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## ► To cite this version:

Abdou Rachid Thiam, Aymeric Chorlay, Lionel Forêt. Origin of gradients in lipid density and surface tension between connected lipid droplet and bilayer. *Biophysical Journal*, 2021, 120 (24), pp.5491-5503. 10.1016/j.bpj.2021.11.022 . hal-03452801

**HAL Id: hal-03452801**

**<https://hal.science/hal-03452801>**

Submitted on 8 Jan 2024

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1 **Origin of gradients in lipid density and surface tension between connected**  
2 **lipid droplet and bilayer**

3

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23

24 **Abstract.**

25

26 We combined theory and experiments to depict physical parameters modulating the phospholipid (PL)  
27 density and tension equilibrium between a bilayer and an oil droplet in contiguity. This situation is  
28 encountered during a neutral lipid (NL) droplet formation in the endoplasmic reticulum. We set up  
29 macroscopic and microscopic models to uncover free parameters and the origin of molecular  
30 interactions controlling the PL densities of the droplet monolayer and the bilayer. The established  
31 physical laws and predictions agreed with experiments performed with droplet-embedded vesicles. We  
32 found that the droplet monolayer is always by a few percent (~10%) less packed with PLs than the  
33 bilayer. Such a density gradient arises from PL-NL interactions on the droplet, which are lower than  
34 PL-PL trans-interactions in the bilayer, i.e., interactions between PLs belonging to different leaflets of  
35 the bilayer. Finally, despite the pseudo-surface tension for the water/PL acyl chains in the bilayer  
36 being higher than the water/NL surface tension, the droplet monolayer always has a higher surface  
37 tension than the bilayer because of its lower PL density. Thus, a PL density gradient is mandatory to  
38 maintain the mechanical and thermodynamic equilibrium of the droplet-bilayer continuity. Our study  
39 sheds light on the origin of the molecular interactions responsible for the unique surface properties of  
40 lipid droplets compared to cellular bilayer membranes.

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43

44 **Significance.**

45

46 Organelles' biogenesis and function rely on membrane mechanics and phospholipid composition.  
47 Lipid droplets (LDs) are unique cell organelles because of their neutral lipid oil core shielded by a  
48 single phospholipid monolayer. LD biogenesis happens in the endoplasmic reticulum (ER) bilayer,  
49 which unzips to encapsulate the developing LD that subsequently buds off in the cytoplasm. The ER  
50 cytoplasmic monolayer leaflet thus equilibrates with that of the forming LD. Experimental data and  
51 simulations support that this equilibration happens with different phospholipid densities and tensions  
52 between the bilayer and monolayer, but the underlining mechanisms remain unknown. A theoretical  
53 framework combined with experiments enabled us to reveal these mechanisms.

54

55

56 **Introduction.**

57 Except for lipid droplets (LDs), most cellular organelles are bilayer bounded (1). LDs are made of  
58 neutral lipids, which are non-membrane lipids and often oily molecules. These neutral lipids are  
59 encapsulated in the core of the LDs, covered by a single phospholipid monolayer in which proteins are  
60 embedded. LD forms in the ER bilayer following a multiple steps process (2, 3). Neutral lipids are  
61 synthesized and sheltered in the ER bilayer's hydrophobic region. The neutral lipids phase separates  
62 from the ER bilayer at a critical concentration and condenses into an LD (4–6). At this stage, the LD is  
63 contiguous with the ER bilayer and can exchange proteins and lipids with its membrane (7). The  
64 nucleated LDs grow by acquiring more neutral lipids and proteins. At maturation, LDs can be  
65 physically disconnected from the ER bilayer but may reconnect with it later to exchange content (8, 9).  
66 Hence, the droplet monolayer surface is functionally connected to the ER bilayer to maintain the  
67 homeostasis of lipids and proteins of the LDs (10–12). A crucial question is how these organelles  
68 manage to share phospholipids and proteins while keeping their respective identity. Answering this  
69 question will help understand LD biogenesis and protein targeting principles (13–17). In particular,  
70 understanding how the density of phospholipids is set between contiguous bilayer and monolayer  
71 interfaces will allow better knowing mechanisms of LD assembly and protein binding (13, 16–18).

72  
73 Important parameters controlling LD biogenesis are the monolayer and bilayer surface tension and  
74 curvature (19–21), which depend on the phospholipid composition of the organelles (16, 22, 23). The  
75 phospholipid density regulates both the LD monolayer surface tension and its accessibility to proteins  
76 (8, 16, 24–28). Firstly, while the ER bilayer can behave as an infinite phospholipid reservoir to the  
77 connected LD, reconstitution approaches, cell data, and molecular dynamics simulations suggest that  
78 the droplet monolayer is less packed with phospholipids than the ER bilayer (25, 29–31). Such a  
79 discrepancy partly explains the higher recruitment of peripheral or monotopic membrane proteins to  
80 LDs than bilayer membranes (24, 25, 30, 32, 33). Secondly, while the ER bilayer is of low surface  
81 tension, circa 0.01mN/m (34), cellular LDs, made from and contiguous with the ER, have a surface  
82 tension above 1mN/m (19, 20), two orders of magnitudes higher. This tension difference is crucial for  
83 the proper emergence of LDs from the ER bilayer (16, 17, 19). Reasons for the discrepancies in  
84 phospholipid densities and surface tensions between LDs and the ER bilayer are unclear as yet.  
85 Understanding the molecular basis of such differences between the two contiguous organelles will  
86 improve our knowledge of their biology.

87  
88 Whether the physical properties of phospholipid bilayers and monolayers are identical was raised  
89 several decades ago (35–37) because both are self-assembled interfaces with essential functions and  
90 applications in biology and physical science. For example, early studies focused on the kinetics and  
91 mechanics of the phospholipid monolayers of air/water or water/oil interfaces for better understanding  
92 the dynamics of lung surfactants (38) or emulsions' stability for digestion purposes (32, 33), etc. In the  
93 meantime, biophysical approaches allowed studying the mechanics and dynamics of phospholipid  
94 bilayers, an important branch of membrane biology (41–43). Altogether, these pioneer focuses on  
95 phospholipid interfaces led to the genesis of several theoretical frameworks describing the mechanics  
96 and thermodynamics of phospholipid-coated fluid interfaces (35, 36, 44). However, because LDs have

97 gained focus relatively lately, little or no application was devoted to studying the case of the  
98 phospholipid monolayer interface covering an LD emerging from the ER bilayer. Mechanisms  
99 controlling the phospholipid density and surface tension equilibrium between a droplet monolayer  
100 jointed to a bilayer are currently unknown. We decided to capitalize on the established theoretical  
101 frameworks to uncover them.

102

103 This paper aims to unveil the molecular origin of mechanisms maintaining the uneven phospholipid  
104 density between a droplet monolayer connected to a bilayer (Figure 1A). We customized macroscopic  
105 and microscopic frameworks of such a situation and established the relationship between the free  
106 parameters of the system.

107

108 Experiments validated our theoretical predictions, and this agreement allowed us to highlight  
109 molecular interactions underlying the difference in phospholipid densities and surface tensions  
110 between the droplet monolayer and bilayer. These interactions involve (a) the adhesion between the  
111 phospholipid monolayer leaflets forming the bilayer, which does not exist on a droplet monolayer, and  
112 (b) a pseudo-surface tension –resulting from the interaction between water molecules and  
113 phospholipid acyl chains of the bilayer– higher than the classical water/oil surface tensions. Keeping a  
114 phospholipid density difference is essential for the mechanical and thermodynamic equilibrium of the  
115 droplet and bilayer contiguity.

116

117

118

## 119 **Results.**

120 The result section is organized as follow. We first present the thermodynamic equilibrium relations,  
121 which govern the droplet morphology and the phospholipid distribution between the droplet monolayer  
122 interfaces and the bilayer. In the second part, we analyze one important and experimentally testable  
123 consequence of the equilibrium conditions: the interdependence between the surface tensions of the  
124 droplet and the bilayer, which reflects the coupling between the monolayer and bilayer phospholipid  
125 densities. The theoretical predictions made from this second part are successfully verified  
126 experimentally in the third part. In the fourth part, we present a microscopic theoretical description of  
127 the contiguous monolayers and bilayer, enabling us to derive the state equations of these interfaces.  
128 These equations are then used to understand the molecular origin of the density difference (part five)  
129 and surface tension difference (part six) between the monolayers and the bilayer. In the last part, a  
130 quantitative comparison between theory and experiments is made to validate the theoretical approach  
131 and its underlying hypothesis.

