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## Fat inclusions strongly alter membrane mechanics

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1 **Fat Inclusions Strongly Alter Membrane Mechanics.**

2

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21 **Abstract:**

22 Neutral lipids (NLs) are apolar oil molecules synthesized in the endoplasmic reticulum (ER)  
23 bilayer upon diverse biological stimuli. NLs synthesized are released in the hydrophobic core  
24 of the bilayer. At a critical concentration, NLs condense by phase separation and nucleate a  
25 lipid droplet (LD). After an LD forms, a fraction of NLs can be present in the bilayer but at a  
26 concentration below that of the nucleation. Here, we study whether and how the accumulation  
27 of NLs alters a lipid bilayer's mechanical properties. In synthetic systems, we found that NLs  
28 proffer unusual bilayer stretching capacities, especially in the presence of negatively curved  
29 phospholipids. This impact becomes spectacular when a lipid droplet is contiguous with the  
30 bilayer and supplies it with NLs. The tested NLs markedly decrease the bilayer area expansion  
31 modulus, significantly increase lysis tension but had opposite effects on membrane bending  
32 rigidity. Our data unveil how NL molecules modify overall membrane mechanics, the alteration  
33 of which may be linked to pathologies or anticancer treatments targeting NLs.

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35

36 **Significance:**

37 Unlike phospholipids, neutral lipids (NLs) are non-surfactants, apolar fat, or oil molecules.  
38 They are synthesized in the endoplasmic reticulum (ER) bilayer and diffuse within the bilayer's  
39 hydrophobic region. At a critical concentration, the NLs phase separate within the bilayer to  
40 form an organelle called a lipid droplet (LD). However, even if they condense into LDs, a  
41 fraction of NLs remains in the bilayer. Also, under diverse pathological conditions associated  
42 with alterations in the ER structure or composition, NLs accumulate in the membrane, failing  
43 to condense efficiently into LDs. Their removal is crucial to preserve the multiple ER bilayer  
44 functions that rely on membrane mechanics. Here, we are interested in understanding how the  
45 presence of NLs impacts such mechanics.

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## 51 **Introduction**

52 Organisms from protists to humans store excess energy in carbon-rich molecules that are often  
53 neutral lipids (NLs) (1, 2). NLs are apolar fat molecules, different from phospholipids, which  
54 are the building blocks of cellular membranes. The most abundant NLs are triacylglycerols,  
55 which are structurally the closest NL to phospholipids, and sterol esters, which are the most  
56 relative to cholesterol, another membrane constituent (3). However, other NLs are found  
57 depending on cell type and metabolic condition, such as retinyl ester, phytyl esters, squalene,  
58 acylceramides, etc. (2, 4–7). Most of these NLs are synthesized in the bilayer of the  
59 endoplasmic reticulum (ER) (8, 9). Because of their hydrophobicity, NLs accumulate within  
60 the bilayer's hydrophobic region as dissolved, mobile hydrophobic inclusions(10). When their  
61 concentration reaches a critical value, NLs phase separate within the bilayer and nucleate an oil  
62 lens (11, 12), which grows and buds off to become an independent organelle called a lipid  
63 droplet (LD) (8). In the context of nucleation phenomena, the formation of an LD consists of  
64 packaging a large fraction of the NLs into a condensed phase, in equilibrium with a dilute NL  
65 phase in the bilayer (7, 11).

66

67 Avoiding the accumulation of NLs in the ER membrane ensures its proper functioning. NL  
68 accumulation could increase the bilayer's thickness, which would likely cause membrane  
69 stresses and affect the stability of transmembrane proteins. Indeed, such an accumulation may  
70 alter membrane structure, fluidity, lipid distribution, and stability, which can cause various  
71 forms of membrane stress. Most of these disturbances can explain the onset of diseases or cell  
72 death when NL molecules accumulate in the ER bilayer and are not properly removed and  
73 packaged into LDs.

74

75 The assembly of triacylglycerol LDs is facilitated by membrane curvature, composition, and  
76 specific proteins (10, 13–18) that decrease the critical nucleation concentration (10, 19–21).  
77 Accordingly, when the ER's curved structure is lost due to the dysfunction in the ER shaping  
78 proteins, triacylglycerols accumulate in the bilayer, unable to effectively condense into LDs  
79 (10, 18). These conditions are associated with several diseases, such as spastic paraplegia (22).  
80 Other factors such as the ER membrane phospholipid composition and surface tension can also  
81 regulate the concentration of the bilayer-dissolved NLs (14) and their propensity to condense  
82 into LDs (23). Likewise, the NL chemistry determines its tendency to remain in the bilayer or  
83 condense into an LD (10, 14). Squalene, an intermediate molecule in the synthesis of sterols, is  
84 an example of an NL that abnormally accumulates in membranes (6, 14, 24). Increasing the

85 proportion of squalene relative to triacylglycerols leads to the formation of larger LDs (14),  
86 probably because squalene increases the nucleation concentration (6, 24) and decreases the  
87 budding propensity of LDs (14). Interestingly, many anticancer and antifungal drugs target the  
88 squalene epoxidase (25–27), leading to reduced cellular cholesterol and, inevitably, an  
89 accumulation of squalene. These two consequences may concomitantly contribute to cellular  
90 death.

91

92 In this paper, we ask the fundamental question of how NL molecules, referred to as fat or oil  
93 molecules hereafter, modulate the mechanical properties of a phospholipid bilayer. To answer  
94 this question, we used model membranes based on the formation of giant unilamellar vesicles  
95 (GUVs) containing neutral lipids, such as those from droplet-embedded vesicles (28, 29) or  
96 inverted emulsion vesicles (30). We found that oil molecules' presence substantially modifies  
97 the bilayer response to stretching and bending, depending on the phospholipid composition and  
98 oil type.

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105 **Results**

106 **Oil droplets expand the critical bilayer area strain while decreasing permeability.**

107 We opted for osmotic swelling as a straightforward means to probe the expansion capacity of a  
108 giant unilamellar vesicle (GUV) bilayer (31–34) (**Figure 1A, top**). To investigate if NLs alter  
109 this expansion, we used the droplet-embedded vesicle system (DEV) (35), consisting of a giant  
110 unilamellar vesicle (GUV) containing an oil droplet made of triolein, the major triacylglycerol  
111 NL (**Figure 1A, bottom**). In the DEV system, the oil droplet equilibrates within seconds with  
112 a constant fraction of triolein molecules mobile in the bilayer (**Figure S1A-E**)(10).

113

114 We prepared GUVs and triolein oil-based DEVs (**Figure 1B, bottom**), both made of DOPC  
115 phospholipid, and we subjected them to the same osmotic shock. Both systems showed swelling  
116 and bursting cycles during which their bilayer underwent stretching and relaxation phases (31)  
117 (**Figure 1A-B**). To describe and understand the systems' response, we focused on the bilayer  
118 surface area strain  $\epsilon$  for both GUVs and DEVs (**Figure 1C**). For DEVs, the bilayer area is  
119 calculated at each time point by withdrawing the space occupied by the droplet from the area  
120 of the sphere fitting the bilayer (**Figure 1C, Figure S1F-I**). Furthermore, the DEVs' swelling  
121 time took a few minutes (**Figure 1C,D**), much longer than the time it takes for triolein oil  
122 molecules to partition between the droplet and the bilayer (10). Therefore, the swelling process  
123 of DEVs occurred at equilibrium.

124

125 Triolein-DEVs had a slower strain rate than GUVs (initial slope, **Figure 1D**), which underwent  
126 many cycles within the same time-lapse. The strain rate is determined by the water flux, i.e.,  
127 the permeability of the bilayer: the higher the permeability, the higher the strain rate.  
128 Accordingly, we found a permeability higher for GUVs,  $24.8 \pm 8.3 \mu\text{m}\cdot\text{s}^{-1}$ , than triolein-DEVs,  
129  $9.0 \pm 2.6 \mu\text{m}\cdot\text{s}^{-1}$  (**Figure 1E left, Figure S1J-M, supplemental text**) (36). Squalene-DEVs were  
130 less permeable than triolein-DEVs (**Figure 1E left, Figure S1J,L,M, supplementary text**),  
131 consistent with permeability studies with droplet interface bilayers (37).

132

133 The critical strain  $\epsilon_{\text{max}}$ , reached when the bilayer bursts, was higher for DEVs than GUVs  
134 (**Figure 1D, Figure 1E right, Figure S1J**). A burst occurs when a critical pore size appears in  
135 the membrane. GUVs stretched at a slower strain rate have a higher probability of reaching the  
136 critical pore size (31) and, therefore, a lower  $\epsilon_{\text{max}}$ . This correlation is not verified with DEVs,  
137 which had slower strain rates than GUVs while higher  $\epsilon_{\text{max}}$  (**Figure 1D, Figure 1E right**).  
138 Therefore, other factors absent in GUVs favor the increase in  $\epsilon_{\text{max}}$  for DEVs. We hypothesized

139 that oil molecules recruited to the bilayer are responsible for this higher critical bilayer strain  
140 for DEVs.

141

142 To test our hypothesis, we decided to vary the phospholipid composition, which controls the  
143 bilayer's oil concentration (14). We worked with different phospholipid mixtures, including  
144 DOPE (PE), DOPA (PA), DPPC, or PufaPC (18:0, 20:4). For each condition, we determined  
145 the critical bilayer strain,  $\epsilon_{\max}$ , and compared it between GUVs and DEVs (**Figure 1F, Figure**  
146 **S1N-R**). Irrespective of composition, triolein-DEV bilayers withstood larger expansions than  
147 GUVs (**Figure 1F, Figure S1R**). Interestingly, the critical bilayer strain of triolein-DEVs was  
148 systematically higher in the presence of negatively curved lipids such as PA and PE, between  
149 3 and 5 times higher than for GUVs (**Figure 1F, Figure S1R**). We found that squalene enabled  
150 a higher membrane expansion than triolein in PC conditions and, more strikingly, in PufaPC  
151 (**Figure 1F, Figure S1R**). This particular increase in PufaPC may pertain to  $\pi$ - $\pi$  interactions  
152 between the numerous unsaturations in the squalene's carbon skeleton and those in the PufaPC  
153 acyl chain. However, compositions containing PA and PE showed no difference between the  
154 two oils (**Figure 1F, Figure S1R**), suggesting that these phospholipids' contribution masked  
155 the difference between the two oils.

156

### 157 **Stretching increases the bilayer oil concentration depending on the phospholipid** 158 **composition.**

159 Based on the above results, we hypothesize that the oil droplet buffers oil molecules to the  
160 bilayer, which has more material to withstand more significant expansions. Following this  
161 hypothesis, PA and PE would lead to more oil molecules in the bilayer.

162

163 To test our model, we used a fluorescently-labeled "triolein", triolein-NBD, mixed with triolein  
164 at 0.05% (w/w) to report for triolein localization (**Figure 2A**). The resulting DEV was then  
165 monitored during the osmotic swelling (**Figure 2B, left**). We found that the bilayer's triolein  
166 signal increased concomitantly with the bilayer stretching (**Figure 2B, right**). Then, the bilayer  
167 concentration sharply decreased following a rapid burst of the DEV (**Figure 2B, right**); the oil  
168 molecules almost spontaneously re-equilibrated between the droplet and the bilayer after this  
169 burst (**Figure 2B, Figure S2A**). These results show that increasing the bilayer surface tension  
170 by osmotic swelling raises the amount of oil in the bilayer, agreeing with previous *in silico*  
171 results (14) and confirmed here by using micropipettes to vary surface tension (**Figure S2B,C**).  
172 To test if squalene behaves like triolein, we used Bodipy, which labels the bilayer's

173 hydrophobicity, as we could not find available fluorescent squalene reporters. We observed a  
174 stronger Bodipy signal in the bilayer during osmotic swelling, most likely due to an increase in  
175 squalene in the bilayer (**Figure S2D**).

