibrium domain shapes (Benvegnu 1992), and found values that varied between 0.1~10 pN, depending on the film pressure. As expected, line tension decreased towards the critical point of phase coexistence for this system. The distorting shear flows used in (Benvegnu 1992) were replaced by laser tweezers in (Wurlitzer 2000), to yield line tensions at liquid expanded / gas phase boundaries of a surfactant monolayer. Analysis of thermally induced domain shape fluctuations gave line tensions at lipid monolayer fluid domain boundaries in the range of ~0.1pN near the critical point (Goldstein 1994). Theoretical examination of domain line tension in lipid monolayers has been based on both continuum (Muller 1991;
Benvegnu 1992) and microscopic models (Akimov 2004; Kuzmin 2005; Hu 2006), the latter giving values around ~1-10 pN.

Lipid *bilayer* line tensions can be divided into those that are measured at a bilayer membrane edge (vesicle pore) and those that are found at the domain boundary of phase separated but otherwise continuous membranes. These edge tensions were determined both experimentally (Zhelev 1993; Karatekin 2003; Puech 2003) and theoretically (de Joannis 2006), with values near ~10 pN. Recently, shape analysis of giant unilamellar vesicles yielded rough line tension estimates for fluid / fluid phase coexistence in the range of ~1 pN (Baumgart 2003; Baumgart 2005). Here line tension values were estimated by determining dimensionless fit parameters through comparison of model membranes shapes and computed shapes using theory developed by Juelicher and Lipowsky (Kuzmin 2005). Rescaling of these fit parameters with independently determined bending stiffness, and vesicle dimension, allowed estimating lateral tensions, bending stiffness differences, and line tension in vesicles with fluid phase coexistence. The problem with generalizing this approach is the fact bending stiffness and other mechanical properties depend on the composition of coexisting phases. These compositions are determined by the tie lines in phase diagrams that describe phase coexistence in lipid mixtures with fluid phase coexistence (Veatch 2005). These tie line directions have thus far been measured only with selected compositions (Veatch 2004; Veatch 2006). Also, to our knowledge, no attempts
have yet been made to measure absolute values of bending stiffness in phase separated lipid membranes. In principle, as in the monolayer systems, domain shape relaxation of equilibrium or non-equilibrium shape fluctuations could be employed to measure fluid domain boundary line tension. However, membrane domain viscosities, which is strongly composition dependent (see e.g. (Kahya 2003)) and would have to be measured independently, contributes to relaxation kinetics (Benvegnu 1992).

Here, we describe a mechanical equilibrium method that determines line tension without the need for independently obtained auxiliary membrane properties, compare the measured values along a compositional trajectory in the quasi-ternary phase diagram of cholesterol, DOPC, and egg sphingomyelin (ESM), and relate the magnitude of line tension to a ternary system where DOPC and ESM were replaced by DiPhyPC and DPPC, respectively. We find that line tension decreases near the critical consolute point in the phase diagram. Furthermore we describe lateral tension controlled, line tension mediated budding phenomena.

The problem with generalizing this approach is that bending stiffness and other mechanical properties depend on the composition of coexisting phases, which are thus far only known for selected average compositions (Goldstein 1994; Akimov 2004). Also, to our knowledge, no attempts have yet been made to measure absolute values of bending stiffness in domains of phase separated lipid membranes. In principle, domain shape
relaxation of equilibrium or non-equilibrium shape fluctuations can be used to measure fluid domain boundary line tension (Kuzmin 2005). However, membrane domain viscosities, which are strongly composition dependent (see e.g. (Hu 2006)) and would have to be measured independently, influence relaxation kinetics (Kuzmin 2005).

2.1.2 Materials and methods:
DOPC, ESM, cholesterol, DPPC, DiPhyPC and the ganglioside GM1 were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and used without purification. Lipid stock solutions were prepared in chloroform or chloroform / methanol solution, the concentrations of which were determined by standard phosphate assay, which was repeated on at least a bi-weekly basis. Fatty-acid free bovine serum albumin (BSA) was from Sigma (St. Louis, MO). Texas Red-DPPE (TR-PE) and fluorescence labeled (Alexa-488) cholera toxin subunit B were purchased from Molecular Probes, Inc. (Eugene, OR).

2.1.2.1 Preparation of giant unilamellar vesicles
GUV were prepared by the method of electro formation as described (Mathivet 1996). Briefly, 100 μl lipid solution (1μM bulk lipids in chloroform, 1 mol% GM1, 0.5 mol% TR-PE) were spread on the conducting surface of ITO glass slides at 60°. The slides were
subsequently evacuated for at least 2h. Two slides were then sandwiched with an O-ring spacer enclosing 100 mM sucrose solution (additionally containing 2mM dithiothreitol (DTT) to reduce photochemical effects and 0.02% sodium azide), and then were incubated at 60° in the presence of an AC field (2V/mm, 5 Hz) applied for 2h. After GUV formation, we added 1% v/v CTB stock solution (0.1 mg/ml in 100 mM sucrose solution) to the GUV dispersion to fluorescently label primarily the Lo phase. CTB labeling was preferred over Lo phase labeling with polycyclic aromatic hydrocarbons (Hammond 2005), since excitation of membrane imbedded perylene or naphthopyrene over the typical time course of our experiment (~1s illumination every 3s over several minutes) lead to photo-induced shape changes that were absent with Alexa-488 CTB excited at 488 nm.

2.1.2.2 Preparation of micropipettes

Micropipettes (WPI, Sarasota, Florida) were fabricated by means of a pipette puller, and pipette tips were then clipped off using a microforge. The inner diameter of the micropipettes was in the range of 1 – 3 μm. Pipettes were filled with 100 mM sucrose solution using a MicroFil needle (World Precision Instruments, Inc. Sarasota, FL). To prevent irreversible membrane/pipette adhesion, we conditioned the pipette tip in 2% fatty-acid-free BSA solution for 5s, and / or added BSA to the vesicle swelling solution.
2.1.2.3 GUV aspiration

100μl GUV dispersion was diluted 1-fold (to decrease GUV concentration) and injected into a chamber designed to minimize solution convection, with a U-shaped opening for the micropipette. The chamber size was 10×10×1mm. Pipettes were operated by a motorized manipulator system (Luigs & Neumann, Germany). The aspiration pressure was controlled by adjusting the water level of a reservoir connected to the micropipette, and was accurately measured via a pressure transducer with DP-20 diaphragm (Validyne Engineering, Los Angeles, California). The typical aspiration pressure range was 0.4 – 6 Pa. The maximum convective bulk flow in the chamber was 1.3 μm/s. This flow velocity causes a maximum pressure difference, due to Stokes friction, of about 0.06Pa at the mouth of pipette, for a vesicle with radius around 8 μm. Because the smallest pressure we could measure was roughly 0.18 Pa, a measurable artifactual contribution to the mechanical equilibrium of aspirated GUVs caused by bulk flow can be neglected. Due to the relatively low aspiration pressures used in our experiments, particular care was exerted regarding zero pressure calibration. We calibrated the zero pressure by the standard method of observing small (fluorescent) particles move in the pipette. The pressure where particles were observed to show diffuse but no detectable convective motion over a time course of several tens of seconds was defined as the zero pressure $p_0$. The pressure $p_0$ was recalibrated after every vesicle aspiration and after every spatial translation of the pipette.
2.1.2.4 Imaging

Vesicles were imaged by fluorescence confocal microscopy (Olympus, FV300), using a 60x, 1.2 NA water immersion objective (Olympus) and excitation at $\lambda = 488$nm and 543 nm, for Alexa 488 and TR-PE, respectively. Image analysis was performed using Matlab (Mathworks, Natick, MA).

2.1.3 Results

After identifying dumbbell shaped giant vesicles (i.e. vesicles deflated from spherical shape) in the vesicle dispersion, GUVs were micropipette aspirated (see Fig. 2.1.1) to initially yield a spherical vesicle shape outside the pipette and a tongue (projection) within the pipette. Aspiration via the $Ld$ phase was avoided in routine measurements since it often led to fragmentation during elongation of the cylindrical aspirated projection. Fragmentation, i.e., budding transitions of the projection, had to be avoided due to potential pressure drops in the micropipette interior caused by congesting budded daughter vesicles that were observed to slowly move away from the mother vesicle into the pipette. Aspiration of GUVs into the pipette interior was observed to be a two-step, pressure dependent process.

Typically, at low pressures, vesicles were observed to attach to the pipette mouth showing
membrane curvature in the membrane patch covering the mouth not significantly different from the nonaspirated vesicle (i.e., almost zero projection lengths were found, see Fig. 2.1.1e). Upon increasing the suction pressure (by lowering the water reservoir connected to the pipette), a sudden (within less than 1 s) transition from approximately zero projection length to a maximal length was observed at the critical aspiration pressure and the aspirated dumbbell became spherical during this transition. For the vesicle depicted in the sequence Fig. 2.1.1b–e, this critical aspiration pressure was 16.1 ± 0.2 Pa. This instability is similar to earlier micropipette aspiration of membranes without line tension contribution, as in red cells (Rand 1964). The suction pressures where these instabilities were observed were larger when aspirating the $Lo$ phase, compared to the $Ld$ phase. For example, for vesicles with composition #3 (see below) the ratio of critical aspiration pressures of $Lo$ and $Ld$ phase (measured with the same pipette in domains of the same vesicle) was 2.5 ± 0.23 (for 10 vesicles). This observation is likely due to higher bending stiffness of $Lo$ versus $Ld$ phase (Baumgart 2003; Baumgart 2005).

After initial aspiration, projection lengths were typically decreased to yield dumbbell shaped vesicles similar to the GUVs shown in Fig. 2.1.1. Projection lengths were observed to be reversibly dependent on the applied suction pressure. Vesicles depicted in Fig. 2.1.1 all refer to mechanical equilibrium. The projection lengths decreased after decreasing suction pressure until a new mechanical equilibrium was reached. Projection lengths could
not be decreased beyond a critical value dependent on area fraction and reduced volume of the aspirated vesicle. Below this value, mechanical stability of a cylindrical aspirated vesicle membrane typically could not be reached and the projection would retract from the pipette interior, with the vesicle remaining attached to the pipette mouth at equilibrium (see Fig. 2.1.1e). The critical releasing pressures associated with these instabilities were significantly smaller than those needed for initial formation of a cylindrical aspirated vesicle domain: compare the critical aspiration pressure of 16.1 ± 0.2 Pa and critical releasing pressure of 0.9 ± 0.2 Pa for the vesicle depicted in Fig. 2.1.1b-e. Both Figs. 2.1.1a and 2.1.1d show projection lengths immediately before the critical releasing instability is reached.

**Figure 2.1.1** a) Demonstration of a typical line tension measurement. One domain
(typically the Lo domain, as depicted in green) of a dumbbell shaped vesicle was aspirated with a micropipette. The Lo domain was labeled with CTB-Alexa 488, and the Ld phase was labeled by Texas Red-DPPE (TR-PE). $R_p$ and $R_b$ are the radii of the pipette and the phase boundary; $R_1$ and $R_2$ are curvature radii of partially aspirated and non-aspirated domain, $\psi_1$ and $\psi_2$ are the tangent angles immediately before and after the phase boundary, the tip of the aspirated vesicle projection is marked with a vertical arrow. b-e) Time-lapse series of an experiment where one vesicle was aspirated using three different aspiration pressures: b) 2.7 Pa; c) 1.9 Pa; d) 1.0 Pa and e) 0.9 Pa (all values ±0.2 Pa). For these aspiration pressures, the line tension obtained from vesicle geometry, pipette diameter and aspiration pressure was roughly the same: b) 3.1±0.2 pN; c) 3.0±0.3 pN; d) 3.0±0.6 pN. e) Vesicle beyond critical releasing instability. Scale bar: 5 μm.

We developed an analysis scheme based on the measurement of the two meridional tangent angles $\psi_1$ and $\psi_2$ of Lo and Ld phase at the phase boundary, boundary and pipette radius $R_b$ and $R_p$, respectively, and suction pressure $\Delta P = P_o - P_p$, where $P_o$ is the pressure outside vesicle and pipette, and $P_p$ is the pressure inside the pipette (see Fig 2.1.1a). Additional geometric vesicle parameters are the radii $R_i$ of near-spherical domains $i = 1,2$, outside the pipette. In the following, we assume dumbbell vesicle mechanical equilibria to primarily depend on the following mechanical parameters: lateral tensions $\Sigma_i$, suction
pressure $\Delta P$, vesicle normal pressure difference outside the pipette $\Delta P_v = P_i - P_o$ (where $P_i$ is the inner vesicle pressure), and line tension $\sigma$, whereas bending stiffness contributions were neglected. This approximation is based on the zero order solution of a recent boundary layer analysis (Allain 2006), which is accurate if phase boundary energies are significantly larger compared to the bending energy. Assuming that the lateral membrane tension $\Sigma_l$ within the domain that is aspirated is equal inside and outside the pipette (Kwok 1981), the suction pressure is related to $\Delta P_v$ through $\Delta P / \Delta P_v = R_i / R_p$, where $R_1$ is the radius of the domain adjacent to the pipette mouth (see Fig 2.1.1a). A force balance within the plane containing the domain boundary yields $\sigma R_b = \Sigma_1 \cos \psi_1 - \Sigma_2 \cos \psi_2$, from which we obtain with $2 \Sigma_i = \Delta P_v R_i$ (for each domain $i$) a relationship between line tension and $\Delta P_v$, $\sigma = 0.5 \Delta P_v R_b^2 (\cot \psi_1 - \cot \psi_2)$ (Baumgart 2003; Allain 2006). Replacing $\Delta P_v$ by $\Delta P$ and eliminating $R_1$ yields the following linear relation between $\Delta P$ and $\sigma$:

$$\sigma = \Delta P \frac{R_b^2 R_p \sin \psi_1}{2(R_b - R_p \sin \psi_1)} (\cot \psi_1 - \cot \psi_2) \quad (2.1.1)$$

Eq. 2.1.1 was used to relate the adjusted suction pressure and the resulting vesicle geometry to the line tension in a particular vesicle. Before we examined the composition dependence of line tension for mixtures referring to a quasi-ternary phase diagram with fluid phase coexistence, we first tested the reliability of this method with different vesicle geometries, obtained from varying aspiration pressures. A vesicle was aspirated at a range
of suction pressures from 1 to 2.7 ± 0.2 Pa, as shown in Fig 2.1.1b-d. Identical line tension values were obtained from the same vesicle in different geometries, within the errors of our approximations and measurement. We then proceeded to demonstrate quantitatively the composition dependence of phase boundary line tension. We examined the line tensions of GUVs made of DOPC: Chol: ESM mixtures with five compositions within the Lo/Ld phase coexistence region as Fig 2.1.2 shows. In this partial phase diagram, we determined the upper (referring to high cholesterol content) boundary of the binary miscibility gap (Veatch 2005), and the area fractions of ordered and disordered phases, from which the location of a critical mixing/demixing (consolute) point could be estimated. Five compositions were chosen, one near the critical point and the remaining ones along a line roughly orthogonal to the expected tie line directions (see Fig 2.1.2). This compositional trajectory roughly crosses the middle of the expected tie lines (Veatch 2005), since the majority of vesicles showed similar areas of Lo and Ld phase for all five compositions (data not shown). Typically, a significant (roughly 10%) spread of area fractions was found in each vesicle preparation, indicating compositional differences among individual vesicles. This difference in composition is also reflected in the spread of line tension value histograms for every measured composition, as shown in Fig 2.1.3.
Figure 2.1.2 Partial room temperature phase diagram of the DOPC: Chol: ESM mixture. The circles demonstrate the upper boundary of the liquid coexistence region. Open circles indicate homogeneous vesicles, pie diagrams indicate phase separated vesicles, in which gray color quantifies the average area fraction of the disordered phase. From the area fractions and the phase boundary the approximate location of an upper critical consolute point can be identified. Five compositions were chosen including this area as well as four more compositions lying on a trajectory orthogonal to the expected tie line directions. The ratios of DOPC:Chol:ESM for each composition from the top to bottom are: (1): 0.34:0.4:0.26; (2): 0.34:0.33:0.33; (3): 0.34:0.28:0.38; (4): 0.34:0.23:0.43; (5): 0.34:0.16:0.5, respectively, as indicated by crosses.

For each composition, 20 vesicles were examined. Histograms demonstrating the large spread of individual measurements summarize our results in Fig 2.1.3a-e. Average values of line tension are shown in Fig 2.1.3f. These average values decreased from 3.3 pN
(farthest away from the critical point) to 0.5 pN (closest to the critical point) see Fig 2.1.3f.

Average line tension values therefore change over almost one order of magnitude, depending on the vesicle composition, and decrease continuously towards the mixing/demixing critical point.

**Figure 2.1.3** a-e) Histograms of line tension values for the five compositions (1) to (5). 20 vesicles were measured for each composition. f) The average value of line tensions are plotted versus the mole ratio of cholesterol and ESM. From left to right are the compositions (1) to (5), and the corresponding average line tension values are 0.5, 0.9, 1.3, 2.3, 3.3 pN, respectively. g) Histogram of line tensions of 20 vesicles with composition
Recently, a closed loop fluid/fluid miscibility gap has been described in a similar ternary lipid mixture (Veatch 2005). In case of a closed loop miscibility gap, in addition to a high cholesterol critical point, an additional critical point is expected near our lowest cholesterol composition (Veatch 2005). In that case, line tension along the compositional trajectory chosen for our measurements should assume a maximum in the middle of the miscibility gap, and decrease towards both critical points. Clearly, this behavior is not reflected by our measurements (Fig 2.1.3f). However, the data of reference (Veatch 2005) show that a closed loop miscibility gap exists above the chain melting temperature $T_m$ of the long chain saturated, ordered phase preferring lipid, whereas below $T_m$, a three phase triangle, where Lo, Ld and a gel phase coexist, neighbors the Lo/Ld phase binary miscibility gap. The phase diagram obtained in Ref. (Veatch 2005) indicates that the terminal tie line of the binary Lo/Ld coexistence region adjacent to the three phase triangle refers to a significant compositional difference between Lo and Ld phase. The line tension associated with a phase boundary in vesicles with composition referring to this terminal tie line therefore can be large. The large line tension obtained from our measurements in vesicles with lowest cholesterol composition therefore suggests that at room temperature, a three phase triangle borders the Lo/Ld coexistence region in the DOPC:Chol:ESM phase
diagram. Since our measurements were performed at room temperature, significantly below $T_m$ of ESM, this interpretation is in accordance with Ref. (Veatch 2005). Three phase coexistence regions are typically difficult to identify by fluorescence microscopy imaging alone (Veatch 2005).

Next we examined to what extent line tensions depend on the lipid species used for ternary and quasi-ternary lipid mixtures. To that end, we measured line tension within the DPhyPC:Chol:DPPC mixture, see Fig 2.1.3g, for a composition roughly referring to the middle of the Lo/Ld miscibility gap at room temperature (Veatch 2005). The line tension of the composition referring to the middle of the miscibility gap in the DOPC:Chol:ESM mixture (1.3 pN) is significantly larger than line tensions measured for vesicles consisting of DPhyPC:Chol:DPPC (0.7 pN). Membrane phase boundary line tensions are due in part to a thickness mismatch of Lo and Ld phase (Akimov 2004). It might be possible to relate the composition and species dependence of line tension to this thickness difference by means of atomic force microscopy measurements of the membrane height profile across the phase boundary.

2.1.4 Conclusion

Our method to measure line tensions is suitable for magnitudes of this parameter that will deform vesicles into dumbbells with domain shapes that approximate truncated spheres.
This situation is found when bending energy is small compared to phase boundary line energy, i.e. \( \kappa \ll \sigma R_0 \), where \( \kappa \) is the bending stiffness and \( R_0 \) is the vesicle radius. Accordingly, our method is expected to be accurate for line tensions larger than on the order of 0.1 pN. We have shown that line tensions in quasi-ternary lipid mixtures are composition dependent and decrease towards a critical mixing/demixing point of the Lo/Ld miscibility gap. We expect that our approach can contribute to the identification of membrane minority components that influence line tension, i.e. line active components. Such molecules could be important as regulators of lateral heterogeneity in biological membranes.

### 2.2 Calcium-induced Domain Shapes and Dynamics within Polyanionic Vesicles

**Subject to External Chemical Stimuli**

Aiwei Tian, David A. Christian, Dennis E. Discher, Tobias Baumgart

2.2.1. Introduction:

Membrane phase heterogeneity is of great interest in various biological processes such as vesicle trafficking and signal transduction. However, it is difficult to elucidate the role that phase separation plays in maintaining cellular function due to the compositional
complexity of biomembranes and the dynamic nature of lipid structures. In turn, understanding the phase behavior of model vesicle membranes is possible due to their controlled simplicity and robustness. Bilayer vesicles (e.g. liposomes, polymersomes) can also encapsulate therapeutics, showing promise in drug delivery applications (Juliano 1981; Christian 2009). The investigation of phase behavior of vesicle membranes has the potential to advance biomedical applications.

Lipid membranes in the form of giant unilamellar vesicles are one of the most intensively studied model membranes, and their phase behavior in thermodynamic equilibrium has been well characterized (Baumgart 2003; Veatch 2003; de Almeida 2007). Domain dynamics have been examined mostly under physical stimuli such as, temperature (Bagatolli 2000; Dietrich 2001; Veatch 2003), osmotic pressure (Yanagisawa 2008) and curvature (Heinrich 2010). However, domain dynamics subject to chemical stimuli have not been well-investigated since lipid membranes are unstable and fusogenic.

Polymersomes made with high molecular weight amphiphilic diblock copolymers offer some advantages. On one hand, polymersomes are highly stable compared to liposomes (Discher 1999; Discher 2002; Discher 2006). This high stability makes domain morphology investigation upon chemical perturbations feasible. One the other hand, interactions between block copolymers containing a weak polyelectrolyte are dynamic with changes in chemical factors like pH and ionic strength. Numerous kinetic
investigations on the effects of pH and salt on assemblies composed of weak polyelectrolytes were carried out involving block copolymer brushes, micelles and vesicles. (Currie 2000; Lee 2002; Konradi 2004; Geng 2005; Konradi 2005; Cui 2007; Shen 2008; Fernyhough 2009).

In an earlier contribution, we described mesoscopic phase separation in polymersomes made of a binary diblock copolymer mixture, neutral poly(ethylene oxide) - polybutadiene (OB18) and negatively charged poly(acrylic acid) - polybutadiene (AB1) (Christian 2009). Divalent cations (e.g. calcium, copper) in solution electrostatically cross-bridge the anionic AB1 chains and induce the lateral segregation of AB1 chains into domains that are stable for years at room temperature. The phase separation in such polymer mixtures was seen in a surprisingly narrow range of pH and calcium concentrations, which suggests that while the phase separation of the system is stable with time, it is sensitive to pH and calcium fluctuation. We therefore examined the dynamic aspects of domain transition in spotted polymersomes upon changes in pH and calcium concentration.

In this contribution, polymersomes are formed within the known region of phase separation, and then shift the system out of the lateral segregation regime by varying the external calcium concentrations and/or pH. Domain morphologies were imaged by confocal microscopy during chemical addition. We obtained domain-mixing kinetics upon removing calcium from the outer leaflet of AB1-rich domains by adding EDTA, which is a
strong calcium chelating reagent with high affinity due to its high denticity. Viscous fingering, domain bulging, and AB1-rich domain area fraction decreasing were seen after increasing pH by adding NaOH, which indicates the swelling of AB1-rich domains and slow mixing of AB1/OB18* phases upon losing H⁺ from PAA chains. We also found that small amount of EDTA accelerated the domain boundary roughening process induced by NaOH.

2.2.2. Materials and methods:

2.2.2.1 Materials

Block copolymers of poly(ethylene oxide)-poly(butadiene) (PEO80-PBD125) denoted as OB18 ($M_w = 10,400$ g/mol) and poly(acrylic acid)-poly(butadiene) (PAA75-PBD103) abbreviated as AB1 ($M_w = 10,050$ g/mol) were made as described (Christian 2009). OB18 was labeled with tetramethylrhodamine (TMR) and was denoted as OB18*.

