show no signs of folding, at this surface tension, even after 450ns of simulation time. As mentioned earlier, in the smallest system size, it is also true that only peptide-containing monolayers fold under a small negative surface tension. These results indicate that the surfactant peptides play a significant role in the folding process and provide a larger driving force for folding than does the unsaturated phospholipid POPG.

3.3.6 Bicelle Formation, Fusion, and Re-expansion

As the folds continue to grow, eventually a bicelle desorbs, leaving a monolayer surface behind (Figure 3.10). When the folds from each monolayer come close to one another, the peptides from each fold can interact (Figure 3.3(b)), leading to the fusion of the two folds into a lipid bridge. The formation of this lipid bridge resulting from peptide-peptide interaction demonstrates the fusogenic ability of the peptides.

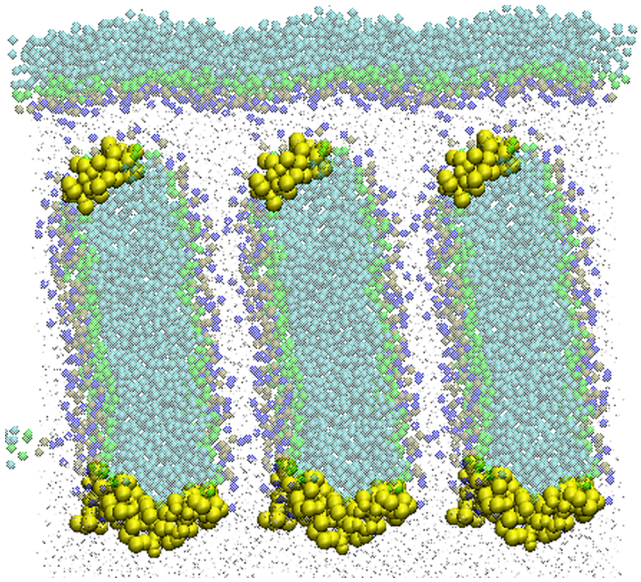


Figure 3.10: Bicelle formation. A bicelle that has desorbed from one monolayer containing 256 DPPC molecules and 4 mutant peptides (MUT2) after 275ns under small negative surface tension. Periodic images are shown for clarity. This simulation was started with peptides initially in a line configuration.

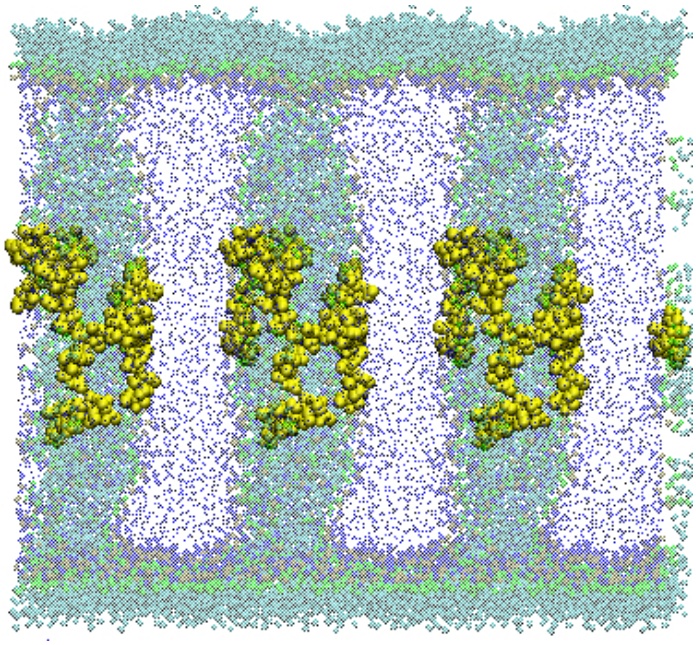
To test the respreadability of the bilayer folds we imposed a negative tangential pressure (positive surface tension) onto the folded systems containing 512 DPPC molecules and 8 peptides (SP-B_{1-25} or SP-C). When a tangential pressure of 5bar is applied (γ=25mN/m), each system, with either SP-B_{1-25} or SP-C , quickly (with 50ns of simulation) respread to form a monolayer. During re-expansion the folds re-spread by the same experimental mechanism described by Lee and co-workers [159, 163], with the folds "unzipping" back into the monolayer.