176

177 Our hypothesis suggests that the number of oil molecules incorporated into the bilayer during  
178 swelling determines the extent of membrane expansion. Since the critical bilayer strain  
179 depended on the phospholipid type (**Figure 1F, Figure S1R**), we measured the triolein increase  
180 in the bilayer for each phospholipid condition. We found an increase in the triolein signal during  
181 swelling for all lipid compositions but to different extents (**Figure 2D, Figure S2E**). We  
182 defined the bilayer triolein enrichment as the ratio between the triolein level in the bilayer at  
183 the maximum expansion (high tension) and the initially unstretched bilayer (low tension)  
184 (**Figure 2C**). We found that DEVs containing PA or PE, which had the most remarkable  
185 expansion (**Figure 1F**), displayed the highest triolein enrichment (**Figure 2D**). In contrast,  
186 triolein-DEVs made with PufaPC-PC, which did not significantly increase the critical bilayer  
187 strain (**Figure 1F**), exhibited a low triolein enrichment. These data are consistent with our  
188 model that, during stretching, triolein molecules can behave as membrane building blocks, like  
189 phospholipids, and allow for more bilayer expansion.

190

191 If our model is correct, then the enrichment level of triolein in the bilayer should dictate the  
192 critical bilayer strain. To test this prediction, we considered for each phospholipid composition  
193 the ratio of the critical bilayer strain of DEVs over GUVs (**Figure 1F**). This normalization  
194 cancels the contribution of phospholipids to the membrane expansion and highlights the  
195 contribution of triolein molecules. Consistent with our prediction, we found that the amount of  
196 triolein injected into the bilayer during stretching increases linearly with the critical bilayer  
197 strain ratio (**Figure 2E**).

198

199 In summary, an oil droplet connected to a bilayer equilibrates with residual oil molecules in the  
200 bilayer. The concentration of this residual oil increases as the bilayer is stretched. Phospholipids  
201 with negative spontaneous curvature favor a higher delivery of oil molecules from the droplet  
202 to the stretched bilayer and, thereupon, a higher critical bilayer strain.

203

204 **The sole presence of oil molecules in a bilayer increases the critical area strain.**

205 To fully demonstrate that the oil molecules favored the increase in the critical bilayer strain, we  
206 decided to repeat the previous experiments with oil molecules in the bilayer without any droplet



207 reservoir. To do so, we used the water-in-oil inverted emulsion method (30) by using a triolein  
208 oil phase containing triolein-NBD (**Figure 3A, left**). With this approach, indeed, we found that  
209 the resulting GUVs, called triolein-GUV (To-GUV), carried only triolein molecules in their  
210 bilayer (**Figure 3A, right**). The bilayer triolein concentration is defined as the triolein-NBD  
211 bilayer signal divided by Cy5-PE, reporting phospholipids (**Figure 3B**). Based on this  
212 normalization, triolein-GUVs and -DEVs composed of PC, and not subjected to an osmotic  
213 swelling, had a similar bilayer triolein signal (**Figure 3C**).

214

215 We first compared GUVs and triolein-GUVs, both made of PC, subjected to osmotic swelling.  
216 We found that the critical bilayer strain,  $\epsilon_{\max}$ , for triolein-GUVs was significantly higher than  
217 for GUVs (**Figures 3D**), consolidating our model that the sole presence of NLs is sufficient to  
218 increase the membrane expansion limit.

219 We next compared triolein-GUVs to triolein-DEVs. Triolein-DEVs had a 1.45 fold higher  
220 membrane expansion limit than triolein-GUVs in PC (**Figure 3D**). The presence of PA  
221 exacerbated this difference, by 3.2 fold higher (**Figure 3E, S3A-B**). These observations also  
222 agree with our model. Indeed, the bilayer of triolein-GUVs had a fixed number of oil molecules  
223 and, therefore, less material to withstand larger expansions; in contrast, the bilayer of triolein-  
224 DEV can be continuously supplied with oil molecules by the droplet. PA highlighted further  
225 this difference because it induced the most substantial triolein release into the bilayer of DEVs  
226 (**Figure 2D-E**). Finally, squalene had a similar impact than triolein in squalene-GUVs vs.  
227 squalene-DEVs (**Figure 3F**).

228

229 Overall, these data show that the oil molecules' presence increases the bilayer's ability to  
230 withstand more extensive stretching than a pure phospholipid membrane. If an NL droplet is  
231 physically connected to the bilayer, it will buffer NLs to the bilayer, further increasing its  
232 expansion. A dramatic consequence of such an increase in the bilayer stretching is that the area  
233 per phospholipid can be increased by several folds by NLs, especially in the presence of  
234 negatively curved phospholipids (**Figure 1F, Figure. 3D-F**).

235

### 236 **Oil molecules modify the mechanical properties of a bilayer.**

237 We wanted to know whether the mechanical properties of the bilayer are influenced by the  
238 presence of oil, as for solvents (38). The critical bilayer surface area strain is determined by the  
239 appearance of a critical pore size, which depends on membrane stretching and bending factors  
240 (31). We probed how NLs altered these mechanical factors independently.

241

242 First, we studied if NLs altered the lysis tension of DEVs, i.e., the surface tension at which a  
243 pore opens in the bilayer. We used micropipette suction to increase the surface tension of PC  
244 GUVs and PC DEVs made of triolein or squalene. The micropipette aspiration (MPA)-applied  
245 bilayer tension was increased continuously by pulling on the bilayer at a rate of approximately  
246 2-3 mbar/sec (**Figure S4A**). The lysis tension was taken as the MPA-applied bilayer tension  
247 was recorded right before the bilayer's burst (**Figure S4A**). In PC, the lysis tension of GUVs  
248 was  $8.1 \pm 1.9$  mN/m, in the range of previously reported values (36, 39). Triolein increased this  
249 lysis tension to  $9.7 \pm 1.9$  mN/m, and squalene DEVs led to a more significant increase, to  $10.8$   
250  $\pm 2.2$  mN/m (**Figure 4A**). A similar trend was found for a PC/PA membrane composition  
251 (**Figure S4B**). This increase in the lysis tension can partly explain the higher critical bilayer  
252 area strain proffered by oil molecules (**Figure 1F**, **Figure S1R**).

253

254 Secondly, we determined the impact of the oil molecules on the area expansion modulus. For  
255 surface tensions over 0.5 mN/m, sufficient to smoothen thermal membrane fluctuations, the  
256 apparent area expansion modulus is given by Hooke's law (40, 41) (**Figure 4B**).  
257 Experimentally, we increased the bilayer area with a micropipette while recording its MPA-  
258 applied surface tension (**Figure 4B**). The slope of the resulting MPA-applied bilayer tension-  
259 area curve (**Figure S4C-D**) yields the apparent area expansion modulus of the bilayer ( $Y_{app}$ )  
260 (41). With this approach, we could only work with GUVs containing oils (**Figure 3A**) since, in  
261 DEVs, the droplet has a contribution in elasticity difficult to model. GUVs made of PC had an  
262 apparent expansion area modulus of  $194 \pm 25$  mN/m (40), while those bearing triolein and  
263 squalene had lower values,  $89 \pm 31$  mN/m and  $111 \pm 27$  mN/m respectively (**Figure 4C**). This  
264 marked decrease in the apparent area expansion modulus is consistent with the overall increase  
265 in the critical bilayer strain observed when the NL oils are present in the bilayer (**Figure 3D-**  
266 **E**). However, no significant difference was found between squalene and triolein, suggesting  
267 that the apparent area expansion modulus does not solely account for the disparity in the bilayer  
268 expansion limit between these two oils (**Figure 1F**).

269

270 Finally, we determined the bending rigidity ( $K$ ) by nanotube pulling experiments (**Figure 4D**  
271 **and S4E-I**). We found that the presence of triolein in a PC GUV bilayer decreased the bending  
272 rigidity ( $12 \pm 4$  k<sub>B</sub>T) roughly by a factor of two compared to the value for a bare GUV ( $21 \pm 4$   
273 k<sub>B</sub>T) (**Figure 4E**); a similar trend was found when measuring the rigidity by fluctuation analysis

274 (42). Surprisingly, squalene slightly increased the bending rigidity ( $27 \pm 4 \text{ k}_B\text{T}$ ), illustrating that  
275 oil molecules do not necessarily change membrane properties in the same way. We used the  
276 bending rigidity values to compute the absolute area expansion moduli (**Figure 4F**) (36). PC  
277 GUVs had an expansion area modulus of  $214 \pm 25 \text{ mN/m}$  (40), while GUVs containing triolein  
278 or squalene had a lower value of  $101 \pm 35 \text{ mN/m}$  and  $120 \pm 28 \text{ mN/m}$  (**Figure 4F**).

279

280 In conclusion, the above data illustrate how and which membrane mechanical properties are  
281 altered by NL oil molecules. NLs can have similar or opposite effects on membrane mechanical  
282 properties, depending on the NLs' chemistry.

283

## 284 **Discussion**

285 Precise control of the biophysical properties of membranes is crucial for cell survival.  
286 Phospholipids and membrane proteins often control these properties. Here, we consider the  
287 impact of neutral lipids, which can behave as hydrophobic membrane inclusions. We found that  
288 neutral lipids proffer unusual mechanical properties to the bilayer membrane. Such an effect  
289 depends on the nature of the hydrophobic inclusion but also the phospholipid composition.

290  
291 We worked with triolein, the main component of cellular LDs, and squalene, which can  
292 significantly accumulate in cells upon drug treatments targeting the squalene epoxidase (25–  
293 27). For the phospholipid conditions that we tested, we found that these neutral lipids increased  
294 the lysis tension of a bilayer and significantly decreased its area expansion modulus. While  
295 triolein decreased the bilayer bending stiffness, squalene increased it. Overall, these mechanical  
296 alterations are consistent with the greater membrane expansion proffered by the neutral lipids.  
297 At the molecular level, the increased critical membrane strain likely results from the insertion  
298 of neutral lipids between phospholipids when the membrane is stretched, i.e., when tension  
299 increases. Thus, the neutral lipids may adopt different molecular conformations when tension  
300 varies (43–45). At low tension, triolein molecules could remain perpendicular to the  
301 phospholipids but parallel to them at high tension, exposing their glycerol backbone to water  
302 (44). This molecular adaptability would allow phospholipids to be spaced further apart than in  
303 a pure bilayer.

304  
305 Squalene is a long terpene with numerous unsaturation, chemically different from triolein.  
306 Despite contrasting chemistry, the two neutral lipids yielded similar elasticity. As for triolein,  
307 squalene may also adopt phospholipid-parallel or antiparallel orientations in the bilayer (46),  
308 depending on the surface tension, in a way that decreases the area expansion modulus.  
309 Concerning the membrane lysis tension, both neutral lipids increased it, especially squalene.  
310 This difference may pertain to the interaction spectrum of NLs with phospholipids. In  
311 particular, squalene can make multiple interactions  $\pi$ - $\pi$  (47) with phospholipids, making it act  
312 like a molecular glue, “cross-linking” the bilayer leaflets and delaying the opening of a pore.  
313 Interestingly, squalene increased the bilayer bending rigidity, whereas triolein decreased it,  
314 relative to GUVs.

