Supplementary Material

Lowering line tension with high cholesterol content induces a transition from macroscopic to nanoscopic phase domains in model biomembranes

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1. Fast cooling technique to acquire desirable size of lipid domains

When GUVs are synthesized with certain lipid compositions, the size of macroscopic Ld + Lo domains may be too large for line tension measurements. Fast cooling is useful for GUV samples in order to break up large macroscopic domains into desirable sizes around 5 μm. Detailed information of applying fast cooling on GUVs in our study is presented in Table S1.

Table S1. Fast cooling techniques used in different four-lipid systems

Four lipid systems	Compositions	ρ values	Fast cooling setup
DSPC/DOPC+POPC/Chol	0.4: 0.4: 0.3	0.15-0.3	Cooled to room temperature over 12 hours, followed by immediate visualization
		0.4-1.0	Cooled to room temperature over 12 hours, re-heated up to 50 ^{o}C and quickly cooled to 0 ^{o}C
BSM/DOPC+POPC/Chol	0.53: 0.2: 0.27 0.5: 0.2: 0.3 0.47: 0.2: 0.33 0.44: 0.2: 0.36 0.42: 0.2: 0.38	0.5-0.7	Cooled to room temperature over 12 hours, followed by immediate visualization
		0.8-1.0	Cooled to room temperature over 12 hours, re-heated up to 50 ^{o}C and quickly cooled to 23 ^{o}C

2. Criteria for line tension measurements

To acquire accurate line tension measurements with minimum deviation, specific criteria are applied for finding appropriate Ld + Lo lipid domains, as described below.

1. Circular Ld + Lo lipid domains with sharp phase-contrast boundary are preferred.

2. Diameters of Ld + Lo lipid domains should be no larger than 1/5th of the GUV diameters.

3. The chosen Ld + Lo lipid domains should be located within the central area of GUV top surfaces. The domains on GUV bottom surfaces must be avoided, due to the interference the GUV surface touching the glass slide.

4. One separate lipid domain on the focused central area is preferable. Having two or more adjacent lipid domains in the focused area significantly interferes with natural fluctuations of Ld/Lo boundary.

5. A diameter of lipid domains around 5 μm should be used to acquire domain images with clear Ld + Lo phase boundary, especially for certain lipid compositions with higher line tension.

6. During line tension measurements using Matlab's Canny edge detection with each 500 frames (the images taken by the microscope), valid frames of each lipid domain must be more than 300 in order to be counted as a valid measurement.

3. Subset analysis

Since the concentration of C12:0 DiI of 0.2 mol % is rather high to achieve sharp boundary contrast between the Ld + Lo phases, light-induced artifacts may occur and cause variation during measurements. Light-induced artifacts can cause breakup, fusion, or an amplitude/frequency change of phase boundary fluctuations. Therefore, it is required to test for any light-induced artifacts by measuring the change in line tension over time. For each lipid

domain, the data were split into successive 5 subsets of 100 frames (successive subsets of each time interval for 3 seconds), starting at the first subset from time 0-3 s, the second subset from time 3-6 s, and so forth. For a given domain, the line tension value for each subset was normalized to the line tension value in the first 100 frames. These data were then averaged together over all domains at each ρ value. The subset analyses of line tension measurements for BSM/DOPC/POPC/Chol are presented in Fig. S1. If the variation of normalized line tension is maintained within ± 0.2, line tension can be accurately determined without light-induced artifacts.