132

### 133 **Thermomechanical equilibrium of a lipid droplet connected to a phospholipid bilayer**

134 We consider a droplet of fixed volume, above tens of nm in size, connected to a phospholipid (PL)  
135 bilayer (Figure 1A). At this scale, the contribution of the bending elasticity and long-range interactions  
136 of the different interfaces is negligible (17, 19). Hence, the PL bilayer and the droplet interfaces can be  
137 described as homogeneous fluid interfaces joining at the droplet edge (17). A line tension arises at this  
138 contact, but its contribution to the order of tens of pN is negligible with respect to surface tension  
139 forces (17) (Discussion of the origin and influence of the line tension in Supplement text sections 1 and  
140 3). The free energy of the droplet embedded in a bilayer is then,

141

$$142 \quad F = A_b f_b(\rho_{be}, \rho_{bi}) + A_{me} f_m(\rho_{me}) + A_{mi} f_m(\rho_{mi}), \quad (1)$$

143

144 where  $A_b$ ,  $A_{me}$ ,  $A_{mi}$  are the area of the three interfaces (bilayer and monolayers covering the LD at the  
145 “external” and “internal” side);  $\rho_{be}, \rho_{bi}$ ,  $\rho_{me}, \rho_{mi}$  are the PL surface density in each bilayer leaflet and  
146 each monolayer (Fig. 1C);  $f_b$  and  $f_m$  are the free energy per surface unit of a PL bilayer and a PL  
147 monolayer at the water/oil interface. (A more thorough discussion of the assumptions leading to  
148 equation (1) in Supplement text section 1). We consider that PLs freely diffuse and equilibrate  
149 between the droplet and the bilayer (Figure 1A). PL flip-flop, which takes several hours or days under  
150 passive conditions (45, 46), is much slower than the lateral diffusion of PLs ( $\sim \mu\text{m}^2/\text{sec}$ ) (21) and is  
151 negligible at relevant time scales (Figure 1A). Also, experimentally, PLs are insoluble in the neutral  
152 lipid oil phase, which is why they self-assemble into droplet interface bilayers (19); therefore, PL  
153 exchange does not occur through the droplet in the relevant time scales. Under these conditions, the  
154 PL number of each leaflet is constant,

155

$$156 \quad N_e = \rho_{me} A_{me} + \rho_{be} A_b = \text{constant} \quad \text{and} \quad N_i = \rho_{mi} A_{mi} + \rho_{bi} A_b = \text{constant}, \quad (2)$$

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158 where  $N_e$  and  $N_i$  can be different.

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At equilibrium, the morphology of the connected droplet and bilayer, as well as the distribution of PL between the droplet and the bilayer, minimize the free energy (equation (1)), under the constraints of equation (2) and of fixed droplet volume. This minimization yields the usual equilibrium conditions presented in the following.

The droplet and bilayer shapes obey the laws of three-fluid phase wetting systems (47). The interfaces must have a constant mean curvature (Laplace law). We focused on axisymmetric geometries for which the droplet interfaces are spherical caps, and the bilayer can either be flat or bear a constant curvature. The droplet morphology is fully characterized by the contact angles between the droplet interfaces and the bilayer,  $\theta_e$  and  $\theta_i$  (Figure 1B). These angles are fixed by the balance of the forces acting at the contact line (Figure 1B):  $\vec{\gamma}_{me} + \vec{\gamma}_{mi} + \vec{\gamma}_b = 0$  or,

$$\gamma_{me} \cos \theta_e + \gamma_{mi} \cos \theta_i + \gamma_b = 0, \quad \gamma_{me} \sin \theta_e = \gamma_{mi} \sin \theta_i, \quad (3)$$

where  $\gamma_{me}$ ,  $\gamma_{mi}$ ,  $\gamma_b$  are respectively the surface tension of the external and internal monolayers of the droplet ( $me$ ,  $mi$ ), and the bilayer ( $b$ ). In vitro experiments (20) confirmed the validity of equation (3) to describe the morphology of large membrane-embedded droplets. In the particular case, where the bilayer is tensionless,  $\gamma_b = 0$ , the angles are not unequivocally determined but must be such that  $\theta_e + \theta_i = 180^\circ$ , which implies that the droplet is spherical.

The PL partitioning between the bilayer and droplet monolayers (Figure 1C) is controlled by the chemical potentials equilibrium,

$$\mu_{me} = \mu_{be} \quad \text{and} \quad \mu_{mi} = \mu_{bi}, \quad (4)$$

where  $\mu_{me}$ ,  $\mu_{mi}$ ,  $\mu_{be}$ ,  $\mu_{bi}$  respectively denote the chemical potential of the external and internal monolayers of either the droplet ( $me$ ,  $mi$ ) or the bilayer ( $be$ ,  $bi$ ). A monolayer and its contiguous bilayer leaflet have equal chemical potentials. Because of the rapid diffusion of PLs, the chemical potential equilibrium is quickly reached, much faster than the growth rate of a cellular LD (~minutes) or the time scale of perturbations imposed in droplet-embedded vesicles (16, 20).

Surface tensions and chemical potentials are functions of the PL densities:  $\gamma_{me} = \gamma_m(\rho_{me})$ ,  $\gamma_{mi} = \gamma_m(\rho_{mi})$ ,  $\mu_{me} = \mu_m(\rho_{me})$ ,  $\mu_{mi} = \mu_m(\rho_{mi})$ ,  $\gamma_b(\rho_{be}, \rho_{bi})$ ,  $\mu_{be} = \mu_b(\rho_{be}, \rho_{bi})$ ,  $\mu_{bi} = \mu_b(\rho_{bi}, \rho_{be})$ , where  $\gamma_m$ ,  $\mu_m$ ,  $\gamma_b$ ,  $\mu_b$  can be expressed in terms of the free energy densities  $f_m$  and  $f_b$  defined in equation (1) and their derivatives, equations (MM1- MM3) (Materials and Methods). It follows that the relations (3) and (4) are interdependent: a variation in PL densities modulates tensions, which alter the contact angles (equation (3)) and interface areas, as the droplet volume is fixed. Conversely, changes in morphology, and, hence, in interfacial areas, redistribute PLs because of mass conservation (equation (2)) and, subsequently, modify chemical potentials (equation (4)).

199

200 In conclusion, for a given oil volume, number of PLs in each membrane leaflet and bilayer tension, the  
201 PL densities,  $\rho_{me}$ ,  $\rho_{mi}$ ,  $\rho_{be}$ ,  $\rho_{bi}$ , and the contact angles,  $\theta_e$ ,  $\theta_i$ , adjust in a coupled manner to fulfill  
202 the thermomechanical equilibrium conditions (equations (3), (4)).

203

#### 204 **Coupling between phospholipid densities and between surface tensions**

205 The above equilibrium relations impose an interdependence between the different interfaces' PL  
206 density and, thus, the droplet and bilayer surface tensions via two distinct mechanisms.

207

208 The first coupling mechanism pertains to the chemical potential equilibriums, equation (4). Indeed, a  
209 slight variation in surface tension of one interface will modify the density of this interface. In response,  
210 PLs will redistribute between the adjacent interfaces to re-establish the equilibrium of the chemical  
211 potentials (equation (3)). Such an adjustment will change the respective PL densities and, hence, the  
212 surface tensions. The link between variations of surface tension and chemical potentials of an  
213 interface, due to variation of its PL density, is expressed by the Gibbs-Duhem relations,

214

$$215 \quad d\gamma_m = -\rho_m d\mu_m \quad \text{and} \quad d\gamma_b = -\rho_{be} d\mu_{be} - \rho_{bi} d\mu_{bi} . \quad (5)$$

216

217 Combining these relations and the differentials of equation (4),  $d\mu_{me} = d\mu_{be}$ ,  $d\mu_{mi} = d\mu_{bi}$ , we obtain  
218 a general link between surface tension variations,

219

$$220 \quad d\gamma_b = \frac{\rho_{be}}{\rho_{me}} d\gamma_{me} + \frac{\rho_{bi}}{\rho_{mi}} d\gamma_{mi} . \quad (6)$$

221

222 This first coupling due to the chemical potential equilibrium (4) is the unique coupling in two practical  
223 and biologically interesting situations: a symmetrical bilayer and an infinite bilayer reservoir.

224 In a symmetrical membrane,  $\rho_{be} = \rho_{bi} = \rho_b$  and  $\rho_{me} = \rho_{mi} = \rho_m$ , the balance of chemical potentials  
225 (equation (4)) reads  $\mu_m(\rho_m) = \mu_b(\rho_b)$  and therefore imposes an unequivocal relation between bilayer  
226 and monolayer tensions,  $\gamma_b(\rho_b)$  and  $\gamma_m(\rho_m)$ . Thereupon, equation (6) becomes,

227

$$228 \quad d\gamma_m = \frac{1}{2} \frac{\rho_m}{\rho_b} d\gamma_b \quad (7)$$

229

230 This relation indicates that the droplet interfacial tension increases with the bilayer one with a slope  
231 proportional to the ratio of their PL densities.

232

233 For a droplet embedded in a large ( $A_b \gg A_{me,mi}$ ) and possibly asymmetrical membrane, the bilayer  
234 behaves as an infinite PL reservoir of fixed PL density, independent of the state of the droplet. This  
235 configuration is more relevant in cell conditions when nascent LDs form in the ER membrane. In this  
236 case, a similar relation as equation (7) holds,

237



238 
$$d\gamma_{me} = \beta_e \frac{\rho_{me}}{\rho_{be}} d\gamma_b \quad (8)$$

239

240 and  $d\gamma_{mi} = \beta_i \frac{\rho_{mi}}{\rho_{bi}} d\gamma_b$  with  $\beta_i = 1 - \beta_e$  (derivation of equation (8) and definition of  $\beta_e$  in Supplement  
 241 text, section 6.3). This equation shows that the monolayer tension should also increase linearly with  
 242 the bilayer ones as for a symmetrical membrane, but with a numerical pre-factor depending on the  
 243 membrane asymmetry;  $\beta_e > 1/2$  if the external leaflet is less dense than the internal one and vice-  
 244 versa.