Setting the surface tension to 50mN/m to re-expand the folds after a lipid bridge had formed between the two monolayers (Figure 3.11(a)), results in the reincorporation of more peptides into one monolayer than the other (Figure 3.11(b)). Since the initial formation of the lipid bridge resulted form peptide-peptide interactions, this process can be thought of as the peptide-mediated transfer of surface-active material between interfaces. The formation of a lipid bridge by peptide-mediated fusion and the ensuing peptide re-distribution upon re-expansion demonstrate the fusogenic abilities of the peptides and their role in surface refining.

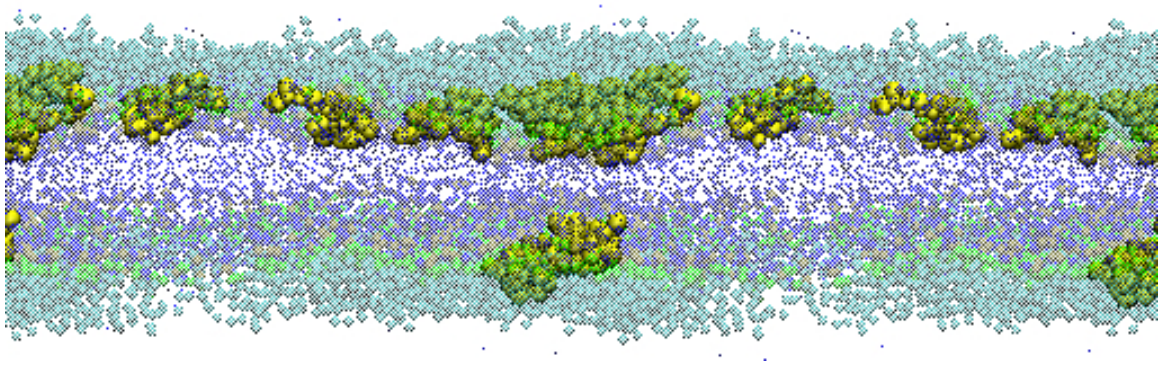
3.4 Discussion

3.4.1 Folding Mechanism

The mechanism of collapse depends on the phase morphology, which is determined by temperature, surface pressure, composition, and compression rate. The collapse of highly compressed monolayers can occur by a variety of mechanisms including folding, crystallite formation, and nanoscale budding [164]. Factors that favor more fluid structures, such as higher temperature, promote collapse by forming discs, multilayers, or vesicles [164, 206]. For example, Gopal and Lee [206] showed that for a 7:3 DPPC/POPG monolayer at low temperatures (below 28°C) the monolayer is biphasic and collapses by forming reversible large-scale folds (up to millimeters in length),



(a)



(b)

Figure 3.11: An example of peptide-mediated surface refining. (a) A lipid bridge has formed between two monolayers originally containing 256 DPPC and 4 mutant peptide (MUT1) molecules each. (b) After the lipid bridge is re-expanded, there are more peptides incorporated into one monolayer than the other. Periodic images are shown for clarity.

while at high temperatures (above 33.5°C) the monolayer is homogenous and collapses by forming micron-scale vesicular structures, and at intermediate temperatures (28-33.5°C) collapse occurs by forming both folds and vesicles. As the temperature is increased, the fraction of disordered phase increases and the fluidity therefore also increases, resulting in a monolayer that collapses on a smaller length scale by form-

ing vesicle-like structures. Faster compression rates favor smaller, more numerous, collapse structures [207]. Reduced compression rate lead to a slightly increased disc size in collapsed calf lung surfactant extract (CLSE) films [164].