BSM/(DOPC+POPC)/Chol=0.53/0.2/0.27

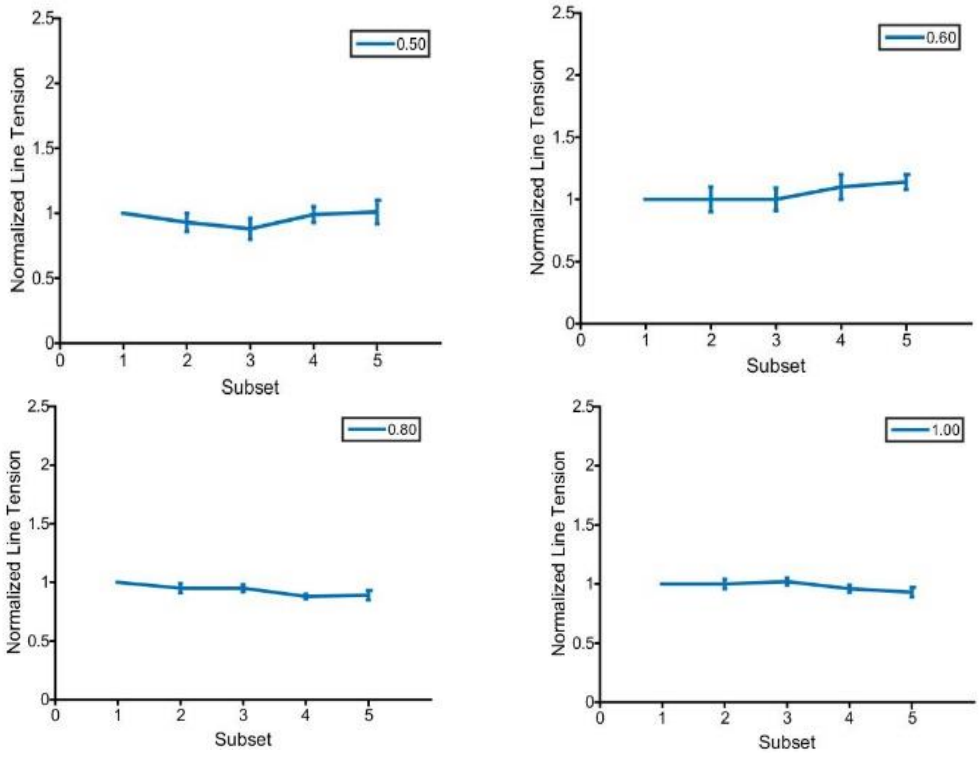
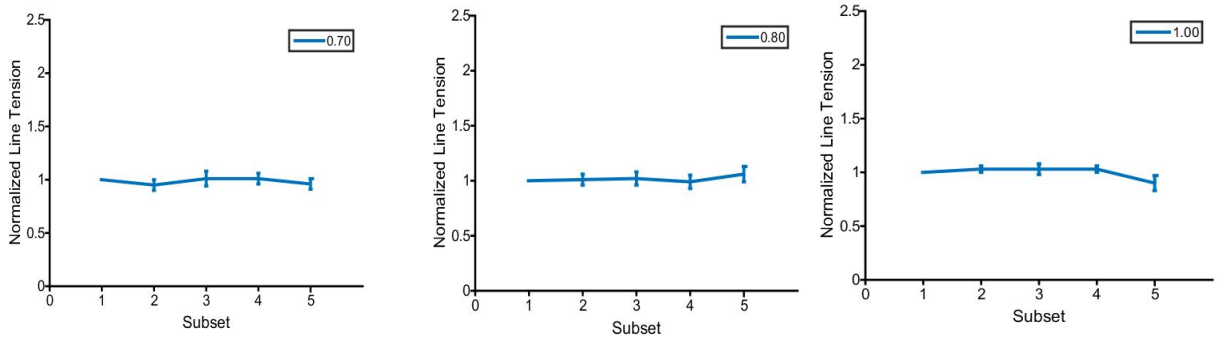


Fig. S1. Subset analyses for detecting light induced artifacts during line tension measurements. An average line tension of each successive subset of 100 frames is normalized by the line tension in the first 100 frames. The legend indicates the system's ρ value.