245

246 A second density coupling mechanism exists for small (bilayer area comparable to droplet area) and  
 247 asymmetrical membranes. This coupling arises from the PL number conservation (equation (2)) and  
 248 the mechanical equilibrium (equation (3)), together linking the PL distribution to the system's  
 249 morphology. A change in surface tension affects the morphology (equation (3)), i.e., the surface area  
 250 of the interfaces, which in turn impacts the densities (equation (2)), and hence tensions. No simple  
 251 mathematical relations resembling equation (6) expressing this coupling can be derived.

252

### 253 **Experimental determination of the interdependence between surface tensions**

254 We wanted to experimentally test the theoretical findings by probing the interdependence between the  
 255 droplet and bilayer surface tensions. For this purpose, we used the droplet-embedded vesicle system  
 256 (DEV) (19, 48) (Figure 2A), which mimics the theoretically described situation of an oil droplet in  
 257 contiguity with a bilayer. DEVs were formed by inserting triolein droplets within the bilayer of giant  
 258 unilamellar vesicles made of DOPC (1,2-dioleoyl-sn-3glycero-3-phosphocholine) (48).

259 The external droplet monolayer and the bilayer of the DEV were captured by two micropipettes (Figure  
 260 2B). We selected DEVs with a symmetrically positioned droplet, *i.e.*, identical contact angles  $\theta_e = \theta_i$ ,  
 261 which, consequently, have similar tension,  $\gamma_{me} = \gamma_{mi}$  (equation (3)). These DEVs meet the symmetric  
 262 condition leading to equation (7), which can be tested experimentally. To establish the tension  
 263 dependency predicted by this equation, we imposed the DEV bilayer tension step by step, by fixing the  
 264 aspiration pressure of the micropipette, and concomitantly measured the micropipette-aspiration-  
 265 applied surface tension of the external monolayer of the droplet (Figure 2B). We found that the  
 266 monolayer tension increased with the bilayer tension (Figure 2C), agreeing with the theoretical  
 267 description: increasing the bilayer tension decreases the chemical potential in the bilayer leaflets; to  
 268 keep the chemical potential equilibrium, the droplet must, therefore, supply PLs to the bilayer, resulting  
 269 in its tension increase.

270 Interestingly, the tensions' dependency followed a linear trend line (Figure 2C). Based on equation (7),  
 271 this linear dependency suggests that the ratio of the monolayer-to-bilayer PL density did not vary,  
 272 independently of the bilayer tension. The slope  $\frac{\rho_m}{2\rho_b}$  of  $0.45 \pm 0.02$ , indicates that the bilayer PL density  
 273 is slightly higher than the monolayer one, agreeing with our previous fluorescence and droplet  
 274 tensiometer measurements where this value was of 0.435 (30).

275

276 The PL density is maximum, i.e., close to full packing, in a tensionless bilayer. Since the droplet  
277 monolayer PL density evolves like the bilayer one, one may speculate that it also reaches near close  
278 packing when the bilayer is tensionless. Thereupon, the monolayer tension would also be close to  
279 zero. Such assumption was not valid as, when the bilayer tension fell to almost zero ( $<0.01$  mN/m),  
280 the monolayer tension was non-negligible, around 1.5 mN/m (Figure 2C). We initially hypothesized  
281 that the non-zero tension of the monolayer could be due to the nature of the tensionless bilayer of the  
282 DEV, which may have been unable to supply PLs to the droplet. To test this hypothesis, we changed  
283 the system and put the droplet in contact with another type of PL reservoir.

284  
285 At the tip of a tensiometer apparatus, we generated a triolein oil-in-buffer droplet, over-supplied with  
286 PLs in the oil (0.5% w/w to triolein) (Figure 2D, left). In this setting, the oil phase acts as a PL reservoir  
287 to the droplet interface. As soon as the droplet was formed in the buffer solution, the surface tension  
288 dropped down, due to the spontaneous adsorption of PLs to the interface, to reach an equilibrium  
289 value of 1.15 mN/m (Figure 2D, Figure S1D); without PLs, the triolein/buffer tension is  $\sim 34$  mN/m.  
290 Thus, even with this reservoir, the monolayer tension was non-zero and close to the  $\sim 1.5$  mN/m  
291 obtained with DEV (Figure 2C). Next, starting from the equilibrium tension at 1.15mN/m, we  
292 mechanically compressed the PL monolayer of the droplet by sucking off the droplet volume (Figure  
293 2D, Figure S1E). Even under this action, surface tension decreased but was still not close to zero,  
294  $\sim 0.6$  mN/m, compared with the tensionless bilayer ( $<0.01$  mN/m). These results indicate that, unlike a  
295 bilayer, a droplet monolayer has specific features, detailed hereafter, that prevent it from reaching a  
296 high PL density and near-zero surface tension.

297  
298 Altogether, the experiments with DEVs are consistent with the theoretical prediction (equation (7)). For  
299 symmetrical conditions, the monolayer tension responds linearly to an increase in bilayer tension,  
300 implying that the phospholipid density ratio is constant between the bilayer and the monolayer.  
301 However, our results revealed two essential aspects: (i) through the bilayer and the droplet are  
302 connected, the PL density is lower for the droplet monolayer than the bilayer, and (ii) the tensions of  
303 the droplet and the bilayer are largely different in general; even in contact with a bilayer of very low  
304 tension, the droplet remains at relatively high tension (above 1 mN/m). In the following sections, we  
305 delve deeper into these findings with a theoretical approach, subsequently validated by a quantitative  
306 analysis of the surface tensions curves of DEVs.

307

### 308 **Microscopic description of the contiguous droplet-bilayer system**

309 To pinpoint the origin of the molecular interactions responsible for the discrepancy in tension and PL  
310 density between the monolayer and bilayer, we developed a microscopic description closely following  
311 the approach and assumptions of refs. (36, 44). The goal is to express the equations of state relating  
312 surface tensions, chemical potentials, and densities, for a monolayer and a bilayer.

313

314 The different contributions to the free energy of a monolayer and a bilayer are depicted in (Figure 3A).  
 315 The free energy per surface unit of a monolayer at a water/oil interface function of the PL density can  
 316 be written as,

$$317$$

$$318 \quad f_m(\rho) = \gamma_{o/w} + f_{\text{int}}(\rho) + f_{\text{hyd}}(\rho) + f_{\text{mo}}(\rho) . \quad (9)$$

319 The four terms account respectively for the bare oil/water surface tension, lateral PL - PL interactions,  
 320 hydrophilic interaction of PL head groups with water, PL tails interaction with oil. The free energy  
 321 density of the bilayer is,

$$322$$

$$323 \quad f_b(\rho_{be}, \rho_{bi}) = f_{\text{int}}(\rho_{be}) + f_{\text{hyd}}(\rho_{be}) + f_{\text{phob}}(\rho_{be}) + f_{\text{int}}(\rho_{bi}) + f_{\text{hyd}}(\rho_{bi}) + f_{\text{phob}}(\rho_{bi}) + f_{\text{mm}}(\rho_{be}, \rho_{bi}) . \quad (10)$$

$$324$$

325 The functions  $f_{\text{int}}$  and  $f_{\text{hyd}}$  in equation (10), which account for the lateral interactions between PLs and  
 326 from PL head – water in a bilayer leaflet, are identical to the ones in equation (9) for the monolayer.  
 327 The term  $f_{\text{mm}}$  accounts for the *trans* interaction between the two leaflets, *i.e.*, the interaction between  
 328 the tails of PLs belonging to different leaflets. Lastly,  $f_{\text{phob}}$  accounts for the hydrophobic interaction of  
 329 PL tails with water, responsible for the cohesion of bilayer in water. Following ref. (37), this contribution  
 330 is supposed to be negligible for monolayer (and is thus absent in equation (9)) and nearly constant  
 331  $f_{\text{phob}}(\rho) = \gamma_{\text{phob}}$  for bilayer, with the parameter  $\gamma_{\text{phob}}$  of the order 34 to 39 mN/m for DOPC. The  
 332 contributions  $f_{\text{mo}}$  and  $f_{\text{mm}}$  are assumed to be linear in PL densities (37),  $f_{\text{mo}}(\rho) = \varepsilon_{\text{mo}}\rho$  and  
 333  $f_{\text{mm}}(\rho_{be}, \rho_{bi}) = \varepsilon_{\text{mo}}(\rho_{be} + \rho_{bi})$ , *i.e.*, the free energy per PL molecule due to PL tail interaction (with oil  
 334 in a monolayer and with the tails of PL in the other leaflet in a bilayer),  $\varepsilon_{\text{mo}}$  and  $\varepsilon_{\text{mm}}$  are constant  
 335 parameters.