SP-B is known to promote the transition from 2D to 3D structures such as small independently nucleated collapse structures [53, 158, 160, 162, 163, 165–167] and macroscopic folds [158, 161, 163, 190]. SP-B_{1-25} [158–162] and dimerized SP-B_{1-25} [162, 165] have also been found to induce these transitions in phospholipid monolayers. SP-C also promotes the formation of collapse structures. While SP-B is associated with the formation of small disc like protrusions, SP-C is associated with sheets of bilayers stacked on top of each other[53, 161, 163, 166–172, 174, 175]. SP-B, SP-B_{1-25} and SP-C are excluded from the condensed phase and localize within the fluid phase [158, 160, 161, 163, 166–170, 172, 173, 208, 209]. Therefore, small disc-like protrusions and multilayer stacks [53, 161, 163, 166–169, 172] have been reported to originate in the expanded phase regions, as observed in our simulations of folding of liquid-expanded phase monolayers. These small collapse structures are sometimes called surface aggregates. Also, the term "squeeze-out" is sometimes used to describe the formation of these collapse structures. However, the "squeeze-out" of these collapse structures does not necessarily imply that the surface is refined to contain almost pure DPPC, as held by classical squeeze-out theory.

Another type of collapse structure, macroscopic folds, has also been reported for model surfactant mixtures. Lee and co-workers [158, 161, 163, 190] found that films containing SP-B in DPPG, DPPG/POPG, or in DPPC/POPG/PA as well as pure lipid films consisting of DPPG/POPG or DPPC/POPG/PA exhibited a flat monolayer coexisting with a buckled monolayer, rendering collapse reversible and enabling rapid respreading upon expansion. The folds consisted of coexisting LC-LE domains that extended several microns into the subphase, and had the same average composition and morphology as the monolayer [158, 161, 163, 190]. Lee and co-

workers [158, 163, 190] suggest that coexistence of LC and LE phase is an essential feature required for reversible collapse via the macroscopic folding transition. They propose that the coexistence of LC and LE phases provides the monolayer enough flexibility to bend and enough cohesiveness to prevent loss of material to the subphase.

Both types of collapse structures - discs or multilayers and macroscopic folds - are reversible. Macroscopic folds are 100μm-1mm in length and extend several μm into the subphase. Bilayer discs are typically <10-500nm in diameter with a height corresponding roughly to the thickness of a single bilayer. Multilayers are typically <0.1-10μm in size with discrete steps in heights corresponding to roughly to the thickness of a bilayer (5-7nm). The formation of surface aggregates such as disc-like protrusions or multilayers occurs near the equilibrium spreading pressure (π_e), while large-scale collapse such as macroscopic folds occurs at the surface pressures near the collapse pressure (π_c). Electron micrographs of thin sections of rabbit lungs have revealed that portions of the alveolar film are multilamellar [157]. Multilamellar films can form not only by collapse but also by adsorption [210, 211]. Reversible discs and/or multilayers have been identified in BLES (bovine lipid extract surfactant) [30, 53] and CLSE [164]. It has been suggested that the extended irregularly shaped multilayers identified in CLSE may have originated from multiple folding events and/or the fusion of smaller stacks [30]. There is also experimental evidence for the formation of multilayers during BLES adsorption [212].To the best of our knowledge, there is no evidence of macroscopic folds in surfactant extracts without added PA. However, the lung surfactant Survanta, which contains a significant portion of PA (8.5% w/w), displays a rich variety of collapse structures, including reversible macroscopic folds that maintain the same morphology as the monolayer [161, 173], larger multilayers (800μm wide) [173], and smaller lipid-protein aggregates [165, 173]. It has been suggested that the collapse of Survanta could occur by a complex mechanism involving the detachment of the buckled region followed by reattachment to form more complex

multilayered structures [173]. However, experiments involving the injection of BLES underneath pre-formed films of either BLES or DPPC suggest that reattachment to a monolayer at equilibrium does not occur readily [212].