BSM/(DOPC+POPC)/Chol=0.5/0.2/0.3



BSM/(DOPC+POPC)/Chol=0.47/0.2/0.33

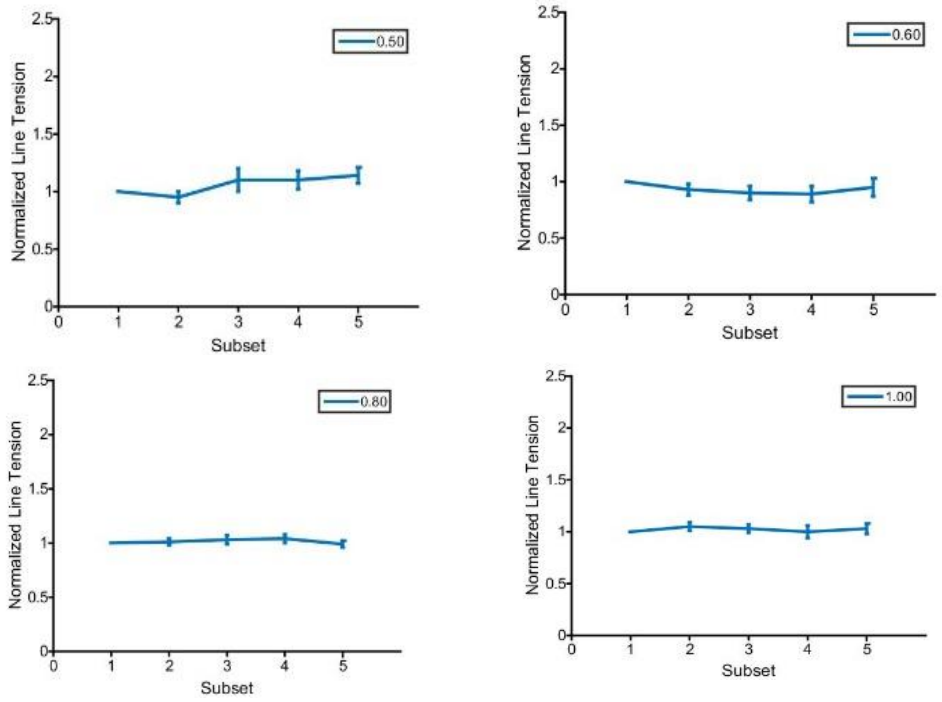
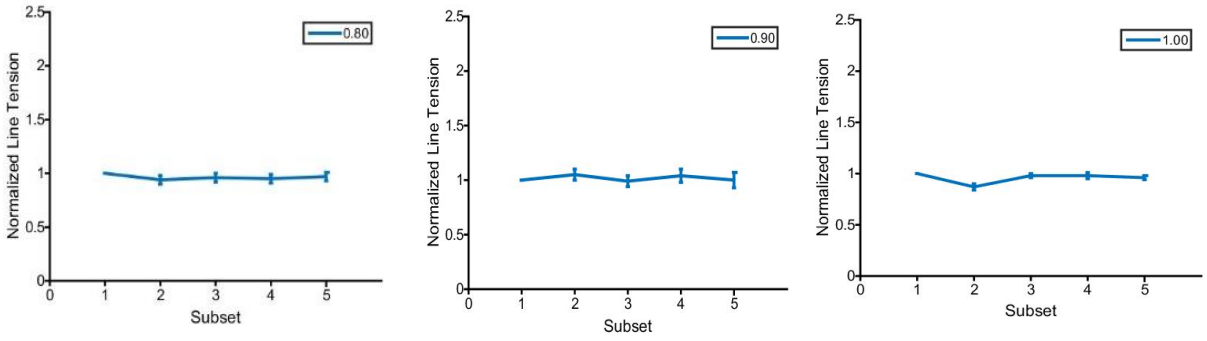


Fig. S1 (continued). Subset analyses for detecting light induced artifacts during line tension measurements. An average line tension of each