315  
316 The bending rigidity ( $\kappa$ ) depends on the bilayer thickness ( $d$ ), the area expansion modulus ( $Y$ ),  
317 and the coupling constant ( $\beta$ ) of the monolayer leaflets composing the bilayer:  $\kappa \propto \beta \cdot d^2 \cdot Y$  (48).

318 Triolein and squalene decreased by two, the area expansion modulus,  $Y$ , (**Figure 4F**), which  
319 tends to reduce the bending rigidity, but not for squalene (**Figure 4E**). Squalene particularly  
320 increases membrane thickness, more than triacylglycerols (37, 38, 49, 50). Also, via  $\pi$ - $\pi$   
321 interactions developed with phospholipids (47), squalene likely increases the monolayer  
322 coupling  $\beta$ . Consequently, the contribution of  $\beta \cdot d^2$  for squalene may compensate for the  
323 decrease in  $Y$ , sufficiently to overall increase the bending rigidity  $\kappa$ .

324

325 Based on the above analysis and the rigidity measurement (Figure 4E), curving squalene-rich  
326 membranes is more difficult. Squalene accumulation is promoted by several antifungal and  
327 anticancer drugs (25–27) and, therefore, the subsequent alteration in membrane mechanics  
328 could contribute to the death of fungal or cancer cells. Inducing the synthesis and membrane-  
329 accumulation of specific neutral lipids could be a strategy to prompt cancer cells to die.

330

331 Finally, our data show that a droplet connected to a bilayer can supply neutral lipids to the  
332 membrane, especially when the bilayer experiences stretching. The amount of neutral lipids  
333 delivered to the bilayer depends on the bilayer phospholipid composition. Thus, in a cellular  
334 context, LDs should be disconnected from the ER bilayer to avoid residual neutral lipids in the  
335 membrane. If not, the neutral lipid accumulation would hinder the ER 's multiple functions that  
336 rely on the precise regulation of its mechanical properties, involving membrane tubulation,  
337 stretching, budding, or fusion.

338

339 In conclusion, our study provides important insights into how neutral lipids can change  
340 membranes' biophysical properties. This study is relevant to better understand the ER  
341 membrane mechanics during fat storage, particularly in pathological conditions where neutral  
342 lipids fail to efficiently phase separate into LDs. Our approaches can be adapted to test the  
343 behavior of more exotic lipids.

344

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349

350 **Competing interests**

351 The authors declare no competing interests.

352

353 **Author contributions**

354 ART designed research. AC and AS performed experiments and analyzed the data with LF.

355 AC, AS, and ART wrote the manuscript.

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496 **Figure 1: Oil droplets connected to a bilayer expand the critical membrane area strain.**  
497 **A) Top:** Schematic representation of the behavior of a GUV under hypotonic shock. GUVs undergo  
498 swelling and burst cycles. **Bottom:** Schematic representation of a triolein DEV (Droplet Embedded  
499 Vesicle) system compared to a GUV which also undergo swelling and burst cycles. **B) Time-lapse** of  
500 triolein DEV and GUV during a hypotonic shock experiment. Black dashed circles report maximum  
501 vesicle radius. Scale bar is 10 $\mu$ m. **C) Top:** Green arc underlying the bilayer area for an uninflated and  
502 inflated DEV. **Bottom:** Formula used to compute the bilayer strain,  $\epsilon$ , defined as the relative expansion  
503 of the bilayer area. **D)** Corresponding bilayer strain  $\epsilon$  variations of triolein DEV and GUV over time of  
504 Figure 1B. Swelling and burst cycles are clearly visible for GUVs (blue points; 6 cycles) and DEVs  
505 (green points; 1 cycle). **E) Left:** Plot of the initial water permeability of PC bilayer of GUVs (n=8),  
506 triolein-DEVs (n=9) and squalene-DEVs (n=9). Mean +/- SD. **Right:** Plot of the critical bilayer strain  
507 ( $\epsilon_{max}$ ) of GUVs (blue, n=28), triolein-DEVs (green, n=29) and squalene-DEVs (grey, n=21) for PC  
508 bilayer. Mean +/- SD. **F)** Plot of the critical bilayer strain ( $\epsilon_{max}$ ) of GUVs (blue), triolein-DEVs (green)  
509 and squalene-DEVs (grey) for various phospholipid compositions of the bilayer. Mean +/- SD.

511 **Figure 2: Stretching increases the oil concentration in the bilayer depending on the phospholipid**  
512 **composition.**

513 **A)** Schematic representation of the triolein DEV formation protocol. To report triolein distribution,  
514 triolein was supplemented with 0,05% (w/w) triolein-NBD. **B) Left,** confocal images of PC triolein  
515 DEVs under hypotonic shock with triolein tagged by triolein-NBD. Line scans show a triolein-NBD  
516 signal colocalizing with the bilayer. Scale bar is 5 $\mu$ m. **Right,** corresponding quantification of triolein-  
517 NBD signal and bilayer area variation. During bilayer stretching, triolein-NBD signal increased, and  
518 quickly dropped after deflation. **C)** Representative confocal fluorescence images of a PC triolein-DEVs  
519 at the maximum bilayer expansion state (high tension) and at the "unstretched" state (low tension).  
520 Linescans allow for quantification of triolein-NBD fluorescence intensity in the bilayer in each state.  
521 'Triolein enrichment' was computed by taking the fluorescence ratio between stretched state and  
522 unstretched state. Scale bar is 5  $\mu$ m. **D)** Triolein enrichment measured for PC (n=8), PA-PC (n=5), PE-  
523 PC (n=4), DPPC-PC (n=3) and PufaPC-PC (n=8) triolein-DEVs. Mean +/- SD. **E)** Plot of the critical  
524 bilayer strain ratio between DEV and GUV against the triolein enrichment for previous phospholipid  
525 compositions. Statistical analysis revealed a significant slope different from zero for a linear fit (dotted  
526 lines). Mean +/- SD.

528 **Figure 3: The sole presence of oil molecules increases the critical bilayer strain.**

529 **A) Left,** illustration of the formation of triolein-GUVs (To-GUVs) using a water-in-oil inverted  
530 emulsion method. The triolein was tagged with 0,05% (w/w) of triolein-NBD. **Right,** fluorescence  
531 confocal imaging of the resulting triolein-GUVs. Significant triolein-NBD signal colocalize with the  
532 bilayer reported by Cy5-PE. Scale bar: 5 $\mu$ m. **B) Top:** three different model systems: a bilayer with no

533 neutral lipids (GUV) , a bilayer supplemented with triolein molecules (To-GUV) and a bilayer  
534 supplemented with triolein molecules provide by a triolein droplet (To-DEV). **Bottom:** to assess the  
535 concentration of triolein in the bilayer of the three model systems, we compute the ‘triolein in bilayer’  
536 by taking the fluorescence ratio between triolein-NBD signal and Cy5-PE signal; microscope settings  
537 were kept constant. **C)** Quantification of the triolein in bilayer for GUVs (n=6), triolein-GUVs (n=13)  
538 and triolein-DEVs (n=49) made with PC phospholipids. Mean +/- SD. T-test show no significant  
539 difference of triolein in bilayer between triolein-GUVs and triolein-DEVs. **D)** Quantification of the  
540 critical bilayer strain ( $\epsilon_{\max}$ ) of PC GUVs (n=28), triolein-GUVs (n=41) and triolein-DEVs (n=29),  
541 showing higher stretching for DEVs and GUVs containing oil. Mean +/- SD. **E)** Plot of the critical  
542 bilayer strain ( $\epsilon_{\max}$ ) for PC-PA GUVs (n=9), triolein-GUVs (n=15) and triolein-DEVs (n=6) showing  
543 higher stretching for DEVs and GUVs containing oil. Mean +/- SD. **F)** Quantification of the critical  
544 bilayer strain ( $\epsilon_{\max}$ ) of PC GUVs (n=28), squalene-GUVs (Sq-GUV) (n=7) and squalene-DEVs (Sq-  
545 DEVs) (n=21), showing higher stretching for DEVs and GUVs containing oil. Mean +/- SD.

546

547 **Figure 4: Oil molecules modify the mechanical properties of a bilayer.**

548 **A)** Bilayer lysis tension measured for GUVs (n=43), triolein-DEVs (n=24) and squalene-DEVs (n=41),  
549 made with PC. Mean +/- SD. **B)** Illustration of the experimental setup to determine the apparent area  
550 expansion modulus of a bilayer. Thanks to micropipettes, tension is measured while increasing the  
551 bilayer area. Apparent area expansion modulus (Y) is computed from the ratio between surface tension  
552 and area variation. **C)** Apparent area expansion modulus of PC GUVs (n=5), triolein-GUVs (n=7) and  
553 squalene-GUVs (n=5). Mean +/- SD. The presence of neutral lipids in the bilayer (triolein or squalene)  
554 decreases apparent area expansion modulus. see also (**Figure S4 C-E**). **D)** Experimental determination  
555 of the bending modulus (K) by pulling a nanotube from the bilayer. The bending modulus is directly  
556 linked to the nanotube radius and the membrane tension. For further details see (**Figure S4E-I**). **E)**  
557 Bending modulus of GUVs (n=7), triolein-GUVs (n=4) and squalene-GUVs (n=3) made with PC. Mean  
558 +/- SD. The presence of triolein in the bilayer results in a significant bending modulus decrease.  
559 Squalene increases the bending modulus. **E)** Area expansion modulus of PC GUVs (n=5), triolein-  
560 GUVs (n=7) and squalene-GUVs (n=5). Mean +/- SD. The presence of neutral lipids in the bilayer  
561 (triolein or squalene) decreases area expansion modulus.

562

563

564

## 565 **MATERIALS AND METHODS**

566 All experiments were performed in the following HKM buffer: 50 mM Hepes, 120 mM K-  
567 acetate, and 1 mM MgCl<sub>2</sub> (in Milli-Q water) at pH 7.4 and 280±15 mOsm.

568

### 569 **Preparation of GUVs.**

570 GUVs were prepared by electro-formation. Phospholipids and mixtures thereof in chloroform  
571 at 0.5µM were dried on an indium tin oxide (ITO) coated glass plate. The lipid film was  
572 desiccated for 1 h. The chamber was sealed with another ITO coated glass plate. The lipids  
573 were then rehydrated with a sucrose solution (280±15 mOsm). Electro-formation is performed  
574 using 100 Hz AC voltage at 1.0 to 1.4 V<sub>pp</sub> and maintained for at least 2h. This low voltage was  
575 used to avoid hydrolysis of water and dissolution of the titanium ions on the glass plate. GUVs  
576 were either stored in the chamber at 4°C overnight or directly collected with a Pasteur pipette.

577

### 578 **GUVs composition for used Phospholipids mixtures:**

579 PC GUVs were 100 % PC, PE-PC GUVs were 50% PE and 50% PC, PA-PC GUVs were 30%  
580 PA and 70% PC, DPPC-PC GUVs were 40% DPPC and 60% PC, PufaPC-PC GUVs were 30%  
581 PufaPC and 70% PC.