2.2.2.2 Preparation of phase separated polymersomes

Phase separated polymersomes were formed via film hydration as described (Christian 2009) at pH 3.5 with $[\text{Ca}^{++}] = 0.1$ mM at a total polymer concentration of 0.1mg/ml.
2.2.2.3 Leaflet asymmetric treatment of phase separated polymersomes

After phase separated vesicle formation, ~50 μl vesicle dispersion was transferred to an imaging chamber. A single phase-separated vesicle was immobilized by means of micropipette aspiration while imaged by confocal microscopy over a time course of minutes to hours. Vesicle imaging and micromanipulation were performed as described (Tian 2009). EDTA or [EDTA:NaOH=1:8] aqueous solution was injected into the chamber through a 10 μl syringe (Hamilton, Reno, NV) while imaging. Due to the slow kinetics of NaOH treatment, vesicles were allowed to incubate with NaOH in Eppendorf tubes for more than 4 hrs (typically overnight) and then transferred to the imaging chamber.

2.2.2.4 Imaging

Phase separated polymersomes were imaged by $xyz$ scan using a laser scanning confocal fluorescence microscope (Olympus, FV300) with a 60X water immersion objective (Olympus). OB18* was excited using 543 nm laser.

2.2.2.5 Image analysis

Hemisphere projections of vesicles are obtained by combining $z$-stack images using
Olympus Fluoview300. Quantitative analysis of images was performed through MATLAB (Mathworks, Natick, MA) and ImageJ (National Institutes of Health, Bethesda, MD).

2.2.3 Results:

Polymer vesicles have thick and highly stable membranes that are especially impermeable to ion transfer (Opsteen 2004; Battaglia 2006; Discher 2006) when compared to lipid membranes. Therefore, changes in ion concentration and pH in the solution outside the polymer vesicle result in a highly asymmetric treatment of the membrane as the aqueous lumen of the vesicle – which contacts the inner leaflet – remains unchanged. This ionic impermeability also provides stability to the vesicle structure during changes in the concentration of the outer solution, which makes the observation of changes in domain morphology feasible. Here, we have tested the effects of EDTA and/or NaOH on polymersome domains by monitoring domains after asymmetric addition of EDTA and/or NaOH solutions. As Fig. 2.2.1 illustrates, EDTA and NaOH remove Ca$^{2+}$ and H$^+$, respectively, from the outer leaflet of AB1-rich domains of polymer vesicles, and these changes are shown to have dramatic effects of domain morphology.
2.2.3.1 EDTA treatment

Calcium cross-bridges weak polyanion chains (e.g. PAA) in polyelectrolyte gels (Horkay 2000), brushes (Konradi 2005), and also the hydrophilic brush of PAA-PBD vesicles (Christian 2009). This cross-bridging between PAA-PBD chains mediates domain formation in polymersomes containing neutral PEO-PBD (Christian 2009), and so to test the stability of such domains after removed calcium from the outer leaflet of the membrane, excess EDTA (0.36 mM ~ 0.96 mM) was added to the vesicle dispersion (with 0.1 mM Ca^{2+}). Upon EDTA addition, AB1 chains in the AB1-rich domains began to
dissociate and mix with OB18*. The kinetics of dissolution and the mechanism of mixing depended on the size of each AB1-rich domain.

Generally speaking, small dark AB1-rich domains (domain area 0.1~1 μm²) mix with OB18*-rich domains mainly through an increase in fluorescence intensity that is uniform throughout the AB1-rich domain; this is unlike, for example, diffusion into a photobleached region in studies by fluorescence recovery after photo bleaching (FRAP) in the dark region (illustrated by the schematic drawing in the upper region of Fig. 2.2.2d).

Vesicles with only small AB1-rich domains become homogeneous within one hour after EDTA addition. In the case of large AB1-rich domains (domain area 1~5 μm²), domain mixing occurs by an area-shrinking process. Phase separation in vesicles with large domains persists more than 2 hrs after EDTA treatment.

To quantify AB1-rich domain mixing kinetics, vesicles with area fraction of AB1-rich domains about 25% were examined. Due to the two distinct domain mixing mechanisms described above (fluorescence intensity increase and area shrinkage), we analyzed vesicles with small domains and those with large domains differently as seen in Fig. 2.2.2.

2.2.3.1.1 EDTA treatment – OB18* fluorescence intensity increase in small AB1-rich domains

A typical vesicle with multiple small AB1-rich domains (~20% of vesicle population) and
the dynamics of such domains upon EDTA addition are shown in the first row of Fig. 2.2.2. 

The increase of OB18* intensity in unlabeled AB1-rich domains is a result of diffusion of OB18* into the AB1-rich domain, and reflects the mixing of the OB18*-rich and AB1-rich domains. While the fluorescence intensity of the AB1-rich domains increases, the domain area remained constant (insets in Fig. 2.2.2a,b). This increasing intensity but constant area seems to suggest that domains register across the bilayer. We propose that asymmetric addition of EDTA first removes the calcium cross-bridging in the outer leaflet which results in mixing of the outer leaflet only. In small domains the mixing of the outer leaflet disrupts the calcium cross-bridging of the inner leaflet, and thus the inner leaflet eventually becomes homogeneous to remain in registration with the outer leaflet. This process is illustrated in the schematic drawing in Fig. 2.2.2c. Inner and outer leaflets are coupled originally before EDTA treatment as shown in stage (i). At stage (ii), the outer leaflet of AB1-rich domain dissociates and mixes with OB18*, whereas the inner leaflet domain persists. At stage (iii), the inner leaflet also mixes and results in a homogeneous vesicle. The phenomenon of asymmetric leaflet treatment leading to the change of both leaflets is also observed by the Keller group while applying b-cyclodextrin to deplete cholesterol from the outer leaflet of giant lipid vesicle (Veatch 2003), as well as Samsonov.et.al who found that the addition of cholesterol oxidase to one side of a supported bilayer affected phase behavior in both monolayers (Samsonov 2001).
To measure the kinetics of domains mixing, a diffusion model was fit to the plot of AB1-rich domain fluorescence intensity (normalized with respect to the OB18*-rich domains) versus time (Fig. 2.2.2d) – an analysis commonly used in FRAP experiments (Soumpasis 1983). The inter-diffusion coefficient of OB18* into the AB1-rich domain was obtained as \( D = (6 \pm 2) \times 10^{-4} \, \mu m^2/s \) (\( N_{domain} = 28, N_{vesicle} = 3 \), average domain size is \( 0.91 \pm 0.37 \, \mu m^2 \)).

Figure 2.2.2. Domain dynamics after EDTA chelation of calcium from the outer leaflet. First (a-c) and second (d-f) rows display the intensity increasing and area shrinking phenomena of small and large domains upon EDTA treatments, respectively. (a,b)
Confocal z-stacks displaying the top hemisphere of a phase-separated vesicle (AB1:OB18* = 25:75) with multiple small domains before (a) and 15 min after (b) adding 0.96 mM EDTA to the vesicle dispersion. The OB18* domain appears red, and the black is the unlabeled AB1 domain. Scale bar: 5 μm. Enlarged images of the AB1 domain pointed by arrows are highlighted in inserts of panels (a-b) with scale bar as 1 μm, which displays increasing fluorescence intensity resulting from diffusion of OB18* into the domain after EDTA addition. The constant area of the AB1 domain is a result of the temporary persistence of an AB1-rich domain in the inner leaflet and is suggestive of domain registration across the bilayer. (c) AB1-rich domain fluorescence intensity $I_{AB1}$ versus time normalized to intensity of OB18*-rich domain $I_{OB18}$ ($N_{domain}=28$, $N_{vesicle}=3$, average domain size is $0.91 \pm 0.37$ μm$^2$). The solid line is the fitting of AB1 domain intensity versus time using the diffusion model as described (Soumpasis 1983), from which an interdiffusion coefficient of OB18* $D = (6.2 \pm 3.2) \times 10^{-4}$ μm$^2$/s is obtained. The schematic drawings in the upper corner of panel (c) illustrate the process of domain intensity increasing mechanism. Circles represent top views of AB1 domains, rectangles depict side views of the outer (top) and the inner (bottom) leaflets of AB1 domains. Black and red denote AB1 and OB18* respectively. Stage i) is the AB1 domain before EDTA treatment; ii) is the intermediate stage, where the outer leaflet is mixed with OB18* and inner leaflet domain persists; iii) is the final stage, where AB1 domain in both leaflets are mixed with
OB18*. (d,e) Confocal z-stacks displaying the top hemisphere of a phase separated vesicle (AB1:OB18* = 25:75) with multiple large domains (average domain size 2.9 μm²), 1min (d) and 69 min after (e) adding 0.83 mM EDTA to the vesicle dispersion. Scale bar: 5μm. Enlarged images of the AB1 domain pointed by arrows are highlighted in inserts of panels (d-e) with scale bar as 1μm, which displays decrease of AB1 domain areas resulting from the diffusion of OB18* into the domain after EDTA addition. (f) Plot of domain area change (A - A₀) versus time upon EDTA treatment. The solid line is the fitting using Eq.2, from which we calculated inter-diffusion coefficient of OB18* as \( D = (5.9 \pm 1.5) \times 10^{-5} \) μm²/s (\( N_{domain} = 6, N_{vesicle} = 2 \) with an average area as 2.7 ± 1.4 μm²). The schematic drawings in the upper corner of panel (f) illustrate the process of domain area shrinking mechanism. \( R_0 \) is the original AB1 domain radius at \( t = 0 \), \( R_m \) is the radius of the melting AB1 boundary. Stage i) shows the AB1 domain before EDTA treatment; Stage ii) and iii) demonstrate the process of OB18*-rich domain invading AB1-rich domain with time.

2.2.3.1.2 EDTA treatment - Large AB1-rich domain area shrinking

In Fig. 2.2.2e-h, a typical vesicle with large AB1-rich domains and dynamics of such domains during EDTA addition are depicted. In contrast to small domain mixing, large domains display a domain-shrinking process without obvious increases in domain fluorescence intensity (Fig. 2.2.2e and f). Therefore it is postulated that large domains
maintain leaflet registration under asymmetric EDTA treatment as illustrated in the schematic drawing of Fig. 2.2.2g.

To describe the area decreasing mechanism of mixing of large domains, a simple diffusion model according to Fick’s second law is applied to a cylindrical system:

\[
D \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) = \frac{\partial C}{\partial t} \tag{2.2.1}
\]

Where \(C\) is the concentration of OB18* in the mixing AB1-rich domain, \(r\) is the radius of AB1-rich domain, and \(D\) is the inter-diffusion coefficient of OB18*. Together with the continuity equation, diffusion can be calculated as (for details, refer Appendix I):

\[
D = \frac{1}{2\pi} \frac{A - A_0}{t} \tag{2.2.2}
\]

By fitting Eq. 2 to the plot of AB1-rich domain area versus time (Fig. 2.2.2h), we calculated the inter-diffusion coefficient of OB18* as \(D = (5.9 \pm 1.5) \times 10^{-5} \, \mu m^2/s\) \((N_{\text{domain}} = 6, N_{\text{vesicle}} = 2\) with an average area as \(2.7 \pm 1.4 \, \mu m^2\)). This inter-diffusion coefficient is one order of magnitude smaller than the diffusion coefficient obtained from the intensity analysis for small domains.

Because of the high molecular weight of the diblock copolymers and the resulting chain entanglement in the vesicle bilayer (Bermudez 2004), the rate-limiting step of domain mixing can be considered to be polymer inter-diffusion instead of removal of calcium cross-bridges by excess EDTA. Inter-leaflet domain registration is suggested from the images and kinetics of domain mixing after asymmetric EDTA treatment. In both small
and large AB1-rich domains, inter-diffusion coefficients of OB18* are orders of magnitude smaller compared to OB18* self-diffusion (0.0024 μm²/s (Lee 2002)). This difference could be a result of the difference of outer leaflet only and bilayer driven diffusions. The diffusion mechanism and speed are strongly domain size dependent, which could be related to the different energy penalties caused by dissociating inner leaflet calcium cross bridging. However, the detail interpretation of outer leaflet driven bilayer inter-diffusion requires the understanding of domain registration which is still unclear. Regardless, the EDTA effects confirm that calcium cross-bridging of negatively charged poly(acrylic acid) chains plays an essential role in the phase separation of polymer vesicles.

2.2.3.2 NaOH treatment

In addition to calcium, another key factor controlling phase behavior is pH, which could modulate calcium condensation onto PAA chains at a critical range (Christian 2009). To test the domain stability upon losing H⁺ from the PAA chains in the outer leaflet of vesicle membrane, we increased pH by adding NaOH at concentrations ranging between 0.15mM and 6.25 mM. As seen with excess EDTA, no obvious concentration dependence of domain change is found with NaOH. However, the stability of phase-separated vesicles is strongly dependent on NaOH concentration and time. The speed of vesicle disintegration
accelerates as the NaOH concentration increases. Near the upper bound of this range (6.25 mM) a resulting of pH of 7.9 causes vesicles to break down to membrane patches or worms within hours. In this paper, we focus on the domain stability and morphology. The integrity of the vesicle is thus not further investigated here but will be mentioned in the discussion later.

Upon addition of NaOH, the increasing pH leads to deprotonation of the carboxylic groups of PAA chains, and hence PAA chains become increasingly negatively charged. The removing of H⁺ leads metal ions (from the solution) to bind to PAA chains. However, as the early investigation pointed out, the entropy penalty (favoring mixing) associated with confining counterions in PAA-rich domains increases with counterion concentration (Christian 2009). The high counterion entropy favors mixing and PAA chains become negatively charged again. To test the effect of sodium ions, NaCl solution was added to vesicle dispersions. No significant phenomenon was observed (data not shown). Therefore, pH increase is the major contribution of adding NaOH. Domain persistence under asymmetric NaOH treatment allowed observation of dramatic changes in domain morphology such as viscous fingering, bulging AB1-rich domains, in addition to a change in domain area fraction.

2.2.3.2.1 Fingering domains – mixing/demixing transition and AB1-rich domain in plane
swelling

Asymmetric treatment of phase separated polymersomes with NaOH for several hours results in a small number (~5%) of vesicles developing fingering domain boundaries (Fig. 2.2.3a,b). This fingering pattern could be caused by the swelling and softening of AB1-rich domains upon increasing inter-chain electrostatic repulsion. As previously shown, AB1-rich domains are calcium cross-bridged gels that cannot be aspirated into a micropipette at low aspiration pressures (<200 Pa) (Christian 2009). Furthermore, AB1-rich domains show irregular rigid creases under osmotic deflation (see insets in Fig. 2.2.3c), which also suggests gel-like AB1-rich domains. In contrast, incubation with NaOH softens AB1-rich domains to a liquid-like state as evidenced by aspiration into the micropipette at a pressure of ~150 Pa (Fig. 2.2.3c). The AB1-rich domain deformed while being projected into the pipette and later disassembled resulting in failure of the vesicle integrity (data not shown). Therefore liquid-like AB1-rich domains are not mechanically stable after NaOH treatment due to the electrostatic repulsion between chains.

As AB1-rich domains soften from a solid to liquid phase, their phase properties become increasingly close to the liquid OB18*-rich phase (Christian 2009). The phase boundary line tension thus decreases as the difference between two phases become smaller. Phase boundary line tension acts to minimize the AB1/OB18* domain interface length and reduce the interfacial energy penalty, which gives rise to compact domains with circular
boundaries. Addition of NaOH increases the electrostatic repulsion between AB1 chains, which not only decreases domain line tension but also maximizes the distances between AB1 chains and favors sprawling domains. Therefore, both decreases in line tension and increases in electrostatic repulsion lead to the growth of AB1/OB18* domain interface length and results in fingering domain patterns.

The fingering pattern is similar to that found in the phase separated giant lipid vesicle systems close to a temperature-dependent mixing/demixing critical point (Veatch 2003). Similarly, the evidence of the AB1-rich phase softening that leads to the decrease of phase property differences between AB1/OB18* phases, it is likely that these NaOH-treated phase-separated polymersomes are approaching a mixing/demixing critical point. Although the mechanisms of mixing/demixing are different (chemically versus physically induced), the phase separated polymersome and the giant liposome display a similar phenomenon as they reach a mixing/demixing critical point.
**Figure 2.2.3.** Domain morphologies with weight ratio AB1:OB18* = 50:50 after adding NaOH (a,b) Confocal Z-stack hemispheres displaying fingering domains imaged 24hrs (a) and 48hrs (b) after adding 0.6 mM NaOH to the vesicle dispersion. (c) Equatorial section of a dumbbell shaped vesicle imaged 24 hrs after 0.6 mM NaOH addition. The AB1-rich phase is aspirated into the micropipette at 150 Pa, which suggests a softening of the AB1-rich phase. Inserts in panel (c) are vesicles before NaOH treatment and under osmotic deflation. The creased, folded configurations reveal the original solid feature of
AB1-rich domains. (d) AB1-rich domain bulging induced by the chain repulsion in the outer leaflet after 24hr NaOH treatment. Inserts in panel (d) are vesicles before NaOH treatment, from which we see original flat and inwards bending AB1 domains. Scale bar = 5 μm.

2.2.3.2.2 Bending outwards – 3 dimensional AB1-rich domain swelling

As Fig. 2.2.3d shows, in addition to viscous fingering, nearly 10% of vesicles have AB1-rich domains bulging outwards after the addition of NaOH. In comparison, before treatment the majority of AB1-rich domains have no curvature or negative curvatures (Fig. 2.2.3d, insets). We propose that the change in curvature of AB1-rich domains results from the difference in the interfacial area of AB1 molecules in the outer and inner leaflets. As NaOH is added to asymmetrically alter the pH, the repulsion between PAA chains in the outer leaflet leads to area expansion whereas the inner leaflet area remains constant. When the outer leaflet has more area compared to the inner leaflet, the domain bends outward. Therefore, AB1-rich domain shape changes to more positive curvature may therefore reflect the leaflet asymmetry after NaOH treatment.
Figure 2.2.4. (a-c) Equal area surface mapping tool for vesicle area fraction analysis. (a) Confocal z-stack hemispheres of a representative phase separated vesicle with the mixing ratio: AB1:OB18* = 50:50. Scale bar = 2 μm. (b) Schematic demonstration of the working principle of the mapping tool. We estimated the center point and the vesicle radius from the z-stack image of the vesicle and generate a sphere accordingly. The intensities on the vesicle surface were obtained by searching along a line (indicated in red) perpendicular to the sphere surface for the maximum intensities. (c) 2D equal area mapping of the phase
separated vesicle shown in panel (a), of which AB1 domain (black domain) area fraction is calculated as 0.518. (d) AB1-rich domain area fraction plot as a function of AB1 weight fraction before (solid squares) and after (open squares) NaOH treatments. The average AB1 area fraction values are: 48 ± 2% \( (N_{\text{vesicle}} = 11, \text{before NaOH}) \) and 33 ± 4% \( (N_{\text{vesicle}} = 19, \text{after NaOH}) \) for AB1 weight fraction of 50%, as well as 22 ± 5% \( (N_{\text{vesicle}} = 5, \text{before NaOH}) \) and 10 ± 1% \( (N_{\text{vesicle}} = 5, \text{after NaOH}) \) for AB1 weight fraction of 25%. Errors are obtained using standard error of the mean: S.E. = S.D. / \( (N_{\text{vesicle}})^{1/2} \), where S.D is the standard deviation. Solid and dash lines are the linear fitting of the solid and open squares, respectively. The parallel trend of these two lines indicates the decrease of domain area fraction is roughly identical and independent of compositions.

2.2.3.2.3 Decreasing area fraction – slow mixing of AB1/OB18*

After adding NaOH, the area fraction of AB1-rich domains decreases due to the partially mixing of AB1-rich and OB18*-rich domains. To estimate the extent of mixing, we quantify the area fraction of each phase per vesicle. A surface-mapping tool was developed, which projects the 3D vesicle surface to an equal area 2D map using the software MATLAB. The working principle of the algorithm is illustrated in Fig. 2.2.4b. Briefly, coordinates and fluorescence intensities of the vesicle are obtained by searching the maximum intensity along lines normal to the surface of the sphere calculated from the
estimation of the center and the radius of the vesicle. An example is depicted in Fig. 2.2.4a and c, where a confocal z-projection of a vesicle (Fig. 2.2.4a) with a mixing weight ratio (which is approximately equal to the molar ratio) of AB1:OB18* = 50:50 is mapped to a 2D surface (Fig. 2.2.4c), from which AB1 area fraction was calculated to be 51.8%.

With the help of the mapping tool, domain area fractions were calculated at different mixing ratios. AB1-rich domain area fractions of vesicles were measured to be 22% ± 5% ($N_{vesicle} = 5$) and 48% ± 2% ($N_{vesicle} = 11$) for AB1 weight fractions of 25% and 50%, respectively. However, after NaOH treatment, the AB1-rich domain area fractions are reduced as seen from area fraction plots in Fig. 2.2.3d. Average values of AB1-rich domain area fractions were significantly decreased to 33% ± 4% ($N_{vesicle} = 19$, $p < 0.005$) for 50% AB1 weight fraction and 10% ± 1% ($N_{vesicle} = 5$, $p < 0.05$) for 25% AB1 weight fraction of 50% and 25%, respectively. Here, standard error of the mean: $S.E. = S.D. / (N_{vesicle})^{1/2}$, where $S.D$ is the standard deviation, is considered to be the error of area fraction. The decrease of AB1 area fraction reveals the increase of solubility of AB1 in OB18*-rich domains and hence the two polymers become more miscible after NaOH treatment. The mixing behavior is caused by the increase of counterion entropy after pH increasing (Christian 2009).
Figure 2.2.5. Domain roughening process within minutes is observed upon both EDTA and NaOH addition. (a) Time lapse images upon adding 0.09mM EDTA and 0.72 mM NaOH. Scale bar: 2μm. (b) Circularities of OB18* domains decrease as a function of time ($N_{\text{domain}} = 4$), which is calculated using $\text{Circularity} = 4\pi (\text{area}/\text{perimeter}^2)$. Black line is the exponential fitting of the data $\text{circularity} = e^{-t/\tau}$, from which the half decay time is determined: $\tau = 25$s as indicated using the dash line.

2.2.3.3 EDTA plus NaOH treatment

In addition to adding EDTA and NaOH independently, a mixture of EDTA and NaOH at a molar ratio of 1:8 was added to 60% AB1 phase separated polymersome dispersions. We found that a small amount of EDTA accelerates the effects of NaOH on domain
morphology. EDTA added here (0.09mM) is much less concentrated compared to the previous EDTA alone treatment in excess (0.36 mM ~ 0.96 mM). As Fig. 2.2.5a shows, domain morphology deviates from circular domains with smooth boundaries within minutes, which is much faster compared to the kinetics of NaOH treatment. To quantitatively measure the loss of the circular domain morphology upon addition of EDTA and NaOH, the circularity of domains was calculated as $circularity = 4\pi(area/\text{perimeter}^2)$ (Fig. 2.2.5b). The decrease of the domain circularity with time reveals decreasing line tension at the domain boundary as well as an increase in the electrostatic repulsion between AB1 molecules. The decreasing circularity is fitted with an exponential decay resulting in $circularity = e^{-t/25} (N_{\text{domain}} = 4)$. As the small decay constant indicates, a fast (within 10 minutes) boundary roughening process is achieved with the help of EDTA addition compared to NaOH alone treatment (more than 4 hours). In addition to changes in circularity, changes in the vesicle surface area and domain areas were also observed. The increasing of the AB1-rich domain area (indicated using arrows in Fig. 2.2.5a) may result from the swelling of PAA chains upon both losing calcium cross-bridging and increased inter-chain repulsion with deprotonation. Using the mapping tool, we calculated that the total AB1-rich domain area in Fig. 2.2.5a increased 128 $\mu$m$^2$. The increase of AB1-rich domain area leads to the increase of vesicle surface area. As seen in Fig. 2.2.5a, vesicle surface area increased approximately 126 $\mu$m$^2$ correspondingly, which agrees with the
expansion AB1-rich domain area.