336  
 337 The surface tension and the chemical potential of a monolayer, functions of the PL density, can be  
 338 deduced from the free energy equation (9) and equation (MM1) (material and method):

$$339$$

$$340 \quad \gamma_m(\rho) = \gamma_{o/w} - \pi(\rho) \quad (11)$$

341 and

$$342 \quad \mu_m(\rho) = \int \frac{\pi'(\rho)}{\rho} d\rho + \varepsilon_{\text{mo}} , \quad (12)$$

343 where the prime denotes the derivative of the function. The quantity  $\pi(\rho)$ , named the lateral pressure,  
 344 is defined as  $\pi(\rho) = \rho (f_{\text{int}}'(\rho) + f_{\text{hyd}}'(\rho)) - f_{\text{int}}(\rho) - f_{\text{hyd}}(\rho)$ : it arises from lateral PL - PL interactions  
 345 and hydrophilic PL head interactions. This lateral pressure can be measured and well described  
 346 theoretically by classical equations of state of two-dimensional fluids, such as the van der Waals  
 347 equation of state (37), see below.

348 The surface tension of a bilayer function and the chemical potential of one of its leaflets, functions of  
 349 the leaflet densities, are deduced from equation (10) and equations (MM2-MM3) (Materials and  
 350 Method), and read,

$$351 \quad \gamma_b(\rho_{be}, \rho_{bi}) = 2\gamma_{phob} - \pi(\rho_{be}) - \pi(\rho_{bi}) \quad (13)$$

352 and

$$353 \quad \mu_b(\rho_b) = \mu_m(\rho_b) + \varepsilon_{mm} - \varepsilon_{mo} \quad (14)$$

354 (The chemical potential of a leaflet is independent of the density of the other leaflet under our  
 355 assumptions.)

356

357 A central parameter in these state equations (11 – 14) is the lateral pressure  $\pi(\rho)$ . By using the  
 358 pending drop experiment, described Fig. 2C and ref. (30), we obtained  $\pi(\rho)$  for a DOPC monolayer at  
 359 the water – TO interface (Figure 3B, Figure S2A); it was well described by a van der Waals state  
 360 equation,  $\pi(\rho) = \frac{kT\rho}{1-a\rho} - u\rho^2$ . The parameter  $a$  characterizes the excluded area due to steric repulsion  
 361 and  $u$  the strength of the lateral interactions between PL molecules (Materials and Methods, Figure  
 362 S2B). The determination of  $\pi(\rho)$  enabled us to determine (up to a constant)  $\mu_m(\rho)$  using equation (12)  
 363 (Figure 3C). Note that the exact profile of  $\pi(\rho)$  depends on the PL type, yet the global features of the  
 364 curves in Fig. 3B and C should be generic.

365

366 Combining the state equations (11 - 14) with the equilibrium conditions (4), we discuss in the following  
 367 two sections the origin of the density difference between the connected monolayers and bilayer, and  
 368 then the basis of their surface tension difference. Additionally, these equations of state allow  
 369 performing a complete quantitative analysis of the surface tension curves obtained for DEVs, in  
 370 particular in the case of asymmetric DEVs (last section).

371

### 372 **Droplet-bilayer phospholipid equilibration following the microscopic description**

373 PL partitioning is governed by the equilibrium condition (4): the PL densities in adjacent monolayer  
 374 and bilayer leaflet,  $\rho_m$  and  $\rho_b$ , are such that  $\mu_m(\rho_m) = \mu_b(\rho_b)$ . The chemical potentials of the  
 375 monolayer and bilayer leaflet,  $\mu_b$  and  $\mu_m$ , functions of the PL density, as given by the state equations  
 376 (12) and (14), are shown in Figure 3C. The two curves have the same profiles, but are shifted by  
 377  $\varepsilon_{mm} - \varepsilon_{mo}$ . Then, for the chemical potentials to be equal, the densities of contiguous monolayer and  
 378 bilayer must be different in general,  $\rho_m \neq \rho_b$ , as long as  $\varepsilon_{mm} \neq \varepsilon_{mo}$ . Thereupon, the microscopic  
 379 description (equation (14)) pinpoints the parameter  $\varepsilon_{mm} - \varepsilon_{mo}$  as the primary lever imposing the  
 380 phospholipid density difference between the monolayer and bilayer. We remind that this parameter  
 381 quantifies the difference in interactions of PL tails with their underneath environments which are: the  
 382 tails of PLs in the opposite leaflet for the bilayer, and the NL oil phase for the droplet monolayer  
 383 (Figure 3A). Depending on the sign of  $\varepsilon_{mm} - \varepsilon_{mo}$ , the monolayer lipid density is always larger or  
 384 smaller than the bilayer leaflet density.

385

386 However, according to the surface tension measurements (Figure 2C) and equation (5), the PL  
 387 density is expected to be lower at the droplet interface  $\rho_m < \rho_b$ , meaning that  $\varepsilon_{mm} - \varepsilon_{mo} < 0$ .  
 388 Consequently, trans-interactions between PL tails in the bilayer are energetically more favorable than  
 389 the interactions of PL tails with oil molecules in the droplet.

390

391 Assuming a slight relative difference of PL density between the droplet monolayer and bilayer leaflet,  
 392 and expanding the equilibrium relation  $\mu_b(\rho_b) = \mu_m(\rho_m)$  to the linear order (Supplement text, section  
 393 8), one obtains

$$394 \quad \frac{\rho_b - \rho_m}{\rho_b} \simeq \frac{\varepsilon_{mo} - \varepsilon_{mm}}{\pi'(\rho_b)} \quad (15)$$

395

396 This equation shows that the relative density difference between the bilayer and droplet monolayer is  
 397 modulated by  $\varepsilon_{mm} - \varepsilon_{mo}$  (Figure 3A). A bilayer is stable only in a narrow range of PL density, near  
 398 close packing, and can be stretched by only about 5% (49, 50). Consequently,  $\pi'(\rho_b)$  should be very  
 399 large and, according to equation (15), the relative difference in density between a bilayer leaflet and its  
 400 contiguous droplet monolayer should be relatively small. This interpretation agrees with the density  
 401 ratio obtained from the slope of Figure 2C using equation (7) and our recent experimental finding in  
 402 DEVs (30), suggesting that for 100 PLs on a bilayer leaflet, droplet monolayer in contiguity with this  
 403 leaflet has ~90 PLs.

404

#### 405 **Surface tensions comparison following the microscopic description**

406 Combining the previous results on the PL distribution with the state equations (11, 13) allows  
 407 understanding how the droplet tension compares with the bilayer one. In particular, we wanted to  
 408 understand why the droplet remains with a non-zero tension while being in contact with a tensionless  
 409 bilayer (Figure 2C).

410 According to equation (11), the droplet surface tension is maximal in the absence of PLs, equal to  
 411  $\gamma_{o/w}$ , and decreases when the PL density or the lateral pressure increases. The bilayer tension has a  
 412 similar dependency on the PL density (equation 13), with  $\gamma_{phob}$  being reminiscent of  $\gamma_{o/w}$ , though they  
 413 have different molecular origins (Figure 3A). As found above, the bilayer PL density should always be  
 414 slightly higher than the droplet monolayer one. Consequently, the monolayer should be of lower lateral  
 415 pressure than the bilayer leaflet, i.e.,  $\pi(\rho_b) > \pi(\rho_m)$ .

416 At zero bilayer tension, the mechanical equilibrium condition (3) imposes the same tension and thus  
 417 the same PL densities between the two droplet monolayers. It follows from equation (4) that the two  
 418 bilayer leaflets also have the same density,  $\rho_b^0$ . Under this condition, the lateral pressure in the bilayer  
 419 balances exactly the hydrophobic contribution in equation (11),  $\gamma_{phob} = \pi(\rho_b^0)$ . On the droplet  
 420 monolayer side, the lateral pressure is lower, i.e.,  $\pi(\rho_m^0) < \pi(\rho_b^0)$ , and too small to completely cancel  
 421 the tension of the droplet interface (equation (10)),  $\gamma_m^0 = \gamma_{o/w} - \pi(\rho_m^0) \neq 0$ . Assuming a slight relative  
 422 difference in PL density between a monolayer and a bilayer leaflet (30), the tension of a droplet  
 423 embedded in a tensionless bilayer can be approximately expressed by (Supplement text, section 8),

424

425 
$$\gamma_m^0 \simeq \gamma_{o/w} - \gamma_{phob} + \rho_b^0(\varepsilon_{mo} - \varepsilon_{mm})$$
 (16)

426

427 This equation shows that  $\gamma_{o/w} - \gamma_{phob}$  and  $\varepsilon_{mo} - \varepsilon_{mm}$ , which controls the monolayer-bilayer density  
428 mismatch, are the two key parameters keeping the droplet monolayer tension to a non-zero value  
429 when the bilayer is tension-free.