582

### 583 **Preparation of DEVs**

584 To prepare the oil droplets, 5 µL of the oil (triolein or squalene) was added to 70 µL of HKM  
585 buffer. The mixture was sonicated. The diameter of the resulting droplets is on the order of a  
586 few hundred nanometers. To make DEV, GUVs were then incubated with the LDs for 5 min.  
587 To make DEV with triolein-NBD, the oil was composed of 99,95% of triolein and 0,05% (w/w)  
588 of To-NBD. The GUV-LD mixture was then placed on a glass coverslip (pretreated with 10 %  
589 (w/w) BSA and washed three times with buffer) and observed with a confocal microscope.

590

### 591 **Preparation of GUVs by the inverted emulsion technique**

592 Two phospholipids mixtures were diluted in chloroform at 0.5 µM and dried. A 150µL oil  
593 volume was poured onto one mixture and 200µL of HKM-buffer onto the other one. These  
594 solutions were sonicated for 5 minutes to facilitates the solubilization of phospholipids. A  
595 100µL volume of the oil-phospholipid mixture was deposited on the HKM-phospholipids  
596 solution. Then, the remaining 50µL of the oil-phospholipid mixture was mixed with 4µL of an  
597 HKM-sucrose 1:1 (w/w) solution to generate a water-in-oil inverted micro-emulsion which was

598 then added to the HKM-contacting-oil solution. The resulting mixture was centrifuged for three  
599 minutes. The oil-containing GUVs were next collected in the aqueous volume.

600

### 601 **Confocal microscope images**

602 All micrographs of DEV were made on a Carl ZEISS LSM 800 with an X63 oil immersion  
603 objective or X10 air objective, and observed samples were held by Glass coverslips (Menzel  
604 Glaser (24x36mm #0)).

605

### 606 **Hypotonic shock**

607 DEVs were produced following the techniques described above. The resulting solution was  
608 placed on a coverslip (pretreated with 10 % (w/w) BSA and washed three times with buffer)  
609 and observed with a confocal microscope (x10 objective). Spherical DEVs with no thermal  
610 fluctuation of the bilayer have been selected so that the surface of the bilayer can be easily  
611 determined. Next, 50 $\mu$ L of MQ water was added to 120 $\mu$ L of DEV solution to applied an  
612 osmotic shock of  $\Delta C=80$ mOsm. The osmotic gradients applied were always the same  
613 ( $\Delta C=80$ mOsm for **Figure 1,2,3 and Figure S1,S2,S3**) except for (**Figure 1D,E and Figure**  
614 **S1J-M** due to permeability protocol precautions) where the  $\Delta C$  was 145mOsm and for (**Figure**  
615 **2B and Figure S2A** to report rapid triolein partitioning) where  $\Delta C$  was 210mOsm. The DEVs  
616 were then imaged overtime to follow the evolution during swelling. The same protocol was  
617 used to apply hypotonic shock to GUVs or Oil-containing-GUVs (**Figure 1F, 2D, 3D,E,F**).

618

### 619 **Determination of the critical bilayer strain $\epsilon_{max}$**

620 The maximum expansion of the bilayer is given by the following equation:

$$621 \quad \epsilon_{max} = \frac{A_{max} - A_0}{A_0}$$

622 where  $A_{max}$  and  $A_0$  are respectively the maximal areas of the bilayer before burst (stretched  
623 state) and the area before starting the swelling experiments (unstretched state). This area was  
624 determined by fitting a circle onto the DEV (GUV) bilayer and recording its radius. The radius  
625 of the embedded droplets was also recorded for DEV swelling experiments to determine the  
626 corrected surface area of the DEVs bilayer (see the section below).

627

### 628 **Bilayer surface area of the DEVs**

629 To consider only the surface of the DEV's bilayer, the surface occupied by the embedded  
630 droplet had to be removed. To do this, we calculated the surface of the sphere formed by the

631 bilayer and subtracted the surface of the spherical cap corresponding to the droplet (Figure  
632 S1C). This gave the following expression of the surface area of the DEV bilayer (A)

633

$$634 \quad A_{DEV} = 2\pi R_g^2 \cdot \left(1 + \sqrt{1 - \left(\frac{R_d}{R_g}\right)^2}\right)$$

635

636 where  $R_g$  and  $R_d$  are respectively the DEV radius and droplet radius.

637

### 638 **Permeability assay**

639 To determine permeability, we made osmotic shock experiments thanks to an applied osmotic  
640 gradient between the outside and inside of the vesicles of  $\Delta C=145\text{mOsm}$ . To do so, we added  
641  $20\mu\text{L}$  of DEVs (or GUVs) sucrose solution ( $280\pm 15\text{ mOsm}$ ) into  $100\mu\text{L}$  HKM buffer. Then,  
642 we added  $100\mu\text{L}$  of Milli-Q water to induce an osmotic shock. During the experiment, we  
643 maintained the DEVs (or the GUVs) thanks micropipettes, to have a clear determination of both  
644 vesicle radius and droplet radius (See **Figure 1C, and supplemental material 1**). This enables  
645 to determine the permeation area and the volume of the vesicle as a function of time during the  
646 swelling experiment.

647

### 648 **GUVs, triolein-DEV,s and squalene-DEVs initial permeability computation**

649 See supplemental material 1 for the computation of the initial permeability.

650

### 651 **Determination of the triolein concentration in the bilayer and following of this** 652 **concentration as a function of time**

653 To determine the concentration of triolein molecules in the bilayer, we used triolein-DEV  
654 (triolein GUVs). The triolein mixture was made of 99,95% triolein and 0,05% (w/w) of triolein-  
655 NBD. For all our experimental conditions involving these measurements (**Figures 1,2 and 3**),  
656 we chose DEVs (triolein GUVs) which radius was between 5 and 15 microns with micrometric  
657 triolein droplets. DEVs snap were taken by keeping the same settings for each DEVs. Then,  
658 fluorescence profiles were drawn perpendicular to the bilayer of triolein-DEVs (triolein GUVs)  
659 and averaged over 15 pixels. The maximum value of the profiles was recorded and averaged  
660 over multiple DEVs. The same protocol was used for Cy5-PE fluorescence signals  
661 measurements (**Figure 3C**). For monitoring of triolein concentration in the bilayer (**Figure**  
662 **S1D-E**), we captured images every 5 minutes to avoid bleaching of the triolein-NBD signal.

663

664 **FRAP experiment, triolein-NBD bleaching in the Bilayer**

665 FRAP experiments were performed by bleaching a part of the DEV bilayer (**Figure S1B-C**).  
666 To-NBD signal was bleached, then, the fluorescence signal recovery was monitored. The FRAP  
667 curve is normalized by the fluorescence before bleaching DEVs and just after the bleach (in  
668 non-bleached regions). GraphPad Prism was used to fit the FRAP recovery curve with a non-  
669 linear regression and the exponential “one-phase association model”.

670

671 **Bodipy fluorescence in squalene DEVs during hypotonic shock**

672 To follow bodipy fluorescence signal during hypotonic shock upon squalene DEVs (**Figure**  
673 **S2F**), 0,5μL of bodipy was added in 100μL of HKM buffer containing Squalene DEVs. After  
674 10 minutes, 20μL of water was added to the coverslip and the evolution of bodipy signal in the  
675 bilayer of the Squalene DEV was monitored.

676

677 **Micromanipulation & surface tension measurements by micropipette aspiration applied**  
678 **bilayer tension (MPA-applied bilayer tension).**

679 Micropipettes were made from capillaries drawn out with a Sutter Instruments pipette puller.  
680 They were used to manipulate GUVs, DEVs, or GUVs containing oil. The pipettes were  
681 incubated for 30 min in a 5% BSA solution before use, to prevent droplets and membranes from  
682 adhering to the glass.

683

684 Additionally, surface tensions of bilayers were measured using the same pipettes. Using  
685 Laplace’s law, and measuring the pipette inner radius, the GUV radius, and suction pressure,  
686 the surface tension of the bilayer can be determined by:

687 
$$\gamma = \frac{\Delta P_{suc}}{2 \left( \frac{1}{R_p} - \frac{1}{R_g} \right)}$$

688 where  $\Delta P_{suc}$ ,  $R_p$ , and  $R_g$  are the suction pressure, the pipette inner radius, and the vesicle radius,  
689 respectively

690 The suction was carried out using a syringe. The resulting pressure was measured with a  
691 pressure transducer (DP103 provided by Validyne Eng. Corp, USA), the output voltage of  
692 which was monitored with a digital voltmeter. The pressure transducer (range 55 kPa) was  
693 calibrated before the experiments. Micro-pipettes made from capillaries  
694 (1.0oDx0.58iDx150Lmm 30-0017 GC100-15b Harvard Apparatus) with a micropipette puller  
695 (Sutter instrument model P-2000). Micromanipulation (Eppendorf TransferMan! 4r). Pressure  
696 measurement unit (DP103 provided by Validyne eng. corp, USA).



697

698 For bending rigidity measurements (see below, **Bending rigidity measurements**), that needed  
699 a better measurement precision, the suction was applied thanks to a water reservoir whose  
700 height was control with a micrometer gauge (precision 0.005 mm). The resulting suction  
701 pressure was determined thanks to the measurement of the water level variation.

702

### 703 **Lysis tension measurements**

704 To determine an estimation of the bilayer lysis tension of GUVs, DEVs, or GUVs containing  
705 oil, we used the micropipette aspiration technique. Thanks to a slight aspiration, a bilayer  
706 tongue was sucked in the micropipette. The aspiration was then increased at an approximate  
707 rate of 2-3 mbar/sec, causing a proportional increase in the bilayer surface tension (see above,  
708 **Micromanipulation & surface tension measurements**). At a certain tension, the bilayer  
709 ruptured because of a pore opening. The measured lysis tension was taken as the higher tension  
710 reached just before bilayer rupture.

711

### 712 **Bending rigidity measurements**

713 First, nanotubes were pulled from GUVs or oil-GUVs: Streptavidin-coated microspheres were  
714 added to the bulk on the glass coverslip. Then, the GUV was gently captured by one of the two  
715 micro-pipettes to control surface tension  $\gamma$ . One streptavidin-coated microsphere was caught by  
716 the other micro-pipette and it was slowly moved toward the GUV. Upon contact, the  
717 biotinylated phospholipid of the GUV interacted strongly with the streptavidin-coated  
718 microsphere surface. Finally, a nanotube was pulled by removing slowly the micropipette in  
719 the opposite direction to the GUV.

720

721 The bending rigidity was obtained with the following equation:

722

$$R_t = \sqrt{\frac{\kappa}{2\gamma}}$$

723 Where  $\kappa$  is the bending rigidity of the membrane which has to be determined with the  
724 experimental measurements of the bilayer tension  $\gamma$  (see **Micromanipulation & surface  
725 tension measurements by micro-aspiration** section) and nanotube radius  $R_t$ .

726

727 To obtain  $R_t$ , we vary the tube length at a constant displacement velocity of  $2,5\mu\text{m}\cdot\text{s}^{-1}$  at  
728 constant surface tension, which keeps the radius constant based on the previous equation.

729 Therefore, for a given tension, we have many couples of tube length  $L_t$  and tongue length  $L_p$  in  
730 the micropipette. The conservation of surface yields:

$$731 \quad L_p = \delta \cdot L_t + b$$

732 Thus, from this conservation, the slope ( $\delta$ ) was extracted by measuring different couples of  $L_p$   
733 and  $L_t$ . We also checked that we do not detect hysteresis on these curves (**Figure S4H-I**) to  
734 ensure that we reached equilibrium for the oil partitioning (for triolein-GUV and squalene-  
735 GUV) between the nanotube and the GUV.