successive subset of 100 frames is normalized by the line tension in the first 100 frames. The legend indicates the system's ρ value.

BSM/(DOPC+POPC)/Chol=0.44/0.2/0.36



BSM/(DOPC+POPC)/Chol=0.42/0.2/0.38

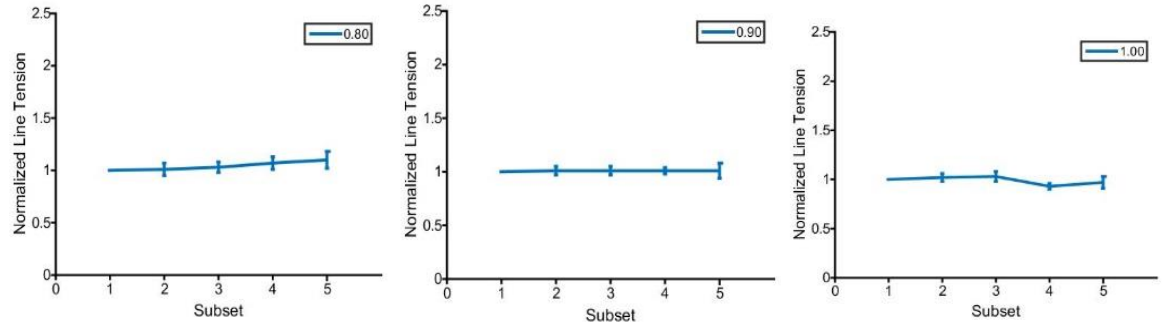
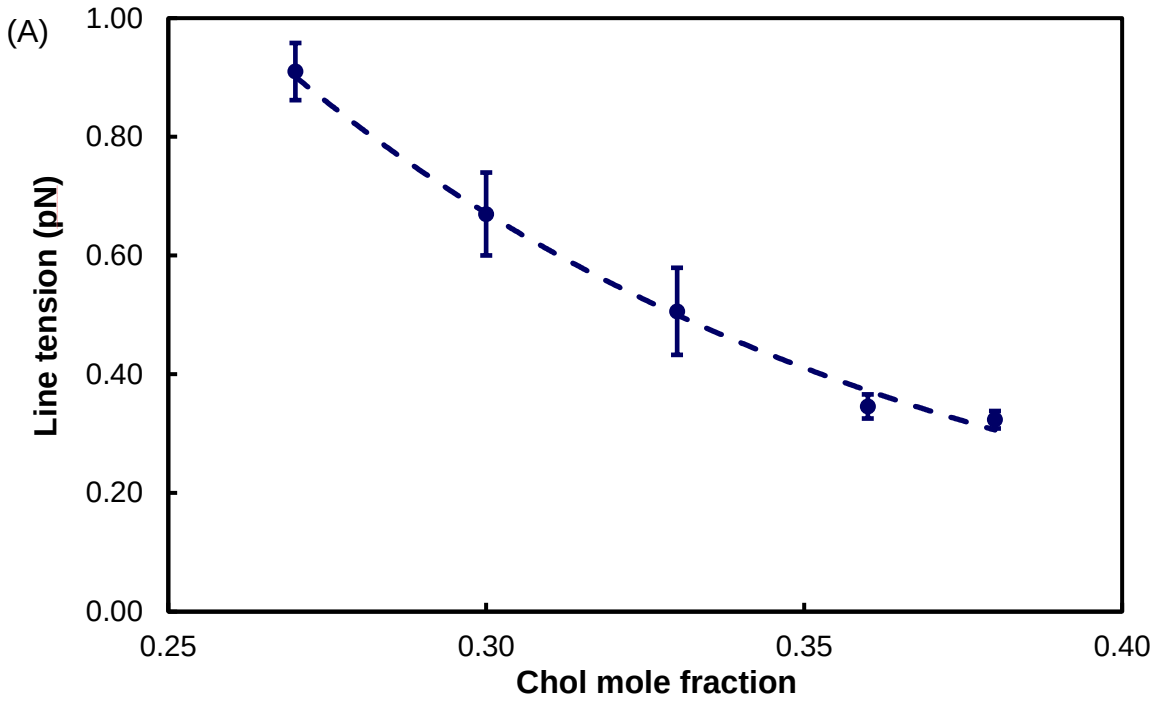