2.2.4 Discussion and conclusion

As the schematic phase diagram in Fig. 2.2.6 illustrates, phase separated vesicles (White star) were treated with external chemical stimuli, by which the system was shifted out of the region of phase separation (pink region) in roughly three directions: decreasing [H\(^+\)] (black line), decreasing [Ca\(^{2+}\)] (blue line), and decreasing both [H\(^+\)] and [Ca\(^{2+}\)] (red line). Decreases in [Ca\(^{2+}\)] were achieved by the addition of excess concentrations of the calcium-chelating agent EDTA. Once calcium was removed from the AB1-rich domains in the outer leaflet of the vesicle, domains began mixing within minutes. Addition of NaOH increases pH and subsequently inducnes decreases in AB1-rich domain area fractions, viscous fingering patterns in AB1-rich domains, and changes in AB1-rich domain curvature. A commonality in both EDTA and NaOH treatments is the mixing behavior of AB1-rich and OB18*-rich domains. In both cases, domain mixing results from a disruption of the calcium cross-bridging that induces the lateral segregation of AB1 into domains – either by increasing calcium entropy or removal of calcium by chelation. These different mechanisms for cross-bridge disruption result in the difference in mixing kinetics, where the increase in fluorescence intensity and decrease in area of AB1-rich domains
upon EDTA addition show a fast (minutes-hours) totally mixing, and the area fraction
decrease induced by NaOH features a slow (hours-days) partially mixing. The difference
in mixing speed reflects the sensitivity of AB1-rich domain (changing from solid to fluid)
upon external stimuli and is consistent with our previous finding that the effective rigidity
of AB1 membrane is more sensitive to changes in calcium concentration than in pH
(Christian 2009).

As seen in the gray region of Fig. 2.2.6, vesicles become unstable within minutes after
treatment as they disassemble to form worms or membrane patches. We propose that the
strong electrostatic repulsion between PAA chains at large increases in pH (pH 3.5 to 7.9)
gives rise to leaky vesicles at this regime. Importantly, at physiological pH (pH 7.4)
phase-separated polymersomes are still stable and undergoing slow domain mixing. This
stability presents promise for application of spotted polymersomes in controlled drug
release under physiological conditions.

The domain behavior upon external stimuli has thus far been shown to be concentration
independent which could be due to the limited concentration range explored in our
experiment (stars). Therefore, large range of chemical concentrations (especially at low
concentration) and the corresponding long-term domain examination is expected for
future study.
The complexity and variety of phenomena in different vesicles shown under the same chemical treatment reveal the metastable status of the system. Due to their high molecular weight and nonergodic nature (Battaglia 2005), polymer chains are trapped in local energy minima and cannot reach equilibrium at short times after treatment, thus domains have a very long lifetime in metastable phases.

**Figure 2.2.6.** Phase diagram illustration of pH and [Ca$^{2+}$] shift. The pink area denotes the hydration solution conditions where phase separated polymer vesicles composed of AB1
and OB18* are formed. The white star denotes the hydration condition of phase separated vesicles used in this study, which is pH 3.5 and [Ca$^{2+}$] = 0.1 mM. We added chemicals to vesicle dispersions reaching an external condition as denoted by the colored stars. The black line denotes the treatment with NaOH, leading to an increase in pH and domain change that occur within hours to days. Red lines represent the treatments with NaOH plus a small amount of EDTA that is 12.5% of the NaOH concentration. Boundary roughening which happens within minutes is accelerated by the small amount of EDTA. The blue line depicts EDTA treatment that chelates calcium from the outer leaflet of the vesicles, thus inducing fast domain mixing within minutes. The gray region of the phase diagram designates the limit of vesicle stability upon adding EDTA and/or NaOH.

2.2.5 Comparison of phase behavior in giant liposomes and polymersomes

The phase separation of polymersomes offers new insights to the GUV system. As mentioned in section 1.1.1.1, it is still controversial whether large-scale phase separations could be observed in GUV membranes made with PS/PC or PA/PC. Lateral segregation was found in polymer vesicles made with binary neutral and charged diblock copolymers at rather narrow pH and calcium ranges. The interactions among polymer amphiphiles are larger and the entropy of polymer mixtures at constant area is smaller than those of lipids. Large entropy favors mixing in opposition to the interactions among molecules.
Regardless, one PAA chain has 75 carboxylic groups (75 negative charges), which provides numerous calcium binding and cross-linking sites, whereas PS or PA only have one negative charge. Therefore, it is likely that phase segregation of binary mixtures by electrostatic interaction is easier to observe in polymersomes than in giant lipid system. However, it is also possible that a similar narrow pH and calcium range exists, where GUVs are able to form phase heterogeneous membranes with neutral and negatively charged lipids, but that it has not yet been found.

Surprisingly, circular domain boundaries were observed in polymer vesicles with gel/fluid coexisting phases, whereas lipid gel domains show irregular shapes. Unlike fluid/fluid coexisting lipid domains, no domain coarsening is observed for both lipid and polymer vesicles with gel/fluid coexisting phases in the absence of external physical or chemical perturbations. Diffusion coefficients of lipids in fluid domains are 3 or 4 order of magnitudes higher (1~10 μm²/s) (Korlach 1999) than OB18 polymer in fluid phase (~ 0.0024 μm²/s) (Lee 2002). The circular shape in polymer gel/fluid phase boundary could be due to the small line tension at the fluid/gel domain boundaries, which results from high viscosity and slow diffusion of the fluid phase, and/or the weak gelation of calcium crosslinked domains.

Domain registration across the inner and outer leaflets of polymer bilayer membranes is suggested from asymmetric EDTA treatments. Further confirming the domain registration
hypothesis, after mapping 3D vesicle z-stack projections to 2D area maps no third intensity level was observed before or after NaOH treatment. Domain registration is generally accepted in lipid systems such as cell membrane ‘rafts’, planar lipid bilayers, as well as giant unilamellar lipid vesicles (GUV). While several hypotheses of transbilayer coupling exist for lipid membranes – including cholesterol translocation, chain interdigitation, domain interfacial tension, membrane curvature, and electrostatic potential across the membrane (Collins 2008) – a definitive theory remains elusive. For polymer vesicles, polymer translocation is not possible as flip-flop is prevented by the high molecular weight of the polymer.

In conclusion, calcium chelation from the outer leaflet reveals the different kinetics of large and small domains (see the schematic illustration in Fig. 2.2.2). The different mechanisms could be due to different interfacial energy penalties for small and large domains during leaflet uncoupling. Although leaflet coupling is maintained in both cases as vesicles become homogeneous, decoupling of inner and outer leaflet is seen only when remixing is fast for small domains. Since these high molecular weight amphiphiles give impermeable membranes and are not likely to flip-flop, leaflet coupling seems more structural but collective. Thus it appears that domain interfacial tension between leaflets is an important factor for domain registration. Small domains go through a leaflet-uncoupling process potentially due to the small interfacial energy penalty for
unregistered domains when compared to large domains. Another possibility for domain registration suggested here is the domain curvature. The phenomenon of different curvatures shown in different types of domains confirms the domain registration hypothesis as well as suggests a curvature induced inter-leaflet coupling mechanism. This is consistent with Naumann group, who suggests that inter-leaflet coupling of polymer-tethered lipid bilayers is caused by polymer-induced bilayer deformations (Deverall 2008). Studies of polymersome trans-bilayer coupling may aid the understanding of cell membrane leaflet coupling and offer new insights into this field.
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CHAPTER 3
CURVATURE SORTING MEMBRANE COMPONENTS

Aiwei Tian, Michael Heinrich, Ben Capraro, Cinzia Esposito and Tobias Baumgart

3.1 Sorting of DiI dyes and Cholera Toxin in Membrane Curvature Gradients

3.1.1 Introduction

The sorting of both lipids and proteins in trafficking pathways lies at the heart of cellular organelle homeostasis. Most membrane-bound molecules do not require specific signals to effectively recycle to the cell surface (Mayor 1993). However, the significantly different protein and lipid composition of various cellular membranes (Gennis 1989) requires sorting in cellular trafficking centers, such as the trans-Golgi network, the plasma membrane, endosomes and the endocytic recycling compartment. Precisely how sorting occurs for components that lack proteinaceous recognition sequences is still largely unknown (Holthuis 2005; De Matteis 2008; Bonifacino 2004; Maxfield 2004)

Direct evidence for non-homogenous intracellular lipid distributions and cellular lipid sorting can be obtained from microscopy imaging of fluorescent lipids (Kuershner 2005; Maier 2002) or lipid-like fluorophores (Maier 2002; Mukherjee 2000; Kok 1991). By
alternative methods, the unconventional lipid lysobisphosphatidic acid (LBPA) has been shown to be enriched in endosomes of highly curved multivesicular morphology. LBPA is assumed to be crucial for regulating the membrane geometry of this organelle (Kobayashi 1998; Matsuo 2004). A coupling of membrane curvature to the translocation of cellular lipids has furthermore been invoked to explain changes in lipid distributions associated with fusion pore formation (Ostrowski 2004).

In addition to vesicular transport intermediates, the intra-cellular sorting of lipids and proteins crucially involves tubular membranes (De Matteis 2008; Maxfield 2004). These are essential components of both secretory and endocytic pathways. Tubular membranes are morphological features characteristic of the trans-Golgi network, as well as sorting endosomes (Maxfield 2004). Furthermore, the endocytic recycling compartment is essentially a collection of tubular membranes with diameters around 60 nm that are associated with microtubules (Maxfield 2004). The endoplasmic reticulum also shows regions of interconnected tubular membranes (Shibata 2006). Cellular membrane tubes have recently received significantly increased attention in part due to the discovery of tubular inter-cellular connections (“tunneling nanotubes”) (Rustom 2004; Sowinski 2008; Pontes 2008). Furthermore, intra-cellular membrane tubes have come to a forefront since biophysical mechanisms and the proteins that are involved in shaping these membranes are beginning to be identified (Hu 2008; Upadhyaya 2004; Koster 2003; Leduc 2004;
Lipid membrane tubes were first investigated by biophysicists in 1973 (Hochmuth 1973). Since then, the quantitative understanding of tube mechanics, in particular, has been advanced both by experimental (Hochmuth 1982; Bo 1989; Song 1990; Waugh 1992; Heinrich 1996; Raphael 1996; Rossier 2003; Cuvelier 2005; Cuvelier 2005; Koster 2005), and theoretical (Hochmuth 1982; Bozic 1992; Evans 1994; Waugh 1987; Bukman 1996; Powers 2002; Derenyi 2002) analyses, and also through simulations (Harmadaris 2006). Most of these studies have focused on single-component membranes. Recently, however, membrane tubes consisting of lipid mixtures with the propensity for fluid-fluid phase separation have been approached experimentally (Roux 2005; Baumgart 2003) and theoretically (Allain 2004), and mechanical consequences of lipid / protein interactions in membrane tubes are beginning to be addressed (Hu 2008; Roux 2006).

The research described in this contribution is motivated in part by seminal findings from Maxfield’s group (Mukherjee 1999). In order to investigate sorting of lipids in live cells, the authors incubated CHO cells with three different fluorescent dyes from the dialkyllindocarbocyanine (DiI) series. It was found that those dyes, which all had the same head group but different lipid chain regions, were internalized equivalently. However, shortly after uptake, a dye with long and saturated chains (DiIC\textsubscript{16}(3)) was sorted away from both a short saturated chain lipid analog dye (DiIC\textsubscript{12}(3)) and an unsaturated chain...
dye (FAST DiI) (Mukherjee 1999).

Essentially two (non-mutually exclusive) hypotheses were put forward to explain the underlying sorting mechanisms. One of them assumed that lipids laterally partition between membrane domains that vary in phase state (and degree of fluidity) (Mukherjee 1999). The second hypothesis was based on the molecular shape of these lipids (Mukherjee 1999). This latter explanation has frequently been invoked (Maxfield 2004; Gruenberg 2004; Koivusalo 2007; Sonnino 2007), and is the focus of the present contribution.

There is evidence for lipid sorting from a biophysical perspective, obtained from self-assembled lipid model membranes of giant unilamellar vesicles (GUVs) with fluid-fluid phase coexistence, namely of the liquid ordered (Lo) and liquid disordered (Ld) phases. In ternary membrane mixtures consisting of egg sphingomyelin (ESM), dioleoylphosphatidylcholine (DOPC) and cholesterol, phase segregation in membranes with curvature gradients was seen to lead to curvature-dependent segregation of fluid domains, in starfish vesicles and connected strings of membrane beads (Baumgart 2003). Similar curvature sorting was also observed in membrane tubes pulled from giant vesicles, where Ld phase markers were shown to enrich in nanoscale tubes, relative to giant vesicle membranes with essentially negligible curvature (Roux 2005). These sorting phenomena in domain-forming membranes are likely to result from the differing bending stiffnesses of
Lo and Ld domains (Baumgart 2003; Baumgart 2005; Semrau 2008).

An additional means of sorting established by model membrane research is through differential partitioning of lipids among membrane domains. The partitioning of numerous membrane fluorophores and proteins among Lo and Ld domains has been investigated (Baumgart 2007; Baumgart 2007). The biological relevance of phase partitioning associated with fluid phase coexistence is currently a matter of intense biophysical interest (Veatch 2005; Simons 1997).

The goal of the present contribution is a quantitative analysis of the extent to which individual lipids can be sorted among laterally connected high curvature (tubular), and small curvature (vesicular) membranes. The manuscript is organized as follows. We first demonstrate that a stepwise elongation of tethers pulled via a micro-bead from pipette-aspirated giant vesicles allows measurement of membrane tube diameters. We find in all cases that simple quadratic continuum theory describes tether mechanics. We examine the curvature partitioning of lipid-like fluorophores by measuring tether fluorescence intensity as a function of tether radius. In addition to quasi-single component membrane with trace fluorophores, we investigate demixing phenomena in tubes from lipid mixtures near phase boundaries. We proceed with a measurement of lateral distributions of the protein Cholera toxin subunit B (CTB) in response to changing membrane curvature. CTB curvature partitioning is of interest due to the fact that it is a
widely used tool to study trafficking and cellular function (de Haan 2004; Chinnapen 2007). A paradigm emerging from our work is that individual lipids (as opposed to lipid domains) are not effectively curvature sorted by membrane curvature differences of magnitudes found in intracellular membranes, and even down to extremely small tube curvature radius of roughly 10 nm. For membranes that show cooperative demixing effects, however, curvature amplifies demixing. Finally, peripheral membrane proteins can segregate significantly between membrane regions of different curvature.

3.1.2 Materials and methods

3.1.2.1 Materials

Palmitoyloleoylphosphatidylcholine (POPC), Cholesterol (CHOL), 1,2- Dioleoyl- sn-Glycero- 3- Phosphocholine (DOPC), 1,2- Dipalmitoyl- sn- Glycero- 3- Phosphocholine (DPPC), Distearoylphosphatidylethanolamine- N- [Biotinyl(Polyethylene Glycol)2000] (DSPE-Bio-PEG2000), and ganglioside GM1 were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Fatty-acid free bovine serum albumin (BSA) was from Sigma Chemical Co. (St. Louis, MO). Cholera toxin subunit B (CTB)-Alexa Fluor 488(A488), CTB-A555, C18:2-1,1'-dilinoleyl-3,3',3'- tetramethylindocarbocyanine perchlorate (FAST DiI), C12:0-DiI (DiIC12(3)), C16:0-DiI (DiIC16(3)), 3,3'- dihexadecyloxacarbocyanine

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perchlorate (DiOC$_{16}(3)$), Texas Red- 1,2- dihexadecanoyl- sn- glycerol- 3- phosphoethanolamine triethylammonium salt (TR-DHPE) and $N$- 4,4- difluoro- 5,7- dimethyl- 4- bora- 3a,4a- diaza- s- indacene- 3- propionyl (BODIPY)-DHPE were purchased from Invitrogen (Carlsbad, CA).

3.1.2.2 Preparation of giant unilamellar vesicles (GUVs)

GUVs were prepared by electroformation as described (Mathivet 1996). Briefly, we spread 50 µl lipid solution (POPC 1mM in chloroform, 1-3 mol% GM1, 0.3 mol% TR-DHPE or 0.1 mol% BODIPY-DHPE) on the surface of ITO glass slides (Delta Technologies Ltd, Stillwater, MN), at 60 ºC. The slides were subsequently evacuated for at least 2 h. Two slides were then combined with a silicone spacer (Grace Bio-Labs, Bend, OR) to enclose 100 mM sucrose solution, and then were incubated at 60 ºC in the presence of an AC field (2 V/mm, 5 Hz) for 2 h. After GUV formation, we added 1% v/v CTB stock solution (0.1 mg/ml in phosphate buffered saline (PBS)) to GUVs containing GM1. DiI dyes were complexed with BSA for outer leaflet labeling as described (Mukherjee 1999).

3.1.2.3 Preparation of micropipettes

Micropipettes (World Precision Instruments, Inc. Sarasota, FL) were fabricated with a pipette puller, and the tips were clipped using a microforge. The inner diameters of the
micropipettes were ~3 µm. To prevent irreversible membrane/pipette adhesion, micropipette tips were incubated with 0.05 % fatty-acid-free BSA dissolved in PBS using a MICROFIL needle (WPI, Sarasota, FL), and the pipettes were then filled with a 120 mM sucrose solution.

3.1.2.4 Formation of solid supported membranes

Supported bilayers were formed on glass surfaces by vesicle fusion. Small unilamellar vesicles (SUV) were prepared by first depositing 500 µl of a 1mg/ml lipid solution in chloroform on the wall of a 50 ml round bottom flask. The flask was evacuated for at least 2 h. 1 ml PBS was added to the flask, which was vortexed until all lipid was suspended. The lipid dispersion was extruded 21 times through polycarbonate filters with 50 nm pores mounted in a mini-extruder (Avestin Inc., Ottawa, Canada).

Glass substrates were washed with detergent, rinsed excessively with DI water, and then dried under air. The substrates were subsequently plasma cleaned. 100 µl of SUV solution was deposited on the glass surfaces, followed by incubation for 5 min. Surfaces were then rinsed with PBS. For membranes to be incubated with CTB, 1 µl of 0.2 mg/ml CTB solution in PBS was added after rinsing, and the supported membranes were then incubated for another 5 minutes before rinsing a second time.
Figure 3.1.1  

a) Schematic configuration of a vesicle aspiration and tube pulling experiment, where $P_i$, $P_o$, and $P_p$ are the pressures inside vesicle, outside vesicle and in the micropipette, respectively; $R_t$, $R_v$, and $R_p$ are the radii of tube, vesicle and pipette, respectively; $L_t$ and $L_p$ are the lengths of tube and vesicle projection in pipette, respectively; and $f$ indicates pulling force. 

b) Combined transmitted light/confocal fluorescence microscopy image of aspirated vesicle and membrane tether which is pulled by means of a bead; scale bar: 5 µm. 

c) Cross section image of a membrane tether obtained by confocal line scan as indicated by the gray area in b; scale bar: 1 µm. 

d) Stepwise pulling and releasing demonstration of tether radius measurements. Tether radius is 44 nm. 

e) Tether intensities recorded under varying laser powers. Tether radius: 22 nm.
3.1.2.5 Chamber preparation and tether pulling

GUV dispersions were diluted 1:10 in 120 mM sucrose solution. Diluted GUV dispersion (80 µl) and 0.5 µl streptavidin coated polystyrene bead solution (Polysciences, Warrington, PA, diameter ~6 µm) were injected into a measurement chamber constructed from microscope slides and cover slips that allowed access by two micropipettes oriented at an angle of 90º; see Fig.3.1.1b. Additionally, 1µl 10X PBS had to be added to the measurement chamber as, in its absence, beads would not robustly bind to vesicular membranes. Micropipettes were operated by a motorized manipulator system (Luigs & Neumann, Germany). A vesicle was pipette-aspirated with an initially small suction pressure (5-12 Pa), while a second pipette held a bead with aspiration pressure of roughly 60 Pa; see Figs. 3.1.1a and 3.1.1b. The aspiration pressure was controlled by adjusting the water level of a reservoir connected to the micropipettes, and was measured via a pressure transducer with a DP-28 diaphragm (Validyne Engineering, Los Angeles, CA). The bead was slowly moved towards the aspirated vesicle until contact, and was then moved away from the vesicle to pull an initial membrane tube of about 5 µm in length.

3.1.2.6 Tether radius measurement

We obtained tether radius from stepwise horizontal elongation of the membrane tube at
constant lateral membrane tension (adjusted by the pipette aspiration pressure) by moving the pipette-aspirated bead with a micromanipulator (Dai 1998), and concomitantly determining the associated change in the length of the pipette-aspirated vesicle projection (see Figs. 3.1.1a and 3.1.1d). A similar method was used in Ref. (Bo 1989) where tethers were elongated vertically by means of a freely suspended membrane-attached bead which exerted a constant pulling force. Even earlier measurements extended the tube in a continuous dynamic fashion, which allowed researchers to also measure membrane viscosity (Hochmuth 1982).

To measure tether radius, the tether was typically elongated in five steps (each of 20 μm) to a maximum length of 105 μm and was then released to the beginning position using the same step distance (Fig.3.1.1d). For the lowest pressures, the number of steps occasionally had to be decreased because of insufficient excess area available for tube extension in the pipette-aspirated vesicle projection (see below). The tether length range was chosen based on the following considerations. At constant lateral membrane tension, tube elongation occurs at constant pulling force (Koster 2005; Powers 2002; Derenyi 2002), provided the following conditions are met. First, the tether has to be long enough to have overcome a force barrier for tube formation (Cuvelier 2005; Koster 2005). This initial elongation step associated with variable pulling force is expected to cover a range of less than 50 times the tether radius \( R_t \) (Derenyi 2002) and is below our initial pulling length of 5 μm. Second, the
pipette-aspirated projection of the vesicle used for tube pulling has to be long enough to provide enough excess membrane area (relative to the area for a spherical vesicle) over the entire tether elongation experiment (Cuvelier 2005). This requirement is met if, for the longest tether length, the projection within the pipette still consists of a cylindrical element terminated by a spherical cap (with inner pipette radius $R_p$; see Fig.3.1.1a). Only in that case is the lateral membrane tension $\sigma$ related by the following formula (Waugh 1979) to the pipette aspiration pressure $\Delta P = P_o - P_p$, where $P_o$ and $P_p$ are the hydrostatic pressures outside and inside the pipette:

$$\sigma = \Delta P \frac{R_p}{2(1 - R_p/R_v)}$$

where $R_v$ is the vesicle radius. We used Eq. 1 to compute lateral membrane tension. Third, for long tethers pulled from the vesicle, the resulting area difference between outer and inner membrane leaflet in tubes with high curvature will measurably influence force equilibria (Waugh 1992; Bozic 1992; Bukman 1996). This effect can be neglected for tethers with lengths in the range of and below 100 $\mu$m (Bo 1989; Waugh 1992; Bukman 1996). Finally, sticking of the membranes to the pipette walls has to be avoided. All of the above mentioned complications would lead to deviations from a linear relationship (see below) between projection length and tether length change, as well as to unreliable bending stiffness values, as deduced from the relationship $k_c = 2\sigma R_v^2$ (Derenyi 2002). Experiments with such outcomes were not considered further. A successful tube pulling
experiment is shown in Fig.3.1.1d that clearly indicates a linear relationship between tether length $L_t$ and projection length $L_p$. Also indicated in Fig.3.1.1d is the required condition of reversibility comparing tube elongation and release cycles.

After each pulling step, the length of the vesicle projection $L_p$ within the micropipette was determined by image analysis. Tether radius were determined based on these tether and projection length pairs. Following Hochmuth et al. (Hochmuth 1982; Hochmuth 1982), the tether radius is proportional to the ratio of projection length change ($\Delta L_p$) and tether length change ($\Delta L_t$), i.e., the slope in Fig.3.1.1d. The radius can be derived from this slope and the pipette radius $R_p$, as well as vesicle radius $R_v$ (see Fig.3.1.1a):

$$R_t = -\frac{\Delta L_p}{\Delta L_t} \left(1 - \frac{R_p}{R_v}\right) R_p$$

3.1.2

This relationship results from conservation of membrane area and constant vesicle volume (Hochmuth 1982) over the time course of the experiment. Linearity and reversibility in Fig.3.1.1d indicate that those conditions are met. We estimate the error in pipette radius measurements by confocal microscopy imaging to be roughly 10%. By means of the partial derivative method, we obtained errors in tube radius and bending stiffness on the order of 20% and 50%, respectively.