The "folds" observed in our simulations occur in LE phase monolayers, and our simulation box size is smaller than the size of a single experimental LE phase domain. Therefore, the "folds" observed in our simulations are similar to the small collapse structures originating from the protein rich LE phase rather than macroscopic folds. Still, even the "small" collapse structures seen experimentally ($\sim$35nm, ref [162]) are larger than our folds, and so direct comparisons of our simulated collapse structures to the experimental ones must be made with caution.

Our simulations do suggest, however, that folding can occur through the amplification of undulations (buckling). We find that folding can also occur by nucleation of a fold about a defect, which will be addressed later in this discussion. Folding by amplification of undulations has been observed previously by CG molecular dynamics [29] and is in agreement with experimental observations and the general wrinkle-to-fold transition mechanism described previously by Lee and co-workers [213, 214]. According to Lee and co-workers, when a membrane undergoes compression the amplitudes of the undulations grow until a critical compression is reached, at which an instability leads to rapid amplification of one or a few wrinkles, while the rest decay to zero [213, 214]. However, as noted previously, our folds are on a much smaller length scale than the macroscopic folds reported by Lee and co-workers, which span across both LC and LE phase domains and maintain the original biphasic morphology of the monolayer.

Small (nm scale) globular structures have been identified in model surfactant mixtures at low surface pressures and are thought to be nucleation sites for subsequent multilayer formation [166, 167, 174]. Small nuclei have also been identified in CLSE films, which grow into larger collapse structures [164]. The folds observed in our

simulations could be similar to these pre-collapse nuclei.

Variations in the composition of the monolayer can lead to variations in spontaneous curvature and bending modulus between domains [191]. It has been suggested that as the monolayer is compressed the curvature at domain boundaries can grow, causing the monolayer to overlap itself, resulting in the formation of a multilayer [191]. Due to the finite system size, finite simulation time, and the absence of domains of varying heights in our simulations we do not observe monolayer overlap. Instead we observe folds extending into the subphase.

Visualization of collapse structures commonly requires deposition of the film onto a substrate, which could result in ambiguities about the actual shape and orientation of experimental collapse structures. In the past there has been some ambiguity about whether aggregates collapse into the subphase or into the air. All folds observed in our simulations occurred into the water subphase. This is in agreement with the observations of Baoukina et al. [32], which suggest that buckling into the water subphase is more favorable because it results in a lower free energy than collapse into the vacuum. Their simulations show that although a lipid bilayer patch in the water subphase is stable, an inverted bilayer patch placed in the vacuum subphase is unstable and readily fuses with the adjacent monolayer [32]. Although these results suggest that collapse occurs into the water subphase rather than into the air, we cannot discount the fact that the simulation conditions differ somewhat from physiological conditions.

3.4.2 Component Effects

Fluidizing agents promote collapse by decreasing the bending rigidity of the monolayer and increasing the mobility of the constituent lipids. The decrease in bilayer bending modulus with increasing unsaturated lipid content has been demonstrated previously [29]. Our results indicate that the addition of fluidizing agents such as

POPG, SP-B and SP-C assist collapse out of the LE phase, which occurs experimentally through the formation of surface aggregates near π_e. Conversely, condensing agents resist this collapse, providing access to higher surface pressures where collapse may occur by a different mechanism. We propose that the proteins further assist collapse by acting as defects in the monolayer about which collapse structures can nucleate. The peptides appear to provide a larger driving force for folding than the unsaturated phospholipid POPG, allowing fold formation to occur for peptides containing monolayers under conditions for which it does not occur in the presence of POPG alone. The extent of the perturbation of the peptide-containing monolayers (and the occurrence of folding) is determined by the hydrophobicity of the proteins.