430

431 The parameters  $\gamma_{o/w}$  and  $\varepsilon_{mo}$ , are a function of the oil type. Thus, changing the oil type should vary the  
432 tension of the droplet when the bilayer is tensionless (equation (11)). To test this prediction, we  
433 established the monolayer-bilayer tension diagram of DEVs made with different oils. Namely, we used  
434 a triolein-sterol ester mixture or squalene (Figure 3D-E). Independently of the oil type, a linear trend  
435 was observed for symmetric DEVs, as expected from equation (7). The slopes were also conserved,  
436 confirming that these oils' monolayer-bilayer densities were similar (30). However, the droplet tension  
437 at zero bilayer tension was significantly different (Figure 3E), agreeing with our prediction from the  
438 theoretical analysis (equation (16)). At very low bilayer tension ( $<0.1$  mN/m), the squalene droplet had  
439 a lower tension than the triolein one (Figure 3E). Since  $\gamma_{o/w}$  was respectively 23.7 mN/m and 34 mN/m  
440 for squalene and triolein, the term  $|\gamma_{o/w} - \gamma_{phob}|$  would be lower for squalene, which, following  
441 equation (16), would partly explain the lower droplet monolayer tension value for squalene.

442

#### 443 **Validation of the microscopic model by the quantitative analysis of the DEV system**

444 We wished to compare quantitatively experimental data obtained with the DEV system and theory  
445 output to go beyond the qualitative agreement between theoretical predictions and experimental  
446 observations. A quantitative agreement would eventually validate our theoretical approach and the  
447 underlying hypothesis.

448

449 We built a complete theoretical description of the DEV system based on the thermodynamic approach  
450 developed in the preceding sections (Supplementary text, section 7). The model allows us to compute  
451 all the characteristics of a DEV, in particular the surface tension of the different interfaces, which are  
452 experimentally measurable and tunable observables. The parameters of the model are of two origins:  
453 geometrical and physicochemical. The geometrical parameters (oil volume, vesicle inner volume and  
454 leaflet asymmetry in PL number) are specific to each DEV and are experimentally obtained by image  
455 analysis. The physicochemical (or microscopic) parameters are the ones in the state equations (11-14)  
456 and only depend on the PL and oil types composing the DEV. The physicochemical parameters  $\gamma_{o/w}$   
457 and the lateral pressure were determined by the drop tensiometer method (Figure S2B). The only  
458 unknown physicochemical parameters of the model are hence  $\gamma_{phob}$  and  $\varepsilon_{mo} - \varepsilon_{mm}$  (Figure 3A). We  
459 adjusted these two parameters to fit the model to the surface tensions data of Figure 2C, obtained with  
460 a symmetric DEV. We found that  $\gamma_{phob} = 38.6$  mN/m, close to reported values for DOPC (36), and  
461  $\varepsilon_{mm} - \varepsilon_{mo} = -4.4$  pN.nm =  $1.1 k_B T$  (Table Figure 4B). The value of  $\varepsilon_{mm} - \varepsilon_{mo}$ , looks rather low but  
462 sufficient enough to proffer a higher PL packing to the bilayer than the monolayer (equation (14)). This  
463 means that the small differences between the PL tail - NL interactions on the droplet and the tail-tail

464 interactions between PLs on opposite leaflets of the bilayer are sufficient to induce a PL density  
465 gradient between the bilayer and the monolayer.

466

467 By definition, the above values of the different physicochemical (or microscopic) parameters should  
468 only be set by molecular interactions, exclusively depending on the PL and oil type and not on DEV  
469 geometry. Therefore, the values of these parameters should allow fitting the model for any DEV  
470 geometry. To test this prediction, we considered DEVs, made of DOPC vesicles and triolein droplets of  
471 different sizes, and different asymmetries in DOPC number between their internal and external  
472 leaflets. PL asymmetry is obvious at low bilayer tension, as the droplets are spherical (16, 20) and  
473 differently positioned in the bilayer (16, 20): the droplets are either centered,  $\theta_e = \pi/2$ , or emerged  
474 outward,  $\theta_e < \pi/2$ , or inward,  $\theta_e > \pi/2$ , (Figure 4A, Figure S3 A-D). We took advantage of this  
475 variability in DEV geometry, i.e., in size and asymmetry, to test whether the microscopic parameters'  
476 values (Table Figure 4B) allow us to predict the correct DEVs tensions for all DEV geometry.

477

478 For each DEV (Figure S3 A-D), including the one in Figure 2C, we measured the tension of the  
479 external droplet monolayer  $\gamma_{me}$  against the bilayer one  $\gamma_b$  (Figure 4A, data points); we also determined  
480 by image analysis the DEV geometrical parameters (size and droplet position asymmetry). Then,  
481 using the microscopic parameters' values (Table Figure 4B), we computed  $\gamma_{me}$  as a function of  $\gamma_b$  for  
482 each DEV imposing specific geometrical constraints (Figure 4A, model, solid lines). Strikingly, the  
483 computed curves were perfectly superposed to each of the experimental data they modeled, without  
484 any adjustment of the microscopic parameters (Figure 4A). Given the number of experimental data  
485 points, the perfect match with the model, with the pre-adjustment of only two microscopic parameters,  
486 i.e.,  $\gamma_{phob}$  and  $\varepsilon_{mo} - \varepsilon_{mm}$ , supports the validity of the theoretical approach.

487

488 Two additional features also support the validity of our model. First, at zero bilayer tension, the  
489 tensions of the droplet interfaces are the same for all the DEVs (Figure 4A), which had different  
490 geometries. This observation agrees with equation (14), which predicts in this regime that the tensions  
491 of the LD interfaces should exclusively depend on the microscopic parameters and be independent of  
492 DEV size and morphology. Second, the slope of  $\gamma_{me}(\gamma_b)$  curve depends on the droplet position at zero  
493 bilayer tension (Figure 4A). The more the droplet budded inward, the steepest was the slope  $d\gamma_{me}/$   
494  $d\gamma_b$ . The exact expression of the slope  $d\gamma_{me}/d\gamma_b$  at  $\gamma_b = 0$  predicted by the theory is provided in  
495 supplement text, section 6.4 and, indeed, confirms that the larger  $\theta_e$  at zero tension, the larger  
496  $d\gamma_{me}/d\gamma_b$ .

497

498 In conclusion, the comparison of the DEV experimental data to the theory output showed a  
499 quantitative agreement, which supports the validity of our theoretical approach and its underlying  
500 hypothesis.

501

502

503 **Discussion.**

504 Lipid droplets have multiple biological functions that rely on the targeted proteins to their surface and  
505 their surface tension. The phospholipid monolayer of lipid droplets represents a barrier for proteins to  
506 access to lipid droplets and determine their tension (13, 14, 18, 19, 51). Since lipid droplets share a  
507 hemimembrane with the endoplasmic reticulum bilayer, at least during the steps of lipid droplet  
508 assembly (52), understanding how phospholipids partition between the bilayer and the droplet  
509 monolayer is crucial.

510

511 We developed macroscopic and microscopic models to establish the relationship between the  
512 system's free parameters composed of an oil droplet embedded in a phospholipid bilayer. We  
513 subsequently set up experiments based on droplet-embedded vesicles to test the theoretical outputs  
514 and found strong agreements. Such achievement led us to pinpoint molecular parameters or  
515 interactions responsible for the unequal phospholipid density and surface tensions between the  
516 droplet and the bilayer.

517 The phospholipid density discrepancy between the droplet monolayer and the bilayer in contiguity  
518 essentially pertains to the different environments underneath a PL layer in the bilayer and the droplet  
519 monolayer. For the bilayer, the PL-tails of a leaflet interact with the PL-tails of the counterpart leaflet  
520 (the interaction energy per molecule is  $\epsilon_{mm}$ ) while in the monolayer, the PL-tails interact with the NL  
521 oil phase beneath the monolayer (the interaction energy per molecule is  $\epsilon_{mo}$ ) (Figure 3A, C). At a  
522 constant bilayer tension, increasing  $\epsilon_{mm} - \epsilon_{mo}$ , for example by increasing the affinity between oil  
523 molecules and phospholipid acyl chains, will result in a phospholipid flux from the bilayer to the  
524 droplet, thereby increasing the droplet phospholipid density (Figure 4C,  $\rho_m$ ). Such an improvement in  
525 phospholipid cover will induce a decrease in the droplet tension and, consequently, the spreading of  
526 the droplet, according to equation (3). Quantitatively, a 2 pN.nm variation of  $\epsilon_{mm} - \epsilon_{mo}$ , at fixed bilayer  
527 tension, will result in a variation in density of the order of a few percent (Figure 4D).

528 Should the bilayer present an asymmetry of phospholipid composition between its two leaflets, the  
529 droplet's inner and outer phospholipid monolayers may experience different interactions with oil  
530 molecules and therefore have different densities. Such an asymmetric monolayer phospholipid  
531 distribution will promote the droplet emergence to the better-covered monolayer (16).

532 The bilayer tension also impacts the phospholipid densities (Figure 4E). If the bilayer tension is  
533 increased, the densities of the bilayer and monolayer will decrease simultaneously. However, the  
534 relative difference between the droplet density and the bilayer density remains almost constant.