3.1.2.7 Tether intensity measurement

In order to investigate curvature sorting, we related the change in the fluorescence
intensity to the change in tube diameter and probe concentration in the tubular membrane. The tether fluorescence measurements are illustrated in Fig. 3.1.1b and 3.1.1c. The fluorescence intensity of the tether was recorded after the pulling steps to measure the tether radius. A z-scan across the tether cross section was taken at a step width of 0.15 µm to yield a total depth of 6 µm. The resulting cross-sectional intensity profile was background-corrected, and intensity was measured in an elliptical region of interest defined by the point spread function of the microscope with image processing software FLUOVIEW (Olympus, Center Valley, PA). After each intensity measurement the pipette suction pressure was changed, resulting in a different tube radius, and we repeated measurements until the tether ruptured or detached from the pulling bead.

We choose our laser power range in the following method. In order to test the reliability of the intensity recording and analysis approach, we measured intensities of a tether with fixed radius at varying laser powers. At laser powers below ~4 µW (measured at the objective back-aperture), a linear relationship between tether intensity and laser power was observed (Fig. 3.1.1e), while at larger laser power, the curve deviates from the linear trend (see inset of Fig. 3.1.1e). The linear range below 4 µW laser power was subsequently used to reliably measure tether intensity.

For normalization of the tether fluorescence intensity, we determined vesicle fluorescence intensity from the average of five randomly chosen circular regions of interest with equal
area on the membrane contour of the equatorial plane.

3.1.2.8 Imaging

Vesicles were imaged by fluorescence confocal microscopy (FV300 scanning system integrated with a motorized inverted microscope IX81, Olympus, Center Valley, PA), using a 60x, 1.2 NA water immersion lens with cover slip correction collar (Olympus). Image analysis was performed using IMAGEJ (National Institutes of Health, Bethesda, MD).

3.1.2.9 Diffusion measurements via fluorescence recovery after photobleaching (FRAP)

For FRAP measurements on the membrane tube, a tether of length 19 µm was pulled from the vesicle and the entire tether was bleached via full power illumination with a 488 nm laser. Pre- and post-bleach intensities were measured using excitation with attenuated laser (0.1% to 0.3% of full power) at 488 nm. For diffusion measurements in supported bilayers, a circular area with a diameter of 3-14 µm was bleached by simultaneous full intensity illumination with 488 nm and 405 nm lasers of our confocal microscope setup. Fluorescence recovery was imaged with attenuated excitation intensity (0.1% to 0.3%) of the 488 nm laser. Photobleaching recovery data were analyzed according to Soumpasis (Soumpasis 1983).
3.1.3 Results

One of the main objectives of this study was to establish the partitioning of DiI dye derivatives between connected membranes with a steep curvature gradient. DiI probes are amphiphilic molecules with a headgroup composed of an indocarbocyanine ring system and two hydrophobic alkyl chains (Fig. 3.1.1f). We tested three DiI derivatives: DiIC\textsubscript{16}(3), DiIC\textsubscript{12}(3), and FAST DiI. These dyes differ in their alkyl chains, but have identical headgroups. They have been suggested to segregate differentially among membranes with differing phase state or curvatures (Mukherjee 1999).
Figure 3.1.2. Partitioning of DiI derivatives in POPC membrane tethers. (a–c) Plots of double leaflet labeled DiI dyes from the same experiments with shared figure legend shown in a. (d–f) Plots of outer leaflet labeled DiI dyes from the same experiments with shared legend shown in d. (a, b, d, and e) Relationship of DiI dye intensities in tethers and tether radius. (a and d) Tether intensities normalized with respect to the intensities of vesicles from which tethers were pulled. Black solid lines, gray solid lines, and black dashed lines are the trend lines of DiIC16, FAST DiI, and DiIC12 data points, respectively. (b and e) Tether intensity to vesicle intensity ratios of panels a and b respectively scaled with respect to corresponding membrane curvature plotted versus tether radius. (c and f) Bending stiffness at varying tether radius

Fig. 3.1.2 shows results of DiI dyes symmetrically (Fig. 3.1.2, first column) and asymmetrically (Fig. 3.1.2, second column) incorporated in trace amounts into quasi single-component (POPC) membranes. Symmetric labeling was achieved by addition of fluorophores to lipid solutions before vesicle swelling. In tubular membranes, the outer and inner leaflets have mean curvature with opposing signs. Consequently, in membranes labeled both in outer and inner leaflets, lateral partitioning of molecules in curvature gradients could compensate each other such that the measured tube fluorescence intensity remains constant. We therefore labeled vesicles asymmetrically (in outer leaflets only) by
forming GUVs in the absence of fluorophore and subsequent addition to the GUV dispersion of a DiI/BSA complex solution (Mukherjee 1999) (5% v/v; see Materials and Methods). Tether radii were determined as explained in the methods section, by stepwise elongation (relaxation) of the tether and measurement of the resulting decrease (increase) of the vesicle projection within the aspiration pipette.

Fig. 3.1.2, a and d, indicate linear dependency of fluorescence intensity on the tube radius extrapolating approximately to the origin of the graphs. As we discuss below, if fluorophores were measurably curvature sorted, we would expect a vertical translation of the linear relationships in Fig. 3.1.2, a and d along the ordinate. Fluorophore enrichment in high curvature membrane tubes would lead to an upward shift, and vice versa for fluorophores that are depleted from high curvature membranes. To quantify the relationship of intensity and radius, we fitted linear equations to these intensity curves. The intercept of the intensity plot linear fit is used to determine the partitioning of fluorescent molecules. For example the intercept of the Fast DiI curve in Fig. 3.1.2 d is $-0.022 \pm 0.015 \ (n = 4)$. The uncertainty of intercept (obtained from the least squares fitting uncertainty) within one data set is consistently smaller than that between different data sets and has been neglected. For Fast DiI outer leaflet labeling, the intercept is $-0.014 \pm 0.039 \ (n = 4)$. Because the intercept value is small and the uncertainty is large, displacements in the case of DiI dyes in POPC bilayers are not detected with sufficient
accuracy. Absence of such displacements (Fig. 3.1.2, a and d) suggests inefficient curvature sorting of lipid-like fluorophores as we quantitatively examine below. For Fig. 3.1.2, a and d, variable slopes are apparent comparing different fluorophores. Repeated experiments for tubes pulled from different vesicles containing the same fluorophore indicated (data not shown) that slopes did not depend on fluorophore type but on conditions such as the vesicle radius and the exact location of the region used to determine vesicle fluorescence intensity. These conditions varied slightly among experiments, explaining the different slopes in Fig. 3.1.2, a and d. To amplify potential deviations from linearity for small tether radius, we scaled the tube fluorescence intensity ratios in Fig. 3.1.2, a and d, by the inverse of the tube radius (Fig. 3.1.2, b and e). Both figures indicate that the ratio of fluorescence intensity to tube radius is independent of tube curvature. We thus conclude that curvature-induced sorting is not detectable in either leaflet of a high curvature membrane tube attached to a GUV with small curvature.

The accuracy of the indirect determination of tether radius by tube elongation for quasi-single component (POPC) membranes can be evaluated through computation of the tether bending stiffness $k_c$. These $k_c$ values are related to lateral membrane tension $\Sigma$ and the tube radius $R_t$ via $k_c = 2\sigma R_t^2$. Every measured tube radius therefore yields a bending stiffness; examples are displayed in Fig. 3.1.2, c and f. We determined a stiffness mean value of $9.2 \times 10^{-20}$ J with a standard deviation of $3.0 \times 10^{-20}$ J for tubes from 22 POPC
vesicles with different labels. These values are in the range of published numbers (Heinrich 1996; Zhou 2005). We did not observe statistically significant differences in bending stiffness comparing symmetric labeling and outer leaflet DiI dye labeling. Furthermore, we did not observe bending stiffness to depend on tether radius for single component membranes (Fig. 3.1.2, c and f), in agreement with earlier observations (Bo 1989; Song 1990), and with recent molecular dynamics simulations of tethers with radius that approached bilayer thickness (Harmadaris 2006). This indicates that the bending energy is a simple quadratic function of tube radius for single component membranes over the entire range of our measurements.

In membranes of ternary lipid mixtures with composition near a liquid ordered mixing/demixing phase boundary, we have measured a significant decrease of bending stiffness with increasing curvature (not shown). Those results, obtained with the same method as described above, will be published in a forthcoming contribution.

We caution that our fluorescence intensity analysis is based on assuming a negligible dependence of lipid fluorophore quantum yield on membrane curvature. Because we used headgroup-labeled lipids in all cases, however, we do not expect the environment of our fluorophores to significantly change with membrane curvature. Furthermore, it could be the case that the membrane affinity of our probes is curvature-dependent, which would interfere with our partitioning analysis. Generally, the half-time for desorption of
double-chain lipids is on the order of days (Holthuis 2005), but could increase with membrane curvature. Indeed, an important mechanism of intracellular lipid sorting is based on monomeric lipid transport that involves desorption facilitated by lipid-transfer proteins (Holthuis 2005). Our observation of linear fluorescence intensity / tube radius relationships in our system indicates that in the absence of such proteins potentially curvature-dependent monomeric desorption of double-chained lipids is not likely to be an important sorting mechanism.

In addition to lipid curvature partitioning we investigated the curvature-dependent lateral segregation of a large membrane-bound fluorescently labeled protein Cholera toxin subunit B (structure shown in Fig. 3.1.1 g). Fluorescent CTB is a tool used commonly to investigate intracellular trafficking. However, the mechanisms that underlie its sorting into retrograde pathways are far from being understood (de Haan 2004; Chinnapen 2007). This protein peripherally binds to five GM1 lipids per molecule. To mimic intracellular membrane trafficking, we ideally would examine CTB curvature sorting on the inner membrane leaflet of GUVs. Due to the associated delivery problem, however, we labeled vesicles with CTB bound on the outer leaflet. To investigate protein curvature partitioning, both CTB fluorescence intensity and tether radius were measured. In the experiment depicted in Fig. 3.1.3 a and b, vesicles were incubated with CTB before tether formation. Fig. 3.1.3 a shows CTB-A488 fluorescence in the green channel, and the red channel
refers to the lipid fluorophore TR-DHPE. From the image overlay in Fig. 3.1.3 a it is thus observed that the tether shows predominantly red (TR-DHPE) color whereas the vesicle exhibits more green. This suggests that CTB is segregated from the high curvature tubular membrane to the lower curvature vesicle membrane. To ensure that the depletion of CTB fluorescence on the membrane tube is not specific to the particular choice of fluorescence dyes, we used an opposite color pair in the form of CTB-A555 and BODIPY-DHPE (Fig. 3.1.3 b). In agreement with the observations of Fig. 3.1.3 a, more lipid fluorescence (BODIPY-DHPE, green) is seen in the high curvature tether compared to the low curvature vesicle.

**Figure 3.1.3.** Combined images of red, green and transmitted light channels of POPC (1% GM1) tethers and vesicles coated with CTB. (a) Lipid membrane labeled symmetrically (i.e., in both leaflets) with TR-DHPE, whereas CTB-A488 was added before tether formed;
$R_t = 63$ nm. (b) Membrane symmetrically labeled with BODIPY-DHPE. CTB-A555 was added before tether formed; $R_t = 66$ nm. (c and d) Membrane symmetrically labeled with TR-DHPE; $R_t = 50$ nm, images taken before (c) and 18 min after (d) CTB-A488 was added. Scale bar = 5 μm.

The results summarized in Fig. 3.13, a and b, indicate curvature sorting of CTB. This sorting effect might be explained by the following three hypotheses. First, when a tether is pulled from a vesicle, most of the CTB molecules could remain on the vesicle membrane due to a barrier to CTB transport presented by the tether neck. Second, the membrane affinity of CTB may be a function of membrane tube radius, with high curvature promoting dissociation of CTB from the tether membrane into solution. Third, CTB could dynamically partition laterally between vesicle and tube membrane, with a lateral partition coefficient depending on tube curvature.

To address the first hypothesis, we added CTB to the solution containing TR-DHPE labeled vesicles only after a tether was pulled and tested the binding preference of CTB to high curvature (tube) or low curvature (vesicle) membranes. Fig. 3.1.3 c shows the tether and vesicle before CTB addition, and Fig. 3.1.3 d displays the same tether after CTB had been added. Clearly CTB fluorescence is almost absent in the tether because mostly red lipid dye fluorescence is found in the tube whereas the vesicle shows yellow color due to
simultaneous red and green (CTB) fluorescence (Fig. 3.1.3 d). Thus, even if a CTB diffusion barrier were to exist at the vesicle neck, CTB molecules incubated with a vesicle-attached membrane tether system preferentially are observed to bind from solution to the low curvature vesicular membranes. All experiments in Fig. 3.1.3 indicate qualitatively that CTB is measurably curvature sorted.

We proceed with a quantitative investigation of curvature-dependent partitioning of CTB. Fig. 3.1.4, a and b, indicate decreasing CTB to lipid dye intensity ratio as the tether radius is decreased. As tether radius is reduced, the total amount of any tether component in a given cylindrical tube element of length $\Delta L$ will decrease. In the absence of curvature sorting, this decrease should be equivalent for all components, and thus the fluorescence intensity ratio of two different membrane components should be independent of tether radius. However, Fig. 3.1.4, a and d, suggest that the CTB concentration decreases significantly faster than that of the lipid dyes as the curvature increases. To further evaluate this possibility, we plotted the tube intensities (relative to vesicle intensities) of CTB and lipid dyes versus tether radius separately in Fig. 3.1.4, b and e, and found approximately linear fluorescence/radius relationships for both TR-DHPE and CTB. The essential difference, however, is that the TR-DHPE curve tends toward the origin as in the case of the lipid-like DiI dyes examined above, whereas the CTB curve does not. Instead, it lies significantly below the TR-DHPE curve and the CTB intensity becomes
immeasurably small long before the minimal tube radius is achieved (as indicated by tube rupture). The finding of negligible curvature sorting of TR-DHPE and BODIPY-DHPE is further emphasized in the curvature-scaled plots of Fig. 3.1.4, c and f, respectively. Fig. 3.1.4 thus depicts quantitatively the curvature preference of CTB already qualitatively observed in Fig. 3.1.3, and confirms absence of curvature sorting for lipid-analog dyes.

**Figure 3.1.4.** (a and d) POPC tether membrane fluorescence intensity ratios of (a) CTB-A488 to TR-DHPE and (d) CTB-A555 to BODIPY-DHPE (BODIPY-PE) versus tether radius plots of tethers containing (a) 1% GM1 and (d) 3% GM1. (b and e) Normalized tether intensity ratios with respect to vesicle intensities of (b) CTB-A488 and
TR-DHPE and (e) CTB-A555 and BODIPY-DHPE at varying tether radius of POPC membranes with 1% GM1 (b) and 3% GM1 (e). (c and f) Same data sets as panels b and e, respectively; curvature-scaled tether intensities plotted with respect to tether radius. (a–c) Tether radius was measured from stepwise elongation and releasing as described in Materials and Methods. (d–f) Approximate tether radius calculated from membrane tension by assuming $k_c = 8 \times 10^{-20}$ J.

To exclude potential double labeling artifacts such as fluorescence resonance energy transfer, we tested a vesicle labeled with fluorescent CTB only (no lipid fluorophore). The CTB measurements in Fig. 3.1.5 show the same curvature trend as observed in Fig. 3.1.4, b and e; the fluorescence intensity as a function of tube radius does not pass through the origin (Fig. 3.1.5 a). We furthermore ensured that our CTB experiments were carried out under the condition of mechanical and thermodynamic equilibrium, i.e., in the absence of hysteresis and time-dependent phenomena. These conditions are confirmed by the complete reversibility of tube CTB fluorescence intensity shown in Fig. 3.1.5 a.

Furthermore, intensity ratios scaled by tube radius decrease with increasing curvature (Fig. 3.1.5 b), which means that CTB is sorted away from high positive curvature. As shown in Fig. 3.1.5 c, from tether radius measurements we obtain a constant membrane tube bending stiffness, confirming that changing molar ratios of CTB/lipid do not
measurably influence tether bending stiffness, as the tube radius decreases in quasi-single component membranes.

**Figure 3.1.5** (a and b) Reversibility test of tether intensity at varying tether radius of POPC (2% GM1) membrane labeled only with CTB-A488. Tether intensity is normalized with respect to vesicle intensity. The dashed line refers to the fitting of data points using the spontaneous curvature sorting model, and the gray solid line fitted with the bending stiffness sorting model. (b) Same data as in panel a additionally scaled with membrane curvature. (c) Bending stiffness versus tether radius of POPC (1% GM1) membrane
labeled with both CTB-A488 and TR-DHPE. Note that these bending stiffness values refer to the data set of the first column in Fig. 3.1.4.

Finally, we compared diffusivities of lipid fluorophores and CTB on membrane tethers, as well as on supported bilayers by FRAP measurements. Fig. 3.1.6 depicts the FRAP curves of CTB (Fig. 3.1.6a) and DiOC\textsubscript{16}(3) (Fig. 3.1.6b) on POPC membrane tethers at the same radius and tether length (whole tether is bleached), in which CTB shows a longer (by ~2 times) half-recovery time ($t_{1/2} = 46.7 \pm 8.5$ s, $n = 6$) compared to DiOC\textsubscript{16}(3) ($t_{1/2} = 21.6 \pm 3.8$ s, $n = 5$). The fast lateral diffusion of membrane fluorophores in the pulled tether shown in Fig. 3.1.6 indicates that the tether neck under no condition in our experiment functioned as a measurable diffusion barrier. This contributes to the reversibility of our measurements shown in Fig.3.1.5a. FRAP with the same labeled molecules was also carried out on supported POPC bilayers for comparison to the tube diffusivities. We measured the diffusion coefficients of CTB and DiOC\textsubscript{16}(3) on supported bilayer as $0.35 \pm 0.09 \, \mu m^2/s$ ($n = 8$) and $2.7 \pm 0.5 \, \mu m^2/s$ ($n = 6$), respectively.

From this comparison, we see that CTB diffuses nearly eight times slower than DiOC\textsubscript{16}(3) on supported bilayers, but only two times slower on membrane tethers. Assuming that there is no difference in the CTB membrane binding mode comparing tether and supported bilayer, the ratio of its diffusivity to that of the lipid dye is expected to be similar both on
tethers and on supported membranes. A hypothesis for explaining this difference is that CTB might be more crowded on flat bilayers (Wang 2004) compared to tethers. The clustering of CTB on planar supported membranes could slow its diffusion compared to monomeric CTB molecules, which may be more prominent on highly curved membrane tethers compared to flat membranes. A curvature-dependence of diffusion coefficients has been suggested (Daniels 2007), and could be addressed by the investigation of diffusion as a function of membrane tube radius in future studies.

Figure 3.1.6. Fluorescence recovery after photobleaching data comparing (a) CTB-A488 and (b) DiOC$_{16}$(3) on membrane tethers with similar radius ~65 nm and tether length 19 μm. Whole tethers are bleached in this experiment. Each panel contains five data sets shown with different symbols. CTB is observed to diffuse more slowly compared to DiO on membrane tubes. See the main text for a comparison to diffusion measurements in planar membranes.
By our methodology, lipid-like DiI dyes, as well as the labeled lipids TR-DHPE and BODIPY-DHPE, are not detectably sorted in POPC membranes with cylindrical curvature down to cylinder radius of ~10 nm. In contrast, the peripherally membrane-associating protein CTB was effectively curvature sorted.

The problem of interleaflet lipid sorting in cylindrically shaped bilayer membranes was addressed recently by coarse grained molecular dynamics simulations (Cooke 2006). They were based on a model consisting of a bilayer composed of lipids that were represented by three beads with differing sizes, arranged to form cone or inverted-cone shaped molecules. Those lipids were allowed to flip without restriction among leaflets to minimize curvature energy. Preferential lateral interactions, associated with non-ideal mixing and phase separation, however, were excluded. In accordance with our observations, membrane curvature was not found by those investigators to be an efficient driving force for individual lipid sorting (Cooke 2006).

A simple lattice model (Cooke 2006; Iglic 2006; Kralj-Iglic 2002; Wolf 1985) shows that favorable energetic (curvature energy) contributions to free energy changes associated with sorting of individual lipids are insufficient to overcome the entropic penalty of sorting. More complex free energy models further underline that sorting is amplified near phase transitions and particularly mixing/demixing critical points (Seifert 1993; Leibler 1986). In the following, we develop a model considering individual molecule curvature
sorting based on spontaneous curvature in membrane tubes attached to vesicles. Lateral partition coefficients between large and small curvature membrane regions as a function of lipid shape and membrane curvature are obtained based on equal chemical potentials in tube and vesicle. Free energies for tether $F_t$ (Derenyi 2002) and for vesicle $F_v$ are:

$$F_t = U_t - TS_t - fL,$$  \hspace{1cm} 3.1.3

and

$$F_v = U_v - TS_v,$$  \hspace{1cm} 3.1.4

respectively, where $U_t$, $T$, and $S_t$ are internal energy, temperature, and entropy, respectively of the tube. The last term arises from the pulling force $f$, acting on the membrane tether of length $L$. This free energy expression neglects the normal pressure difference across the membrane, which is an approximation that is appropriate for thin tethers (Waugh 1987). We consider membranes consisting of two types of molecules $\alpha$ and $\beta$ with differing spontaneous curvature of values $C_j$ for molecules $j$ and same molecular cross-section area $a$. The weighted average spontaneous curvature is $C_s = (C_\alpha N_\alpha + C_\beta N_\beta)/N$ (Kozlov 1992), $N$ is the total number of molecules.

We obtained partition coefficients as (for details see Appendix B)

$$K_o = \exp \left( \frac{k_a C_{C_j}}{k_BT} \right) = \frac{\phi_{jot}}{\phi_{jov}},$$

$$K_i = \exp \left( - \frac{k_a C_{C_j}}{k_BT} \right) = \frac{\phi_{jii}}{\phi_{jiv}},$$  \hspace{1cm} 3.1.5
where \( k_c \) is the bending stiffness, \( C = 1/R_t \) is the tube curvature, \( a_j \) is the molecular area, 
are the mole fractions of molecules in tube and vesicle in each leaflet, e.g., in outer leaflet 
\( j = N_j/\text{Not} \). Essentially equivalent expressions have previously been derived for 
interleaflet partitioning in a lipid tube (Cooke 2006). The time scale of Dil dyes' flip-flop 
has been analyzed by fluorescence quenching in large unilamellar vesicles and was found 
to be negligible over at least 4 h at temperatures between 4 and 37°C (Wolf 1985). Measurements in black lipid membranes, however, have suggested higher flip-flop rates 
(Melikyan 1996), and it is also possible that flip-flop rates increase in tubular membranes 
with high curvature (Raphael 1996). Regardless of whether curvature sorting would occur 
in our system primarily by lateral partitioning or by interleaflet flipping, we did not detect 
curvature sorting for the lipid dyes used in this study.

The model reproduces the expectation that molecules are laterally sorted oppositely in the 
inner and the outer leaflet. Furthermore, from Eq. 3.1.5, cone-shaped lipids \( (C_j < 0) \), are 
depleted in the outer tube leaflet relative to the vesicle, whereas inverted cone-shaped 
lipids are enriched. Note that this lattice model neglects the geometric requirement that the 
total lipid amount in the inner tube leaflet be smaller compared to the outer leaflet.

The molecular structures of the Dil dyes used in this study suggest an inverted-cone shape 
for DilC\(_{16}\)(3) and DilC\(_{12}\)(3) (Mukherjee 1999), and a cylindrical or cone shape for FAST 
Dil (Mukherjee 1999). Measurements of the spontaneous curvature of these dyes are not
available; however, the probe DiIC$_{18}(3)$ was observed to lower the transition temperature for formation of an inverted hexagonal phase (Razinkov 1998), indicating an inverted cone shape for that molecule.