Fig. S1 (continued). Subset analyses for detecting light induced artifacts during line tension measurements. An average line tension of each successive subset of 100 frames is normalized by the line tension in the first 100 frames. The legend indicates the system's ρ value.

4. Correlation of line tension and Chol mole fraction for the BSM/DOPC/Chol lipid mixture

In Figure S2 (A), line tension decreases from 0.91 pN to 0.32 pN with overall Chol mole fraction ranging from 0.27 to 0.38. Figure S2 (B) shows the decrease of line tension with elevation of Chol content in the Ld or Lo phase, respectively. At ρ = 1, Chol mole fraction in the Ld phase

increases by 150%, from 0.1 to 0.25, but increases only by about 20% from 0.35 to 0.42 in the Lo phase, as shown by the blue and green arrows, respectively.



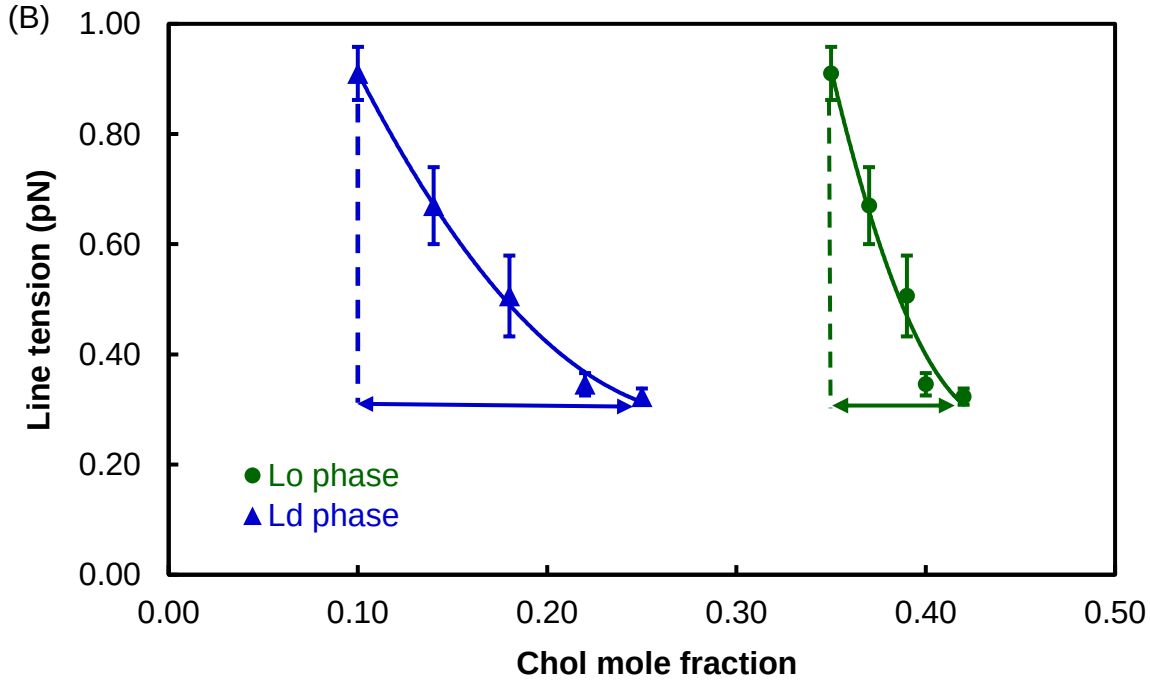


Fig. S2. Line tension determined for the BSM/DOPC/Chol lipid mixture (ρ=1) with Chol mole fraction increasing from 0.27 to 0.38; (A) Line tension decreases with overall Chol fraction increasing from 0.27 to 0.38; (B) The Chol fraction of Ld increases by 150 %, from 0.1 to 0.25, but increases only by about 20% from 0.35 to 0.42 in Lo, as shown by the blue and green arrows, respectively. Error bars correspond to SE.

5. Modulated-phase windows (ρ windows) and line tension of the coexisting Ld + Lo regime determined for the BSM/DOPC/POPC/Chol with different Chol mole fractions from 0.27 to 0.38

Modulated-phase windows for the BSM/DOPC/POPC/Chol with different Chol fractions from 0.27 to 0.38 are presented in Figure S3. As shown in Figure S4, we found a few GUVs with macroscopic Ld + Lo domains for line tension measurements in lipid mixtures containing Chol fractions of 0.36 and 0.38. During electroformation of GUVs, small osmotic pressure variation in the microenvironment of the swelling chamber may induce a small change in vesicle tension of some GUVs. If this small tension increase occurs for a vesicle that has line tension close to the value needed to form macrodomains, visible Ld + Lo domains could abruptly appear. (detailed explanation presented in Figure S16 of Supplemental Information of our previous study [1]).