736 On the other hand, conservation of the volume of the system links the radius of the tube to that  
737 of the GUV ( $R_g$ ) and the pipette through the following equation (51):

$$738 \quad R_t = \delta \cdot \left(1 - \frac{R_p}{R_g}\right) \cdot R_p$$

739 Finally, different values of  $R_t$ , associated with a different value of  $\gamma$  were measured, gave us a  
740 mean value for  $\kappa$  with the first equation of this section.

741

#### 742 **Apparent area expansion modulus measurements**

743 GUVs or Oil-containing-GUVs were gently captured with a micropipette. Then, aspiration in  
744 the micropipette was slowly increased to obtain multiple values of membrane tension  
745 (approximately 20 sec between each point). For each value of MPA-applied bilayer tension the  
746 variation of the length of the bilayer tongue in the micropipette and the radius of the GUV was  
747 measured allowing to determine an approximate variation at the first order of the surface area  
748 of the bilayer of the GUV  $\Delta A$  (36) :

$$749 \quad \Delta A = 2\pi R_p (1 - R_p/R_g) \Delta L_p$$

750 where  $\Delta L_p$ ,  $R_p$ , and  $R_g$  are respectively the variation of the bilayer tongue length in the  
751 micropipette, the pipette inner radius, and the GUV radius.

752 The MPA-applied bilayer tension was then plotted against the relative surface area variation of  
753 the GUV's bilayer. As the GUV was already slightly tensed (initial tension above 0.1 mN/m)  
754 the membrane fluctuation is negligible and the MPA-applied bilayer tension ( $\gamma$ ) can be linked  
755 to the surface area variation ( $\Delta A/A_0$ ) by the apparent area expansion modulus ( $Y$ ):

$$756 \quad Y_{app} = \frac{\gamma}{\Delta A/A_0}$$

757 Therefore, the slope of the MPA-applied bilayer tension -surface area variation curve gave the  
758 apparent area expansion modulus of the membrane ( $Y_{app}$ ).

759

#### 760 **Area expansion modulus computing**

761 Area expansion modulus was determined following the experiment in the “apparent area  
762 expansion modulus measurements” section and previously determined bending rigidity  $\kappa$ . To  
763 plot the MPA-applied bilayer tension  $\gamma$  against the relative surface area variation  $\Delta A/A_0$  as  
764 above, the MPA-applied bilayer tension is the plot as a function of :

$$765 \quad \frac{\Delta A}{A_0} + \Delta\alpha$$

766 With

$$767 \quad \Delta\alpha = -(K_B T / 8\pi\kappa) \ln(\gamma / \gamma_0)$$

768  $K_B$  is the Boltzmann constant, T the room temperature in Kelvin and  $\gamma_0$  the initial MPA-applied  
769 bilayer tension at the beginning of the experiment. Therefore, the slope of the MPA-applied  
770 bilayer tension -  $\frac{\Delta A}{A_0} + \Delta\alpha$  curve gave the area expansion modulus (Y) (36).

771

772

## 773 **QUANTIFICATION AND STATISTICAL ANALYSIS**

### 774 **Statistical analysis**

775 The statistical comparison was made using student a parametric test where we assume a  
776 Gaussian distribution. Then the test is an unpaired t-test. The statistical analysis were made by  
777 GraphPad Prism 7.0a and “\*\*\*\*” indicates  $p < 0.0005$ , “\*\*\*” indicates  $p < 0,005$  and “\*\*”  
778 indicates  $p < 0.05$ .

779 When the bar graph is plotted, values shown in the text and figures are mean  $\pm$  S.D. When only  
780 the data set of one experiment is shown, the values on the graph are absolute value  $\pm$  uncertainty  
781 of measurements.

782

## 783 **EQUIPMENT AND REAGENTS**

### 784 **Equipment**

785 All micrographs were made on a Carl ZEISS LSM 800. Glass coverslips: Menzel Glaser  
786 (24x36mm #0). Micro-pipettes made from capillaries (1.0ODx0.58IDx150Lmm 30-0017  
787 GC100-15b Harvard Apparatus) with a micropipette puller (Sutter instrument model P-2000).  
788 Micromanipulation (Eppendorf TransferMan® 4r). Pressure measurement unit (DP103  
789 provided by Validyne eng. corp, USA).

790

### 791 **Chemical products:**

792 Phospholipids: PC (1,2-dioleoyl-sn-3glycero-3-phosphocholine), PE (1,2-dioleoyl-sn-glycero-  
793 3-phosphoethanolamine), PA (1,2-dioleoyl-sn-glycero-3-phosphate), DPPC (1,2-dipalmitoyl-  
794 sn-glycero-3-phosphocholine), PufaPC 18:0-20:4 (1-stearoyl-2-arachidonoyl-sn-glycero-3-  
795 phosphocholine), biotynil-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl), Rh-  
796 PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl) and  
797 Cy5-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Cyanine 5)) were from Avanti  
798 Polar Lipids. Bodipy 493/503 was from Thermo Fisher Scientific. Triolein and squalene were  
799 from Sigma Aldrich and To-NBD (1,3-Di(cis-9-octadecenoyl-2-((6-(7-notrobenz-2-oxa-1, 3-  
800 diazol-4-yl) amino) hexanoyl) glycerol was from Setareh biotech. Streptavidin-coated  
801 microspheres were from Bangs Laboratories. Hepes (54457–250-F), K acetate (P1190), MgCl<sub>2</sub>  
802 (M8266-100G), BSA 98% (A7906-100G), and sucrose 99.5% (59378-500G) were from Sigma-  
803 Aldrich.

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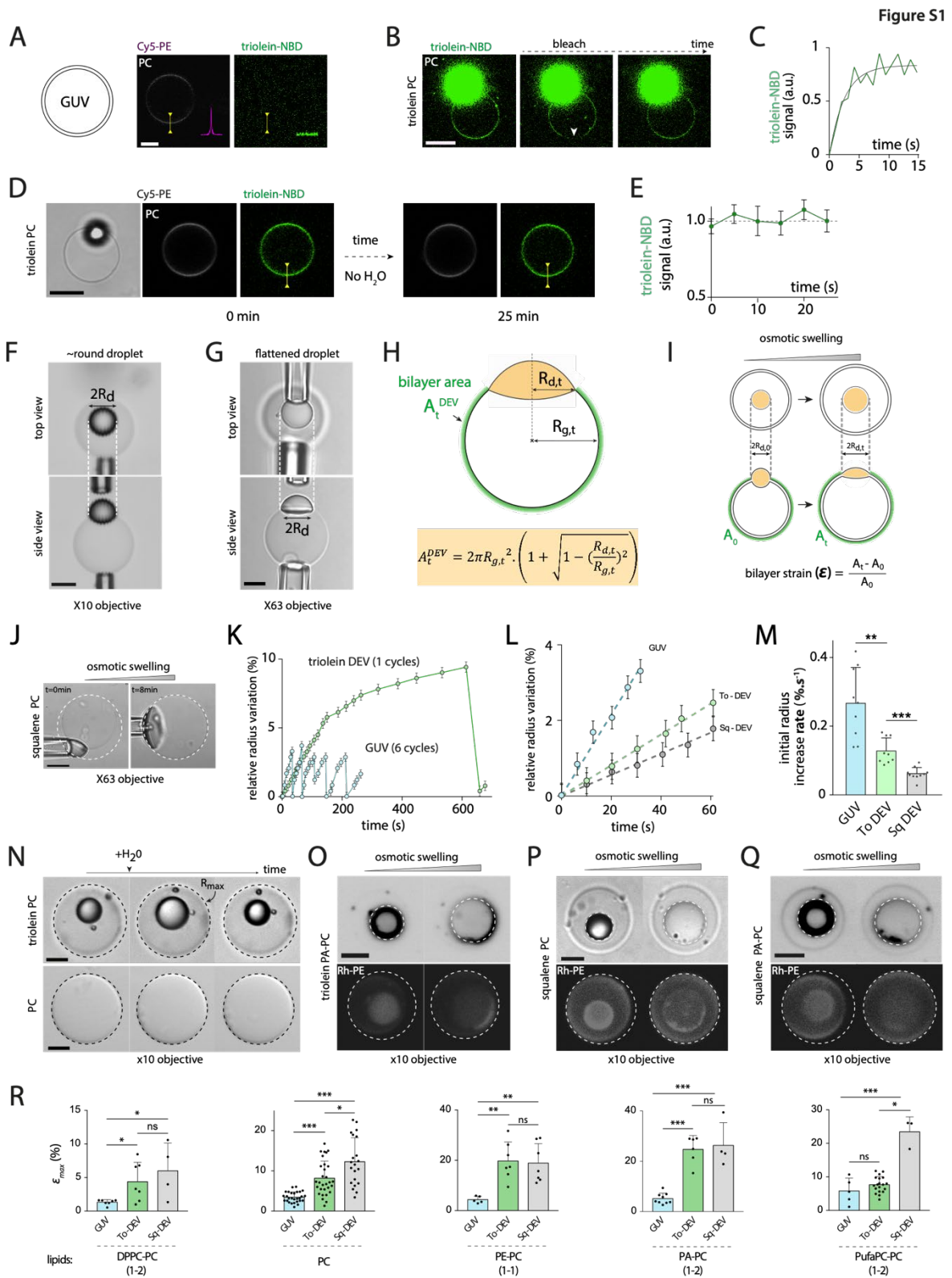
# **Fat Inclusions Strongly Alter Membrane Mechanics.**

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§ These authors contributed equally to this work

# Supplemental figures



### Figure S1: related to figure 1

**A)** Confocal images of a PC GUV marked with Cy5-PE. The same settings as in Figure 2B were used for the green channel. No signal of triolein-NBD was visible here. Scale bar: 5 $\mu$ m.

**B)** Confocal images of PC DEVs of triolein supplemented with 0,05% (w/w) triolein-NBD to report for triolein distribution. Image sequence of a FRAP (Fluorescence Recovery After Photobleaching) experiment on the bilayer. After bleaching the bilayer (see arrowhead), the triolein-NBD signal recovered. The scale bar is 5 $\mu$ m.

**C)** Over time quantification of normalized triolein-NBD fluorescence signal after bleaching showing recovery. The non-linear fit matches the FRAP recovery curve. The fit was done with an exponential “one-phase association model”.

**D)** Confocal images of PC DEVs of triolein supplemented with triolein-NBD. The triolein DEVs were imaged for 25 minutes with no osmotic shock. The scale bar is 10 $\mu$ m.

**E)** Over time variations of the triolein NBD-signal in the bilayer shows no variation of triolein concentration during 25 minutes. Absolute value +/- measurement uncertainty.

**F)** Confocal bright field images captured at x10 objective of single PC DEV of triolein at a low tension state. Top view and side view showing the same droplet radius ( $R_d$ ) used to compute the initial bilayer area of a DEV. The scale bar is 10 $\mu$ m.

**G)** Confocal bright field images captured at x63 objective of single PC DEV of triolein at a high tension state with a flattened droplet. Top view and side view showing the same droplet radius ( $R_d$ ) used to compute the final bilayer area of a DEV. The scale bar is 5 $\mu$ m.