In Fig.3.1.7 $a$, we plot theoretical fluorescence intensity ratios in the outer tether leaflet (in arbitrary units) computed from the following equation:

$$\frac{I_{ot}}{I_{ov}} = \varepsilon R_t K_p = \varepsilon R_t \exp\left(\frac{k_a C_j}{k_b T R_t}\right) \tag{3.1.6}$$

where $\varepsilon$ is an unknown parameter that depends on laser beam waist, imaging methods, and other factors. Two lipids with extreme spontaneous curvature (Zimmerberg 2006): diaclylglycerol (DAG), a cone-shaped lipid with negative spontaneous curvature $C_{\text{DAG}} = -1/1.01$ nm$^{-1}$, and lysophosphatidic acid (LPA), an inverted-cone-shaped lipid with positive spontaneous curvature $C_{\text{LPA}} = 1/2$ nm$^{-1}$ are used for illustration purposes. Also considered is dioleoylphosphatidylethanolamine (DOPE), a phospholipid with rather strong cone shape: $C_{\text{DOPE}} = -1/3$ nm$^{-1}$ (Zimmerberg 2006). The fluorescence intensity ratio that is expected for cylindrically shaped lipids ($C_j = 0, K = 1$) is shown in Fig.3.1.7$a$ as a solid line. Nonzero spontaneous curvature primarily displaces the intensity ratio versus tube radius plots along the ordinate, whereas the slope remains constant at $R_t$ larger than $\sim8$ nm (Fig.3.1.7 $a$). This can be understood from expanding Eq. 3.1.6 to yield for the intensity ratios $I_{ot}/I_{ov}$ the simplified relation
Equation 3.1.7 is linear in the tube radius $R_t$ and causes the intercept to be proportional to the spontaneous curvature. This form explains the shift along the ordinate in Fig.3.1.7. However, such a simplification is only suitable for the case where $K$ is close to unity. Therefore, in the case of CTB, for which $K$ is significantly less than unity, we cannot expand the exponential in Eq. 3.1.6 to obtain a suitable linear form.

Fig.3.1.7 a shows that DOPE partitioning would be experimentally indistinguishable from the case of cylindrically shaped lipids, given the degree of uncertainty of our experimental data (see Figure 3.1.2 and Figure 3.1.4). The simple model underlying the graph shown in Fig.3.1.7 a therefore is in accordance with our DiI dye curvature partitioning measurements that indicate absence of detectable curvature sorting, considering that their spontaneous curvature is expected to be significantly smaller compared to DAG or LPA.

From our simple model, spontaneous curvatures of labeled membrane components can be determined. As we discussed in the results section, based on the small intercept and large uncertainty of linear fitting for DiIC12 and Fast DiI, significant curvature sorting of DiI dyes does not occur. The spontaneous curvatures of DiI dyes therefore cannot be determined accurately. For example, fitting experimental intensity ratios to Eq. 3.1.7 ($a_{DiI} = 0.64 \text{ nm}^2$ (Gullapalli 2008), $T = 295 \text{ K}$, $k_c = 9.2 \times 10^{-20} \text{ J}$), we calculated spontaneous curvatures of DiIC12 and Fast DiI as $C_{DiIC12} = 0.32 \pm 0.47 \text{ nm}^{-1}$ (n = 4) and $C_{Fast \text{ DiI}} =$
$-0.11 \pm 0.41 \text{ nm}^{-1} \ (n = 4)$.

Figure 3.1.7. (a) Relative theoretical outer leaflet tether fluorescence intensity as a function of tether radius calculated from spontaneous curvature sorting model, for two extreme spontaneous lipid curvatures (DAG and LPA), or moderate spontaneous curvature (DOPE), as well as a cylindrical (i.e., zero spontaneous curvature) lipid (solid line). Tether intensity is calculated from Eq. 3.1.6 ($a = 0.5 \text{ nm}^2$, $T = 295 \ K$). Note that the presence of spontaneous curvature primarily shifts the curves along the ordinate (see Eq. 3.1.7). (b) Theoretical tether fluorescence intensity plots calculated from bending stiffness model. Different bending stiffness is applied as legend shows. Tether intensity is calculated based on $I \propto R_t \frac{\phi_{\text{out}}}{\phi_{\text{zov}}}$ in which the molar ratios were obtained from Eq. 3.1.8 ($a = 25 \text{ nm}^2$, $T = 295 \ K$).
However, for CTB, whose intensity curve shows a large displacement from the simple proportional relationship, we are able to determine a spontaneous curvature of the membrane patch covered by CTB. Experimental CTB intensity profiles were fitted to Eq. 3.1.6 ($a_{\text{CTB}} = 25 \text{ nm}^2$ (Wang 2004), $T = 295 \text{ K}$, $k_c = 9.2 \times 10^{-20} \text{ J}$). A representative fit is shown in Fig.3.1.5a (dashed line). We obtained $C_{\text{CTB}} = -0.055 \pm 0.012 \text{ nm}^{-1}$ ($n = 8$).

In addition to our spontaneous curvature sorting model, we developed an alternative theoretical model for CTB curvature partitioning based on bending stiffness differences. In this model, we assume that membrane patches coated by molecules added in trace amount have a bending stiffness $k_\alpha$ different from the bulk lipid $k_\beta$. The weighted average bending stiffness is $k_c = \frac{A k_\alpha k_\beta}{k_\beta a_\alpha N_\alpha + k_\alpha a_\beta N_\beta}$ (Kozlov 1992). The free energies for tether and vesicle are equivalent to those of Eqs. 3.1.3 and 3.1.4. We obtain the partition coefficient for both leaflets as (for details see Appendix B):

$$K = \exp \left( \frac{k_\beta^2 a C^2}{2 k_\alpha T} \left( \frac{1}{k_\alpha} - \frac{1}{k_\beta} \right) \right) = \frac{\phi_{\alpha t}}{\phi_{\alpha v}}$$  

where $a$ is the molecular area of the protein adsorbed to the membrane.

From Eq. 3.1.8, theoretical fluorophore intensity ratios obtained from our bending stiffness model are plotted in Fig.3.1.7b. The graph indicates that the sorting efficiency does not sensitively depend on specific stiffness values if the bending stiffness of the trace
component is significantly larger than that of the lipid membrane. For instance, the plots
for \( k_\alpha = 5 \times 10^{-19} \text{ J} \) and \( k_\alpha = 10^{-5} \text{ J} \) essentially overlap each other (not shown).
Consequently, fitting experimental CTB intensity data to the theoretical curve \((T = 295 \text{ K})\)
gives widely spread CTB bending stiffness values from \( k_{c(\text{CTB})} = 10^{-5} \text{ J} \) to \( k_{c(\text{CTB})} = 10^{-19} \text{ J} \)
\((n = 8)\). A representative fitting result is shown in Fig.3.1.5a (gray solid line).
Despite the fact that both models fit well to the experimental data, the fitting quality from
the spontaneous curvature model is slightly better than that from the bending stiffness
model (eight data sets). Further evidence for the spontaneous curvature model can be
obtained from a quantitative comparison of the data shown in Fig.3.1.5 c, to our sorting
models. Fig.3.1.5 c indicates that the bending stiffness of a membrane covered by CTB
(with a coverage area fraction of ~20%) does not measurably depend on tube radius. In the
context of sorting by bending stiffness, this suggests that this observation implies a
contribution by cholera toxin to the overall membrane bending stiffness too small to allow
for the efficient sorting shown in Figs. 3.1.4, b and e, and 3.1.5 a. This comparison
solidifies our hypothesis of CTB sorting by spontaneous curvature. Moreover, induction
of spontaneous curvature by CTB binding to membranes does not seem to be unreasonable
as we have previously described inward invagination of cholera toxin domains on GUVs
(Baumgart 2007). Furthermore, it has been reported that the related protein Shiga Toxin
induces tubular membrane invaginations (Romer 2007).
We have shown that the CTB/GM1 complex is effectively curvature-sorted. However, we have not yet addressed the curvature partitioning of GM1 itself. This could be achieved by means of GM1 variants labeled with fluorophores in the headgroup region. Based on the partitioning results for the three DiI dyes, as well as the fluorescent lipids TR-DHPE and BODIPY-DHPE, we do not expect GM1 to be curvature sorted in the absence of CTB, unless GM1 by itself significantly clusters, which currently is a matter of debate (Wang 2004; Marushchak 2007; Shi 2007). Recent evidence indicates that clustered GM1, however, inhibits Cholera toxin binding (Shi 2007). We therefore regard it as unlikely that curvature sorting of clustered GM1 underlies the sorting of fluorescent CTB that we describe here.

3.1.4 Conclusion

The main conclusion from our investigation is that individual lipids, in contrast to the peripherally binding protein CTB, do not sense curvature effectively enough to be significantly sorted by their shape. This experimental observation is in accordance with molecular dynamics simulations (Cooke 2006), as well as a simple analytical model for lateral curvature sorting developed here, which is in the spirit of those developed by Cooke and Deserno (Cooke 2006), Iglic et al. (Iglic 2006), and Kralj-Iglic et al. (Kralj-Iglic 2002).
Although the absence of effective lipid sorting by molecular shape is shown, non-ideal mixing lipids display significant curvature sorting, as has been shown clearly in previous model membrane research (Roux 2005; Baumgart 2003; Parthasarathy 2006). In a situation where lipids non-ideally mix to form extended regions with differing bending stiffness (Baumgart 2003; Baumgart 2005), or differing spontaneous curvature (Seifert 1993; Leibler 1986), curvature energy effectively contributes to lipid sorting. Theory predicts that this effect becomes amplified close to a phase boundary and particularly a critical mixing/demixing point (Seifert 1993). If lateral segregation into membrane domains is an important mechanism for sorting of lipids and proteins in cells, then an essential question is whether segregation is present before budding of transport vesicles in intracellular sorting centers, or if segregation occurs during vesicle budding (Holthuis 2005). It is conceivable that membrane curvature may trigger lateral phase segregation (Pencer 2008) in membranes with compositions near a phase boundary (Roux 2005; Lee 2005).

Whereas we did not observe lipids to be individually sorted, the protein CTB showed more effective segregation. Two statistical mechanical sorting models were developed. Comparison of the two models and our data suggests sorting of CTB by spontaneous curvature. Preferential interactions of proteins with lipids could cause lipid sorting as a secondary effect to protein sorting in live cells. The area of lipid and protein trafficking is
one where understanding of fundamental mechanisms remains missing (Holthuis 2005), and we expect model membrane biophysics to be an important contributor in solving some of the essential questions.

### 3.2 Bending Stiffness Depends on Curvature of Ternary Lipid Mixture Tubular Membranes

#### 3.2.1 Introduction

Motivated by the aim to understand how molecular sorting of membrane components is achieved in biological cells (De Matteis 2008; Holthuis 2005), the coupling between membrane curvature and composition has recently been under intensive investigation. In addition to proteins, fluorescent lipids have been used as trafficking markers. Importantly, lipid-like dyes of the indocarbocyanine family with identical head groups were shown to display remarkably different sorting in cells (Mukherjee 1999). The protein Cholera toxin subunit B (CTB) is, furthermore, frequently used to study intracellular trafficking (Chinnapen 2007). We demonstrated that these lipid-like fluorophores with differing intrinsic curvature were not significantly sorted in membranes with steep curvature gradients (Tian 2009). Conversely, we found that CTB is effectively sorted among
connected membranes with differing curvature (Tian 2009).

Several different types of membrane curvature can be distinguished in cells (Zimmerberg 2006; McMahon 2005). Among these, membranes with spherical (e.g. vesicles) and cylindrical curvature (e.g. membrane tubes), are the most prominent long-lived high curvature intracellular membrane morphologies. Our research focuses on biophysical characterization of sorting in tubular membranes. Biological membrane tubes have recently received increasing interest in various differing contexts, including intercellular nanotubes with signaling function (Rustom 2004) and intracellular tubes connecting different organelles (Simpson 2006).

For a quantitative characterization of mechanical contributions to the sorting of membrane components, lipid model membrane systems have proved advantageous. Evidence for the sorting of fluid phase domains with differing bending stiffness came from fluorescence microscopy images of giant unilamellar vesicles with liquid ordered (Lo) and liquid disordered (Ld) phase coexistence (Baumgart 2003; Parthasarathy 2006). Lateral segregation has also been observed in tubular model membranes (Baumgart 2003; Roux 2005). Lipid bilayer membrane tubes have long been of interest to biophysicists (Bo 1989; Dai 1998; Hochmuth 1982), and model membrane tubes with complex composition are receiving increasing attention (Allain 2004; Jiang 2008; Roux 2005; Tian 2009). This contribution investigates how curvature sorting is amplified in non-ideally mixing
multi-component membranes.

The coupling between membrane curvature and composition has thoroughly been theoretically investigated. Several continuum theories have been developed. The earliest analytical exposition is the one by Markin (Markin 1981), who proposed the composition dependence of the models of spontaneous curvature and bending stiffness used in this work. Kozlov developed a thorough thermodynamic analysis of elastic amphiphile mixture interfaces (Kozlov 1989; Kozlov 1992), which forms the basis of the analytical component of the present contribution. Phenomenological continuum theories predicted curvature instabilities (Leibler 1986; Leibler 1987) and curvature-induced phase segregation in vesicles (Seifert 1993), and several more recent contributions have computed membrane composition profiles as a function of curvature (Bozic 2006; Derganc 2007; Mukhopadhyay 2008). In the strong segregation limit, the influence of elastic domain properties on membrane shape has been thoroughly investigated (Harden 1994; Harden 2005) and the weak segregation limit has also been explored (Leibler 1986; Leibler 1987; Taniguchi 1996). Most of these investigations focused on the role of local spontaneous curvature, as opposed to bending stiffness, in coupling membrane shape and composition.

Several microscopic theories have been developed to quantitatively describe the properties of membranes with inclusions (Dimeglio 1985). Furthermore, statistical mechanical
models predicted elastic features of amphiphile mixtures (Henriksen 2004). Simulations have enabled the investigation of the dependence of membrane bending stiffness on composition (Meleard 1997) and the magnitude of sorting that occurs for molecules with intrinsic curvature (Pan 2008). Several previous experimental studies have examined the effect of composition on bending stiffness. Amphiphilic membranes of interest have included emulsions (Hochmuth 1982; Waugh 1979) and lipid bilayers (Esposito 2007; Hochmuth 1982).

The contents of this manuscript are organized as follows. The main focus of our contribution is to demonstrate that the bending stiffness of mixed lipid membranes depends on membrane curvature if lipids show non-ideal mixing due to preferential lateral interactions. We first investigated potential curvature-induced sorting in binary mixtures consisting of the lipids palmitoyloleoylphosphatidylcholine (POPC) and cholesterol (Chol). A constant bending stiffness over the accessible curvature range indicated that significant curvature sorting does not occur in these binary mixtures. We next examined ternary mixtures with compositions near a fluid/fluid mixing/demixing phase boundary. We find that tubular membranes from a large range of ternary mixture compositions show a curvature-dependent bending stiffness, demonstrating a curvature-dependent composition. Close proximity to a demixing point was thus not a requirement for curvature-induced sorting; however, the sorting efficiency increased upon approaching the
critical neighborhood of the phase diagram. We develop a thermodynamic model that quantitatively describes the curvature-dependence of the bending stiffness by means of the ratio of two thermodynamic partial derivatives. We compare this curvature dependent segregation mechanism to sorting by spontaneous curvature.

3.2.2 Materials and Methods

3.2.2.1 Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol (Chol), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-stearoyl-2oleoyl-sn-glycero-3-phosphocholine (SOPC) and distearoylphosphatidyl-ethanolamine-N-[biotinyl(polyethylene glycol)2000] (DSPE-Bio-PEG2000) were from Avanti Polar Lipids Inc. (Alabaster, AL). Fatty-acid free bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO). TEXAS RED-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (TR-DHPE) was purchased from Invitrogen (Carlsbad, CA).

Vesicle preparation, micropipette fabrication, and tether pulling method were as previously described (Tian 2009) (also see supplementary information).
3.2.2.2 Membrane tension, tether radius, and bending stiffness determination

Lateral membrane tension $\sigma$ is obtained from pipette aspiration pressure $\Delta P$, by the following formula (Waugh 1979):

$$\sigma = \Delta P \frac{R_p}{2(1 - R_p/R_v)}$$

in which $R_p$ and $R_v$ are the pipette radius and vesicle radius (of the spherical part of the vesicle outside of the pipette, also see supplementary information) respectively.

Membrane tension determines a corresponding tether radius. The tether radius is obtained from stepwise horizontal elongation and release of the membrane tube as described (Tian 2009). Briefly, the tether was typically elongated in three to five steps (each of 20 $\mu$m) and the associated change in the length of the pipette-aspirated vesicle projection was measured. The tether radius can then be calculated from the ratio of projection length change ($\Delta L_p$) and tether length change ($\Delta L_t$). The ratios (i.e., the slopes) at different membrane tensions are shown in Fig. 2a.

From the conservation of membrane area and total vesicle volume, the following relation can be obtained which we used to calculate tether radii (Hochmuth 1982; Hochmuth 1982):

$$R_t = -\frac{\Delta L_p}{\Delta L_t} \left(1 - \frac{R_p}{R_v}\right) R_p$$

The influence on the determination of $R_t$ of vesicles with prolate spheroid shape is
quantitatively discussed in the supplementary information. Briefly, the error is negligible under all conditions used.

3.2.2.3 Imaging

Vesicles were imaged by fluorescence confocal microscopy (FV300 scanning system integrated with a motorized inverted microscope IX81, Olympus, Center Valley, PA), using a 60x, 1.2 NA water immersion lens with cover slip correction collar (Olympus). Illumination intensities and illumination times were minimized to reduce photoeffects (Esposito 2007). Image analysis was performed using IMAGEJ (National Institutes of Health, Bethesda, MD).

3.2.3 Results

We focused our investigation of curvature-dependent sorting on the ternary lipid mixture DOPC, DPPC, and Chol, the phase behavior of which is well characterized (Veatch 2003; Veatch 2004) (see Fig.3.2.1). We chose six different compositions (labeled I–VI in Fig.3.2.1) made with these three lipid types. Vesicles were prepared with the addition of the fluorophore TR-DHPE, as well as a biotinylated lipid with oligo-ethyleneglycol spacer, which allowed us to couple streptavidin-coated beads to the vesicular membranes. The large majority of vesicles prepared with these six compositions were optically
homogenous as determined by fluorescence microscopy, in accordance with the published phase diagram (Veatch 2003; Veatch 2004). To localize the position of the phase boundary and critical point at our measurement temperature (22 ± 0.5°C) in the ternary phase diagram, we determined approximate area fractions as well as the fraction of phase-separated vesicles. The resulting phase boundary shown in Fig. 3.2.1 is in good agreement with data by Veatch and Keller (Veatch 2003). Only optically homogenous vesicles were examined in the experiments below. We selected two different compositional trajectories: one approximately parallel to the phase boundary (compositions I–III), and one roughly orthogonal to the phase boundary (compositions III–VI); see Fig. 3.2.1. These two compositional directions are expected to lead to differing critical exponents of thermodynamic quantities (Griffiths 1970). According to the phase diagram, compositions III and IV are expected at room temperature to be in the neighborhood of a critical consolute (i.e., mixing/demixing) point (Veatch 2003; Veatch 2004).
Figure 3.2.1. Experimental phase diagram indicating compositions (depicted using stars) of ternary lipid mixture DOPC/Chol/DPPC: I, 70:20:10; II, 55:28:17; III, 40:37:23; IV, 40:40:20; V, 40:46:14; and VI, 40:50:10. Circles mark compositions of vesicles imaged to determine the upper phase boundary and the critical point of phase separation (critical consolute point). Population fractions of vesicles with phase separation are indicated in the left legend. The grayscale line represents the upper phase boundary binodal line at $\sim 22^\circ$C. The binodal line also displays the area fractions of the disordered phase according to the legend on the right. Compositions III and IV are in the neighborhood of the critical point where area fraction is 40–60% (indicated by the thick bar on binodal and right legend). Our compositions I–VI are chosen in the homogeneous phase region outside the miscibility gap and approach the neighborhood of the critical point in two directions: along lines parallel (I, II, and III) and lines perpendicular (VI, V, IV, and III) to the upper phase boundary.

Vesicles were pipette-aspirated and the lateral membrane tension was adjusted by a hydrostatic approach, as described in Tian and Baumgart (Tian 2009). Our approach to determining membrane bending stiffness as a function of curvature is identical to our previous measurements (Tian 2009), and a similar technique has been described by Bo and Waugh (Bo 1989). Vesicles with sufficient excess membrane area (relative to the area of a
sphere with the same volume) were chosen for pipette aspiration. The excess area yields a pipette-aspirated membrane fraction with a length that should exceed the inner aspiration pipette radius under all experimental conditions. After pipette aspiration, a streptavidin-coated bead was brought into contact with the vesicular membrane by means of a second pipette that was moved via a micromanipulator assembly. Moving the bead away from the vesicle generated a cylindrical tether from the vesicular membrane. The curvature of the tether was adjusted by means of the pipette aspiration pressure. We determined the radius by a stepwise extension of the tube length \( L_t \) and measurement of the accompanying decrease of the length \( L_p \) of the pipette-aspirated vesicle projection in the pipette interior (Bo 1989; Tian 2009). Examples for such measurements are shown in Fig. 3.2.2 \( a \) for three different lateral membrane tensions. From the slope of the plot in Fig. 3.2.2 \( a \), the tube radius can be calculated. Examples are shown in Fig. 3.2.2 \( b \), where, to contrast with ternary mixtures, we consider a binary mixture of DOPC/Chol in a molar ratio 2:1. The line in Fig. 3.2.2 \( b \) is a fit to the equation \( R = \sqrt{\kappa/2\sigma} \) (Derenyi 2002; Evans 1994). The excellent agreement between fit and experimental data in Fig. 3.2.2 \( b \) indicates that the binary mixture membrane displays a constant bending stiffness over the entire curvature range, down to \( \sim 10 \) nm. This finding is further demonstrated in the bending stiffness values that were calculated from every radius/tension pair, shown in Fig. 3.2.2 \( c \) over the entire curvature range. Earlier measurements using binary mixtures of
palmitoyloleoylphosphatidylerine and SOPC also did not report a curvature-dependent 
bending stiffness (Song 1990).

**Figure 3.2.2.** Membrane bending stiffness from projection length ($L_p$)/tether length ($L_t$) 
measurements. (a) Demonstration of $L_p$ versus $L_t$ plots at different membrane tensions. 
(Square, $\sigma = 1.2 \times 10^{-4}$ N/m; triangle, $\sigma = 5.2 \times 10^{-5}$ N/m; circle, $\sigma = 3.4 \times 10^{-5}$ N/m; and 
solid and open symbols represent the elongation and relaxation steps of the tether, 
respectively.) This example is chosen from three tether radius measurements of 
composition III. From the slopes of these plots, we obtained tether radii (see Eq. 3.2.2). 
(Square, $R_t = 20.6$ nm; triangle, $R_t = 34.0$ nm; and circle, $R_t = 39.0$ nm.) (b) Relation of 
tether radius and membrane tension of a tether consisting of binary mixture DOPC/Chol = 2:1. Solid line indicates the fit of data points using a single bending stiffness ($\kappa = 7.4 \times$
$10^{-20}$ J. (c) Bending stiffness versus tether radius plot. Bending stiffness is calculated from panel $b$ and is observed to be roughly constant, under changing curvature. (d) Relation of tether radius and membrane tension of a tether from ternary mixture III. Solid and open circles represent decreasing and increasing $R_t$, respectively. Solid line and shaded line are plots at the maximum ($\kappa_{\text{max}} = 1.5 \times 10^{-19}$ J) and the minimum ($\kappa_{\text{min}} = 8.5 \times 10^{-20}$ J) bending stiffness values among these data points. It is thus observed that the bending stiffness decreases with curvature for this mixture.

Fig. 3.2.2 $d$ shows radius measurement examples in a tube with composition III (close to the critical point). The lines in Fig. 3.2.2 $d$ are tube radius values calculated for a constant bending stiffness assuming either the stiffness value ($\kappa_{\text{max}}$) obtained from the radius/tension pair of the largest radius in Fig. 3.2.2 $d$ (solid line), or the stiffness value ($\kappa_{\text{min}}$) obtained from the radius/tension pair of the smallest radius value (shaded line). Clearly, the experimental data cannot be fitted by assuming a constant bending stiffness. In Fig. 3.2.3, we illustrate the bending stiffness values obtained from the radius/tension pairs shown in Fig. 3.2.2 $d$ for composition III in three different representations: as a function of tube radius (Fig. 3.2.3 $a$); as a function of curvature, $C = 1/R_t$, where $R_t$ is the tube radius (Fig. 3.2.3 $b$); and, as a function of the square of the tube curvature (Fig. 3.2.3 $c$). Fig. 3.2.3 $c$ indicates a different slope for the first few data points, compared to those obtained
at larger curvatures. The axis scaling of Fig. 3.2.3c is motivated by an analytical theory (detailed below), which will indicate the bending stiffness to be a function of the squared curvature in the low curvature regime. Fig. 3.2.3d displays data collected for measurements in eight different vesicles over the entire curvature range of composition III.