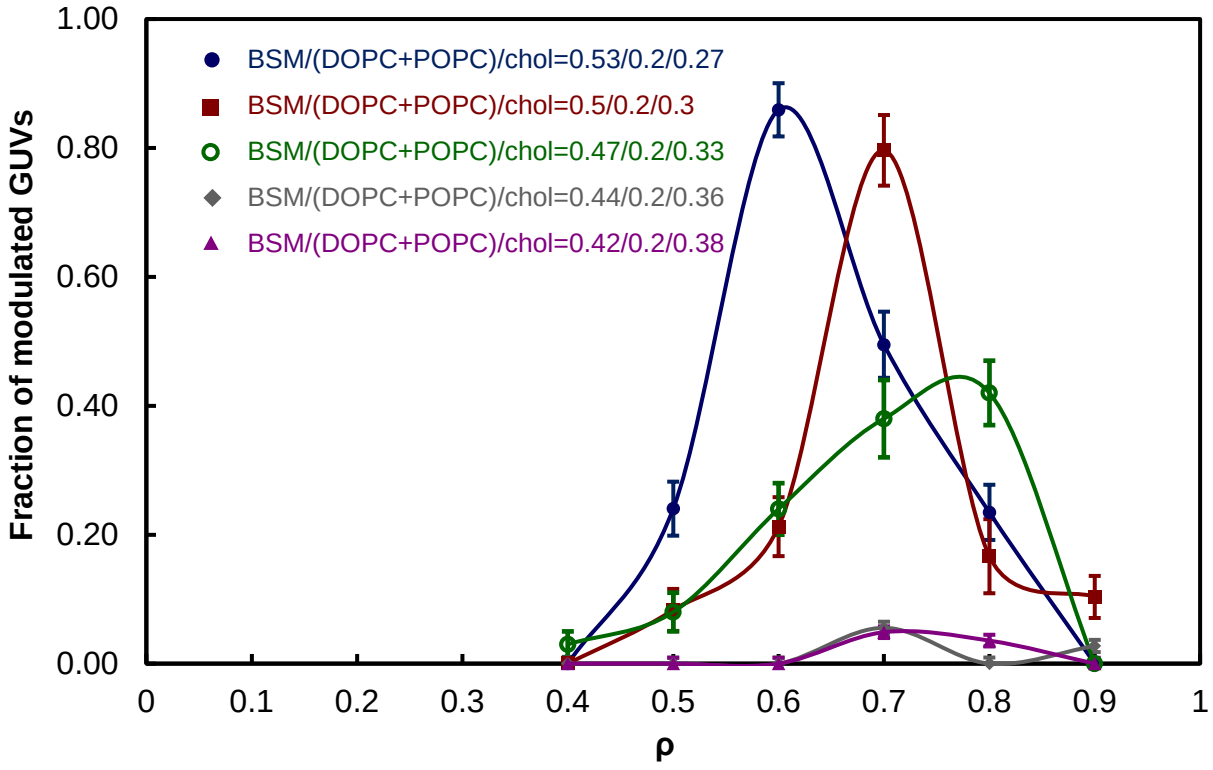


Fig. S3. Modulated-phase windows for the coexisting Ld + Lo regime determined for the BSM/DOPC/POPC/Chol with different Chol mole fractions increasing from 0.27 to 0.38. The peak of the modulated-phase window shifts to higher ρ values from 0.6 to 0.8, with increasing Chol mole fraction from 0.27 to 0.33. The fraction of modulated GUVs drastically decreases to less than 0.1 at higher Chol mole fraction of 0.36 and 0.38. Error bars correspond to SE.

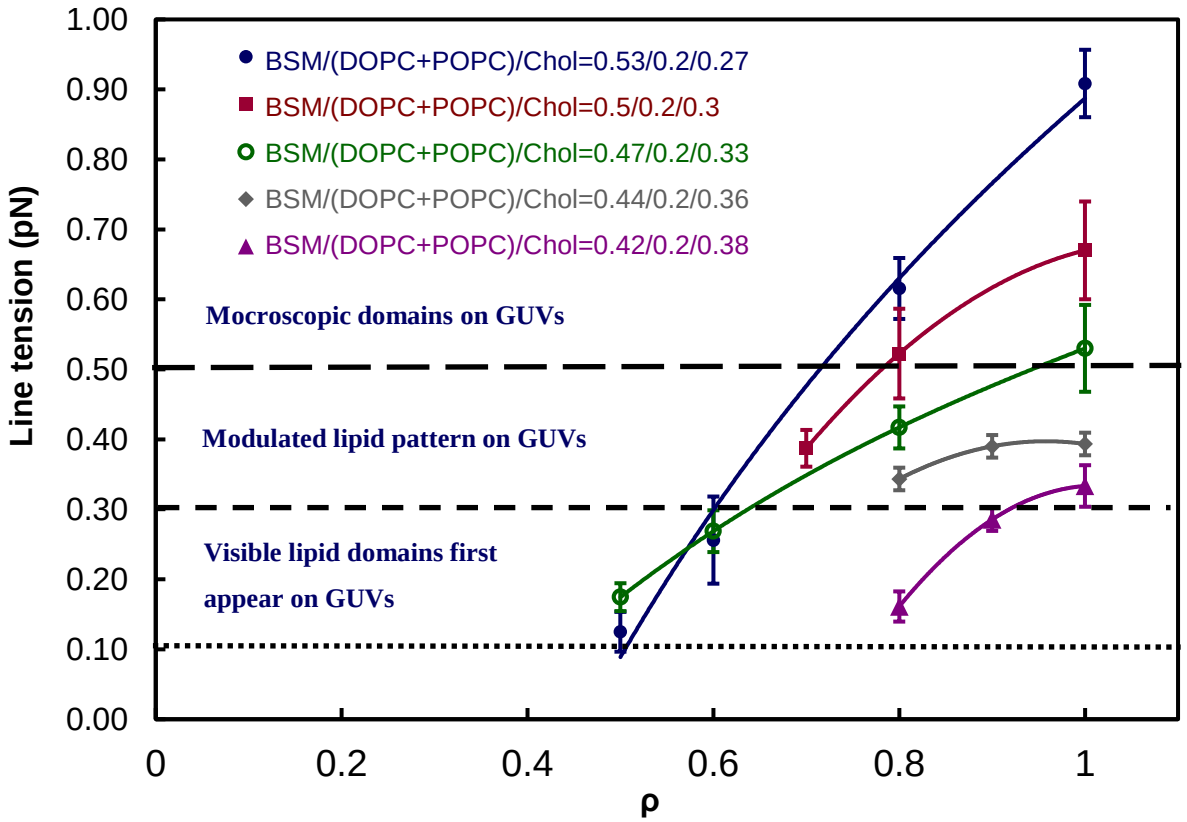


Fig. S4. Line tension increases as ρ increases for BSM/DOPC/POPC/Chol. In all five mixtures, visible domains first appear at line tension ranging from 0.1 to 0.3 pN (dotted line). Modulated GUVs become dominant at line tension between 0.3 to 0.5 pN (dashed line). Above 0.5 pN, macroscopic GUVs with large and round domains account for the majority (long dashed line). Compared to those of the lipid mixtures with 0.27 to 0.33 of Chol mole fractions, line tension of the lipid mixtures with 0.36 and 0.38 of Chol mole fractions at higher ρ values (ρ= 0.8 and 1.0) seem to be relatively lessened. Error bars correspond to SE.