**H) Top:** Illustration of a DEV and needed parameters to compute the DEV bilayer area subtracting the area occupied by the embedded droplet. **Bottom:** Equation used to compute the bilayer area of a DEV.

**I) Top:** Schematic representation of the bilayer area variations during osmotic swelling of a DEV (top view and side view). Green arcs underly the bilayer area. **Bottom:** Equation used to compute the bilayer strain  $\epsilon$ .

**J)** Representative images of a PC squalene-DEV observed at x63 objective during a hypotonic shock experiment. The first image corresponds to the unswollen state and the second to one of the critical bilayer strain before burst. Dashed circles report maximum radius. Scale bar: 10 $\mu$ m.

**K)** Related to Figure 1B. The plot of the relative vesicle radius variations of triolein DEV and GUV over time. Swelling and burst cycles are visible for GUVs (blue points; 6 cycles) and DEVs (green points; 1 cycle). Absolute value +/- measurement uncertainty.

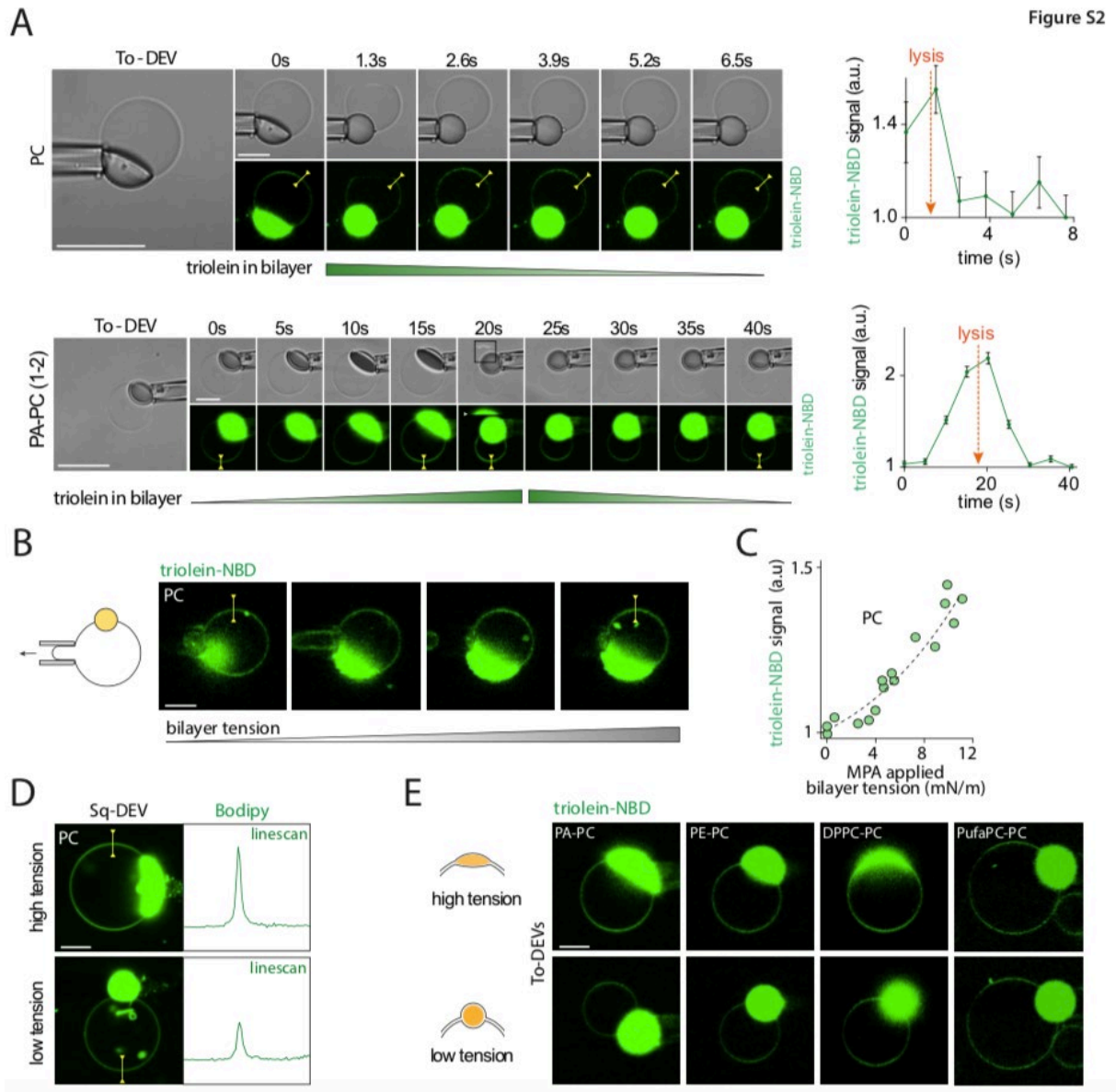
**L)** Plot of the vesicle relative radius variation at the beginning of a swelling experiment of a GUV (blue points), a triolein DEV (green points), and a squalene DEV (grey points). The slope of the linear fit (dotted lines on the graph) was used to determine the initial radius increase rate. Absolute value +/- measurement uncertainty.

**M)** Plot of the initial radius increase rate of PC bilayer of GUVs (n=8), triolein-DEVs (n=9), and squalene DEVs (n=9). Mean +/- SD.

**N)** Time-lapse of triolein DEV and GUV during a

hypotonic shock experiment. Black dashed circles report maximum radius. The scale bar is 5 $\mu$ m. **O)** Representative images of a PC-PA triolein-DEV during a hypotonic shock experiment. The first image corresponds to the unswollen state and the second to the bilayer critical strain before burst. Dashed circles report maximum radius. Scale bar: 10 $\mu$ m. **P)** Representative images of a PC squalene-DEV during a hypotonic shock experiment. The first image corresponds to the unswollen state and the second to the bilayer critical strain before burst. Dashed circles report maximum radius. Scale bar: 10 $\mu$ m. **Q)** Representative images of a PC-PA squalene-DEV during a hypotonic shock experiment. The first image corresponds to the unswollen state and the second to the bilayer critical strain before burst. Dashed circles report maximum radius. Scale bar: 10 $\mu$ m. **R)** Detailed plots of figure 1F. Critical bilayer strain is shown for each phospholipids conditions with a student t-test. Stars indicate if means are significantly different or not. Mean +/- SD.





**Figure S2: related to figure 2**

**A) Top:** Time course of confocal images of a PC triolein-DEV (left) just after the burst of the vesicle. The scale bar is 20 $\mu$ m. Corresponding quantification of the amount of oil (right) in the bilayer shows a fast recovery of low triolein level after burst (few seconds). Absolute value +/- measurement uncertainty. **Bottom:** Time course of confocal images of a PC-PA triolein-DEV (left) before and after the burst. The scale bar is 20 $\mu$ m. Corresponding quantification of the amount of oil (right) in the bilayer shows a fast increase of oil level before burst followed by a rapid decrease of oil level after burst (few seconds). Absolute value +/- measurement uncertainty.

**B) Left,** Illustration of the method used to increase the bilayer tension of a triolein-DEV thanks to the micropipette aspiration technique. **Right,** the triolein-NBD signal in the bilayer of a PC triolein-DEVs during MPA-applied bilayer tension increase. Scale bar: 5 $\mu$ m. **C)** Plot of the triolein-NBD signal in bilayer against MPA-applied bilayer tension for PC triolein-DEVs. n=4 experiments. **D)** Confocal images of PC Sq-DEVs during the swelling experiment with bodipy, a hydrophobic probe. Scale bar: 5 $\mu$ m. A line scan of the bodipy signal in the bilayer is shown both in the stretched and unstretched state. **E)** Visualization of triolein-NBD signal in the bilayer during a hypotonic shock of PA-PC, PE-PC, DPPC-PC, and PufaPC-PC of triolein-DEVs both in the stretched and unstretched state. Scale bar: 5 $\mu$ m.

A

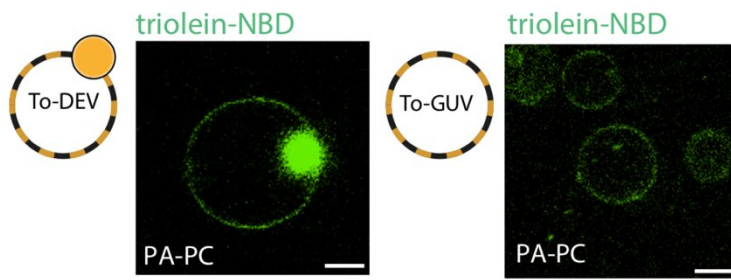
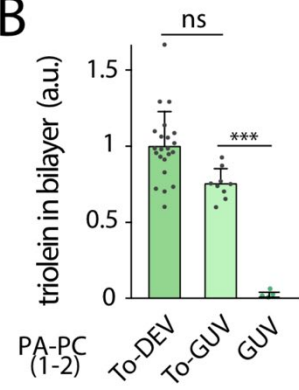