**Figure 3.2.3.** Bending stiffness versus (a) tether radius, (b) tether curvature, and (c) squared curvature plots from the same experimental data shown in Fig. 3.2.2d. The figures clearly indicate the bending stiffness to decrease with increasing curvature. (d) Bending stiffness and squared curvature $C^2$ relation from combination of eight whole-range data sets of composition III. The value $n$ refers to the number of different vesicles examined. Data points in panels c and d are separated into two ranges by dashed
lines at $C^2 = 0.0012 \text{ nm}^{-2}$. The bending stiffnesses obtained at $C^2$ values <0.0012 nm$^{-2}$ were chosen for fitting our thermodynamic model to bending stiffness-curvature relations $\kappa_{\text{eff}} = \kappa_0 - \Omega C^2$, for all compositions examined.

Before we quantitatively examine our data (results shown in Fig. 3.2.4), we outline the theoretical framework of our analysis and contrast description of sorting caused by composition-dependent spontaneous curvature (Kozlov 1992; Leibler 1986; Derganc 2007) to sorting invoked by composition-dependent bending stiffness in membrane mixtures. To apply the theory developed by Kozlov and Helfrich (Kozlov 1992) to our situation, we first approximate the ternary mixture of Fig. 3.2.1 with a binary mixture. Previous work has shown that a quasibinary phenomenological condensed complex model well describes the fluid phase boundaries of experimental ternary phase diagrams (Radhakrishnan 2005). Below, we obtain alternative expressions for ternary mixtures. These expressions are more complex but do not change the interpretation of experimental data. We consider a thermodynamic process that keeps the area of the membrane tube constant but changes the curvature (compare to the Appendix of Kozlov and Helfrich (Kozlov 1992)) and thus define the differential of the tube free energy, $F$, as

$$dF = A \kappa dC + \mu_\alpha dN_\alpha + \mu_\beta dN_\beta = A \kappa dC + N(\mu_\alpha - \mu_\beta) d\phi_\alpha$$

where $A$ is the tube area, $\kappa$ is the bending stiffness of the tube, and $\mu_i$ and $N_i$ are chemical...
potentials and numbers of molecular components $i$, respectively. Expansions with respect to mole fraction $\varphi_i$ (as opposed to molecular component numbers $N_i$) simplify the resulting expressions. Equation 3.2.3 allows us to obtain a Taylor expansion of the tube free energy (Kozlov 1992)) about a flat membrane from expansions of the intensive parameters in Eq. 3.2.3, followed by integration (see Appendix of Kozlov and Helfrich (Kozlov 1992)). We define the diffusion (or exchange) potential as

$$\bar{\mu} = \mu_a - \mu_\beta = \frac{1}{N} \left( \frac{\partial F}{\partial \varphi_a} \right)_{A,C}$$

where $N$ is the total number of molecules in the tube, and the derivative is evaluated at constant tube area and constant curvature. All following derivatives are evaluated at constant area; however, below we drop the index $A$. As Appendix C demonstrates in more detail, an expansion of the quantity $\bar{\mu}$ to first-order in curvature and composition change relative to zero curvature, $\Delta \phi_a = \phi_a(C) - \phi_a(0)$ yields the following relation between these two quantities,

$$\Delta \phi_a = \left( \frac{\partial \bar{\mu}}{\partial C} \right)_0 \left/ \left( \frac{\partial \bar{\mu}}{\partial \varphi_a} \right)_0 \right\} C$$

from which an effective bending stiffness can be obtained (see Appendix C),

$$\kappa_{\text{eff}} = \kappa_0 - \rho \left( \frac{\partial \bar{\mu}}{\partial C} \right)_0 \left/ \left( \frac{\partial \bar{\mu}}{\partial \varphi_a} \right)_0 \right\)$$

where $\rho = N/A$ is an area density that is assumed to be constant. The index 0 in Eqs. 3.2.5 and 3.2.6 indicates that these partial derivatives have to be evaluated for the flat membrane,
as they are Taylor coefficients associated with an expansion about the flat state of the membrane. Note that under our experimental conditions the curvature of the vesicle is 2–3 orders-of-magnitude smaller compared to the curvature of the tube. We therefore regard the vesicle as flat. Furthermore, the number of lipids contained in the vesicle is significantly larger than that in the tube. The vesicle is therefore regarded as a particle reservoir with fixed composition and curvature, and therefore has constant chemical potential. Note that the effective bending stiffness obtained through Eq. 3.2.6 is smaller compared to the bare bending stiffness, but is not a function of curvature. Furthermore, the first derivative in Eq. 3.2.6 will have nonzero values only for membranes with nonzero spontaneous curvature (see Appendix C, which also provides an example of a model for which the thermodynamic derivatives can be evaluated analytically). For both of these reasons, our observations shown in Figure 3.2.3 and Figure 3.2.4 cannot be explained by means of a model that assumes primarily spontaneous curvature to drive the sorting process.
Figure 3.2.4. (a) Linear fits of bending stiffness and squared curvature obtained from multiple data sets in the low curvature range ($C^2 < 0.0012 \text{ nm}^{-2}$) for each composition. Compositions are indicated at the upper-left corner of each graph, and the numbers of tethers included for each composition are shown in the upper-right corner of each plot. Data sets of composition III are the same as those in Fig. 3.2.3 d but display the low curvature region instead of the whole range. (b) Slopes from linear fits for different compositions in panel a. Dashed lines are linear fits depicting the increasing trends of slopes $\Omega$ for compositional trajectories parallel (I, II, and III) and perpendicular (VI, V, IV, and III) to phase boundary approaching the neighborhood of a critical demixing point. The graph indicates that the slope $\Omega$, which determines the sorting efficiency (see Eqs. (3.2.7)
and (3.2.8)), increases upon approaching the critical region. (c) Bending stiffness at zero curvature (intercepts of plots in panel a) for different compositions. Error bars in panels b and c are the uncertainty of linear fits in panel a.

It follows that to obtain a curvature-dependent bending stiffness, the quantity $\bar{\mu}$ has to be expanded to second-order (or, equivalently, the free energy has to be expanded to third-order). From such an expansion, we obtain the composition change as a quadratic function of curvature (see Appendix C for details),

$$\Delta\phi_a = -\frac{1}{2} \left( \frac{\partial^2 \bar{\mu}}{\partial C^2} \right)_0 \left( \frac{\partial \bar{\mu}}{\partial \phi_a} \right)_0 C^2$$  \hspace{1cm} (3.2.7)

allowing us to express the bending stiffness as a function of curvature (see Appendix C),

$$\kappa_{\text{eff}} = \kappa_0 - \frac{3}{4} \left( \frac{\partial \kappa}{\partial \phi_a} \right)_0^2 \rho \left( \frac{\partial \bar{\mu}}{\partial \phi_a} \right)_0 C^2 = \kappa_0 - \Omega C^2$$  \hspace{1cm} (3.2.8)

Equation 3.2.8 is valid at constant chemical potentials fixed by the reservoir. As long as the bending stiffness is composition-dependent, we therefore find the renormalized (effective) bending stiffness to be a quadratic function of curvature. The effective bending resistance is experimentally obtained from $\kappa_{\text{eff}} = 2\sigma R_t^2$, where $\sigma$ and $R_t$ are the experimentally determined quantities. Equation 3.2.8 is the central result of our analysis and is fitted to data in the form $\kappa_{\text{eff}} = \kappa_0 - \Omega C^2$, where $\Omega$ comprises the thermodynamic
derivatives in Eq. 3.2.8. Note that these derivatives are approximately constant for given initial conditions, as they are all evaluated at the flat state.

In both Eqs. 3.2.6 and 3.2.8, the effective bending stiffness depends on a quantity that can be interpreted as a “driving force” to sorting (the first derivative in both equations). This “driving force” is divided by a quantity that resists sorting (second derivative in both equations). The resistance to sorting is composition-dependent (see Appendix C for details including a model for which the thermodynamic derivatives were analytically evaluated).

For membranes that can phase-separate, this derivative will vanish at the spinodal line, in the neighborhood of which sorting is thus expected to be amplified. The spinodal line, which lies within the miscibility gap of the phase diagram, can be approached most closely in the neighborhood of a critical point. This motivates our choice of compositional trajectories (Fig. 3.2.1). We fitted Eq. 3.2.8 to our data within the low curvature regime defined in Fig. 3.2.3. The rationale behind this limited range evolves from the derivation of Eq. 3.2.8. The relation results from Taylor expansions about the flat state. The second-order Taylor expansion of tube free energy in curvature has been shown to be accurate down to curvature radii approaching membrane thickness (Harmandarlis 2006). However, it is likely that the accuracy of a second-order free energy expansion (first-order expansion of the chemical potentials) in composition change is limited to small deviations
from the composition of the flat membrane for the mixing ratios considered in this contribution.

Fig. 3.2.4 summarizes our composition-dependent measurements of bending stiffness for the six compositions of Fig. 3.2.1. Fig. 3.2.4 a shows the low curvature regime of bending stiffness versus squared curvature plots that, according to Eq. 3.2.8, is expected to be linear. Results of linear fits to data obtained from several vesicles (numbers n are indicated in the figure) are displayed in Fig. 3.2.4, b and c. The error bars in Fig. 3.2.4, b and c, result from the uncertainty of slope and intercept of linear fits to the pooled data shown in Fig. 3.2.4 a. Although these error bars are large, Fig. 3.2.4 b suggests that the prefactor $\Omega$ increases upon approaching the neighborhood of the critical point along the trajectory roughly parallel to the phase boundary (compositions I–III). As further discussed below, this may be rationalized by the value of the second derivative in Eq. 3.2.8 becoming smaller along this trajectory. We furthermore observe that approaching the critical region along the trajectory orthogonal to the phase boundary is also associated with an increase of the prefactor $\Omega$. Refined measurements will have to be performed to determine critical exponents in these experiments, because the current uncertainties are large. Our results, however, do indicate that effective sorting, indicated by composition-dependent bending stiffness values, occurs over a large composition range far from the critical point.
Fig. 3.2.4 c displays bending stiffness values for the flat state obtained from fitting Eq. 3.2.8 to our data. The figure indicates that the reservoir bending stiffness $\kappa_0$ increases when approaching the critical neighborhood through compositions I–III. Bending stiffness measurements in ternary mixture membranes are still rare (Semrau 2008; Rawicz 2008). We therefore used a second technique to confirm the composition dependence of the bending stiffness of the vesicle reservoir displayed in Fig. 3.2.4 c. We measured projected membrane area of aspirated vesicles as a function of lateral tension, and determined bending stiffness (Rawicz 2000). We minimized curvature effects in these measurements by using large pipettes and focusing on the low tension regime. We first confirmed (see Table 3.2.1) that our experimental conditions reproduced bending stiffness values for the lipid SOPC, which has frequently been used in pipette aspiration experiments (Rawicz 2000; Ly2002). Previous measurements have shown that bending stiffness often increases while increasing the cholesterol content of fluid phase binary mixture membranes (Meleard 1997; Henriksen 2004). However, interestingly, cholesterol has a surprisingly small effect on the bending stiffness of binary mixtures with DOPC (Pan 2008). This phenomenon is confirmed by our pipette aspiration measurements (Table 3.2.1). Overall, the stiffness values shown in Table 3.2.1 are systematically smaller, comparing pipette aspiration to membrane tethers. This effect has previously been noted (Marsh 2006) and is likely related to the convolution of bending and stretching elasticities complicating
analysis of pipette aspiration data (Henriksen 2004). The table does, however, indicate that pipette aspiration reports a similar composition trend of reservoir bending stiffness values compared to values obtained from fitting our Eq. 3.2.8. We finally note that our DOPC/Chol bending stiffness values are in good agreement with literature values (Pan 2008; Marsh 2006).

<table>
<thead>
<tr>
<th>Composition</th>
<th>$\kappa_0 \times 10^{-19}$ J (Aspiration)</th>
<th>$\kappa_0 \times 10^{-19}$ J (Tether)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$0.91 \pm 0.17$ ($n = 4$)</td>
<td>$1.36 \pm 0.27$ ($n = 5$)</td>
</tr>
<tr>
<td>II</td>
<td>—</td>
<td>$1.84 \pm 0.12$ ($n = 6$)</td>
</tr>
<tr>
<td>III</td>
<td>—</td>
<td>$1.86 \pm 0.47$ ($n = 8$)</td>
</tr>
<tr>
<td>IV</td>
<td>$1.40 \pm 0.52$ ($n = 8$)</td>
<td>$2.16 \pm 0.12$ ($n = 9$)</td>
</tr>
<tr>
<td>V</td>
<td>—</td>
<td>$2.29 \pm 0.11$ ($n = 7$)</td>
</tr>
<tr>
<td>VI</td>
<td>$0.93 \pm 0.13$ ($n = 4$)</td>
<td>$1.36 \pm 0.11$ ($n = 5$)</td>
</tr>
<tr>
<td>DOPC/Chol = 1:1</td>
<td>$0.91 \pm 0.33$ ($n = 10$)</td>
<td>—</td>
</tr>
<tr>
<td>DOPC/Chol = 2:1</td>
<td>$0.86 \pm 0.13$ ($n = 8$)</td>
<td>$1.01 \pm 0.23$ ($n = 9$)</td>
</tr>
<tr>
<td>SOPC</td>
<td>$0.77 \pm 0.14$ ($n = 13$)</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 3.2.1. Bending stiffness of quasiflat membranes by pipette aspiration and tether pulling. Comparison of experimental bending stiffness values using micropipette aspiration method and tether pulling, respectively. Uncertainties result from measurements in $n$ vesicles indicated in parentheses.
We have thus far interpreted our measurements by means of quasibinary mixture models with straightforward interpretation and minimized algebraic effort. In reality, however, both bending stiffness and chemical potentials depend on the concentrations of all three components—DOPC, DPPC, and Chol. The ternary mixture equivalents to Eqs. (3.2.6) and (3.2.8) are obtained from an exercise in matrix algebra, using the two exchange potentials \( \mu_i = \mu_i - \mu_3 \) referenced to the chemical potential of the third component. For the effective resistance to bending in the ternary spontaneous curvature model, we have

\[
\kappa_{\text{eff}} = \kappa_0 - \rho \frac{(\frac{\partial \mu_1}{\partial C})_0^2 (\frac{\partial \mu_2}{\partial \phi_2})_0 - 2 (\frac{\partial \mu_1}{\partial C})_0 (\frac{\partial \mu_2}{\partial \phi_2})_0 (\frac{\partial \mu_1}{\partial \phi_2})_0 + (\frac{\partial \mu_2}{\partial \phi_2})_0^2 (\frac{\partial \mu_1}{\partial \phi_2})_0}{(\frac{\partial \mu_1}{\partial \phi_1})_0 (\frac{\partial \mu_2}{\partial \phi_2})_0 - (\frac{\partial \mu_1}{\partial \phi_2})_0 (\frac{\partial \mu_2}{\partial \phi_1})_0}
\]

3.2.9

i.e., the sole difference between Eqs. 3.2.6 and 3.2.8 is the interpretation of the thermodynamic factor. Equivalently, the curvature- and composition-dependent resistance to bending described by Eq. 3.2.8 reads, for a ternary mixture,

\[
\kappa_{\text{eff}} = \kappa_0 - \frac{3}{4} \rho \left\{ \frac{(\partial \kappa)}{(\partial \phi_1)}_0^2 (\frac{\partial \mu_2}{\partial \phi_2})_0 - 2 (\frac{\partial \kappa}{\partial \phi_1})_0 (\frac{\partial \kappa}{\partial \phi_2})_0 (\frac{\partial \mu_2}{\partial \phi_2})_0 + (\frac{\partial \kappa}{\partial \phi_2})_0^2 (\frac{\partial \mu_2}{\partial \phi_2})_0 \right\} C^2
\]

3.2.10

where the ratio of thermodynamic derivatives is the ternary version of the parameter \( \Omega \) determined from the fits shown in Fig. 3.2.4. Presently, little is known about composition dependence of \( \kappa \) and \( \mu_i \) along lines parallel to the edges of the Gibbs phase triangle representing ternary mixtures. Future experimental measurements of ternary mixture
bending stiffness values across the phase diagram will, we hope, allow to test the detailed form of $\Omega$.

We have approached the critical mixing/demixing region of a ternary lipid mixture from two different compositional directions, one approximately orthogonal and one roughly parallel to the phase boundary. Comparing these two trajectories, Fig. 3.2.4 suggests that along the direction orthogonal to the phase boundary the parameter $\Omega$ decreases more quickly away from the critical point compared to the trajectory asymptotically parallel to the phase boundary. It is a well-known fact that critical exponents associated with the liquid/gas phase transition in a single component system differ, comparing the approach of the critical point asymptotically parallel versus along a direction orthogonal to the coexistence curve in field space (Griffiths 1970). The scaling behavior of mixtures satisfies the same scaling laws as single component systems, if suitable field variables are introduced (Anisimov 1992; Leung 1973), alternative to the densities (mole fractions) of Fig. 3.2.1. A more trivial challenge to the measurement of critical exponents is the large uncertainty in our present measurements.

Bending stiffness values $\kappa_0$ of the quasiflat reservoir were observed to increase when approaching the phase boundary along the trajectory of compositions I–III (Fig. 3.2.4 c and Table 3.2.1), i.e., along a trajectory with increasing DPPC concentration. Importantly, this behavior in approaching a demixing transition phase boundary is opposite to the
trends in bending stiffness near a main phase transition in single component membranes. In DMPC membranes, for example, a significant softening occurs when decreasing temperature toward the phase transition temperature (Chu 2005; Dimova 2000; Mecke 2003). This decreased bending stiffness is likely due to a divergence of the area compressibility modulus at the DMPC main phase transition temperature (Marsh 2006; Evans 1982). On the other hand, the area compressibility modulus of ternary lipid mixtures does not appear to show anomalies near a mixing/demixing phase boundary (Rawicz 2000). In the critical neighborhood of a mixing/demixing transition in ternary mixtures, the area compressibility modulus and bending stiffness thus appear to remain finite, whereas the osmotic compressibility diverges. We therefore argue that the increase of the quantity $\Omega$, defined in Eq. 3.2.8, upon approaching the critical neighborhood, is dominated by the divergence of the denominator, whereas the numerator remains finite and is not likely to show anomalies near the critical point. Equation 3.2.8 indicates that membranes consisting of nonideal lipid mixtures may become mechanically instable toward curvature fluctuations in the neighborhood of the critical mixing/demixing point. In all measurements of curvature/bending resistance relations, the bending stiffness decreased monotonously with increasing curvature and no discontinuous transitions were observed. We thus conclude that the finding of curvature-induced sorting in ternary lipid mixtures is not associated with an abrupt, first-order phase transition. This hypothesis is
supported by the fact that we have not observed any sharp boundaries, which would separate a membrane tube phase from a second phase of the quasiflat (vesicle) reservoir. Accordingly, the composition change as a function of curvature must be a continuous transition toward a phase with a more disordered-phase character, associated with a composition of the tube membrane that does not enter the coexistence region.

In characteristically different regions of the phase diagram than those investigated in this study, in the strong segregation limit far from the critical point, we have, however, observed curvature-dependent nucleation of domains in initially homogeneous phase membrane tubes (not shown). After nucleation, exclusively, a single liquid-disordered domain, with sharp phase boundaries, extended with characteristic square-root growth kinetics that are strongly dependent on curvature. This phenomenon thus shows rather different behavior compared to the measurements described above for the weak segregation limit in the critical neighborhood. A detailed description of these curvature-induced first-order transitions along with an initial theoretical analysis will be the topic of a forthcoming article.

3.2.4 Conclusion

Our results indicate that curvature-induced lateral lipid segregation in membranes with curvature gradients is amplified in nonideal lipid mixtures. Entropic penalties to sorting
that dominate (Tian 2009) the distribution of ideally diluted trace components, and that also dominate in ideal lipid mixtures, can thus be overcome by enthalpic contributions from preferential lipid/lipid interactions. The resistance to sorting, expressed in Eqs.3.2.5-3.2.8, vanishes at the spinodal line of the ternary mixture phase diagram, where sorting thus becomes maximally efficient.

We note that the phenomenon of curvature-induced demixing that we show here seems to be confirmed by recent independent measurements of pulling forces for tubes extracted from vesicles composed of a multicomponent mixture of several different sphingomyelins plus DOPC and cholesterol (Sorre 2009). Our choice of technique yields the tube radius, and thus does not require the use of reference curvatures.

The measurements described in this report have focused on simple model membrane mixtures. Recent contributions have reported phase transitions (Baumgart 2007) and near-critical behavior (Veatch 2008) in vesicles obtained from cellular membranes, indicating strongly nonideal mixing. This suggests that curvature-modulated lateral sorting may be a relevant mechanism for sorting in biological membranes. Trace components, such as Dil dyes (Mukherjee 1999), have been shown to sort differentially among intracellular biological membranes (Mukherjee 1999). Our findings suggest that membrane trace components are sorted by sensing the curvature-dependent membrane bulk composition. This sorting mechanism is different from trace components directly
sensing membrane curvature, a scenario that we (Tian 2009), and others (Cooke 2006; Derganc 2007), have found to be inefficient.

Lipid model membrane systems bear the potential for providing further insight into the biophysical contribution of membrane curvature to biological sorting phenomena.

3.3 Dynamic Sorting of Lipids and Proteins by Curvature: a Moving Phase Boundary Problem

3.3.1 Introduction

The sorting of both lipids and proteins within cells is an essential but poorly understood process. Sorting of intracellular membrane components can be mediated by protein coats (Bonifacino 2006), and involves biophysical mechanisms based on membrane fluidity, molecular shape of components, and membrane phases (Mukherjee 2000). Lateral segregation of membrane lipids likely constitutes an important sorting principle in cellular membranes (Mukherjee 2000; Simons 1997). In model membranes, segregation into the biologically most relevant fluid phase domains - the liquid ordered (Lo) phase and liquid disordered (Ld) phase - is well characterized (Veatch 2003). In curvature gradients, Lo phase membrane domains segregate into regions of low curvature. This
curvature-mediated segregation occurs because Lo phase membranes show larger resistance towards bending, compared to Ld phase domains (Roux 2005; Baumgart 2005; Semrau 2008). The present work considers the strong segregation limit, where membranes consist of domains with sharp phase boundaries (Lipowsky 1992; Parhasarathy 2006; Rozycki 2008). To be distinguished from that situation is the weak segregation limit near critical demixing (consolute) points (Tian 2009; Sorre 2009; Leibler 1986; Seifert 1993). Analysis of intracellular cargo movement has demonstrated that many transport carriers consist of tubes, rather than vesicles (Bonifacino 2005; Derby 2007). Furthermore, parts of several cellular organelles, including the endoplasmic reticulum, the trans-Golgi network, and various endosomal compartments consist of tubular networks (Derby 2007). Numerous mechanisms are known that lead to tube formation in vivo. These include exertion of pulling forces by molecular motors, membrane/cytoskeletal interactions, and the action of curvature-generating peripherally binding and trans-membrane proteins.

Tubes can be formed from model membrane lipid mixtures under controlled membrane tension that regulates tube curvature (Roux 2005; Tian 2009; Sorre 2009; Allain 2004). Phase separation in tubular membranes has previously been induced by photoactivation (Roux 2005; Yuan 2008), exploiting the sensitivity of membrane phase behavior to fluorophore-mediated lipid breakdown (Roux 2005). Here, we induced first-order demixing transitions through membrane curvature of pulled tubes.
Most model membrane research involving lipid and lipid/protein membrane mixtures has focused on thermodynamic equilibrium conditions (Tian 2009; Sorre 2009; Tian 2009). However, since the situation in biological cells is one of steady state and regulated mass transport with continuous recycling of membrane components, here we investigate dynamic aspects of membrane sorting.