535

536 Phospholipid packing voids occur more often when triacylglycerols are infused in a bilayer (25, 27, 29,  
537 32, 49). Such observations already suggested, at least qualitatively, that a lipid droplet connected to a  
538 bilayer would be less packed with phospholipids. This is confirmed by fluorescence measurement  
539 showing that a droplet monolayer connected to a bilayer is ~13% less packed with phospholipids than  
540 the bilayer. Our approach in this paper provided an alternative measurement of the relative  
541 phospholipid densities but, most importantly, it points to the origin of such phospholipid density  
542 gradient.



543

544 Previous experimental and theoretical studies illustrated the relevance of the surface tension  
545 difference between a lipid droplet and its host membrane (16, 17, 20, 53). This tension gap controls to  
546 a large extent the morphology of droplet, its stability and budding capacity. While the tension of  
547 organelles such as the ER is very low (34), the surface tension of LD forming in their membrane is  
548 much larger (19). This could seem surprising since the monolayers at the LD interfaces and the host  
549 membrane leaflets are connected. The present work shows how the surface tensions of the forming  
550 LD and host membrane are coupled in general and reveals the microscopic origin of the different  
551 tensions. Our results explains how a lipid droplet connected to a nearly tensionless membrane can  
552 keep a relatively high surface tension.

553

554 Finally, our theoretical approach may present two limitations. First, our model does not explicitly  
555 consider the partitioning of oil molecules from the droplet to the bilayer hydrophobic region, as seen in  
556 DEVs, which alter the membrane's biophysical properties (49). From a theoretical standpoint, it will be  
557 interesting to include the presence of these oil molecules to gain accuracy in the microscopic  
558 description. Nonetheless, the match between our theoretical predictions and experimental data,  
559 including fluorescent density measurements with DEVs (30), suggests that the oil-free description  
560 captured the dominant mechanisms controlling the phospholipid densities, as well as the  
561 interdependence between surface tensions. Secondly, in the biologically relevant tensions, below 0.1  
562 mN/m (34), the bilayer tension mostly affects the excess area stored in the thermal undulations (54,  
563 55) rather than the intermolecular distance between phospholipids in the bilayer. In other words,  
564 tension impacts the effective density, *i.e.* the inverse of the projected area per molecule, rather than  
565 the real surface density. This effect of thermal undulations is not included in our theoretical description,  
566 in particular in the equation of state (13) for the bilayer tension. Our model might thus be unable to  
567 precisely reproduce  $\gamma_{me}(\gamma_b)$  curves (Figure 4A) for very low bilayer tension, provided such curves  
568 could be obtained experimentally in this range. However, the most important effect expected is that in  
569 the low-tension regime, the real surface density of the bilayer is always very close to the zero-tension  
570 density  $\rho_m^0$  and then the monolayer tension close to  $\gamma_m^0$  (equation 16). Then, the conclusions drawn for  
571 the zero-bilayer tension case should hold more generally in all the low bilayer tension regime.

572

573 In conclusion, our present study highlights critical parameters that may modulate the phospholipid  
574 density at the surface of lipid droplets connected to the endoplasmic reticulum. More generally, our  
575 approach may be upgraded to study how phospholipid mixtures, and even protein mixtures,  
576 redistribute or partition between the bilayer and the droplet, a crucial question in lipid droplet biology.

577

578 **Declaration of Interest.**

579 The authors declare no competing interests.

580

581 **Author Contributions.**

582 AC, LF and ART designed, performed research, analyzed data and wrote the paper.

583

584 **Acknowledgments.**

585 We are thankful to our group members for their valuable comments and critical discussions about the  
586 manuscript. This work was supported the ANR-MOBIL and ANR-21-CE11-LIPRODYN to ART.

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- 723  
724  
725  
726

## 727 **Figure Legends.**

### 728 **Figure 1: Equilibrium conditions of a lipid droplet connected to a phospholipid bilayer**

729 **A)** Schematic view of a lipid droplet embedded in a bilayer. The two phospholipid monolayers covering  
730 the oil droplet are in continuity with the leaflets of the bilayer allowing phospholipid exchanges.

731 **B)** The mechanical equilibrium, resulting from the balance of the surface tension forces acting on the  
732 contact line, imposes the shape of the droplet reported by the two contact angles  $\theta_e$  and  $\theta_i$ . The  
733 bilayer tension, the external monolayer tension and the internal monolayer tension are respectively  
734  $(\gamma_b)$ ,  $(\gamma_{me})$  and  $(\gamma_{mi})$ .

735 **C)** Chemical equilibrium between phospholipids in the bilayer leaflet and in the monolayer. The  
736 equilibrium distribution between phospholipids in contiguous bilayer leaflet and LD monolayer is  
737 determined by the balance of their chemical potentials  $\mu_b$  and  $\mu_m$ . Phospholipids of a given  
738 membrane leaflet are free to exchange by lateral diffusion between the contiguous LD monolayer and  
739 the bilayer leaflet but phospholipid flip-flop is considered irrelevant.  $\rho_{me}$  and  $\rho_{mi}$  are the phospholipid  
740 density in the external and internal monolayer,  $\rho_{be}$  and  $\rho_{bi}$  are the density in the external and internal  
741 leaflet of the bilayer.

742

### 743 **Figure 2: Experimental determination of the interdependence between surface tensions**

744 **A)** Formation of the droplet-embedded vesicle (DEV) system: Giant unilamellar vesicles (GUVs) are  
745 mixed with nano emulsion droplet that are incorporated into the intermonolayer space of the GUVs to  
746 form DEVs.

747 **B)** Left, Schematic view of micropipette measurement of the monolayer surface tension of a DEV while  
748 simultaneously measuring and increasing its bilayer surface tension with a second micropipette. Right,  
749 representative confocal micrograph showing a brightfield image of a triolein-DOPC DEV merge with  
750 the fluorescence image of the phospholipids reported by rhodamine DOPE. Scale bar is 10  $\mu\text{m}$ .

751 **C)** Monolayer-bilayer surface tension diagram of a triolein-DOPC DEV. This DEV was selected with a  
752 droplet symmetrically positioned with respect to the bilayer, i.e. identical contact angles  $\theta_e$  and  $\theta_i$ . See  
753 supplementary figure S1A for confocal images of the experiment. See supplementary figure S1B,C for  
754 monolayer-bilayer tension diagram of DEVs with asymmetric droplet position and of DEVs with other  
755 phospholipidic compositions. A trend line is drawn to highlight the linear dependence of the tensions.

756 **D)** (left) Scheme of the droplet tensiometer method: an oil-in-water droplet is generated at the tip of a  
757 tube, with phospholipids added in excess in the oil phase. Compression is achieved by decreasing the  
758 droplet volume through withdrawing the oil phase. (right) Quantification of the surface tension  
759 of DOPC monolayer at triolein-buffer interface, respectively for a monolayer in continuity with a lipid  
760 bilayer with tension  $< 0.01 \text{ mN/m}$  (DEV), a monolayer in contact with a large phospholipid reservoir  
761 dissolved in the oil phase and a monolayer mechanically compressed. Data are represented as mean  
762  $\pm$ SD. Related to supplementary figures S1 D,E.

763

### 764 **Figure 3: Microscopic description of the contiguous droplet-bilayer system**

765 **A)** Microscopic contributions to the free energy density of a monolayer and of a bilayer. For the  
766 monolayer: the surface tension of the bare water/oil interface ( $\gamma_{o/w}$ ), the interaction of the phospholipid  
767 tails with the oil phase ( $f_{mo} = \varepsilon_{mo}\rho_m$ ), the lateral interaction between phospholipids ( $f_{int}$ ), and the  
768 interaction of the phospholipid headgroups with water ( $f_{hyd}$ ). For the bilayer: the unfavorable  
769 interactions of the PL tails with water ( $f_{phob} = \gamma_{phob}$ ), the trans-interaction between phospholipid tails  
770 ( $f_{mm} = \varepsilon_{mm}(\rho_{be} + \rho_{bi})$ ) of the apposed leaflet, the lateral interaction between phospholipids ( $f_{int}$ ), and  
771 the interaction of the phospholipid headgroups with water ( $f_{hyd}$ ).

772 **B)** Lateral pressure as a function of phospholipid density at the triolein-water interface deduced from a  
773 compression isotherm obtained from a pending droplet experiment (see supplementary Figure S2A,B).

774 **C)** Chemical potential of a bilayer and a monolayer as a function of phospholipid density. The two  
775 chemical potentials vary identically but are shifted by the amount  $\varepsilon_{mm} - \varepsilon_{mo}$ . At equilibrium a  
776 monolayer in contact with a bilayer have the same chemical potential ( $\mu_b = \mu_m$ ) and therefore have  
777 different phospholipid densities, whose values are directly impacted by the  $\varepsilon_{mm} - \varepsilon_{mo}$  gap.

778 **D)** Molecular structure of the different oils used to probe the effect of oil composition on monolayer-  
779 bilayer surface tension diagram.