6. Morphology transition of GUVs composed of different BSM/DOPC/Chol lipid mixtures

Figure S5 shows the phase-morphology images of the GUVs composed of different BSM/DOPC/Chol lipid mixtures. With Chol mole fraction increased from 0.27 to 0.38,

observations suggest that the coexisting Ld + Lo regime turns from macroscopic/modulated domains into nanoscopic domains. As shown in Figure S6, the fraction of visible lipid domains (modulated + macrodomain GUVs) continuously decreased from 1.0 to less than 0.2, while the fraction of uniform GUVs increased with rising Chol content.

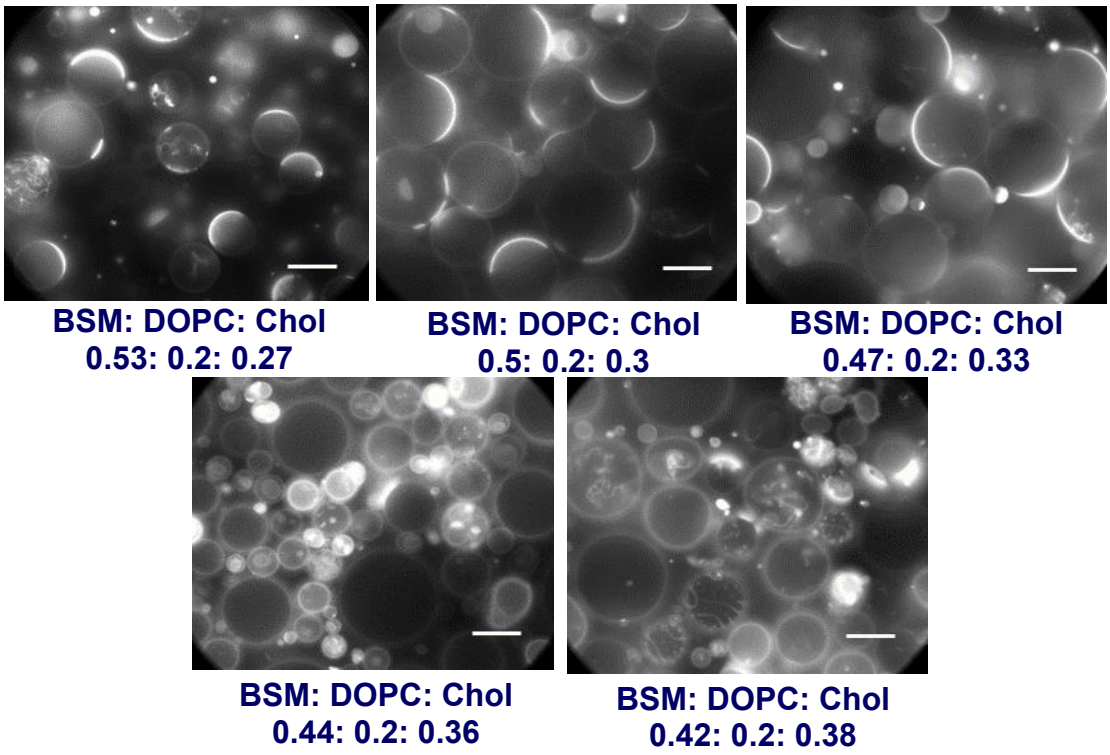


Fig. S5. Morphology of GUVs composed of different BSM/DOPC/Chol lipid mixtures with increasing Chol mole fractions from 0.27 to 0.38. Scale bar: 20 μm.