3.3.2 Materials and Methods

3.3.2.1 Preparation of GUVs.

Vesicles were made via electroswelling(11). Lipid mixtures had composition 3:3:3:3.7 di-oleoyl-phosphatidylcholine (DOPC)/cholesterol (chol)/di-palmitoyl-phosphatidylcholine (DPPC) indicated by the dot in Fig. 3.3.1c. Trace amounts of biotinylated lipid (binding streptavidin-coated beads) and Ld phase-preferring red lipid fluorophore (Texas-Red DHPE) were added (see SI). For Fig. 3.3.1, 1 mol% of the ganglioside GM1 was added.

3.3.2.2 Pipette aspiration and force measurements

Vesicles with radius $R_v$ were aspirated by means of glass capillaries (inner radius $R_p$) at an aspiration pressure of $\Delta P = P_o - P_p$, controlling vesicle lateral tension.
\[ \sigma = \Delta P \cdot R_p / 2(1 - R_p / R_v) \]. Forces \( f \) were measured via displacement \( x \) of pulling bead from center of a laser trap, in combination with measured trap stiffness \( k \): \( f = kx \). To pull tubes, the trapping objective was moved laterally.

3.3.3 Results

To systematically probe the effects of curvature on nucleation and growth of membrane domains, we used a classical vesicle pipette aspiration approach (Hochmoth 1982). Membrane tubes were pulled by beads that were moved either by a second aspiration pipette (Fig. 3.3.1a), or by a laser trap (see Fig. 3.3.1b and materials and methods). From a thermodynamic point of view, vesicles provided a particle-reservoir that fixes lipid chemical exchange potentials (Tian 2009). The lateral membrane tension was controlled by means of the pipette aspiration pressure. Fig. 3.3.1c shows that our vesicle composition was within the Lo/Ld phase coexistence region, in the strong segregation limit of the phase diagram (Veatch 2003). This composition was chosen to yield vesicles with an Lo/Ld area fraction ratio typically larger than one (to facilitate tube pulling from the Lo phase) and with low domain boundary line tension (to avoid tube fission (Allain 2004)).

Vesicles with a single large liquid ordered (Lo) domain coexisting with a single large liquid disordered (Ld) domain (Fig. 3.3.1d) were selected and aspirated from the Ld phase (shown in red in Fig. 1D; pipette shown at the left edge of Fig. 3.3.1d).
Shortly (typically milliseconds to seconds but occasionally up to several minutes) after pulling a tube from the Lo phase of the vesicle (labeled in green by means of the peripherally binding protein cholera toxin (CTB, which binds to ganglioside, GM1) in Fig. 3.3.1d-g), an Ld domain nucleated at the neck of the tube, while the total tube length was held constant. Nucleation times (defined as the time between the end of the process of tube extraction and first observation of a domain) thus were variable, in accordance with thermally activated nucleation.

**Figure 3.3.1.** Curvature-induced first order demixing transitions are observed in tube pulled from lipid mixture membranes. *a*) Illustration of tube pulling by two-micropipette
setup. The liquid disordered (Ld) phase (gray line) of a vesicle with radius $R_v$ is aspirated by micropipette $a$. A streptavidin-coated bead is held by micropipette $b$ to pull a tube from the liquid ordered (Lo) phase (black line) of the vesicle. $Z_0$ represents the length of the Ld phase growing from the tube neck. b) Schematic of a specially designed fluorescence imaging / optical trapping setup. c) Schematic phase diagram of ternary lipid mixture. Loop encloses the Ld / Lo coexistence region. Lipid composition is indicated by an open circle. d-g) Confocal microscopy images of domain growth (at constant tube length) using two-micropipette setup. $d$) A tube is pulled from the Lo phase (green) of a vesicle and imaged before observable Ld phase (red) growth occurred. Scale bar: 2 $\mu$m. e-g) Growing Ld domain nucleated at the tube neck at constant reservoir membrane tension $\sigma = 3.3 \cdot 10^{-5}$ N/m. Growth time is denoted in each panel. The Lo phases of vesicle and tube appear yellow due to enhanced red channel display, used to emphasize the Ld phase. Scale bar: 1 $\mu$m.

Exclusively, only a single Ld domain nucleated in the tube, and it was always located at the tube neck. Both of these observations suggest that curvature/composition coupling (Tian 2009; Sorre 2009) rather than photoeffects (Roux 2005; Yuan 2008) caused phase-separation. This conclusion was further supported by the qualitative observation that increasing curvature (by increasing the vesicle lateral tension) on average decreased the time needed for nucleation (from up to minutes for the lowest lateral tensions to short
(milliseconds) times for the highest tensions considered).

Previous research has shown that CTB bound to the outer leaflet of GUV membranes preferentially partitions away from high curvature tubes pulled from vesicles with equilibration times less than one minute (Tian 2009; Sorre 2009). Furthermore, Ld phase lipid diffusion coefficients in our mixture are in the range of $\sim 3-5 \, \mu m^2/s$ (Scherfeld 2003).

Because green CTB fluorescence is observed for $> 400$ s on the tube (Fig. 3.3.1g), the red Ld domain shown in Fig. 3.3.1e-g serves as a diffusion barrier to protein (and lipid) exchange between tube Lo and vesicle Lo domains. Such diffusion barriers have been postulated to modulate intracellular lipid and protein sorting (Murkherjee 2000).

Curvature-nucleated domains grow in time, i.e. the red Ld phase invades the tube membrane, progressively replacing the green Lo phase (Fig. 3.3.1e-g, time was measured relative to the first observation of fluorescence heterogeneity at the tube neck). The comparison of the time needed for domain boundary movement between first and second (Fig. 3.3.1e,f) versus second and third (Fig. 3.3.1 f,g) frames reveals that the domain boundary velocity decreased with time.

The addition of the CTB/GM1 complex to our membranes so far has served to illustrate the possibility of dynamic protein sorting in Fig. 3.3.1. It has previously been shown, however, that CTB itself is an effective curvature sensor (Tian 2009; Sorre 2009) and furthermore amplifies lipid curvature sorting (Sorre 2009). For the following quantitative
characterization of the dynamic lipid phase behavior phenomena depicted in Fig. 3.3.1d-g, we therefore omitted CTB/GM1.

Figure 3.3.2 Curvature-induced domains show parabolic growth and characteristic pulling force decay. a) Six domains were sequentially nucleated at the tether neck of a single vesicle through step-wise total tube length extension followed by domain growth at constant total tube length (and reservoir tension) as shown in schematic (i). Domain length as a function of time relative to the first moment of detectable fluorescence heterogeneity
at the tube neck (ii). Closed and open circles, closed and open triangles, closed and open squares: domains grown at pressures of 17±0.5 Pa, 23 Pa, 31 Pa, 38 Pa, 50 Pa, and 61 Pa, respectively, corresponding to the lateral tensions in panel b). b) Same data as in panel A) plotted versus $\sqrt{t}$. Linear fits are shown as solid lines. c) Tube force is observed to decrease after domain nucleation and concomitant to growth. Black curve corresponds to the first domain generated (closed circles in panels a), b), and d)); lighter gray curves correspond to domains grown sequentially at increasing aspiration pressures as in a). d) Tube force as a function of domain length; symbols as in panel b). Solid lines are linear fits for domain lengths up to 10 µm. e) Double logarithmic plot (with standard deviations) of the slopes of the linear fits as in panel b) as a function of membrane tension for 6 different vesicles and 26 vesicle tensions. Slope of a linear fit to the binned data yields $0.62 \pm 0.08$. f) Three sequentially nucleated domains grown at constant membrane tension but with different initial lengths of Lo phase tube (schematic in (i)) exhibited essentially identical growth behavior and force decay (ii) for various total tube lengths.

We found a systematic dependence of domain boundary positions (measured as the distance between the intra-tube phase boundary and the tube neck) on both observation time and lateral membrane tension (Fig. 3.3.2a). The domain boundary length to a good approximation showed a square-root time dependence (Fig. 3.3.2b).
Ld phase domains that had been nucleated at the tube neck could be pulled into the tube interior by quickly (typically 30 μm/s) increasing the total tube length by moving the pulling bead. This led to nucleation (at the tube neck) of a new (here called primary) domain (with nucleation times similar to the previously nucleated, here called secondary, domain) in the Lo phase membrane region that was pulled onto the tube (see schematics in Figs 3.3.2a and 3.3.2f). Essentially identical growth dynamics were observed for multiple domains that resulted from successive pulls of the same tube (see Fig. 3.3.2f) when vesicle lateral tension was held constant. These observations allowed us to perform multiple domain growth experiments using the same tube and vesicle, but varying the vesicle (below also called reservoir-) lateral tensions to change tube curvature.

Concomitant to the domain length characterization analyzed in Fig. 3.3.2a,b, we measured the pulling force exerted by the tube membrane on a bead held in an optical trap. Following curvature-induced phase separation, tubes displayed pulling forces that decayed on time scales longer than 100 s. The pulling force decay for the six domains analyzed in Fig. 3.3.2a,b is shown in Fig. 3.3.2c. Intriguingly, pulling forces decreased essentially linearly with domain boundary displacement (Fig. 3.3.2d). We characterized the time scales required for initial mechanical equilibration after membrane tube elongation or aspiration pressure change using homogeneous vesicles (without phase separation). These time scales were less than 2 s to reach a constant pulling force after tube
elongation. Accordingly, the force decay during domain growth (Fig. 3.3.2c) occurs on a time scale significantly longer than needed to reach mechanical equilibrium after initial tube formation.

Fig. 3.3.2e suggests a power-law dependence between the slopes of Fig. 3.3.2b and vesicle lateral tension, with an exponent of $0.62\pm0.08$. This systematic dependence further emphasizes that curvature, rather than photoeffects, is the driving force for phase separation and mass transport. For high vesicle tensions, we confirmed that after long (> 10 min) waiting times, tether membranes consisted of one single Ld domain, consistent with Ref. (Rozycki 2008).

The slowing-down of domain boundary velocities observed in Fig. 3.3.1 and Fig. 3.3.2 suggests that the growing Ld phase presents a mass transport resistance that grows with its length. The parabolic moving boundary ("Stefan problem") behavior that we observe here is displayed by many physical chemical phenomena involving both heat and mass transfer (Stefan 1891; De Groot 1963).
Figure 3.3.3. Schematic of linear irreversible thermodynamics model. a) Vesicle (left hand side) and Lo phase tube domain (right hand side) are considered thermodynamic reservoirs with fixed chemical exchange potential $\mu$ (panel b, difference between reservoirs is $\delta\mu$) and fixed composition $\phi$ (panel c, maximal composition difference in Ld tube due to curvature is $\delta\phi$). Here, $\phi$ is defined as the mole fraction of Lo phase lipid in a quasi-binary mixture; the width of the phase coexistence region is $\Delta\phi$. Due to membrane curvature, the chemical potential in the right hand side reservoir is larger compared to the left hand side reservoir, whereas the composition of the Lo phase in both reservoirs is the same. Linear chemical potential and composition gradients are assumed in the Ld phase connecting both reservoirs. At the intra-tube phase boundary (where the axial coordinate $Z = Z_0$), the mean curvature is $C_t$. At the vesicle/tube phase boundary (where $Z = 0$), the mean curvature is $C_n$. The vesicle has negligible curvature.
We derived a simple thermodynamic transport model that enables us to explain dynamic aspects of the findings shown in Figs. 3.3.1 and 3.3.2. The key features of the model are depicted in Fig. 3.3.3. We assume the vesicle and the Lo phase of the tube are thermodynamic reservoirs (with chemical equilibrium being maintained at their boundaries) and that a linear chemical potential gradient across the thermodynamic system (here the Ld phase tube) connects both reservoirs (De Groot 1963). We reduce the ternary mixture to a quasi-binary mixture. We define the diffusion potential as \( \bar{\mu} = \mu_a - \mu_b \), where \( \mu_i \) is the chemical potential of component \( i \) of the quasi-binary mixture (Tian 2009).

In our model, transport is driven by a curvature-dependent diffusion potential difference \( \partial \bar{\mu} \) between both reservoirs (see Fig. 3.3.3). We will first examine the results of the assumption that \( \partial \bar{\mu} \) is time-independent. In that case, analytically integrating the appropriate continuity equation at the inner tube Ld/Lo phase boundary yields (see SI for details)

\[
Z_0^2 = t \frac{M_{Ld}}{\rho^2 \Delta \phi_e} \left( \frac{\partial}{\partial \phi_e} \right) \left( \frac{\sigma_{Ld}}{\kappa_{Ld}} \right) = tAC^2.
\]

Eq. 3.3.1 is a growth law with parabolic time \( t \) dependence demonstrating increase of domain boundary velocities (phase boundary position is \( Z_0 \)) with lateral tension \( \sigma_{Ld} \) (and therefore curvature \( C \)), which are features in accordance with the experimental observations (Fig. 3.3.2). Here, \( M \) is a mobility, \( \rho \) a lipid density, and \( \Delta \phi_e \) is related to
the width of the miscibility gap (Fig. 3.3.3) as well as the ratio of the tube radii

\[ \frac{R_{Lo}}{R_{Ld}} = \kappa_{Lo} / \kappa_{Ld} \equiv \varepsilon ; \]

\( R_i \) and \( \kappa_i \) are the radius and bending stiffness of phase \( i \), respectively. The quantity \( A \) summarizes the time- and curvature-independent parameters on the left hand side of Eq 3.3.1. It contains the composition-dependence of the Ld phase bending stiffness, assumed here to underlie curvature-mediated sorting (Tian 2009; Sorre 2009). We note that accounting for non-linear concentration gradients affects the numerical value of \( A \), but does not otherwise influence the time-dependence of \( Z_0 \) in this model (Crank 1984). The quantity \( AC^2 \) can be interpreted as a curvature-dependent inter-diffusion coefficient.

We note that Eq. 3.3.1 is based on numerous simplifications required to obtain an analytically tractable model. These include: (A) neglect of area density (\( \rho \)) difference among Lo and Ld phase; (B) assumed absence of friction effects contributed by water (and membrane area) transport among tube and vesicle; (C) spatially fixed vesicle/tether phase boundary (numerically investigated more accurately below); (D) assumption of time-independent tube radii of Lo and Ld phase; and (E) absence of concentration gradient in tube Lo phase. We discuss (D) and (E) immediately below.

The assumption made so far, of an Lo phase tube reservoir with constant curvature at constant reservoir tension, is not in agreement with the experimentally observed pulling force decay, since the pulling force \( f = 2\pi \sqrt{2\kappa_{Lo} \sigma_{Lo}} = 2\pi \kappa_{Lo} C_{Lo} \) (Derenyi 2002), where
mechanical quantities are those near the pulling bead.

We will discuss the following two alternative hypotheses to explain the pulling force decay. The first is that the pulling force decreases due to a change in lateral tension of the tube at fixed (vesicle) reservoir tension, while the composition of the tube domain in the Lo phase remains approximately constant (in accordance with the assumption of an Lo phase reservoir, see Fig. 3.3.3). The second hypothesis is that the bending stiffness of the Lo phase of the tube decreases with time because the imposed curvature field could lead to a change in the composition of the Lo phase near the bead to lower the tube free energy (Tian 2009; Sorre 2009). If the bending stiffness at the end of the tube would decrease through diffusive composition change, then the time scales of the lipid exchange between the end of the tube and the vesicle would be affected by the total tube length.

In support of the first, rather than the second hypothesis, we found negligible influence of the total tube length on domain growth (Figs. 3.3.2f). We furthermore verified that pulling force decays were similar (Figs. 3.3.2f) in serially nucleated domains in the same tube at constant reservoir lateral tension, confirming that the composition at the tube end did not measurably change over the time course of our experiments. Furthermore, secondary (i.e. inner tube) Lo phase domains were observed to reduce their length during the growth process of the primary domain. This is consistent with increasing radius of the secondary domain at constant domain area, due to decreasing lateral tension during the primary
domain growth process.

We thus concluded that the coupling of lateral tension between the vesicle reservoir and the tube changed with time. In additional support of this conclusion, we observed a rapid (< 1 s) force drop associated with the following phenomenon. Occasionally, small (radius on the order of 1 μm) Ld phase domains diffused from the vesicle into the neck region and became irreversibly trapped at the tube neck. Lo phase tubes were pulled from the vesicle, and no curvature-induced Ld phase nucleation was observed before the small domain was trapped in the neck region. The pulling force was constant before the Ld domain approached the neck region (consistent with the expectation of an approximately time-independent tube composition) and dropped rapidly as the Ld phase was trapped in the neck. This force drop indicated that the detailed geometry of the tube neck was pivotal in determining the pulling force exerted by a tube with phase coexistence. After the fast force drop, no additional force decay occurred while the Ld phase invaded the Lo phase tube.

To gain further mechanical insight, the geometry of tube necks was obtained from numerical solutions to the differential shape equations of lipid bilayer membranes with phase separation (Julicher 1996), including an axial pulling force (Derenyi 2002; Bozic 1997). We assumed a membrane suspended from a ring with zero mean curvature (free hinge) boundary condition (Derenyi 2002). In the presence of an axial force, such
membranes will show an approximate catenoid shape (Derenyi 2002). From this catenoid we suspended a tube with cylinder shape boundary condition at its tip (Allain 2006). For all calculated shapes that we show here, the total membrane area was fixed (Baumgart 2005). Shapes such as those in Fig. 3.3.4 were calculated at constant lateral tension in the Lo phase of the catenoid. The catenoid edge radius was variable to allow for changes in tube geometry at fixed total membrane area. The catenoid thus constituted a membrane area-reservoir, to reflect our experiments shown in Fig. 3.3.1 (where the pipette-aspirated membrane fraction of the GUV provided an area-reservoir). The catenoid/tube shape (with phase sequence Lo/Ld/Lo) was parameterized by means of the radius $r$ and the angle $\psi$ of the tangent to the shape, as a function of arclength $s$ (Derenyi 2002) (see inset of Fig. 3.3.4a). A line tension $T$ could be imposed at each phase boundary. Gaussian bending stiffness differences $\zeta$ among phases could also be accounted for (Baumgart 2005; Julicher 1996).

Fig. 3.3.4a shows the profile of a membrane tube without phase separation in order to illustrate a well-known feature: at the tube neck, the radius shows an invagination and a locally increased free energy density (Derenyi 2002). This fact explained why the curvature-induced Ld phase always nucleated at the neck.

We next aimed to calculate shapes of phase-separated tubes. Knowledge of the elasticities of Lo and Ld phase was thus required (Baumgart 2005). We derived bending stiffness
values from pulling force measurements(5) in homogeneous vesicles with several different compositions corresponding to Ld and Lo phases. We found values of roughly 0.8\cdot10^{-19} \text{ J} for the Ld phase, and 3.6\cdot10^{-19} \text{ J} for the Lo phase. This yielded a ratio $\varepsilon \approx 4.5$ of elasticities in Lo versus Ld phase, in agreement with previous estimates (Baumgart 2005; Semrau 2008). With this value for $\varepsilon$, we calculated series of tube shapes mimicking the experimental phase separation process of Figs. 3.3.1-3.3.2, assuming, for the moment, vanishing $T$ (and zero $\zeta$).
Figure 3.3.4. Computational shape series from membrane elasticity theory shows pulling force decay. a) Single phase tube with an Lo phase bending stiffness of 364 pN-nm. Tube radius is minimal in the neck region. Tube force for this shape is 24.0 pN with a lateral tension of 0.020 mN/m (equivalent reservoir tension for all following shapes). Inset illustrates parameterization of shape coordinates. b) Three-phase tube with Ld phase domain (gray) with a bending stiffness of 81 pN-nm (4.5 times less than the Lo phase). The Ld domain tension is higher than both the reservoir and Lo phase tube tensions. c) Tube shape with a large Ld phase domain area. The Ld tension is now close to the reservoir tension. Inset shows leading-edge phase boundary. d) Tube force decreases linearly with domain length until reaching a plateau. Arrows correspond to shapes in panels b-c). Note that lateral tensions of the Lo and Ld phase regions of the tube decrease during domain growth. e) Close-up of neck region for shapes shown in a-c). Vesicle/tube phase boundary moves onto the catenoid during domain growth. f) Mean curvature of Lo (black) and Ld (gray) phases at the catenoid/tube phase boundary as a function of domain length. Both mean curvatures decrease to zero upon reaching the force plateau shown in d). The curvature on Ld and Lo phase side of the phase boundary differ, in accordance with the mechanical jump conditions.

Nucleation of an Ld domain led to further constriction of the neck (Fig. 3.3.4b). To
calculate series of shapes for phase-separated tubes at constant total membrane area, we varied the area fraction of tube Lo phase and tube Ld phase, keeping the area of the catenoid Lo phase constant (Fig. 3.3.4b,c). Note that such shape series do not preserve the total Lo/Ld area fraction. Mass conservation of Ld phase and Lo phase lipids can, in principle, be achieved by adding an additional Ld phase to the nearly flat catenoid (as in the experimental vesicles, see Fig. 1). The area fraction of this additional Ld phase could be varied to fix the total area fraction of Ld phase while varying the tube area fractions (Fig. 3.3.4b,c). Since this amendment in the quasi-flat reservoir would have negligible mechanical consequences, we varied the area fraction of the tube domains only and regarded the catenoid as a thermodynamic particle- and area-reservoir, fixing chemical potentials (instead of particle numbers).

Fig. 3.3.4a – c indicate that during invasion of the tube by the growing Ld phase domain, the left hand side phase boundary moves into the catenoid (note that both phase boundaries thus were not fixed in space but allowed to move to minimize the shape’s total mechanical energy). We note for completeness that the inner-tube phase boundary shows a tangent angle of $\pi/2$ (inset of Fig. 3.3.4c) (Allain 2004). During the tube invasion, the pulling force decays (see Fig. 3.3.4d). Remarkably, the relation between Ld domain length and pulling force is linear in the numerically obtained shape series (Fig. 3.3.4d), as in the experiment (Fig. 3.3.2e). Note that a force plateau is reached before the Ld phase has
completely replaced the tube Lo phase (Figs. 3.3.4c, 3.3.4d).

The reason for the force decay is embedded in the complicated mechanical jump conditions at the vesicle/tube phase boundary (see SI). However, there is a conceptually straightforward explanation. Before phase separation, the lateral tension within the membrane tube is equivalent to the reservoir tension (Fig. 3.3.4a). Near the end of the invasion process, the left hand phase boundary is located in a membrane region with negligible curvature (Fig. 3.3.4c). Fig. 3.3.4e shows a magnification of the catenoid/tube phase boundaries. The mechanical jump conditions therefore dictate the lateral tension in the Ld phase domain to be the same as the reservoir lateral tension (in the absence of line tension). At the right hand (intra-tube) phase boundary, however, the axial force balance requires $\frac{\sigma_{ld}}{\sigma_{lo}} = \frac{\kappa_{lo}}{\kappa_{ld}} = \varepsilon$ (Allain 2004). If both phase boundaries were localized within the tube (as is approximately the case immediately after nucleation of an Ld domain, Fig. 3.3.4b), then the lateral tension within the tube Lo phase would be identical to the reservoir tension. This is because at both phase boundaries the lateral tension would jump, but in opposite directions, according to the axial force balance relation. If only one phase boundary is localized within the tube region, as in Fig. 3.3.4c near the end of the invasion process, the lateral tension within the tube Lo phase must be smaller by an amount that is given by the bending stiffness ratio. During the invasion process, the lateral tension of the Lo phase tube domain therefore monotonically decreased from the reservoir
tension $\sigma_{res}$ to $\sigma_{Lo} = \sigma_{res}/\varepsilon$. In accordance with this interpretation, Fig. 3.3.4\textit{f} shows that the mean curvature at the vesicle/tube phase boundary decreases during domain growth, vanishing at the pulling force plateau of Fig. 3.3.4\textit{d}.

We note that the magnitude of the vesicle/tube phase boundary displacement from the tube neck suggested by Figs. 3.3.4\textit{b-c} is not microscopically resolvable, consistent with Figs. 3.3.1\textit{e-g}.