780 **E)** Monolayer-bilayer surface tension diagram of DOPC DEVs made with different oil composition:  
781 sterol ester-triolein (1:3) (red circles), triolein (black circles) and squalene (blue circles). DEVs were  
782 selected with a droplet symmetrically positioned with respect to the bilayer. Black dot represent the  
783 average monolayer tension of osmotically deflated triolein DEVs (bilayer tension  $< 0.03 \text{ mN/m}$ ;  $n=17$ ).  
784 Blue dot represent the average monolayer tension of osmotically deflated squalene DEVs (bilayer

785 tension < 0.09mN/m ; n=9). See supplementary Figure S2C for monolayer-bilayer tension diagram of  
786 squalene and sterol ester-triolein (1:3) DEVs with asymmetric positions of the droplet.  
787

788 **Figure 4: Validation of the microscopic model by the DEV experimental system**

789 **A)** Monolayer-bilayer surface tension diagram of a triolein-DOPC DEVs having droplets with different  
790 positions relatively to the bilayer: centered droplet ( $\theta_e = \pi/2$ , black dots), droplet budding outward of  
791 the bilayer ( $\theta_e < \pi/2$ , blue dots), or droplet budding inward of the bilayer ( $\theta_e > \pi/2$ , red dots). See  
792 supplementary figure S3 A-D and material and methods for angle determination. Theoretical model  
793 was used to fit the centered experimental data (black line) and enabled to extract the microscopic  
794 parameters. Using the same parameters and only adjusting the droplet's size and position parameters,  
795 the model perfectly fit the data of the other DEVs with asymmetric droplet positions (blue and red  
796 lines).

797 **B)** Main microscopic parameters obtained by fitting the model to the centered experimental data.

798 **C)** Schematic illustration of the effect of the variation of ( $\epsilon_{mm} - \epsilon_{mo}$ ) on phospholipid density at the  
799 surface of a droplet in contiguity with a bilayer. If the bilayer tension is kept constant, the increase  
800  $\epsilon_{mm} - \epsilon_{mo}$  leads to a higher density of phospholipids on the surface of the droplet and therefore a lower  
801 surface tension of the droplets which causes the droplet to spread. This can be achieved by increasing  
802 the affinity of the phospholipid tails for the oil, or decreasing the strength of the trans-interactions  
803 between phospholipid tails in the bilayer.

804 **D)** Theoretical prediction of the effect of the variation of ( $\epsilon_{mm} - \epsilon_{mo}$ ) on the difference between the  
805 density of phospholipids in a bilayer sheet and that at the droplet surface. The bilayer surface tension  
806 ( $\gamma_b$ ) was kept at 0.5mN/m.

807 **E)** Theoretical prediction of the variation of the surface tension of the bilayer ( $\gamma_b$ ) on the phospholipid  
808 density in the bilayer and in the monolayer of droplets.  $\epsilon_{mm} - \epsilon_{mo}$  was kept constant at -4.4 pN.m.

809

810



## 811 **Material and methods: theory.**

### 812 **Definition of the intensive thermodynamic quantities**

813 The surface tension and chemical potential of a PL monolayer are defined by (supplement text,  
814 section 2),

$$815 \quad \gamma_m = f_m(\rho_m) - \rho_m f'_m(\rho_m), \quad \mu_m = f'_m(\rho_m), \quad (MM1)$$

816 where the prime denotes the derivative of the function. For each monolayer,  $\gamma_{me/mi} = \gamma_m(\rho_{me/mi})$  and  
817  $\mu_{me/mi} = \mu_m(\rho_{me/mi})$ . The chemical potential of a bilayer leaflet is

$$818 \quad \mu_b = \partial_\rho f_b(\rho, \bar{\rho}), \quad (MM2)$$

819 where  $\rho$  is the density of the considered leaflet and  $\bar{\rho}$  that of the opposite leaflet, so that,  $\mu_{be} =$   
820  $\mu_b(\rho_{be}, \rho_{bi})$  and  $\mu_{bi} = \mu_b(\rho_{bi}, \rho_{be})$ . The surface tension of the bilayer is defined as,

$$821 \quad \gamma_b = f_b(\rho_{be}, \rho_{bi}) - \rho_{be} \mu_b(\rho_{be}, \rho_{bi}) - \rho_{bi} \mu_b(\rho_{bi}, \rho_{be}). \quad (MM3)$$

822

### 823 **Materials and Methods experimental.**

#### 824 **Preparation of GUVs.**

825 All experiments were performed in the following HKM buffer: 50 mM Hepes, 120 mM Kacetate, and 1  
826 mM MgCl<sub>2</sub> (in Milli-Q water) at pH 7.4 and 275±15 mOsm.

827

828 GUVs were prepared by electro-formation. Phospholipids and mixtures thereof in chloroform at 0.5 μM  
829 were dried on an indium tin oxide (ITO) coated glass plate. The lipid film was desiccated for 1 h. The  
830 chamber was sealed with another ITO-coated glass plate. The lipids were then rehydrated with a  
831 sucrose solution (275±15 mOsm). Electro-formation is performed using 100 Hz AC voltage at 1.0 to  
832 1.4 V<sub>pp</sub> and maintained for at least 1 h. This low voltage was used to avoid hydrolysis of water and  
833 dissolution of the titanium ions on the glass plate. GUVs were either stored in the chamber at 4 °C  
834 overnight or directly collected with a Pasteur pipette.

835

#### 836 **GUVs composition:**

837 DOPC GUVs: 99 % DOPC (1,2-dioleoyl-sn-3glycero-3-phosphocholine) stained with 1% Rhodamine-  
838 DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl).

839 DOPA-DOPC GUVs: 30% DOPA (1,2-dioleoyl-sn-glycero-3-phosphate), 69 % DOPC, and 1%  
840 Rhodamine-DOPE.

841 Pufa-DOPC GUVs: 30% Pufa (18:0–20:4) (1-stearoyl-2-arach- idonoyl-sn-glycero-3-phosphocholine),  
842 69 % DOPC, and 1% Rhodamine-DOPE.

843

#### 844 **Preparation of DEV (GUVs + oil droplet)**

845 To prepare the oil droplets, 5 μL of the oil (triolein or squalene) was added to 45 μL of HKM buffer.  
846 The mixture was sonicated. The diameter of the resulting droplets is on the order of a few hundred  
847 nanometers. To make DEV, GUVs were then incubated with the LDs for 5 min. The GUV-LD mixture  
848 was then placed on a glass coverslip (pretreated with 10 % (w/w) BSA and washed three times with  
849 buffer).

850

#### 851 **Confocal microscope images.**

852 All micrographs of DEV were made on a Carl ZEISS LSM 800 with X10 air objective, and observed  
853 samples were held by Glass coverslips (Menzel Glaser (24x36mm #0)).

854

#### 855 **Micromanipulation & surface tension measurements by microaspiration.**

856 Micro-pipettes were made from capillaries drawn out with a Sutter Instruments pipette-puller. They  
857 were used to manipulate the DEVs in order to get a side view of the system. Pipettes were treated with  
858 an mPEG-Silane solution following the protocol described below.

859 Additionally, surface tensions were measured and modulated using the same pipettes. As shown in  
860 (Figure 2B), the micromanipulation of the external LD monolayer (or the DEV's bilayer) enables the  
861 measurement of the external monolayer surface tension (or the bilayer surface tension). Using  
862 Laplace's law and the measurement of the diameter, droplet (or bilayer) diameter, and suction  
863 pressure, the surface tension of the interface can be determined:

864 
$$\gamma = \frac{\Delta P_{\text{suc}}}{2 \left( \frac{1}{R_p} - \frac{1}{R_d} \right)}$$

865 where  $\Delta P_{\text{suc}}$ ,  $R_p$ , and  $R_d$  are the suction pressure, the pipette radius, and the droplet external radius.  
866 The suction was carried out using a syringe. The resulting pressure was measured with a pressure  
867 transducer (DP103 provided by Validyne Eng. Corp, USA), the output voltage of which was monitored  
868 with a digital voltmeter. The pressure transducer (range 55 kPa) was calibrated prior to the  
869 experiments.

870 External monolayer and bilayer tension of DEV were measured simultaneously while increasing the  
871 bilayer surface tension to obtain the monolayer tension-bilayer tension graph shown in (Figure 2C).

872

#### 873 **Tension measurements of triolein-buffer interface covered with phospholipids**

874 A pendant droplet tensiometer designed by Teclis Instruments was used to measure the interfacial  
875 tension of oil/water interfaces. All experiments were conducted at room temperature. DOPC  
876 phospholipids stored in chloroform were dried under argon and mixed with triolein oil. This mix was  
877 then sonicated and store at room temperature for 2h. To generate a phospholipid monolayer at the oil-  
878 buffer interface, phospholipid-in-triolein droplets (from 5 to 16.0  $\mu\text{l}$ ) were formed at the tip of a J-needle  
879 submerged in 5 ml of HKM buffer. The surface tension of the interface was automatically measured by  
880 the tensiometer.