3.4.2.1 Pure DPPC

DPPC is relatively incompressible, but will eventual collapse once overcompressed [215]. LC monolayers of DPPC have been reported to form highly condensed multilayered collapse structures [216, 217], and nanometer scale folds [215]. However, unlike the discs or multilayer structures formed in the presence of peptides, these collapse structures formed out of the condensed phase and were irreversible. Once the temperature was raised above the main transition temperature of DPPC these collapse structures no longer formed [215, 216]. Other studies have reported that collapse of highly compressed DPPC occurs by reversible nanoscale budding into the air [218]. Yang and Tsay [217] reported irreversible multilayer structures and reversible structures. However, the reversible structures were considered to be elastic deformations rather than true collapse structures.

3.4.2.2 Unsaturated Lipids

In agreement with our observations, the fluidizing effect of POPG on LE phase DPPC molecules has been detected by vibrational sum frequency generation spectroscopy [219]. Additionally, the ability of the unsaturated lipids to form curved structures (because of their fluidity) and preferential interaction between SP-B and the charged headgroup of POPG are thought to promote the formation of multilayered structures [162].

3.4.2.3 Surfactant Proteins

SP-C [166, 167, 208], SP-B [158–160, 166, 167, 208, 209], and SP-B_{1-25} [158–160] have been found to perturb monolayers of DPPC, DPPC/DPPG, and PA, increasing the overall fluidity of the monolayers and producing smaller and more numerous condensed phase domains with a lower line tension between the condensed and expanded phases. Increasing the amount of protein increases the amount of fluid LE phase [158, 160, 166, 208, 209]. SP-B and SP-C also perturb the molecular packing of the fluid phase in which they are distributed [208].

On a per molecule basis, SP-B fluidizes the lipid monolayer more than does SP-C [166, 167, 208], but the reverse is true on a per unit mass basis [208]. Therefore the more pronounced fluidization of the monolayer by SP-B could be due in part to the size difference between the two peptides. We observe that SP-C contains more hydrophobic residues and displays a stronger fluidizing effect than the fragment SP-B_{1-25}, causing a larger drop in the carbon deuterium order parameter. In addition, we observe that the peptides have a localized effect on order. This is in agreement with atomistic simulations [194, 195], which have shown that SP-B_{1-25} significantly decreases the order of nearby fatty acids in a PA monolayer, and less disordering of fatty acids further away. Since the fluidization of the monolayer is localized, and

fluidity promotes collapse, this could result in a higher propensity for collapse in the immediate vicinity of the peptides.

The ability of the peptides to promote collapse in our simulations is in agreement with observations that hydrophobic proteins slightly speed up collapse in monolayers containing the complete set of surfactant lipids at 37°C [189] and in contrast to studies where the proteins were reported to stabilize monolayers against collapse [158–160, 163]. Lee and coworkers [158, 160, 190] suggest that SP-B or SP-B_{1-25} has a synergistic effect with components such as PA and POPG (that have low π_c values), which allows the attainment of high surface pressures (above the π_c values of either component) in lipid-peptide monolayers. However, this stabilization is not expected in DPPC monolayers, since DPPC is on its own capable of sustaining near-zero surface tension (high π_c). We observed no obvious synergy between POPG and SP-B_{1-25} in our 1:1 DPPC:POPG monolayers. Both 1:1 DPPC:POPG monolayers containing SP-B_{1-25} and DPPC monolayers containing SP-B_{1-25} collapse readily, with the former displaying a lower order parameter suggesting only an additive effect of POPG and SP-B_{1-25} on monolayer fluidity. However, the existence of a synergistic effect cannot be ruled out, especially given the length and time scale of our simulations.

It has been recently proposed that surfactant proteins diminish the activation energy barrier to collapse, increasing the rate of collapse above π_e [189, 220]. The proteins could act as a catalyst, promoting reorganization at the interface and lipid exchange between surfactant storages and the interfacial film [189, 220]. Our results also support this conclusion by showing the ability of surfactant peptides to promote collapse and fusion.