In line with the interpretation of a tension-related force decay, the apparent bending stiffness $\kappa_{app}$ calculated from the experimentally determined pulling force and $\sigma_{res}$ (i.e. $\kappa_{app} = f^2/(8\pi^2\sigma_{res})$) continuously decreased from Lo phase values (before invasion) to values approaching those for an Ld phase, here $f_{plateau}^2 = 8\pi^2\sigma_{Lo}\kappa_{Lo} = 8\pi^2\sigma_{res}\kappa_{Ld}$ (Allain 2004), since at the plateau $\sigma_{res} = \sigma_{Ld}$.

We observed that several minutes after primary domain growth was initiated, the lengths of secondary domains remained approximately constant, consistent with the observation of a pulling force plateau at long observation times. Under such conditions, the domain length of a secondary domain was observed to be linearly related to the square root of systematically varied reservoir tension (data not shown). Since the domain radius is inversely proportional to $\sqrt{\sigma_{res}}$ (25), this finding shows that the secondary domain area $A_{sec} \approx 2\pi R_{sec}L_{sec}$ (excluding the phase boundary geometry, see Fig. 3.3.4) was independent of domain curvature. This suggests that the phase diagram is unaffected by curvature (Fig. 3.3.4).
3.3.3c).

In addition to the parameter ε, we measured the line tension $T$ (0.16 ± 0.062 pN, see SI). We then calculated numerical shape series for lateral tensions varying over a range matching the experimentally considered reservoir tensions (see Fig. 3.3.2) and the measured values for ε and $T$. We implemented our transport model (Eq 3.3.1) in differential form: $dZ_0 = dtAC^2/2Z_0$, to calculate a time-axis for the change of geometric and mechanical quantities, taking into account the changing curvature at the inner tube phase boundary during domain growth and choosing a value of $A$ common to all shapes considered.

We note that details of computed shape series differ from the experimental data. These differences likely result from the simplifications of our mechanical and transport models. Improvements may involve phase-field modeling (Lowengrub 2009) or dissipative particle dynamics simulations (Illya 2006), which are beyond the scope of the present work. We note that further support for our mechanical model may be obtained in future experiments where pulling force is clamped due to continuous tether elongation and feedback.

3.3.4 Discussion

The squared slopes in Fig. 3.3.2b can be interpreted as curvature-dependent inter-diffusion...
coefficients. Table S2 shows that their magnitude (0.1 ∼ 10 μm²/s) is in the range of typical lipid diffusion coefficients (from fluorescence correlation spectroscopy (Scherfeld 2003)). Interestingly, comparing the lateral tension dependence of the slopes of plots such as in Fig. 3.3.2b to our transport model (Eq 3.3.1) suggests that the transport coefficient A increases systematically with increasing curvature. This follows from the exponent of 0.62±0.08 obtained from Fig. 3.3.2c, compared to the value of 0.5 expected from Eq 3.3.1. Membrane curvature-dependent diffusion coefficients have been considered in single component membranes before (Daniels 2007), and here are likely influenced by the curvature dependence of the concentration profiles in the tube Ld phase (see Fig. 3.3.3). Our findings suggest a mechanism for intracellular sorting that uses the speed of membrane extraction from a donor reservoir (organelle) as a means for sorting. If high curvature tubes form slowly from a reservoir of essentially flat membranes, then equilibration of chemical potentials between the extracted sheet and the donor organelle is facilitated; in this case, the extracted membranes may show enrichment in Ld lipids and Ld proteins (see Movie S1, where a pulling speed of 0.04 μm/s was used). If, however, membrane patches are extracted quickly, then equilibration may be prevented and Lo rather than Ld membranes may be extracted from the reservoir. Fast pulling is exemplified in Movie S2, using a speed of 1.5 μm/s, which is well within a range of trafficking speeds of intra-cellular cargo membranes (Kural 2005).
3.4 References


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CHAPTER 4 CONCLUSIONS AND FUTURE WORK

4.1 Major findings

4.1.1 Line tension at the domain boundary of fluid coexisting membranes

We developed a method to directly determine line tension at the fluid coexisting domain boundary by micropipette aspiration. According to force balance and Laplace law, line tension was calculated from the suction pressure and the corresponding geometry of an aspirated, dumbbell shaped giant unilamellar vesicle.

Line tension was found to be strongly composition and component dependent. It decreased towards the upper critical consolute point of the fluid coexisting region in the ternary phase diagram, which suggests a three-phase coexisting region at the lower boundary. In addition, line tension decreased as one of the ternary components unsaturated lipid changes to branched lipid.

4.1.2 Calcium-induced domain shapes and dynamics within polyanionic vesicles subject to external chemical stimuli

Triggered dynamics of domains within vesicular membranes is of broad relevance to biomembrane signaling processes and to possible application of patterned vesicles. Here,
we studied domain morphology and mixing kinetics upon external chemical stimuli in phase separated polymer vesicles based on calcium crossbridging of polyanionic amphiphiles.

The calcium chelator EDTA was added to vesicle dispersions, and domain mixing was generally observed within minutes. While confirming that phase separation is due to calcium cross-bridging, the inner and outer leaflets exhibited coupled dissolution dynamics only with large domains that slowly remix. In addition to calcium, the charge of the polyanion also controls domain formation and, surprisingly, chains that are made more negative also remix. Viscous fingering patterns, bulging domains, as well as decreased domain area fraction can be observed after increasing pH by adding NaOH. Domain roughening, a phenomenon subject to NaOH perturbation, can be accelerated by adding additional EDTA. Domain kinetics proves to be much faster with EDTA treatment (minutes-hours) than with NaOH treatment (hours-days), which highlights the strength of calcium cross-bridging. Domain dynamics in spotted polymer vesicles induced by chemical stimuli reveal strengths of interactions and suggest applications in triggered delivery.

4.1.3 Curvature sorting of membrane components
4.1.3.1 Curvature sorting of lipids

By measuring the fluorescence intensity of trace amount molecules as a function of tube radius, DiI (indocarbocyanine) dyes and Texas-Red labeled DHPE curvature partitioning was examined in pure POPC membranes. Individual lipids were found not efficiently sorted by curvature gradients even with rather high spontaneous curvatures. This observation was explained in the framework of a statistical mechanical lattice model that indicates entropy, rather than curvature energy, to dominate lipid distribution in the absence of strong lateral intermolecular interactions.

However, in the present of strong lateral molecular interactions but not enough to cause phase separation, lipids were efficiently sorted in curvature gradients by bending stiffness. Bending stiffness of tube membranes was determined from measuring membrane tension and tether radius. We found that bending stiffness decreased as curvature increased for compositions near the critical point. A simple expression that shows the bending stiffness of an amphiphile mixture to be a quadratic function of curvature was derived. In this analytical model, the degree of sorting is determined by the ratio of two thermodynamic derivatives. Curvature-induced demixing increases upon approaching the critical region of a ternary lipid mixture from two orthogonal compositional trajectories. This observation indicates cooperative lipid demixing amplified by curvature. Our results are likely to be relevant to the molecular sorting of membrane components *in-vivo*. 
In addition, dynamic curvature sorting phases was found in membranes composed of ternary mixture with phase coexistence. Membrane tether was pulled from the Lo phase of a vesicle, and domains were consistently observed to nucleate at the junction between the tube and the pipette-aspirated GUV. This experimental geometry mimics intracellular sorting compartments, since they often show tubular-vesicular membrane regions. We find that Ld domains grow with square root time-dependence and the growth speed is strongly curvature-dependent. A theoretical model was developed which agreed with the parabolic growth behavior. Our investigation suggests a novel membrane sorting principle, which could contribute to the sorting and trafficking in highly dynamic biomembranes.

4.1.3.2 Sorting of CTB in curvature gradients

The protein Cholera Toxin subunit B, which peripherally binds to five ganglioside lipids, is found to prefer low curvatures to high positive curvatures. The curvature preference is caused by its negative spontaneous curvature, which is calculated to be $-0.055 \text{ nm}^{-1}$. The negative spontaneous curvature is consistent with the convex membrane-bending surface of CTB.

4.2 Future directions
4.2.1 Amphiphysin N-BAR domain curvature partitioning

N-BAR domains are found in many proteins (e.g. Amphiphysin, Endophilin) that closely associate with endocytosis. N-BAR has been known to deform membranes to high curvatures (Peter 2004). However, the mechanism of its curvature sensing and generation is still unclear.

Therefore, Amphiphysin N-BAR partitioning and dynamics in tubes will be examined to elucidate its binding behavior in tubes. The fluorescently labeled N-BAR is added to GUV dispersions at different solution concentrations and protein/lipid ratios. Tethers pulled from GUV were rich in Amphiphysin N-BAR due to its positive curvature preference. Diffusion of N-BAR in the tube will be measured through two means, with (fluorescence recovery after tube elongation) and without (fluorescence recovery after photobleach) driving force. A thermodynamic model will be developed to understand the partitioning of Amphiphysin N-BAR to high curvature tubes from vesicle and solution at varying concentrations.

4.3 References

APPENDICES

APPENDIX A

According to Fick’s second law, diffusion like behavior in cylindrical systems is shown in Eq. 2.2.1. Assuming the diffusion is slow enough for the system to reach steady state. Eq. 2.2.1 thus is simplified to the following differential equation:

\[
\frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) = 0. \tag{A1}
\]

and boundary conditions:

\[
C_{\mid r=R_0} = C_0 \quad \text{and} \quad C_{\mid r=R_m} = 0 \tag{A2}
\]

where \( C_0 \) is the OB18* concentration in OB18* domains, as illustrated in Fig. 1f. \( R_0 \) is the original AB1 domain radius at \( t = 0 \), \( R_m \) is the moving radius of the AB1 domain by the invading OB18* phase. Solving Eq. A2, we obtains:

\[
\frac{C}{C_0} = \frac{\ln(r/R_m)}{\ln(R_0/R_m)} \tag{A3}
\]

Given the continuity equation:

\[
-d\frac{R_m}{dt} C_0 = D \left. \frac{dC}{dr} \right|_{R_m}, \tag{A4}
\]

We insert (A4) to (A3), and integrate (A4) over interval \([R_0, R_m]\). It follows that:

\[
\frac{1}{2} (R_0^2 - R_m^2) = D t \tag{A5}
\]

From which we can obtain the diffusion coefficient formula as shown in Eq. 2
APPENDIX B

Curvature sorting based on spontaneous curvature:

For the outer leaflet of the tether, we have the internal energy:

\[ U_{ot} = \frac{1}{2} k_c (C - C_{st})^2 A_t + \Sigma_i A_i \]  
(B1)

The entropy is defined by the following expression:

\[ S_{ot} = -k_B \left( N_{ot} \ln \frac{N_{ot}}{N_{tot}} + N_{flat} \ln \frac{N_{flat}}{N_{ot}} \right) \]  
(B2)

From Eqs. B1 and B2, chemical potentials of molecule \( \alpha \) in outer leaflet of the tether are obtained

\[ \mu_{tot} = \left( \frac{\partial F_{tot}}{\partial N_{tot}} \right)_{N_{flat}} \]  
(B3)

\[ = \frac{1}{2} k_c a (C - C_{st})^2 + k_c a \phi_{flat} (C - C_{st}) (C_{\beta} - C_{\alpha}) + a \Sigma - f \frac{a}{2\pi R} + k_B T \ln \phi_{tot} \]

The mechanical balance equations resulting from partial derivatives with respect to variables describing the tether geometry, are identical to those of single component tethers (Bukman 1996; Derenyi 2002). It can be shown for multicomponent membranes that in the absence of line tension both vesicle and tether are under the same lateral (mean) tension, \( \Sigma \). Similarly, chemical potentials in the outer leaflet of the vesicle are calculated from Eq. 3.1.4. From equal chemical potentials of molecule \( \alpha (\beta) \) on tether and vesicle, it follows that:

\[ k_c a (C - C_{st} + C_{sv})(C_{\beta} - C_{\alpha}) = k_B T \ln \frac{\phi_{tot}}{\phi_{tot}^{flat}} + k_B T \ln \frac{\phi_{flat}}{\phi_{flat}^{tot}} \]  
(B4)
For curvature-partitioning molecules present in trace amounts, we approximate Eq. B4 by considering $C_{sv} = C_{st}$. In addition, $\phi_{aov}$ is close to 0, and we set $C_{\beta} = 0$. Equation B4 thus simplifies to Eq. 3.1.5.

Curvature sorting based on bending stiffness differences:

The internal energy of the tether is a function of the overall bending stiffness $k_{ct}$:

$$ U_{ct} = \frac{1}{2} k_{ct} C^2 A_t + \Sigma_i A_i $$  \hfill (B5)

The whole membrane is considered to be composed of membrane patches with protein molecular area $a$. Thus $k_{ct}$ is simplified as $k_{ct} = \frac{N_{\alpha} k_{\alpha} k_{\beta}}{k_{\beta} N_{a_{\alpha}} + k_{\alpha} N_{\beta_{\alpha}}}$. From Eqs. B5, B2, and 3.1.3, the chemical potential of membrane patch $\alpha$ covered with protein on the tether is obtained

$$ \mu_{\alpha t} = \left( \frac{\partial F_{ct}}{\partial N_{\alpha t}} \right)_{k_{\beta}} = \frac{1}{2} k_{ct} a C^2 + \frac{1}{2} k_{ct} a C^2 \left( 1 - \frac{k_{\alpha}}{k_{\beta}} \right) + k_B T \ln \phi_{\alpha t} $$  \hfill (B6)

Similarly, chemical potentials on the vesicle are obtained from Eq. 3.1.4. At chemical equilibrium, we find:

$$ \frac{1}{2} a C^2 k_{ct}^2 \left( \frac{1}{k_{\beta}} - \frac{1}{k_{\alpha}} \right) = k_B T \ln \frac{\phi_{av}}{\phi_{\alpha t}} + k_B T \ln \frac{\phi_{\beta v}}{\phi_{\beta t}} $$  \hfill (B7)

We consider $\phi_{av}$ close to 0, and assume that $k_{\alpha}$ and $k_{\beta}$ are of the same order of magnitude.

Under those conditions, Eq. B7 simplifies to Eq. 3.1.8.
Enthalpic contributions leading to lipid phase separation can be accounted for by adding i), an interaction term; and ii), a line tension term to the free energy functional, causing a modification of the chemical balance equations and adding a jump in in-plane tension and transverse shear to the mechanical balance equations.
APPENDIX C

Sorting by Spontaneous Curvature

To obtain a relation between the curvature $C$ and the sorting effect $\Delta \phi = \phi_\alpha(C) - \phi_{\alpha_0}$, where $\phi_{\alpha_0}$ is the composition of a flat reservoir, we expand the quantity $\bar{\mu}$ about the flat state ($C = 0$),

$$
\bar{\mu} = \bar{\mu}_0 + \left( \frac{\partial \bar{\mu}}{\partial C} \right)_0 C + \left( \frac{\partial \bar{\mu}}{\partial \phi_\alpha} \right)_0 \Delta \phi_\alpha 
$$

where the index 0 indicates that partial derivatives are evaluated at the flat state. For the spontaneous curvature model, the second derivative in Eq. C1 is a function of the spontaneous curvature of the membrane. Equation 3.2.5 is obtained from considering diffusional equilibrium $\bar{\mu}(C) = \bar{\mu}_0$ of a flat reservoir (approximated by the vesicle) and bent membrane (tube). Equation 3.2.5 can be used to calculate the degree of sorting with specific models, such as the one proposed by Markin (Markin 1981) and Kozlov and Helfrich (Kozlov 1992), who consider an overall spontaneous curvature $C_s$ and bending energy $F^{\text{bend}}$ to be a function of composition.

$$
C_s(\phi) = C_\alpha \phi_\alpha + C_\beta \phi_\beta \quad F^{\text{bend}} = \frac{1}{2} \kappa (C - C_s(\phi))^2 A ,
$$

where $C_i$ values are molecular spontaneous curvatures of molecule $i$. From these equations, the following chemical potential can be calculated (for the outer monolayer and molecule $\alpha$ (Tian 2009)) as

$$
\mu_\alpha = a \kappa (C - C_s(\phi)) \phi_\alpha (C_\beta - C_\alpha) + k_B T \ln \phi_\alpha
$$

(C3)
where \( a = 1/\rho \) is the area per molecule, and \( k_B \) is Boltzmann's constant, such that Eq. 3.2.5 becomes

\[
\Delta \phi_\alpha = \left\{ \left( \frac{\partial \mu}{\partial C} \right)_o \left/ \left( \frac{\partial \mu}{\partial \phi_\alpha} \right)_o \right\} \right\} C = \frac{C}{(C_\alpha - C_\beta) + \frac{k_B T}{a \kappa_0 (C_\alpha - C_\beta)} \left( \frac{1}{\phi_\alpha} + \frac{1}{\phi_\beta} \right)} \quad (C4)
\]

This expression shows that at zero temperature, the curvature \( C \) is equal to the difference of spontaneous curvature in the bent and in the flat state, \( C = C_{s, \text{tube}} - C_{s, 0} \) and that sorting becomes less efficient at higher temperatures. Using the quantity \( \bar{\mu} \), the free energy change of the tube/flat reservoir combination associated with a process at constant area \( A \), is given to second-order by (Kozlov 1992)

\[
\Delta F = \frac{1}{2} \kappa_0 C^2 A + \frac{1}{2} N \left( \frac{\partial \bar{\mu}}{\partial \phi_\alpha} \right)_o \Delta \phi_\alpha^2 + N \left( \frac{\partial \bar{\mu}}{\partial C} \right)_o \Delta \phi_\alpha C. \quad (C5)
\]

Insertion of Eq. C4 leads to the simple form

\[
\Delta F = \frac{1}{2} \kappa_{\text{eff}} C^2 A \quad (C6)
\]

where the effective bending stiffness is expressed as

\[
\kappa_{\text{eff}} = \kappa_0 - \rho \left( \frac{\partial \bar{\mu}}{\partial C} \right)_o \left/ \left( \frac{\partial \bar{\mu}}{\partial \phi_\alpha} \right)_o \right\} = \kappa_0 - \frac{a \kappa_0^2 (C_\alpha - C_\beta)^2}{a \kappa_0 (C_\alpha - C_\beta)^2 + k_B T \left( \frac{1}{\phi_\alpha} + \frac{1}{\phi_\beta} \right)} \quad (C7)
\]

The effective bending stiffness is thus equivalent to the bare bending stiffness \( \kappa_0 \) if both spontaneous curvatures are equal, or if the temperature is high. An equation equivalent to Eq. C7 has been derived in Kozlov and Helfrich (Kozlov 1992) for the case of compressible membranes.
Sorting by Bending Stiffness

For a model that considers a composition-dependent bending stiffness, we have to carry the Taylor expansion of the free energy change comparing flat and bent state up to third-order. A third-order Taylor expansion of \( F \), say in the parameters \( C \) and \( \phi \), will in general include homogeneous third-order derivatives such as \( F_{CC\phi} \) and \( F_{\phi\phi\phi} \), as well as mixed derivatives and their permutations. For these third-order derivatives, we only consider the terms of \( F_{CC\phi} \) of which there are three permutations. Note that we therefore have neglected terms of \( F_{\phi\phi\phi} \), i.e., third-order contributions to an order parameter expansion of \( F \), which may not always be justified (Chaikin 1995), as Fig. 3.2.3 shows.

The terms of \( F_{CC\phi} \) quantify the composition dependence of the bending stiffness, since \( \kappa \) is defined as

\[
\kappa_0 = \frac{1}{A} F_{CC} = \frac{1}{A} \left( \frac{\partial^2 F}{\partial C^2} \right)_0
\]

This definition leads to a relation connecting the third-order derivatives,

\[
\left( \frac{\partial^2 \mu}{\partial C^2} \right) = A / N \left( \frac{\partial \kappa}{\partial \phi_\alpha} \right)
\]

and that thus represents two permutations of \( F_{CC\phi} \). We thus have, for second- and third-order contributions to the Taylor expansion of the free energy that results from Eq. 3.2.2, after inserting intensive parameters expanded to second order, followed by integration (Kozlov 1992),

\[
\Delta F = \frac{1}{2} \kappa_0 C^2 A + \frac{1}{2} \rho \left( \frac{\partial \mu}{\partial \phi_\alpha} \right)_0 \Delta \phi_\alpha A + \frac{1}{2} \left( \frac{\partial \kappa}{\partial \phi_\alpha} \right)_0 \Delta \phi_\alpha C^2 A
\]
Equation C9 is written to obtain, as required at constant curvature, a first-order homogeneous function in $A = 2\pi R_t L$, where $L$ is the length of the tube. We note that this free energy expansion does not contain contributions from line tension (Baumgart 2003), as we did not observe curvature-induced phase transitions with the mixtures examined in this contribution. A free energy functional similar to Eq. C9 has also been considered in Sorre et al. (Sorre 2009).

To obtain a relation between composition change and curvature, the expansion of Eq. C1 carried to second-order leads to

$$C^2 = -2\left(\frac{\partial \mu}{\partial \phi_a} \right)_0 \left(\frac{\partial^2 \mu}{\partial C^2} \right)_0 \Delta \phi_a - \left(\frac{\partial^2 \mu}{\partial \phi_a^2} \right)_0 \left(\frac{\partial^2 \mu}{\partial C^2} \right)_0 \Delta \phi_a^2$$

after consideration of diffusional equilibrium and realizing that the first partial derivative term in Eq. C1 is zero for membranes where sorting occurs by bending stiffness only.

Equation C10 can be viewed as a second-order series expansion of $C^2$ in the parameter $\Delta \phi_a$.

Reverting this series (McQuarrie 2000) yields, to first-order in $C^2$,

$$\Delta \phi_a = -\frac{1}{2} \left(\frac{\partial^2 \mu}{\partial C^2} \right)_0 \left(\frac{\partial \mu}{\partial \phi_a} \right)_0 C^2$$

Equation C11 is used in Eq. C9 to eliminate the composition dependence. To obtain a mechanical balance equation, Eq. C9 is then amended by the term $\sigma A$. Minimizing the resulting functional with respect to radius changes at constant tube length results in

$$\sigma = \frac{1}{2} \frac{\kappa_a}{R_t^4} - \frac{3}{8} \left(\frac{\partial \kappa}{\partial \phi_a} \right)_0 \left(\frac{\partial \mu}{\partial \phi_a} \right)_0 \frac{1}{R_t^4} = \frac{1}{2} \frac{\kappa_{eff}}{R_t^4}$$

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which leads to Eq. 3.2.8 of the main text.

For the bending stiffness composition-dependence model suggested by Markin (Markin 1987) and Kozlov and Helfrich (Kozlov 1992), we have bending stiffness and energy,

\[
\frac{1}{\kappa(\phi)} = \frac{\phi_\alpha}{\kappa_\alpha} + \frac{\phi_\beta}{\kappa_\beta}, \quad F_{\text{bend}} = \frac{1}{2} \kappa(\phi)C^2A
\]  

(C13a,b)

where \( \kappa_1 \) values are the molecular bending stiffnesses. These expressions determine the chemical potential for molecule \( \alpha \) (Tian 2009),

\[
\mu_\alpha = \frac{1}{2}a\kappa(\phi)^2C^2\phi_\beta\left(\frac{1}{\kappa_\beta} - \frac{1}{\kappa_\alpha}\right) + k_B T \ln \phi_\alpha
\]  

from which an effective bending stiffness can be obtained as

\[
\kappa_{\text{eff}} = \kappa_0 - 3a \frac{\rho \left( \frac{\partial \Pi}{\partial \phi_\alpha} \right)_0}{\left( \frac{\partial \kappa}{\partial \phi_\alpha} \right)_0} C^2 = \kappa_0 - 3 \frac{a\kappa_0}{\left( \frac{\kappa_\alpha}{\kappa_\beta} - 1 \right)} C^2
\]  

(C15)

As it should, the sorting effect thus disappears if both molecular bending stiffnesses \( \kappa_1 \) are equal, as well as if the temperature is large. We have expressed the resistance to sorting in Eqs. C14 and C15 as a mere entropic contribution. In the case of phase-separating mixtures, an interaction term can reduce this resistance to zero at the spinodal line. For these cases, the formalism can easily be generalized to more complex free energy functions that include intermolecular interactions.