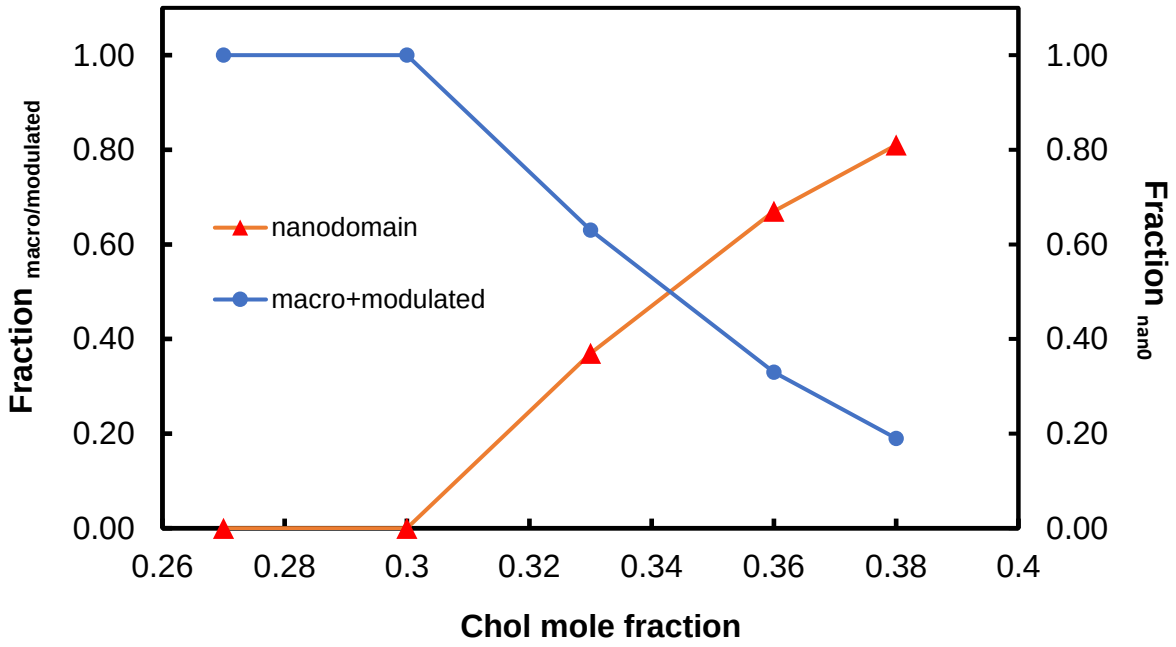


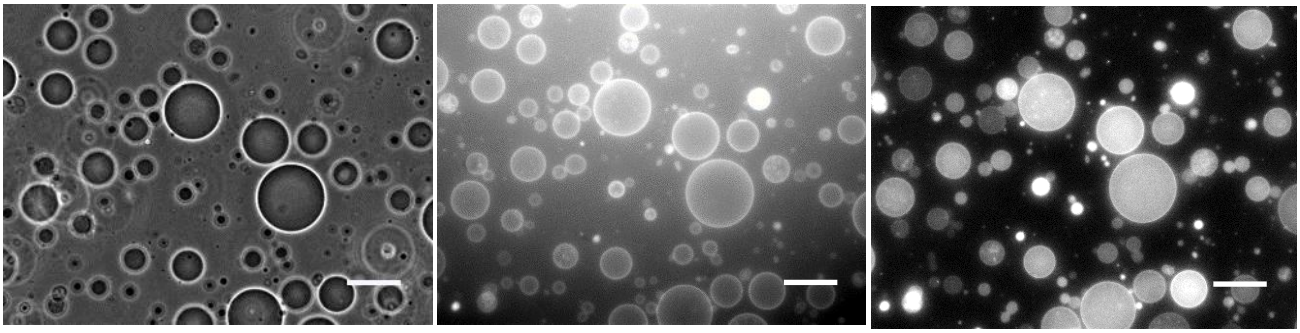
Fig. S6. GUV fractions of nanodomians (orange triangle) and visible domains (macro+modulated; blue circle) in the BSM/DOPC/Chol lipid mixtures with increasing Chol mole fractions.

7. Detection of domain fission between Ld and Lo phases

Macrodomains can bud off from the BSM-containing GUV membrane during or after macroscopic Ld + Lo phase separation. With DiI C12 favorably diffusing into the Ld phase, the budding may lead to an artifact wherein only homogenous GUVs containing the Ld phase *are observed* under fluorescence microscopy, with all Lo domain GUVs being dark. To verify whether the budding effect happened in our study, naphthopyrene was selected for illuminating the Lo phase. To avoid cross-interference of these two fluorescent dyes, excitation wavelengths for naphthopyrene and DiI C12 were chosen at 405 nm and 561 nm, respectively. Figure S7 shows the images of the GUVs containing BSM/DOPC/Chol under bright field and excited

151 fluorescence of 405 nm and 561 nm. All of the images exhibit the same distribution of GUVs.

152 No budding artifact occurred among the GUVs, that would have made pure Lo GUVs invisible.



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154 Fig. S7. Detection of the budding effect among the BSM-containing GUVs:

155 BSM:DOPC:Chol=0.42: 0.2: 0.38. Naphthopyrene: Lo-favoring dye with excited fluorescence at

156 405 nm; DiI C12: Ld-favoring dye with excited fluorescence at 561 nm. Scale bar: 20 μm.

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