We found that in DPPC/SP-C/PA monolayers, collapse did not occur; however collapse occurred in both DPPC/SP-B_{1-25}/PA and DPPC/depalmitoylated SP-C/PA. These observations are in good agreement with experiments, which show that SP-C palmitoylation dramatically increases film stability [221]. Qanbar et al. [221]

found that films containing depalmitoylated SP-C or SP-B were much more prone to instability than films containing SP-C. Furthermore, SP-B is removed from the interface at lower surface pressures than is SP-C as is evident from plateaus in the isotherms of model surfactant mixtures containing these proteins; this has been reported by Nag et al. [208] and many others. Depalmitoylation of SP-C is also thought to impede the formation of multilayers and hinder respreading and adsorption [171, 221].

3.4.2.4 Palmitic Acid

In contrast with unsaturated lipids and surfactant proteins, and in agreement with experiments, we find that PA provides stability against collapse of the LE phase. These results suggest that condensing agents such as PA could act to restrict the formation of the small disc or lamellar collapse structures occurring out of LE phase domains just above π_e. PA is used as an additive in surfactant replacements to enhance monolayer stability and is thought to have effects similar to those of increasing the surface pressure or decreasing the temperature of DPPC-containing monolayers [191, 222]. It is well documented that PA condenses DPPC-containing monolayers by increasing the conformational order of the DPPC tail chains in the LE phase [219], increasing the fraction of LC phase, decreasing the tilt of LC phase DPPC molecules, and increasing the rigidity of the monolayer especially at low surface tensions [161, 222].

Atomistic molecular dynamics simulations of SP-B_{1-25} in PA monolayers suggest that strong electrostatic interactions between the positively charged residues of SP-B_{1-25} and the anionic headgroups of PA anchor the peptide to the monolayer [194, 195] and could provide stability to the fluid PA/SP-B_{1-25} regions of the monolayer [195]. This is in good agreement with the increased resistance to collapse observed in our simulated monolayers containing DPPC with SP-C upon the addition of PA and

with experimental observations. However, the increase in order parameter observed upon addition of PA to DPPC monolayers containing SP-C was absent in DPPC monolayers containing SP-B_{1-25}; instead a decrease was observed.

Lee and co-workers have proposed that in order for reversible macroscopic folding to form the monolayer must be within "the Goldilock window of rigidity"; neither too rigid nor too fluid [190]. They found that addition of components that rigidify the monolayer, such as PA, promote the formation of macroscopic folds, and factors that fluidize the monolayer, such as the addition of monovalent ions to the subphase, or an increase in temperature, lead to the disappearance of the macroscopic folds [161, 190, 206]. Our observations are in agreement with the observations of Lee and co-workers, and suggest that the addition of a condensing agent could restrict the formation of small-scale LE phase collapse structures leading to large-scale collapse at higher surface pressures. We also find that when PA is neutralized it acts as a fluidizing rather than a condensing component, decreasing the order parameter in DPPC monolayers containing either SP-B_{1-25} or SP-C.

3.4.3 Defects and Aggregation

For large systems under a small negative surface tension, folding usually proceeded through amplification of the undulations, while for smaller systems, a defect was always required, about which a fold could nucleate. To test whether fold nucleation about a peptide defect could be reproduced for the largest system size, we replaced four of the peptides in the 2304 DPPC and 32 SP-B_{1-25} monolayers with a pre-formed peptide aggregate, after 0ns and 20ns of simulation (Figure 3.12), under small negative surface tension. For both times we got the same result: fold nucleation about a defect occurred in one of the monolayers and the other monolayer folded by amplification of undulations. Additionally, when monolayers containing 2304 DPPC and 32 SP-B_{1-25} molecules were simulated with surface tension set to zero, fold nucleation around a