#### 881 **Tension of an interface at equilibrium with a large reservoir of PLs dissolved in the oil phase.**

882 Triolein was supplemented with 0.5% (w/w) DOPC to get above the critical micellar concentration. This  
883 mix was used in the pendent droplet tensiometer to form a DOPC monolayer at the oil-water interface.  
884 As the phospholipids exceeded the critical micellar concentration, the oil phase acted as a  
885 phospholipid reservoir in supplying phospholipids to the monolayer. Surface tension was measured  
886 until a plateau was reached (supplementary figure S1D). This plateau of tension is the tension  
887 designated as the tension of a monolayer connected to a phospholipids reservoir.

888

#### 889 **The tension of a mechanically compressed phospholipid monolayer.**

890 Triolein was supplemented with 0.05% (w/w) DOPC. This mix was used in the pendent droplet  
891 tensiometer to form a DOPC monolayer at the oil-water interface. The phospholipids relocated at the  
892 oil-buffer interface resulting in a decrease of surface tension. After tension stabilization, we started to  
893 record tension while gradually sucking up the oil back in the needle. This resulted in the compression  
894 of the phospholipid monolayer at the interface, increasing of the phospholipid density and decreasing  
895 surface tension. At some compression level, a tension plateau was reached (supplementary figure

896 S1E). This plateau of tension is the tension designated as the tension of a monolayer mechanically  
897 compressed at its maximal PLs density.

898

### 899 **Phospholipids monolayer characterization (DOPC).**

900 A DOPC monolayer was formed at the triolein buffer interface using the pendant droplet tensiometer  
901 using the same condition as described in the above paragraph. After tension stabilization, the droplet  
902 area  $A$  was decreased while recording surface tension  $\gamma_m$  (supplementary figure S2A,B). This surface  
903 tension - droplet area isotherm is then fitted by equation (11),  $\gamma_m = \gamma_{o/w} - \pi(A/N)$  with  $\pi(\rho)$  given by  
904 the van der Waals equation of state  $\pi(\rho) = \frac{kT\rho}{1-a\rho} - u\rho^2$  and  $N$  is the number of PL on the droplet  
905 interface. It allows deducing the values of the parameters  $\gamma_{o/w}$ ,  $a$ ,  $u$  (and  $N$ ).

### 906 **Determination of the contact angles between the droplet and the bilayer.**

907 DEVs were imaged at their equatorial plane (side view). Two osculating circles are adjusted to delimit  
908 the droplet and bilayer of the DEV.  $\theta_e$ , the contact angle formed by the two tangents originating from  
909 the intersection point of the two circles (see supplementary figure S3 A-D), can be determined  
910 geometrically using the following equation derived from the law of cosines:

$$911 \quad \cos \theta_e = \frac{d^2 - R_g^2 - R_d^2}{2 R_g R_d}$$

912 Where ( $R_d$ ) is the radius of the droplet auscultating circle, ( $R_g$ ) is the radius of the bilayer auscultating  
913 circle, and ( $d$ ) is the distance between the two centers of the two osculating circles.

914

## 915 **QUANTIFICATION AND STATISTICAL ANALYSIS**

### 916 **Statistical analysis**

917 Unless mentioned, all values shown in the text and figures are mean  $\pm$  S.D.

918

## 919 **EQUIPMENT AND REAGENTS**

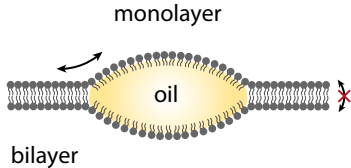
920 **Equipment:** All micrographs were made on a Carl ZEISS LSM 800. Glass coverslips: Menzel Glaser  
921 (24x36mm #0). Micro-pipettes made from capillaries (1.0ODx0.58IDx150Lmm 30-0017 GC100-15b  
922 Harvard Apparatus) with a micropipette puller (Sutter instrument model P-2000). Micromanipulation  
923 (Eppendorf TransferMan® 4r). Pressure measurement unit (DP103 provided by Validyne eng. corp,  
924 USA). Plasma cleaner was purchased from Harrick Plasma (PDC-32G-2 (230V) and used at 0,8 mbar  
925 of air pressure.

### 926 **Chemical product list:**

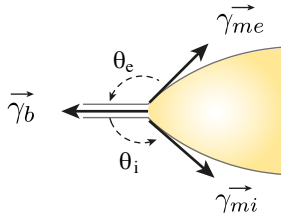
927 Phospholipids:

928 DOPC (1,2-dioleoyl-sn-3glycero-3-phosphocholine), DOPA (1,2-dioleoyl-sn-glycero-3-phosphate, Pufa  
929 (18:0–20:4) (1-stearoyl-2-arach- idonoyl-sn-glycero-3-phosphocholine), Rhodamine-DOPE (1,2-  
930 dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl) were from Avanti Polar  
931 Lipids (Alabaster, AL). triolein (Glyceryl trioleate T7140), Squalene (S3626), sterol-ester (C9253 1G),  
932 Hepes (54457–250-F), K acetate (P1190), MgCl<sub>2</sub> (M8266-100G), BSA 98% (A7906-100G), and  
933 sucrose 99.5% (59378-500G) and mPEG5K-Silane (JKA3037) were from Sigma-Aldrich.

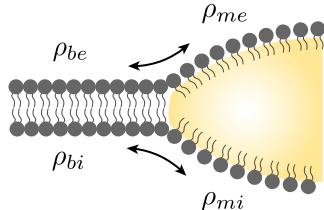
**A** phospholipids distribution

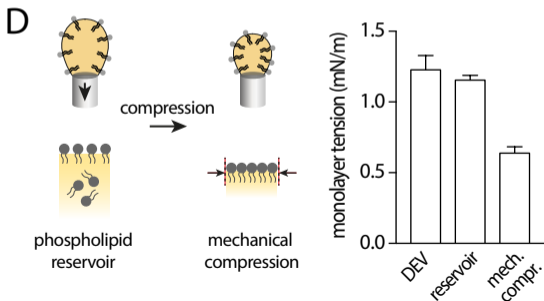
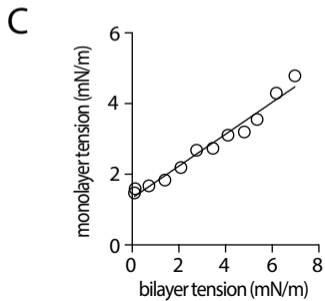
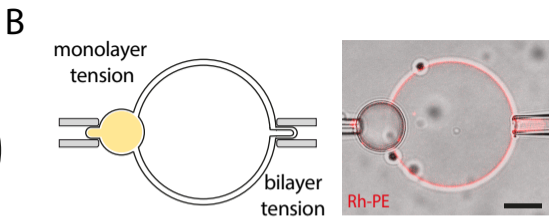
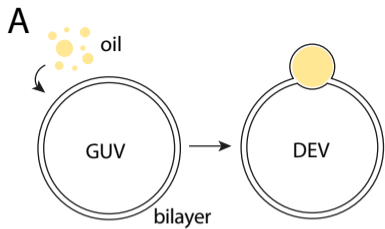


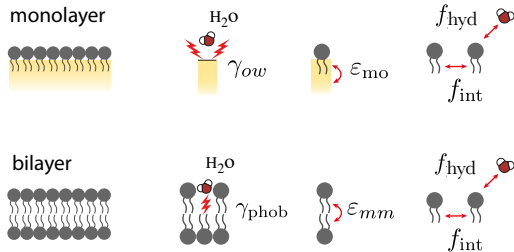
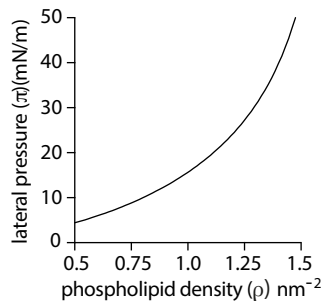
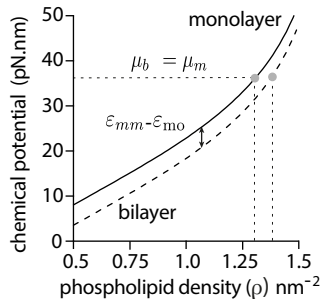
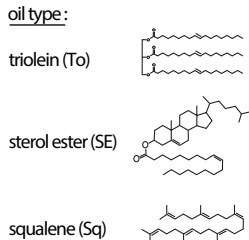
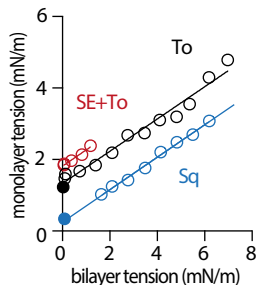
**B** mechanical equilibrium

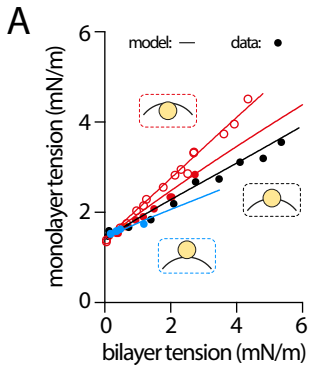


**C** chemical equilibrium:  $\mu_b = \mu_m$





**A****B****C****D****E**



**B**

$\gamma_{low}$	$\gamma_{phob}$	$\epsilon_{mm} - \epsilon_{mo}$
34 mN/m	38.6 mN/m	-4.4 pN.nm