Figure S3

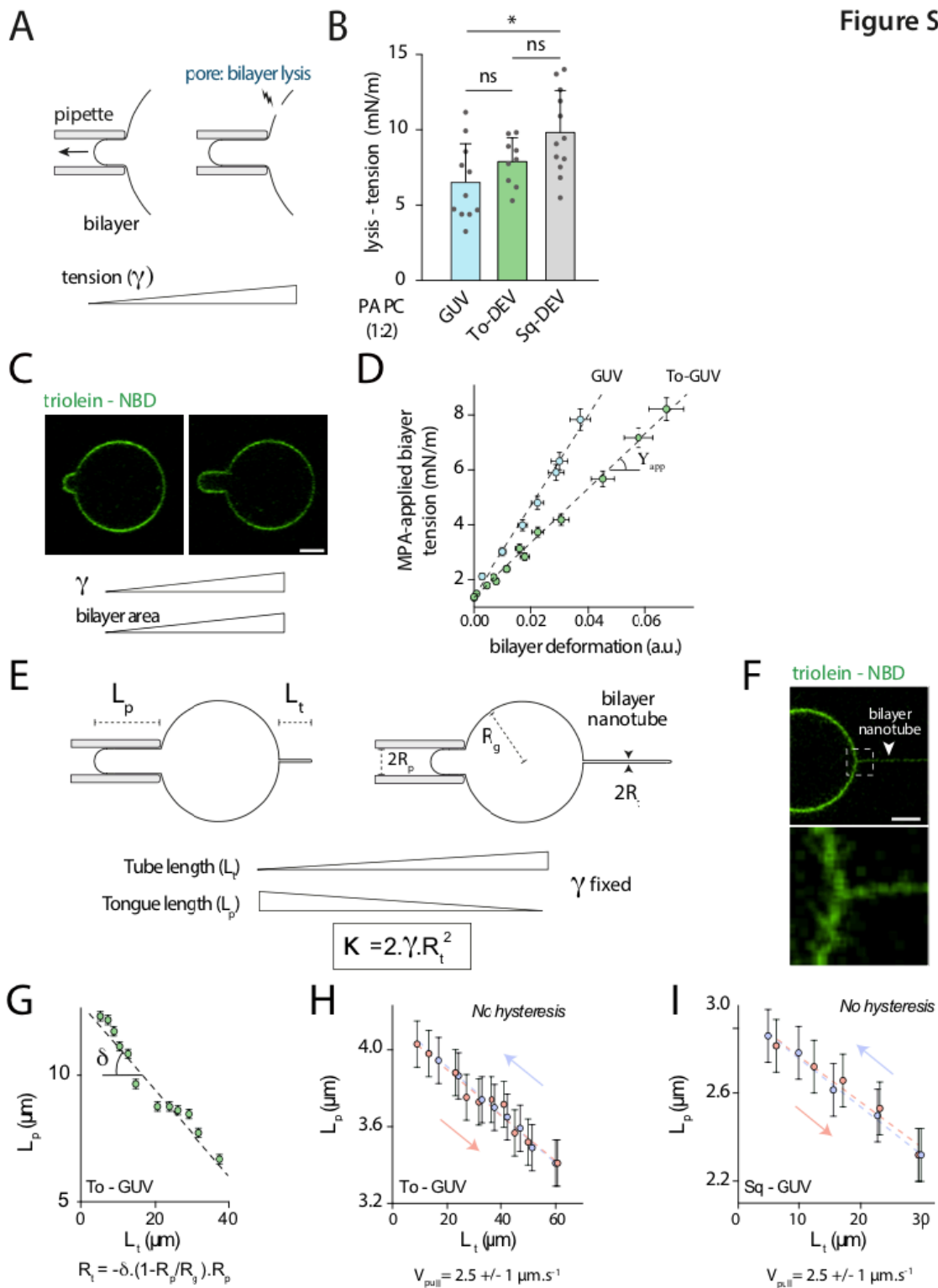
B



**Figure S3: related to figure 3**

**A)** Confocal images of PA-PC triolein-DEV and triolein-GUV with triolein-NBD. Scale bar: 5 $\mu$ m. **B)** Plot of the triolein in bilayer for PC-PA GUVs (n=4), triolein-GUVs (n=9), and triolein-DEVs (n=22). Mean +/- SD.

Figure S4



#### Figure S4: related to figure 4

**A)** Illustration of the method used for lysis tension determination. The bilayer tension of a vesicle is raised with a micropipette until the bilayer ruptures. Lysis tension is taken as the higher sustainable tension just before rupture. **B)** Membrane lysis tension of GUVs (n=11), triolein-DEVs (n=9) and squalene-DEVs (n=12) made with PC-PA. Mean +/- SD. **C)** Confocal images corresponding to the determination of the apparent area expansion modulus on a triolein-GUV with a triolein-NBD signal. Membrane tension is raised and the associated variation of the area is recorded. Scale bar: 5 $\mu$ m. **D)** Plot of the MPA-applied bilayer tension against area relative variation for a PC GUV (blue points) and a PC triolein-GUV (green points). Absolute value +/- measurement uncertainty. The slopes of the linear fits (dotted lines) are the elastic modulus of the corresponding GUV and triolein-GUV. **E)** Illustration of the determination protocol of the bending modulus computed with the membrane tension and the tube radius obtained by varying the tube length and recording the associated tongue length. **F)** Confocal images of a nanotube pulled from a PC triolein-DEV. Scale bar: 5 $\mu$ m. Zoom on the nanotube where a triolein-NBD signal is visible. Scale bar: 1 $\mu$ m. **G)** Example of a tongue length against tube length plot. The slope is linked with the nanotube radius which then allows computing the bending modulus. **H)** Tongue length against tube length plot for a triolein-GUV. The nanotube is pulled (red points) from the triolein-GUV and pushed (blue points) towards the triolein-GUV at a constant velocity of 2.5 +/- 1 $\mu$ m/s. Absolute value +/- measurement uncertainty. No hysteresis was detected indicating that oil partitioning is at the equilibrium during the tube pulling experiment. **G)** Tongue length against tube length plot for a squalene-GUV. The nanotube is pulled (red points) from the squalene-GUV and pushed (blue points) towards the squalene-GUV at a constant velocity of 2.5 +/- 1 $\mu$ m/s. Absolute value +/- measurement uncertainty. No hysteresis was detected indicating that oil partitioning is at equilibrium during the experiment.

## Supplemental Text.

### Supplementary materials: initial permeability determination for GUVs and DEVs

#### A. Fick law: conditions of utilization

Diffusion times of solutes through a phospholipid bilayer are negligible compared to the diffusion time of water. We therefore considered the diffusion of solutes through the bilayer of GUVs (or DEVs) as negligible compared to the diffusion of water. In this condition, Fick law leads to:

$$\frac{dV_t}{dt} = -P \cdot S_t \cdot v_m \cdot \Delta C(t)$$

where  $V_t$  is the volume inside the vesicle delimited by  $S_t$  the area of permeation (ie area of bilayer),  $P$  the bilayer permeability to water,  $v_m$  the molar volume of water ( $18\text{g}\cdot\text{mol}^{-1}$ ) and  $\Delta C(t)$  the difference of solutes concentration between the outside  $C^e(t)$  and the inside  $C^i(t)$  of the vesicle.

$$\Delta C(t) = C^e(t) - C^i(t)$$

#### B. $\Delta C(t)$ : hypothesis and computation as a function of $R_{g,t}$

In our experiments, we considered only the first cycle of swelling and the first minute of experiment because it is difficult to characterize the concentration of solutes inside a GUV or a DEV after the first burst. We assume the concentration of solutes in the buffer to be constant and equal to the initial outside concentration  $C_0^e$  ( $145\text{mOsm}$ ) as the buffer volume ( $220\mu\text{L}$ ) is far greater than the volume of the vesicles having a radius between  $5\mu\text{m}$  and  $15\mu\text{m}$  (between  $1\text{pL}$  and  $15\text{pL}$  in volume). The concentration in the vesicle is equal to the initial one adjusted by the dilution factor  $\frac{V_0}{V_t}$  where  $V_0$  is the initial volume of the vesicle.

$$\Delta C(t) = C_0^e - C_0^i \cdot \frac{V_0}{V_t} = C_0^i \cdot \left( \frac{C_0^e}{C_0^i} - \frac{R_{g,0}^3}{R_{g,t}^3} \right)$$

Where  $R_{g,t}$  and  $R_{g,0}$  are respectively the vesicle radius at the time  $t$  and at the beginning of swelling (**Figure 1**). As we have small variations of radius ( $<10\%$ ). We made the approximation that:

$$R_{g,t} = \delta R_{g,t} + R_{g,0}$$

where  $\delta R_{g,t}$  represent the small variations of vesicle radius.

It follows that:

$$\Delta C(t) = C_0^i \cdot \left( \frac{C_0^e}{C_0^i} - \frac{1}{\left(1 + \frac{\delta R_{g,t}}{R_{g,0}}\right)^3} \right)$$

At the first order we have:

$$\Delta C(t) = C_0^i \cdot \left( \frac{C_0^e}{C_0^i} + \left( 3 \frac{\delta R_{g,t}}{R_{g,0}} - 1 \right) \right)$$

Then we have:

$$\Delta C(t) = C_0^i \cdot \left( \frac{C_0^e}{C_0^i} + 3 \frac{R_{g,t}}{R_{g,0}} - 4 \right)$$

Now that  $\Delta C(t)$  is determine, in next, C&D, we will determine the exact surface and volume of DEV involve in the Fick law.

### C. GUV and DEV permeation surface: hypothesis and computation as a function of $R_{g,t}$

The permeation surface of the GUV ( $S_t^{GUV}$ ) corresponds to the surface of the sphere formed by its bilayer and can be expressed as:

$$S_t^{GUV} = 4\pi R_{g,t}^2$$

In the case of the DEV, the water cannot pass through the oil droplet. Thus to assess the permeation surface of a DEV ( $S_t^{DEV}$ ) we have to subtract the surface area occupied by the droplet  $S_t^{cap}$  from the surface area of the sphere forming its bilayer  $S_t^{GUV}$  (see **Figure 1**) :

$$S_t^{DEV} = S_t^{GUV} - S_t^{cap}$$



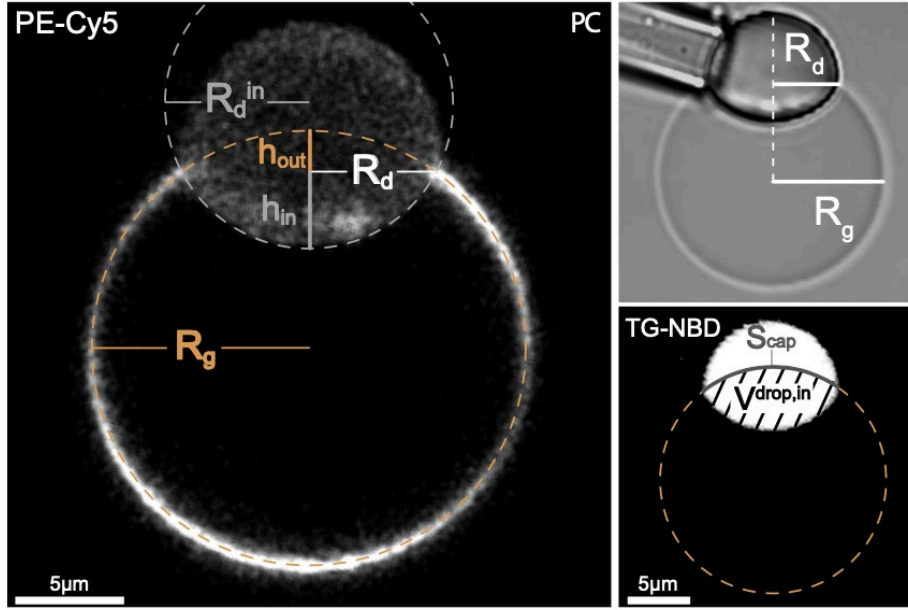


Figure 1: triolein-DEVs of DOPC at x63 objective showing parameters to compute the corrected surface and volume of a DEV.

Note that both  $S_t^{cap}$  and  $S_t^{GUV}$  (Figure 1) are varying over time due to the variations of the vesicle  $R_{g,t}$  and the droplet radii  $R_{d,t}$  (Figure 2).

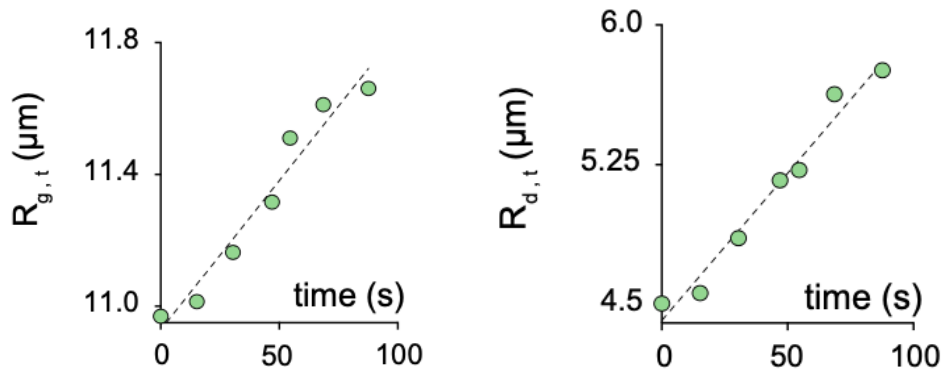


Figure 2: Plot of  $R_g$  and  $R_d$  as a function of time

$S_t^{cap}$  can be calculated as follow:

$$S_t^{cap} = 2\pi R_{g,t}^2 \cdot \left( 1 - \sqrt{1 - \left(\frac{R_{d,t}}{R_{g,t}}\right)^2} \right)$$

Following, we have  $S_t^{DEV}$ :

$$S_t^{DEV} = 2\pi R_{g,t}^2 \cdot \left( 1 + \sqrt{1 - \left(\frac{R_{d,t}}{R_{g,t}}\right)^2} \right)$$

Compared to an equivalent droplet-free vesicle, the permeation area of the DEV must therefore be corrected by the following factor:

$$\beta_t = \frac{S_t^{DEV}}{S_t^{GUV}} = \frac{1}{2} \left( 1 + \sqrt{1 - \left( \frac{R_{d,t}}{R_{g,t}} \right)^2} \right)$$

Correction factor  $\beta_t$ :

As the radius of the vesicle ( $R_{g,t}$ ) and the radius of the droplet ( $R_{d,t}$ ) evolved during the osmotic swelling (**Figure 2**), we calculated  $\beta_t$  over time and found that it only varied from 0,922 to 0,899. These variations are represented in **Figure 3**.

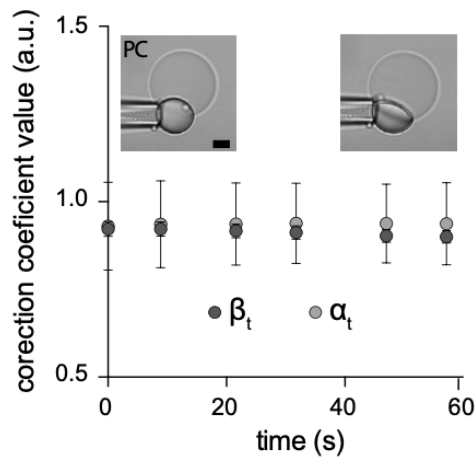


Figure 3: Plot of the correction coefficient  $\alpha$  and  $\beta$

In order to better quantify this small decrease of  $\beta_t$ , we calculated the ratio between the initial  $\beta_t$  and its value after 60 second of each osmotic swelling experiment ( $\beta_{60}/\beta_0$ , Figure 4, right). We find that  $\beta_t$  has very small variations:  $2,15 \pm 0.67 \%$  and  $1,04 \pm 0.69 \%$  respectively for To-DEV and Sq-DEV (**Figure 4, right**). Based on these observations, we considered  $\beta_t = \beta_0$ . We plotted all the value of  $\beta_0$  in (**Figure 4, left**).

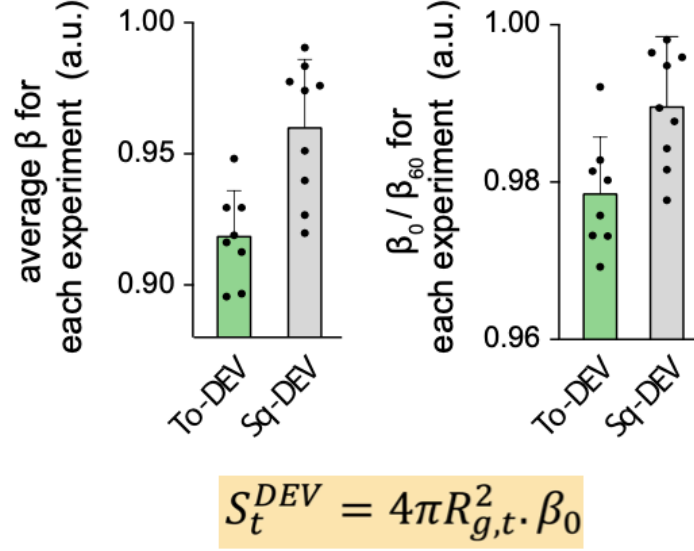


Figure 4: Left: Mean value of  $\beta$  for DEVs permeability assay; Right: Variations of  $\beta$  during permeability experiment.

We thus have a surface of permeation  $S_t$  which can be written as:

$$S_t = \beta_0 \cdot 4\pi R_{g,t}^2 \quad \text{for DEV and} \quad S_t = 4\pi R_{g,t}^2 \quad \text{for GUVs}$$

#### D. GUV and DEV volume: hypothesis and computation as a function of $R_{g,t}$

For the volume of a GUV,  $V_t$  can be expressed as:

$$V_t^{GUV} = \frac{4}{3}\pi R_{g,t}^3$$

The inside DEV volume  $V_t^{DEV}$  has to be corrected because of the presence of the droplet:

$$V_t^{DEV} = V_t^{GUV} - V_t^{drop,in}$$

where  $V_t^{drop,in}$  is the lacking of aqueous volume in the vesicle occupied by the embedded droplet (see Figure S1A).  $V_t^{DEV}$  can be expressed as:

$$V_t^{DEV} = \frac{4}{3}\pi R_{g,t}^3 - \frac{\pi}{3}(h_{out,t}^2(3R_{g,t} - h_{out,t}) + (h_{in,t}^2(3R_{drop,t}^{in} - h_{in,t})))$$

where  $h_{out,t}$ ,  $h_{in,t}$  and  $R_{drop,t}^{in}$  are all defined in **Figure 1**.

It follows:

$$\frac{V_t^{DEV}}{V_t^{GUV}} = 1 - \frac{(h_{out,t}^2(3R_{g,t} - h_{out,t}) + (h_{in,t}^2(3R_{drop,t}^{in} - h_{in,t})))}{4R_{g,t}^3} = \alpha_t$$

For the experiment in **figure 2** the variation of  $\alpha_t$  are plotted as a function of time (**Figure 3**).

Correction factor  $\alpha_t$ :

Where  $\alpha_t$  is the correction factor of the DEV inner volume compared to the one of GUVs. As we did previously for  $\beta_t$ ,  $\alpha_t$  is plotted as a function of time (Figure 3). The variation of  $\alpha_t$  is also very small, from 0,930 to 0,937. We also examined for each experiment the variations of  $\alpha_t$  during the first 60 seconds  $\alpha_{60}/\alpha_0$  (Figure 5, right) which were very small:  $1,11 \pm 0,35 \%$  and  $0,50 \pm 0,48 \%$  respectively for To-DEV and Sq-DEV (**Figure 5, right**). Based on these observations, we considered  $\alpha_t = \alpha_0$ . We plotted all the value of  $\alpha_0$  in (**Figure 5 left**).

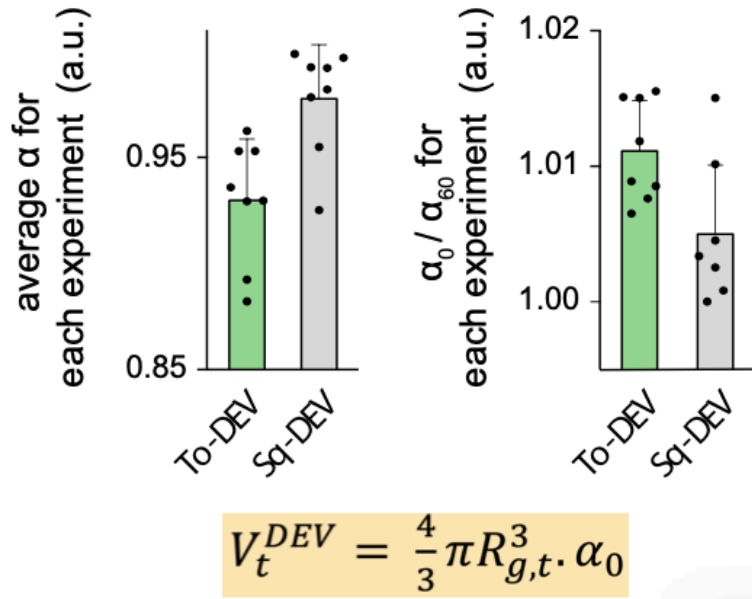


Figure 5: Left: Mean value of  $\alpha$  for DEVs permeability assay; Right: Variations of  $\alpha$  during permeability experiment.

In conclusion, the volume of permeation of a DEV and a GUV write as:

$$V_t = \alpha_0 \cdot \frac{4}{3} \pi R_{g,t}^3 \quad \text{for DEVs and} \quad V_t = \frac{4}{3} \pi R_{g,t}^3 \quad \text{for GUVs}$$

### E. Fick law with $R_{gt}$ as the sole variable:

Considering the expression  $\Delta C(t)$  established in B. one obtains:

$$\frac{dV_t}{dt} = -P \cdot S_t \cdot v_m \cdot C_0^i \cdot \left( \frac{C_0^e}{C_0^i} + 3 \frac{R_{g,t}}{R_{g,0}} - 4 \right)$$

Replacing  $V_t$  and  $S_t$  by their expression obtained in C. and D leads to:

$$\frac{d\left(\alpha_0 \cdot \frac{4}{3} \pi R_{g,t}^3\right)}{dt} = -P \cdot \beta_0 \cdot 4\pi R_{g,t}^2 \cdot v_m \cdot C_0^i \cdot \left( \frac{C_0^e}{C_0^i} + 3 \frac{R_{g,t}}{R_{g,0}} - 4 \right)$$

We obtain a differential equation of the first order with a constant second member:

$$\frac{d\left(\frac{R_{g,t}}{R_{g,0}}\right)}{dt} + \frac{3P \cdot \beta_0 \cdot \alpha_0^{-1} \cdot v_m \cdot C_0^i}{R_{g,0}} \frac{R_{g,t}}{R_{g,0}} = \frac{P \cdot \beta_0 \cdot \alpha_0^{-1} \cdot v_m \cdot C_0^i}{R_{g,0}} \cdot \left(4 - \frac{C_0^e}{C_0^i}\right)$$

So:

$$\frac{d\left(\frac{R_{g,t}}{R_{g,0}}\right)}{dt} + a \frac{R_{g,t}}{R_{g,0}} = \frac{a}{3} \left(4 - \frac{C_0^e}{C_0^i}\right) \quad \text{where} \quad a = \frac{3P \cdot \beta_0 \cdot \alpha_0^{-1} \cdot v_m \cdot C_0^i}{R_{g,0}}$$

As we have linear curve for  $\frac{R_{g,t}}{R_{g,0}}$  for the first 60 seconds (**Figure S1L**),  $\frac{d\left(\frac{R_{g,t}}{R_{g,0}}\right)}{dt} = \omega$  is constant and equal to the slope of  $\frac{R_{g,t}}{R_{g,0}}$ .

$$\omega = \left(4 - \frac{C_0^e}{C_0^i}\right) - 3 \frac{\langle R_{g,t} \rangle}{R_{g,0}} \frac{P_{initial} \cdot \beta_0 \cdot \alpha_0^{-1} \cdot v_m \cdot C_0^i}{R_{g,0}}$$

Here,  $P_{initial}$ , “initial permeability” replaces the permeability because we considered only initial slope at the beginning of experiment. For a given experiment,  $\langle R_{g,t} \rangle$  is the mean of  $R_{g,t}$  in the first 60 seconds.

$$P_{initial} = \frac{\omega \cdot R_{g,0}}{\beta_0 \cdot \alpha_0^{-1} \cdot v_m \cdot \left(4 - 3 \frac{\langle R_{g,t} \rangle}{R_{g,0}}\right) C_0^i - C_0^e}$$

With this expression, we determined the initial permeability to water  $P_{initial}$  for PC GUVs, PC triolein-DEVs and PC squalene-DEVs. (**Principal Figure 1E, left**)

